Transdermal neuromodulation of noradrenergic activity suppresses psychophysiological and biochemical stress responses in humans

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We engineered a transdermal neuromodulation approach that targets peripheral (cranial and spinal) nerves and utilizes their afferent pathways as signaling conduits to influence brain function. We then investigated the effects of this transdermal electrical neurosignaling (TEN) method on sympathetic physiology in response to acute stress induced by classical fear conditioning and a series of time-pressured cognitive tasks. The TEN approach involved delivering high-frequency, pulse-modulated electrical currents to the ophthalmic and maxillary divisions of the right trigeminal nerve and cervical spinal nerve afferents (C2/C3). Compared to active sham stimulation of the same anatomical targets, TEN treatment significantly suppressed sympathetic activity in response to acute stress without impeding cognitive performance. This sympatholytic action of TEN was indicated by significant suppression of heart rate variability changes, galvanic skin responses, and salivary α-amylase levels in response to experimentally induced stress. Our observations are consistent with the hypothesis that TEN acts partially by modulating afferent activity in the locus coeruleus and subsequent noradrenergic signaling. Dampening sympathetic tone using TEN in such a manner represents a promising approach to managing daily stress and improving brain health.

INTRODUCTION

Managing the psychological and physiological effects of stress presents a global challenge for healthcare [1,2]. The negative impact of chronic stress on brain health and cognition has been well defined [3-5]. Unmanaged stress is also known to mediate the breakdown of other biological functions, such as those associated with the immune [6-8] and cardiovascular systems [9,10]. While there are many effective ways to manage stress and treat stress-related disorders [11], each approach suffers from some critical limitation for effectively confronting the negative health consequences of stress.

Pharmacological approaches to managing stress, for example with benzodiazepines, suffer from drawbacks like causing drowsiness, blunting general affect, impairing attention and possibly leading to addiction [12]. Relaxation-based approaches such as meditation, focused breathing, or mindfulness have been shown to be beneficial, but require time investments and training that often prohibit high compliance rates [13]. Exercise is another effective approach to manage stress and has additional health benefits, but again due to the time commitment required and influence of other motivational factors compliance is often low [14]. To address the above referenced limitations, we hypothesized neuromodulation-based approaches to managing stress might provide a viable alternative.

We sought to develop a method for modulating psychophysiological arousal and stress responses by providing electrical signaling waveforms through afferent pathways of cranial nerves to neuromodulatory nuclei in the brainstem. During signal transmission to cortex, incoming sensory signals carried by the trigeminal and facial nerves simultaneously undergo local processing by a series of highly inter-connected structures including the reticular formation (RF) of the reticular activating system (RAS) located in the pons (Fig. 1). These brain stem circuits are a first station of information integration in the brain that support higher consciousness by filtering, integrating, and processing incoming sensory information [15]. Here cranial and spinal nerve activity modulates the nucleus of the solitary tract (NTS), locus coeruleus (LC) and other nuclei responsible for the bottom-up regulation of cortical gain, psychophysiological arousal, and neurobiological responses to environmental stimuli and stressors (Fig. 1) [16-22]. More specifically trigeminal afferent activity can modulate noradrenergic neurons via direct projections from the trigeminal sensory nucleus to the LC [16]. Through these bottom-up pathways, the LC and noradrenergic signaling modulates human behavior, sleep/wake/arousal cycles, and higher cortical functions including attention and cognition [16-22]. Exemplifying functional modulation of this circuitry, trigeminal nerve modulation has been shown to be moderately effective for treating psychiatric disorders like depression [23] and neurological conditions like epilepsy [24,25].

Given the growing interest in electrical and electromagnetic neuromodulation methods, we have spent the past few years developing wearable neurosignaling systems, devices, and approaches that enable the safe and comfortable modulation of

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peripheral and central neural pathways using pulsed electrical waveforms. Norepinephrine (NE) mobilized from the LC plays an integral role in mediating the brain's major sympathetic responses to environmental stressors, making it a promising target for mitigating stress responses. In the present paper, we tested the hypothesis that neuromodulation employing a high-frequency, pulse-modulated transdermal electrical neurosignaling (TEN) waveform targeting afferents (sensory and proprioceptive) of the right trigeminal nerve (V1/V2 distributions), temporal branch of the facial nerve, and cervical spinal nerves (C2/C3) can evoke relaxation while altering the psychophysiological and biochemical responses to experimentally induced acute stress. Our observations suggest this neurosignaling approach may be useful for alleviating the subjective, physiological, and biochemical responses to acute stress by exerting an effect on endogenous noradrenergic signaling.

RESULTS

The influence of TEN on self-reported relaxation

We first conducted a within-subjects experiment designed to assess the influence of TEN on self-reported relaxation. Briefly, all subjects (N = 82) were blinded to the experimental conditions and received both a sham and TEN treatment (14 min each) with a 15 min rest period between sessions (Fig. 2a). Following each treatment, subjects were asked to rate how relaxed they felt. This information was recorded on an 11-point scale, where 0 = not relaxed and 10 = extremely relaxed. A paired sample t-test revealed that subjects were significantly more relaxed following TEN compared to sham (sham relaxation score = 3.70 ± 2.16, TEN relaxation score = 6.42 ± 2.10; $p = 4.07 \times 10^{-17}$; Fig. 3a,b). The effects of TEN treatment were prominent as 77 of 82 subjects (94%) reported it induced greater relaxation than sham treatment (Fig. 3c). Because we had previously observed the effects of TEN can last for up to an hour or more, we designed the experiments described above such that sham stimulation always preceded TEN stimulation. To investigate the degree to which this experimental design may have produced any order effect on self-reported relaxation, we gave a different sample of subjects (N = 53) only a TEN treatment after a fifteen-minute acclimatization period. Compared to the sham relaxation scores of the 82 subjects treated with both sham and TEN, an independent t-test revealed that subjects receiving only TEN experienced significantly higher relaxation scores (TEN only treatment relaxation score = 5.64 ± 2.72; $p = 9.83 \times 10^{-6}$; Fig. 3b). These data bolster our observations that TEN treatment produces significantly greater perceived relaxation than sham treatment. None of the subjects reported either the TEN or sham stimulus as being distressing, uncomfortable, or irritating.

The effects of TEN on Heart rate variability and galvanic skin responses during acute stress

To determine the effect of TEN on physiological stress responses, we tested for group differences (TEN versus sham, N = 10 each) in heart rate variability (HRV), a common biometric of psychophysiological arousal reflecting autonomic function including parasympathetic and sympathetic nervous system activity [26,27]. We found there were no significant differences in average heart rate (HR) between the sham (HR = 67.01 ± 8.47 bpm) and TEN (HR = 69.18 ± 7.34 bpm) treatment groups during the stress trial (p = 0.55; Fig. 4A). Likewise the R-R intervals were not significantly different between the treatment groups during the stress paradigm (sham R-R interval = 922.07 ± 124.88 msec, TEN R-R interval = 885.21 ± 94.34 msec; p = 0.466; Fig. 4b). Analysis of HRV revealed subjects treated with TEN had a significant 34% reduction in SDNN compared to subjects treated with sham during the stress trial (sham SDNN = 105.45 ± 28.91 msec, TEN SDNN = 78.48 ± 16.60 msec; p = 0.02; Fig. 4c). Additionally, subjects in the TEN treatment group had a significant 88% reduction in the power of the LF (0.04 – 0.15 Hz) HRV component of the HRV spectrum compared to subjects treated with sham during the stress trial (sham LF power = 2946.80 ± 1352.12 $msec^2$, TEN LF power = 1567.40 ± 827.24 $msec^2$; p = 0.01; Fig. 4d). A similar trend was observed for the power of the HF component (0.15 – 0.4 Hz) of the HRV spectrum. Subjects treated with TEN had 71% less power in the HF component of the HRV spectrum compared to sham-treated subjects during the stress trial. This difference failed to reach a significant threshold (sham HF power = 2367.70 ± 1534.22 $msec^2$, TEN HF power = 1381.50 ± 804.42 $msec^2$; p = 0.09; Fig. 4f). There were no significant differences in the LF/HF ratios across treatment groups (sham LF/HF = 1.42 ± 0.43, TEN LF/HF = 1.20 ± 0.50; p = 0.30; Fig. 4f).

We next examined how TEN and sham treatment affected changes in electrodermal activity or galvanic skin responses (GSR), which is responsive stress and under the control of sympathetic activity (Fig. 5A,B) [28]. Compared to sham we observed that TEN treatment (N = 10 subjects per group) produced a significant 32% suppression in anticipatory GSR occurring in response to the onset of the fear-conditioning component of the stress trial prior to the delivery of the first unconditioned stimulus (ΔGSR_{fear}; sham ΔGSR_{fear} = 0.54 ± 0.11, TEN ΔGSR_{fear} = 0.37 ± 0.14; p = 0.007; Fig. 5c). Similarly, we observed the average GSR occurring in response to delivery of the unconditioned stimuli (10 electrical shocks per trial) was significantly suppressed by 53% in response to TEN treatment compared to sham treatment (sham ΔGSR_{shock} = 0.19 ± 0.04, TEN ΔGSR_{shock} = 0.09 ± 0.06; p = 0.001; Fig. 5c). These data indicate TEN treatment significantly suppressed sympathetic activity in response to the delivery of unconditioned fear stimuli (electrical shocks) used as an environmental stressor.

The influence of TEN on stress biomarkers: salivary α-amylase and cortisol

The protein enzyme α-amylase is widely recognized as a biochemical marker of sympathetic nervous system activity and sympathoadrenal medullary (SAM) axis activation [29-32]. More specifically, salivary levels of α-amylase directly correlate with plasma norepinephrine (NE) concentrations following the induction of acute stress including when electrical shock is used as a stressor [29-35]. To assess the impact of TEN on sympathetic activity and SAM axis activation, we examined fluctuations in salivary α-amylase (sAA) levels before (baseline) and after the acute induction of stress (Fig. 2b). We found the mean baseline levels of sAA were not significantly different between the sham (N = 8 subject samples) and TEN (N = 10 subject samples) treatment groups (sham = 102.45 ± 39.60 U/mL, TEN = 85.57 ± 41.03 U/mL; p = 0.39; Fig. 6a). Ten minutes after the stress trial, the average levels of sAA for the sham group had increased 0.6% from their baseline values while in the TEN-treatment group they had dropped 24%. However, this was not a significant difference between treatment groups (sham = 103.13 ± 46.43 U/mL, TEN = 68.93 ± 27.26; p = 0.07; Fig. 6a). Thirty minutes after the stress trial, the average sAA levels for the sham group had increased 6.5% from their baseline values while the sAA levels for subjects treated with TEN had dropped 19.8% from baseline values resulting in a significant difference between treatment groups (sham = 110.30 ± 35.11 U/mL, TEN = 68.62 ± 28.93 U/mL; p = 0.01; Fig. 6a).

While sAA is reflective of NE levels and acute SAM axis activity, cortisol is another stress biomarker, which has a slower onset, longer acting time course, and is under hormonal control of the hypothalamic-pituitary-adrenal (HPA) axis. It has previously been shown that sAA and cortisol can exhibit different response profiles that do not always correlate with one another depending upon stressor properties, such as the emotional intensity/valence of a stimulus and the duration of stress exposure [33-36]. Salivary cortisol levels did not significantly differ between sham and TEN treatments at any of the time points tested. At baseline the salivary cortisol concentration for sham was 0.26 ± 0.13 μg/dL and 0.24 ± 0.09 μg/dL for the TEN-treatment group (p = 0.75; Fig. 6b). Ten minutes after the stress paradigm the salivary cortisol concentration for sham was 0.23 ± 0.15 μg/dL and 0.19 ± 0.11 μg/dL for the TEN-treatment group (p = 0.51; Fig. 6b). Thirty minutes after the stress paradigm the salivary cortisol concentration for sham was 0.18 ± 0.09 μg/dL and 0.16 ± 0.06 μg/dL for the TEN-treatment group (p = 0.67; Fig. 6b).

The effects of TEN on cognitive performance or executive processing reaction times

Subjects took three consecutive, time-constrained cognitive tests (Flanker test, n-back, and Stroop task; 2 min each) evaluating attention and working memory to evaluate the effects of TEN on executive processing during stress induction. A series of one-way ANOVAs revealed there were no significant differences between sham and TEN treated subjects in error rates or reaction times for congruent and incongruent tasks (Table 1). These data indicate that while TEN can suppress sympathetic activity in response to acute stress, it does so without impeding general/executive cognitive performance.

TEN did not induce significant side effects

We assessed differences in the incidence (frequency), duration (in minutes), and severity (eight point scale) of the most common side effects associated with electrical neurostimulation using subject self-reports. TEN and sham groups had similar incidence rates of side effects with similar severity and durations (Table 2). Notably, there were no reports of headaches, nausea, or hearing changes. Further, no severe adverse events were reported.

DISCUSSION

Our observations demonstrate the TEN protocol we developed and implemented in this study increased the levels of self-reported relaxation and attenuated psychophysiological and biochemical responses to acute stress. The brain and body's response to stress is complex and not easy to unravel. As discussed below however, an inspection of anatomical circuitry and comparison to well-established neurosignaling pathways indicates the TEN approach we employed acted upon noradrenergic systems to alter psychophysiological and biochemical stress responses via bottom-up mechanisms.

There are several possible mechanisms by which TEN suppressed stress responses. TEN could have both inhibited and activated of cranial nerves and cervical spinal afferents. Perhaps the most parsimonious explanation is that TEN, delivered in pulsed currents ranging from the 7 - 11 kHz suppressed stress responses by inhibiting trigeminal and cervical spinal afferent activity thereby dampening basal sympathetic tone. Such a mechanism would be consistent with other observations using high-frequency electrical currents to modulate neuronal activity. For example, high-frequency (10 kHz) spinal cord stimulation has been found to be effective at blocking low back and leg pain without producing paresthesia [37]. Such an outcome may be due to the fact that high-frequency electrical stimulation (2 - 20 kHz) has been shown capable of producing rapid and reversible blockade of neuromuscular activity [38]. These types of nerve-block mechanisms are attractive to explain the influence of TEN on stress responses primarily due to their simplicity. However skin sensations associated with TEN indicate afferent pathways were intact suggesting nerve activation mediated the attenuation of stress responses observed. In fact, evidence dating back to the dawn of modern electrophysiology has demonstrated high-frequency currents effectively stimulate peripheral nerve through a variety of mechanisms including by avoiding a refractory period [39]. When synthesizing the TEN waveforms used in this study, we chose high frequency pulsing parameters to maximize safety and improve comfort by minimizing efferent stimulation of neuromuscular fibers. We propose the high-frequency TEN currents transmitted via afferent fibers modulated the activity of the locus coeruleus (LC) and norepinephrine (NE) signaling to attenuate stress responses as discussed below.

We originally hypothesized that TEN waveforms targeted to the ophthalmic (V1) and maxillary (V2) divisions of the trigeminal nerve and the temporal branch of the facial nerve innervating the vicinity of the right temple (F8), as well as C2/C3 cervical spinal afferents innervating the neck could attenuate acute stress responses. This hypothesis was derived from the fact that trigeminal and facial nerve afferents give rise to synaptic circuits in the pons that provide inputs to the nucleus of the solitary tract (NTS), reticular formation (RF), and noradrenergic LC as illustrated in Fig. 1. The principal sensory nucleus of the trigeminal nerve provides direct projections to the LC and establishes at least one direct pathway by which trigeminal afferents can modulate noradrenergic activity (Fig. 1) [16]. In addition to trigeminal pathways, sensory afferents from the facial nerve project to the NTS, which also influences the activity of neurons in the RF and LC. The NTS also influences the activity of the dorsal motor nucleus (DMN) of the vagus, which sends preganglionic parasympathetic fibers to various target organs to regulate physiological homeostasis. Afferent projections from cervical spinal pathways provide another pathway by which TEN could modulate the LC [17]. At a bare minimum, the afferent modulation of LC activity alone would alter the mobilization and release of NE to mediate the brain's response to environmental threats and stress [40]. In fact, it has been shown that sensory stimulation rapidly increases the firing rates of NE neurons in the LC [41,42]. In turn, the modulation of NE signaling would directly influence sympathoadrenal medullary (SAM) axis activity and regulate the body's acute response to stress. What lines of evidence support the ability of TEN to act via this mechanistic pathway?

The LC exhibits two essential types of neural discharges (tonic and phasic) that modulate cognition and behavior. These phasic and tonic LC discharge patterns favor different modes of signal processing and behaviors in a manner dependent upon the level of afferent drive [19,43,44]. In response to sensory and environmental cues, neurons in the LC have been shown to undergo shifts from tonic to phasic firing [45]. Endogenously the magnitude of phasic LC activity depends on the level of tonic LC activity (Fig. 7a). During stress tonic activity is high and phasic activity is diminished (Fig. 7a) [44,46,47]. Likewise when tonic activity is extremely low, such as during slow wave sleep, phasic activity is also diminished [44,46,47]. In fact it has recently been demonstrated using optogenetic control of mouse LC neurons *in vivo* that tonic and phasic activity produces robust changes in sleep/wake cycles, motor behaviors, and general arousal in a frequency-dependent manner [48]. These observations reinforce the notion that appropriate levels of arousal stemming from the balance between tonic and phasic LC/noradrenergic activity are necessary for optimal behavioral flexibility and performance [19,43,44]. Based on the transitional behaviors of LC activity discussed above, we hypothesize that through cranial nerve afferent pathways TEN drives LC activity into a sympatholytic phasic firing mode by diminishing high levels of stress-induced tonic activity (Fig. 7b). This increase in phasic activity in turn may result in higher levels of α2 adrenoceptor activity as discussed further below. The outcome of these actions would be a net decrease in sympathetic tone similar to the basic observations we made regarding changes in HRV, GSR, and sAA levels in response to acute stress (Figs. 4 - 6). Future investigations aimed at neurophysiologically elucidating the transfer function(s) between TEN stimulus parameters and LC tonic/phasic activity will be critical to gaining a complete understanding of how TEN can attenuate stress responses. Additional evidence in the literature however supports our hypothetical framework.

Thirty minutes following the stress trial we observed subjects treated with TEN had significantly lower levels of sAA compared to subjects receiving sham treatment (Fig. 6a). This biochemical observation suggests TEN suppressed stress responses by acting partially upon noradrenergic signaling since several studies have shown sAA levels to be predictive of plasma NE concentration and not necessarily to correlate with the temporal dynamics of cortisol mobilization [32-36,49,50]. For example, stress induced by environmental exposure to extreme cold or heat has been found to increase human sAA levels and plasma NE concentrations without affecting cortisol concentrations [49]. More direct evidence stems from the observation that NE infusions in men caused a significant increase in sAA levels [50]. As observed by Chatterton and colleagues (1996), we found TEN affected sAA levels without influencing cortisol [65]. Our observations are also consistent with the findings of others reporting that electrical shock used as a stressor influences sAA levels without affecting cortisol levels [33-35]. Changes in sAA levels have also been shown to correlate with changes in HRV. Psychosocial stress in humans has been shown to significantly increase the LF power of HRV spectra and sAA levels without affecting cortisol concentrations [36]. Compared to TEN treated subjects, we found stress caused subjects treated with sham to have significantly greater sAA levels (Fig. 6a) and significantly more LF HRV power (Fig. 4d). Due to TEN exerting effects on afferent pathways impinging on the brainstem and LC, the HRV and sAA data taken together suggest that subjects treated with TEN protocols had lower plasma NE concentrations following exposure to stress.

There are several NE signaling pathways that exert an influence on psychophysiological arousal and stress responses [51-54]. On interesting candidate is the α2 adrenoceptor, which provides negative feedback to NE neurons by hyperpolarizing presynaptic terminals to prevent the further release of NE [55,56]. The α2 adrenergic receptor agonist clonidine inhibits the tonic activity of neurons without suppressing phasic activity [57]. Our physiological and biochemical data indicate TEN may partially act by producing an increase in α2 adrenergic receptor activity. Clonidine blocks sAA secretion triggered by sympathetic fiber stimulation [58]. Similarly, we observed that TEN significantly attenuated the levels of sAA following stress induction as discussed above (Fig. 6). Clonidine also induces sympatholytic (anxiolytic) and sedative effects while stabilizing cardiovascular activity [59-62]. Here clonidine has been shown to exert some of its actions by significantly decreasing LF HRV while affecting the HF component of HRV to a lesser degree in humans [63]. Oral administration of clonidine to healthy subjects has also been shown to significantly reduce the LF component of HRV, as well as lower plasma concentrations of NE [64,65]. Similar to the actions of clonidine, we found that TEN treatments produced a significant decrease in the LF component of HRV during the stress trial compared to sham (Fig. 4d,e). Also consistent with α2 adrenocepter activation, we found that subjects were significantly more relaxed following TEN treatments compared to sham (Fig. 3). Several other observations indicate TEN acts like clonidine to modulate α2 adrenergic receptors.

During fear-conditioning we observed subjects treated with TEN had significantly attenuated GSR (electrodermal) responses compared to subjects receiving sham treatments (Fig. 5). Clonidine has been shown to inhibit sympathetic-cholinergic activity as indicated by its ability to significantly suppress reflexive and electrically evoked electrodermal activity in cats [66,67]. Abnormal α2 adrenergic receptor activity has been implicated in several mood and stress disorders. Patients with panic disorder have been shown to possess fewer binding sites for α2 adrenergic receptors compared to normal subjects, exhibit significantly larger skin conductance changes (GSR), and experience larger increases in plasma NE concentration following exposure to stress [68]. In addition to α2 adrenergic receptor signaling pathways, other noradrenergic and neuromodulatory pathways may have also contributed to the effects we observed. For example, the beta-blocker propranolol has been shown to suppress sAA increases due to acute stress [31]. Since the raphe nucleus forms part of the RF, serotoninergic modulation could have mediates some of the effects we observed. In fact, there is evidence suggesting that serotonin may mediate some aspects of stress responses [69,70], though evidence for a role of serotonin in stress responses has been far more elusive than the effects of NE. Further experiments involving human pharmacology will be required to fully delineate the molecular mechanisms of action underlying the ability of TEN to inhibit stress responses and induce subjective relaxation. These will be important studies given the growing interest in electroceuticals [71].

The influence of other peripheral pathways should also be considered. For example the vagus, the tenth cranial nerve, plays known roles in regulating neurophysiological responses to environmental stimuli and stressors. However vagal afferents do not innervate the cutaneous regions we targeted. Vagal afferents projecting to the NTS carry information from the ear including the external meatus and tympanic membrane, the throat, and thoracic and abdominal viscera from the heart to the distal small intestine [72]. Thus, modulation of regions around the ear might be presumed to exert different outcomes on psychophysiological arousal by recruiting vagal mechanisms and parasympathetic signaling pathways. This seems likely given vagal loops and NTS modulation of the dorsal motor nucleus of the vagus. The DMN sends preganglionic parasympathetic efferent fibers to visceral organs in the thoracic and abdominal cavities to regulate physiological homeostasis, such as gastric motility. Due to their anatomical localization, we do not believe vagal afferents had a primary role in mediating the major effects of TEN on stress responses described above. Admittedly however, it could be difficult to distinguish some noradrenergic responses from vagal parasympathetic ones. This is especially true since TEN could have affected NTS activity through facial nerve pathways, which in turn would influence the DMN (Fig. 1). In any case our observations provide clear evidence that TEN can alter the balance of sympathetic and parasympathetic activity, as exhibited by its ability to exert a braking action on acute stress responses.

The safety of TEN is supported by a long history of peripheral electrical neurostimulation obtained over more than four decades. Legally marketed electrical nerve stimulation devices are already commercially available and have output levels far greater than the ones implemented here. These devices intended for over-the-counter cosmetic applications of transcutaneous electrical nerve stimulation (TENS) target similar anatomical regions. One example device is the Bio-medical Research (BMR) Face device, which is an over-the-counter TENS device designed to target the trigeminal nerve and provide neuromuscular electrical stimulation (NMES) to encourage facial rejuvenation for aesthetic purposes. A recent study examined the safety and efficacy of this device at a peak current intensity of 35 mA when used five days per week for 20 minutes each day for 12 weeks [73]. There were 56 subjects in the active treatment group and 52 in the placebo sham-control group. There were no significant adverse events in this study and the only reported side effects were minor skin redness following stimulation, which disappeared with 10-20 minutes following use [73]. Consistent with these results, on an acute level we found that TEN did not elicit any significant side effects (Table 2). Further, TEN did not have any significant effect on cognitive performance or reaction times (Table 1). Other observations demonstrating the chronic safety profile of TEN are currently being prepared for publication. Based on all our observations, we conclude TEN provides a safe and reliable approach to modulating psychophysiological arousal.

In summary our observations demonstrate that, compared to sham, TEN treatment induces significantly greater relaxation and significantly suppresses physiological and biochemical responses to acute stress. The reduction of LF HRV, decreased levels of sAA, and suppressed GSR in response to acute stress are consistent with the hypothesis that TEN modulation of trigeminal (V1/V2) and cervical spinal (C1/C2) afferent pathways act partially through bottom-up mechanisms by modulating noradrenergic activity in a first station of information processing in the human brain. While further experiments including pharmacological manipulations will be required to test this hypothesis and to investigate the influence of TEN on signaling cascades downstream of NE, our observations have profound implications for stress management and brain health. Further, the capability of being able to rapidly modulate noradrenergic activity by stimulating the human locus coeruleus is a powerful research probe and can uniquely provide lifestyle approaches to minimizing the impact of acute stress in a chemical-free manner.

FIGURES

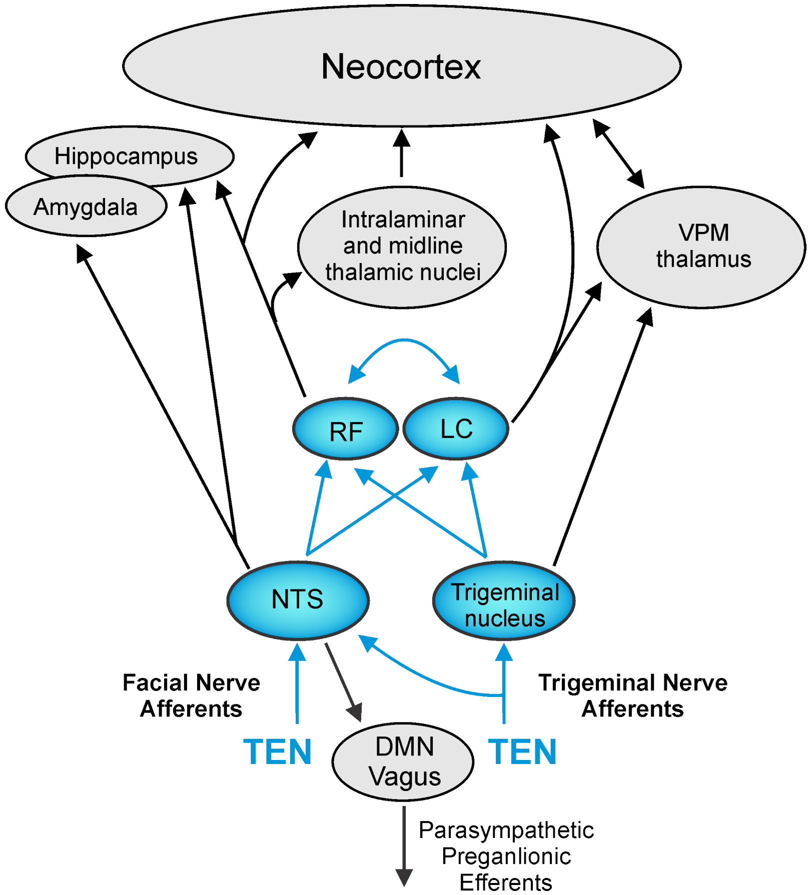

Figure 1. Cranial and spinal afferent pathways provide direct inputs to neural circuits involved in the bottom-up regulation of psychophysiological arousal and stress responses. The circuit diagram illustrates trigeminal and facial nerve afferents impinging on the nucleus of the solitary tract (NTS) and trigeminal nuclei in the brainstem. There are direct projections from the NTS and trigeminal nucleus to the noradrenergic locus coeruleus (LC) and the reticular formation (RF). The major pathways and circuitry highlighted in *blue* was targeted using high frequency (7 – 11 kHz), pulsed modulated transdermal electrical neurosignaling (TEN) waveforms delivered through trigeminal, facial, and C2/C3 cervical spinal afferent fibers projecting to the pons of the brain. Trigeminal and facial nerve afferents (sensory and proprioceptive) can modulate the activity of the LC, NTS, and RF to affect psychophysiolgical arousal, the activity of the sympathoadrenal medullary (SAM) axis, biochemical stress responses, and parasympathetic preganglionic fibers projecting from the dorsal motor nucleus (DMN) of the vagus. The bottom-up neurosignaling pathways illustrated serve mechanisms for the regulation of cortical gain control and performance optimization by influencing a broad variety of neurophysiological and cognitive functions, as well as primary sympathetic responses to environmental stress [43,74].

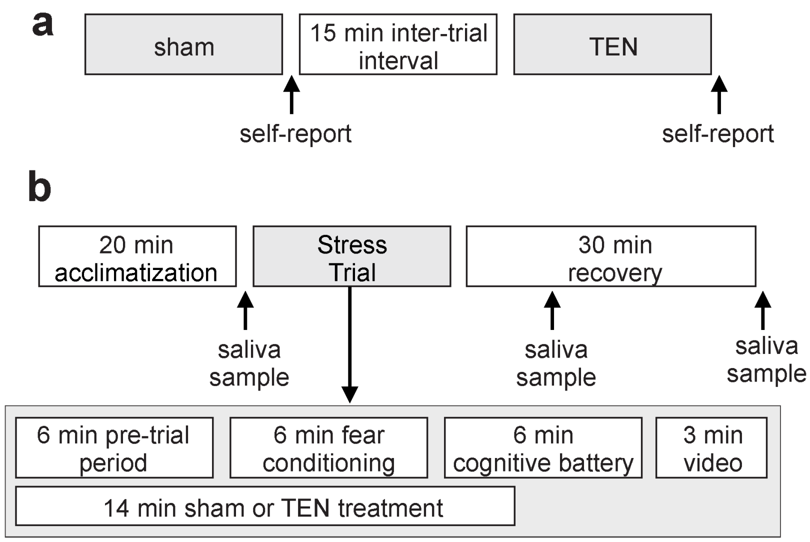

Figure 2. Experimental approach used to assess the effects of TEN on relaxation and acute stress responses. a, The illustration depicts the experimental approach used to evaluate the influence of TEN on self-reported relaxation. In a within-subjects design, 82 subjects received both a 14 min sham treatment and a 14 min TEN treatment separated by a 15 min rest period. The order of sham and TEN treatments were counterbalanced across subjects. At the end of each treatment, subjects self-reported their perceived level of relaxation on a 10-point scale. **b**, The illustration depicts the experimental approach used to evaluate the influence of TEN on psychophysiological and biochemical responses to stress. In a between-subjects design, 20 subjects received either a 14 min sham treatment (N = 10) or a 14 min TEN treatment (N = 10) beginning 20 min after an acclimatization period and immediately before the onset of a stress trial. The composition of the stress trial is expanded for clarity (bottom). To assay stress biomarkers, saliva samples were acquired before the stress trial, 10 min after the stress trial, and 30 min after the stress trial as indicated. We recorded heart rate and galvanic skin resistance during the stress trial for measurement of psychophysiological arousal. For additional details, see *Methods*.

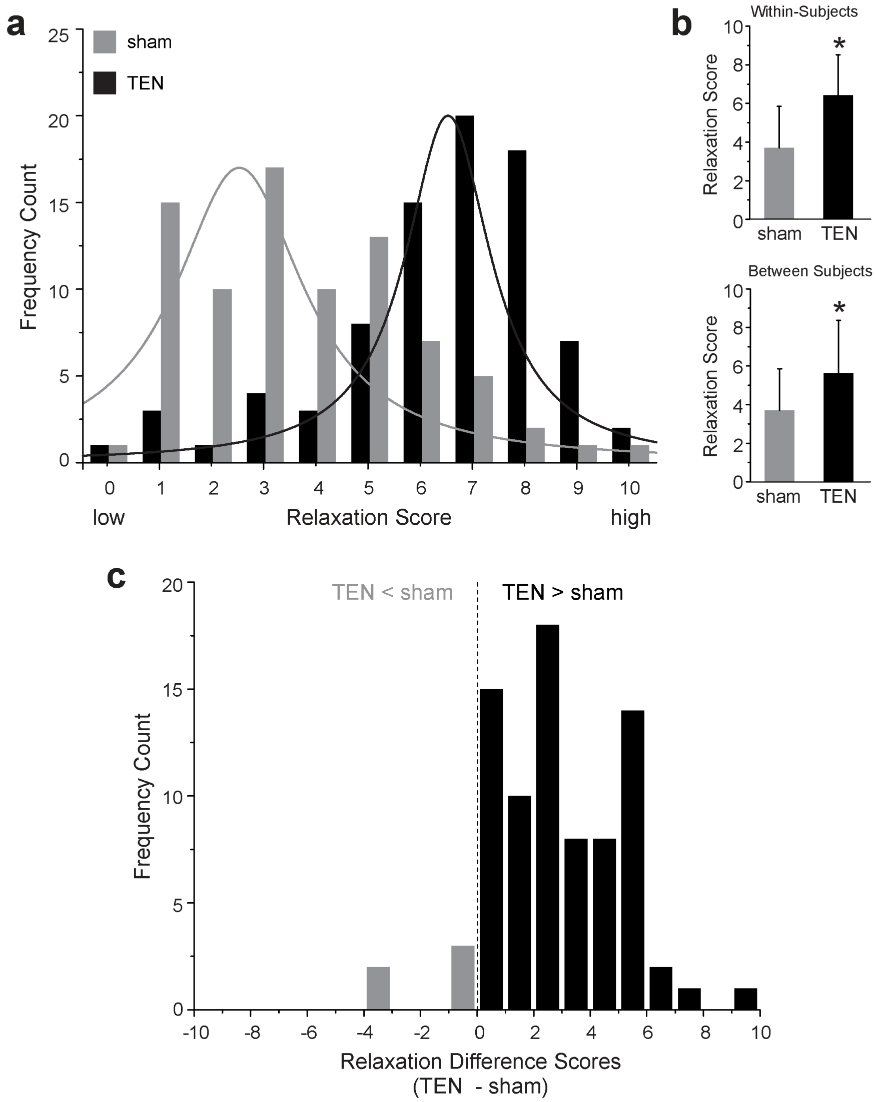

Figure 3. TEN induces significantly greater subjective relaxation compared to sham. a, The histograms illustrate the frequency distribution of self-reported relaxation scores recorded by subjects (N = 82) after receiving sham (grey) and TEN (black) treatments in a within-subject design. **b**, The top histograms illustrate the average relaxation scores for subjects receiving sham and TEN in the with-subjects design (Fig. 2A). The bottom histograms illustrate a between subjects comparison of the average relaxation scores from the sham treatments of the within-group subjects (N = 82) and a different group of subjects (N = 53) only receiving a TEN treatment. Both within-subjects and between subjects comparisons illustrate that TEN produced significantly greater relaxation compared to sham as illustrated. **c**, The histograms illustrate the frequency distribution of difference scores (TEN – sham) obtained on the relaxation self-reports from each subject in the within-subjects experiment. A positive value indicates a subject's relaxation score for TEN > sham while a negative value indicates a relaxation score for TEN < sham.

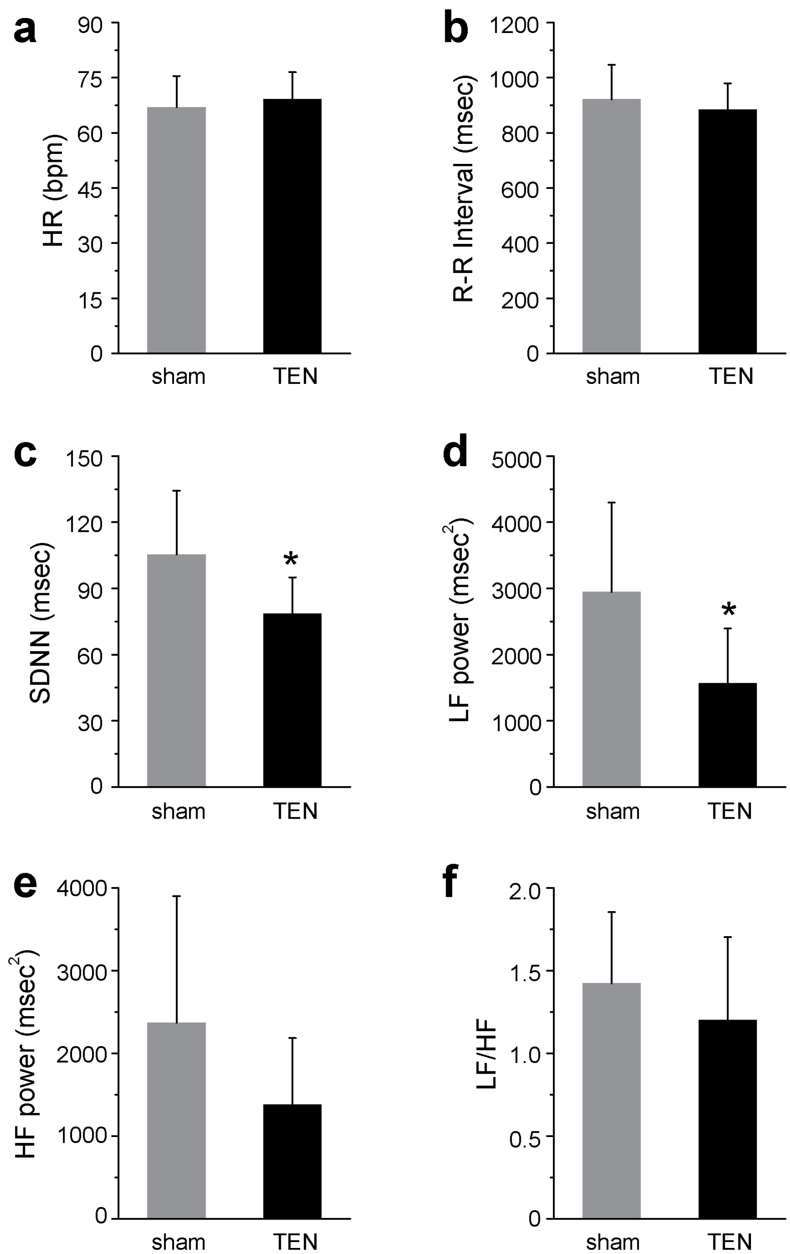

Figure 4. TEN suppresses changes in HRV induced by experimentally induced stress. The histograms illustrate the average HR (**a**), R-R interval (**b**), SDNN (**c**), LF power (**d**), HF power (**e**), and LF/HF (**f**) HRV responses mediated by sham and TEN treatments in response to the induction of acute stress. An asterisk indicates a significant difference with $p < 0.05$.

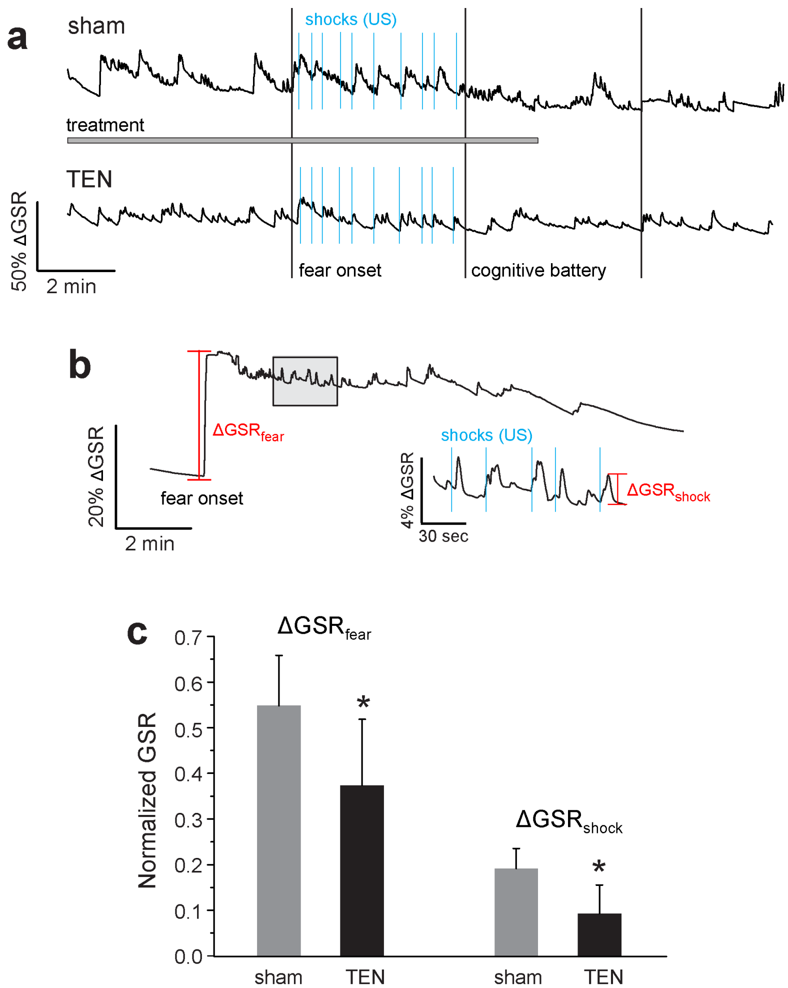

Figure 5. TEN attenuates anticipatory and stimulus-locked changes in GSR during classical fear-conditioning. **a**, Representative GSR traces obtained from a subjects treated with sham or TEN during the experimental paradigm designed to induce acute stress (Fig. 2B). The onset of the classical fear-conditioning paradigm (fear onset), delivery of unconditioned stimuli (US; electrical shocks; blue), and onset of the time-pressured cognitive battery are marked. Note differences in the amplitude of US-evoked GSR responses between the treatment groups illustrate TEN-mediated reduction in sympathetic drive. **b**, The line plot illustrates a different GSR profile obtained from a subject treated with TEN during the fear-conditioning component of the stress trial. As also shown in panel **a**, all subjects exhibited the same type of response where there was a sharp anticipatory rise in GSR (ΔGSR_{fear}) at the onset of the fear-conditioning component of the stress trial before the delivery of the first US. All subjects also exhibited transient GSR increases (ΔGSR_{shock}) in response to the delivery of each US as illustrated. For clarity, the area marked by the *shaded rectangular box* is shown at a higher temporal resolution and amplitude scale (inset). **c**, The histograms illustrate the average GSR changes for the TEN and sham treatment groups obtained during the fear-conditioning phase of the stress trial. An asterisk indicates a significant difference with $p <$ 0.05.

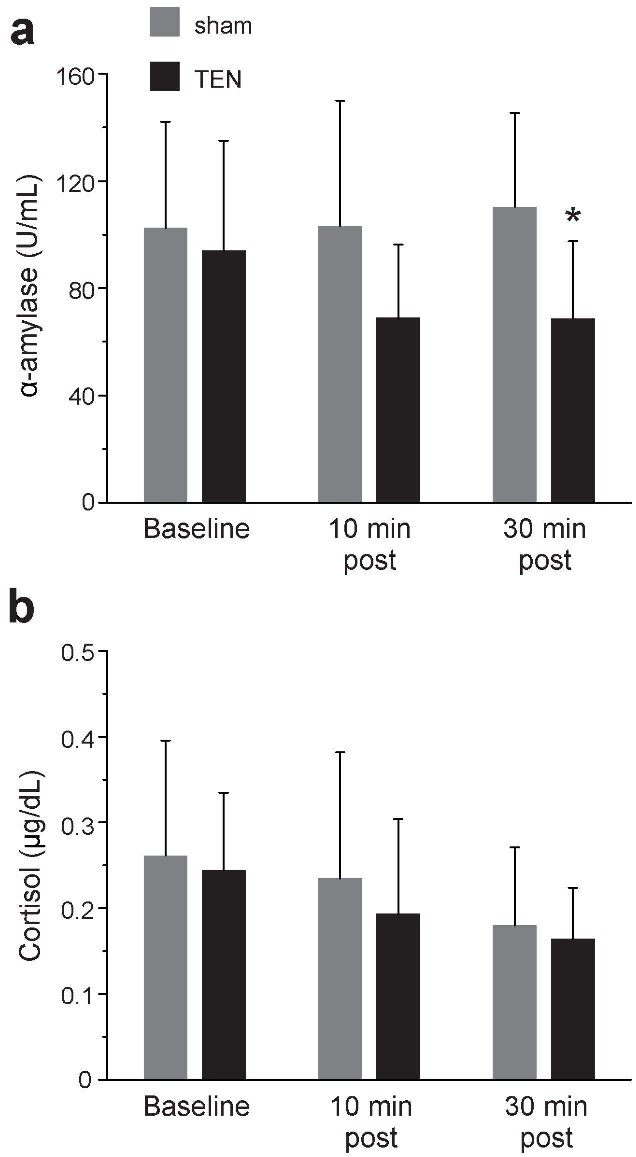

Figure 6. TEN reduces salivary α-amylase levels in response to acute stress. The histograms illustrate the average levels of α-amylase (**a**) and cortisol concentration (**b**) in saliva samples taken before (baseline), 10 min, and 30 min after the stress trial for sham and TEN treatment groups. An asterisk indicates a significant difference with $p < 0.05$.

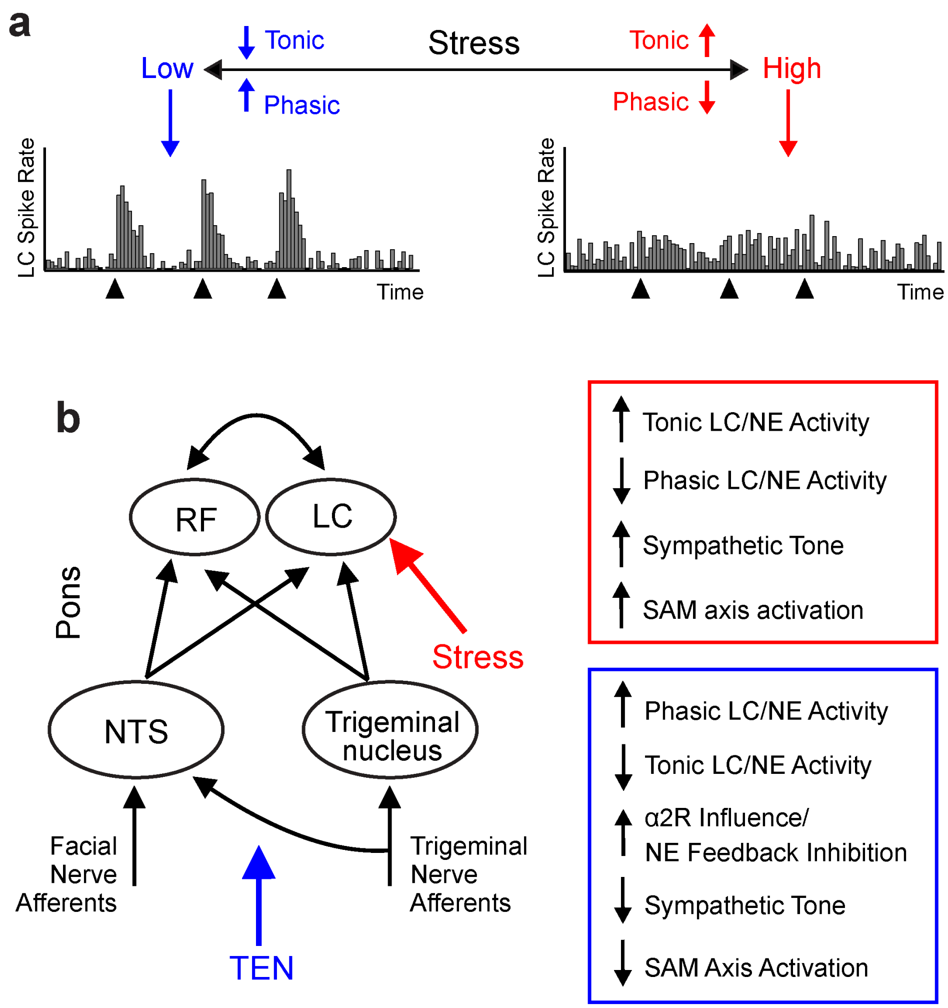

Figure 7. Neurons in the LC dynamically shift between tonic and phasic firing modes as a function of afferent activity and stress. a, The figure illustrates a working model of neuronal activity patterns exhibited by the LC under different levels of stress and arousal. This model incorporates observations that stress leads to high levels of tonic activity in the LC while sensory stimulation (black triangles) triggers phasic activity especially when tonic activity is low [41,42,44-47]. **b**, Facial and trigeminal nerve afferents, as well as the pons portion of the circuit diagram from Fig. 1 are shown (left). As illustrated stress (red) acts on LC neurons to increase their tonic firing rates thereby driving sympathetic activity and increasing SAM axis activation. We hypothesize that TEN (blue) biases LC activity through afferent pathways in a manner that increases phasic firing, reduces tonic activity, and suppresses sympathetic tone and SAM axis activation. Our observations suggest this partial mechanism of action, at a first station of information processing in the brain, may involve increased α2 adrenergic activity as depicted.

TABLES

Table 1. Outcomes of cognitive battery.

	TES			**Sham**			**Comparison**	
	N	M	SD	N	M	SD	ANOVA	*p*
Flanker								
% Correct	10	100.00	0.00	10	99.00	2.11	F (1, 19) = 2.25	0.15
Congruent RT	10	612.37	249.27	10	570.43	190.43	F (1, 19) = 0.18	0.68
Incongruent RT	10	585.92	170.93	10	618.00	182.06	F (1, 19) = 0.17	0.69
Stroop								
% Correct	8	98.75	3.54	6	97.82	2.79	F (1, 13) = 0.28	0.61
Congruent RT	8	973.41	305.72	6	931.88	132.58	F (1, 13) = 0.10	0.76
Incongruent RT	8	1247.82	289.62	6	1102.69	170.31	F (1, 13) = 1.18	0.30
N-Back								
% Correct	8	78.75	10.69	9	83.75	13.42	F (1, 16) = 0.71	0.41
Average RT	8	705.80	103.74	9	713.25	133.01	F (1, 16) = 0.02	0.90
Combined Time	8	921.80	245.53	9	890.29	291.78	F (1, 16) = 0.06	0.81

M = mean, SD = standard deviation, RT = reaction time in milliseconds.

Table 2. Side effects elicited by TEN and sham treatments.

	TES						Sham Stimulation					
	N	Presence	Severity		Duration		N	Presence	Severity		Duration	
		% (Count)	M	SD	M	SD		% (Count)	M	SD	M	SD
Skin Irritation	9	33 (3)	3.50	2.12	5.00	0	10	50 (5)	4.20	2.49	2.65	4.90
Headache	10	0 (0)	--	--	--	--	10	0 (0)	--	--	--	--
Dizziness	10	10 (0)	--	--	--	--	10	10 (1)	3.00	--	0.17	--
Vision Changes	10	10 (1)	3.00	--	10.00	--	10	0 (0)	--	--	--	--
Hearing Changes	10	0 (0)	--	--	--	--	10	0 (0)	--	--	--	--
Nausea	10	0 (0)	--	--	--	--	10	0 (0)	--	--	--	--

M = mean, SD = standard deviation

METHODS

Participants

All experimental procedures were conducted on human volunteers using protocols approved by an independent Institutional Review Board (Solutions IRB, Little Rock, AR). All subjects provided written informed consent prior to experimentation. In Experiment 1, designed to study the influence of TEN on self-reported relaxation, the sample consisted of 82 healthy right-handed subjects (34 male, 48 female) between the ages of 18 and 50 (mean age = 24.07 ± 7.07 years) that were recruited from the greater Boston, MA area. These subjects received both a sham and TEN treatment as described below. Forty three percent of participants were Caucasian, 35% were Asian, 12% were African-American, and 6% were Hispanic. These participants were fairly educated: 60% completed some college, 22% had a college degree, and 16% completed some post-graduate work or had a post-graduate degree. Another sample of subjects (N = 53) was included in Experiment 1, but they only received a TEN treatment. This sample consisted of 24 males and 29 females aged 18 to 43 (mean age = 22.62 ± 4.93 years). Of these subjects 47% were Caucasian, 25% were Asian, 9% were African-American, and 8% were Hispanic. Their educational background was that 70% completed some college, 21% had a college degree, and 8% completed some post-graduate work or had a post-graduate degree. For all experimental conditions, exclusion criteria were as follows: neurological or psychiatric disorder, cranial or facial metal plate or screw implants, severe face or head trauma, recent concussion or brain injury, recent hospitalized for surgery/illness, high blood pressure, heart disease, diabetes, acute eczema on the scalp, and uncorrectable vision or hearing. In Experiment 2, designed to study the influence of TEN on psychophysiological arousal and the mobilization salivary biochemicals in response to acute stress, 20 male subjects were yoked from the previous sample. We used males to avoid the introduction of confounds related to hormonal variance across menstrual cycles on stress biochemical profiles. The subjects were between the ages 19 to 27 (mean age = 22.3 ± 2.2 years). Fifty percent of the subjects were Asian, 35% were Caucasian, 10% were African American and 5% were Hispanic. Forty percent of participants had completed some college, 40% had a college degree and 15% had completed some post-graduate work. Fifty percent were employed and of those who indicated that they were unemployed 50% were students.

Transdermal electrical neuromodulation

Prior to this study, we spent two years developing and investigating a variety of electrical neuromodulation waveforms and approaches. The transdermal electrical neuromodulation (TEN) waveform developed for use in this study was a pulse-modulated (7 – 11 kHz), biphasic electrical current producing average amplitudes of 5 – 7 mA for 14 min. The sham waveform was an active stimulation control as described above, but pulsed at a variable frequency of 1 - 2 kHz (< 4 mA average for 14 min) to mimic skin sensations similar to those experienced throughout the real TEN stimulation protocol (Table 2). Subjects were not able to distinguish any differences between the sensations elicited by the real TEN or sham waveforms. During both the real TEN and sham stimulus protocols, subjects were instructed to adjust the current output of a wearable TEN device (Thync, Inc., Los Gatos, CA) using an iPod touch connected to the device over a Bluetooth low energy network such that it was comfortable. TEN and sham waveforms were delivered to the right temple (10/20 site F8) and base of the neck (5 cm below the inion) using custom-designed electrodes comprising a hydrogel material and a conductive Ag/AgCl film secured to the wearable TEN device. The anterior electrode positioned over F8 was a 4.9 cm² oval having a major axis of 2.75 cm and a minor axis of 2.25 cm while the posterior electrode was a 12.5 cm² rectangle with a length of 5 cm and a height of 2.5 cm. The average current density was < 2 mA/cm² at all times to keep in accordance with general safety practices to prevent any damage to the skin. Subjects were assigned to experimental conditions using a randomization method or a counterbalancing approach. Subjects were always kept blind to all experimental conditions.

Relaxation self-reports

As illustrated in Fig. 2a, 82 participants blinded to condition received a 14 min sham treatment and a 14 min TEN treatment in a within-subjects design. Due to the duration of effects produced by TEN, which we have previously observed we designed the experiment such that sham treatment always preceded TEN treatment. In between the treatment session subjects had a 15 min rest period. An additional group of subjects (N = 53) only received a TEN treatment for comparisons against the sham results above in a between-subjects fashion to account for any potential order confounds of delivering sham treatments first in the within-subjects design (Fig. 3b). Immediately following each treatment subjects were asked to record their level of perceived relaxation on a 11-point scale, where 0 = not relaxed and 10 = extremely relaxed.

Acute stress induction

In Experiment 2, we implemented an acute stress induction paradigm to study the effects of TEN on physiological and biochemical stress responses. Subjects received either TEN or sham treatment in a between-subjects design (N = 10 subjects per group). All participants were tested between the hours of 13:00 and 16:00 to limit variability introduced by circadian fluctuations in salivary analytes (*see Salivary collection and stress biomarker assays below*). Following informed consent, subjects were allowed to acclimate for 20 min before providing a baseline saliva sample. After providing the initial saliva sample, subjects were connected to a wearable TEN device as described above, a peripheral nerve stimulator (MiniStim MS-IVA, Life-Tech, Inc., Dallas, TX) was positioned over the median nerve of the right wrist, and a Shimmer3 (Shimmer, Dublin, Ireland) optical heart rate (HR) monitor and galvanic skin resistance (GSR) sensor was placed on the index, middle, and ring fingers of the opposite hand. The timing, presentation of stimuli, and acquisition of HR and GSR data was accomplished using Attention Tool (iMotions, Inc., Cambridge, MA).

The stress trial (Fig. 2b) commenced at the onset of TEN or sham treatment. There was a 6 min pre-trial period to give the TEN or sham treatment time to begin exerting an effect before the induction of acute stress began. The stress trial comprised a 6 min classical fear-conditioning paradigm immediately followed by a 6 min time-constrained cognitive battery. Immediately following the time constrained cognitive tests the stress trial concluded with a 3 min neutral video of a nature scene. Both the classical fear-conditioning paradigm and the time-constrained series of cognitive tests are known to induce acute stress and increase sympathetic activity [75-78]. Participants were instructed that when the computer monitor they were seated in front of began to flash still images that they would be given an electrical shock every time an image of lightning appeared. This induced an anticipatory increase in acute stress as reflected by instantaneous changes in GSR (Fig. 5b). During the fear-conditioning component of the stress trial participants were randomly presented with 40 still images of nature scenes for 6 seconds each: 10 images of lightning paired with electrical shock (0.5 sec, 4 - 6.5 mA) and 30 neutral nature scenes.

The second component of the stress trial included a time-constrained series of three cognitive tests (2 min each) including a Flanker test, n-back working-memory test, and a Stroop task. The Flanker task is a selective attention task in which participants indicate the direction of a target stimulus that is flanked by stimuli that are oriented in the same direction (congruent), in the opposite direction (incongruent), or a neutral direction as the response target. The n-back, which assesses working memory, has participants view a sequence of stimuli and indicate when the current stimulus matches the stimuli from n steps earlier in the sequence. In this case, subjects were instructed to get to 2 back as quickly and accurately as possible. The Stroop task tests sematic memory by having participants indicate the color of the ink in which a color word is written as fast as possible. Trials can be congruent, the text color and the word refer to the same color, or incongruent, the ink color and the word refer to different colors, which can lead to frustration and itself induce acute stress [77,78]. Reaction times and accuracy were measured for all tests and analyzed off-line in subsequent analyses. Following the stress trial, there was a 30 min. recovery period during which subjects reported the presence, duration (in minutes) and severity (0 to 8 scale) of skin redness or irritation, headache, dizziness, nausea, and vision or hearing changes and two additional saliva samples were collected as described below.

Heart rate variability and galvanic skin resistance metrics

We acquired cardiac activity and electrodermal activity during the stress trial using a Shimmer3 optical heart rate monitor integrated with a GSR sensor. Raw electrocardiogram data were collected using Attention Tool before being processed offline using Kubios HRV (University of Eastern Finland, Kuopio, Finland). From these data we quantified the average HR, R-R interval, standard deviation of the normal-to-normal heartbeat (SDNN), power in the low-frequency (0.04 – 0.15 Hz) and high-frequency (0.15 – 0.4 Hz) bands of the HRV spectra, and the LF/HF ratio in response to TEN and sham treatments during the stress trial. All GSR data were acquired using Attention Tool and stored offline for analysis using Igor Pro (Wavemetrics, Inc., Portland, OR). All GSR data were baseline-normalized (30 sec) for each subject to facilitate comparisons across subjects and treatment conditions. We quantified the peak-to-peak changes for GSR occurring at the onset of the fear-conditioning component of the stress trial (ΔGSR_{fear}; Fig. 5b) and the average peak-to-peak change in response to the delivery of the 10 unconditioned stimuli or electrical shocks (ΔGSR_{shock}; Fig. 5b) for each subject.

Salivary collection and stress biomarker assays

Prior to arrival, participants were instructed to not brush their teeth within 45 minutes, eat within one hour, consume caffeine or alcohol within 12 hours or have dental work performed within 24 hours of their scheduled appointment. After providing informed consent, participants rinsed their mouths in preparation for contributing a saliva assay and were then seated in a quiet room during which they self-reported basic demographic information. After 20 minutes, participants provided a baseline saliva sample via the passive drool method. As per manufacturer's instructions (SalivaBio, Inc., State College, PA), saliva is pooled at the front of the mouth and eased through a tube, centered on the lips, directly into a cryovial, and immediately stored at -20° C. The same collection procedure was used to collect additional saliva samples 10 min and 30 min following the end of the stress trial. Saliva samples were coded and sent to Salimetrics, LLC (State College, PA) where ELISA methods were employed to assess α-amylase (Salimetrics 1-1902) and cortisol levels (Salimetrics 3002) in a blinded manner.

The protein α-amylase is widely recognized as a biochemical marker of sympathetic nervous system activity and sympathoadrenal medullary (SAM) axis activation. More specifically, salivary levels of α-amylase directly correlate with plasma norepinephrine (NE) concentrations following the induction of acute stress including when electrical shock is used as a stressor [29-35]. Cortisol is a prototypical stress hormone reflective of hypothalamus pituitary adrenal (HPA) axis activation, which has slower onset and longer lasting effects compared to SAM axis activation.

Statistical analyses

Unless otherwise indicated all statistical analyses were conducted using independent t-tests with IBM SPSS Statistics Software (IBM Corporation, Armonk, NY). Using SPSS the self-report data were analyzed using a paired t-test or an independent t-test where indicated. The cognitive data were analyzed using a series of one-way ANOVA's with SPSS. Thresholds for statistical significance were set at $p < 0.05$. All data reported and shown are mean ± SD.

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DISCLOSURE

WJT is a co-founder of Thync, Inc. WJT, JC, DW, and SKP are inventors and co-inventors on issued and pending patents related to methods, systems, and devices for neuromodulation. All authors are shareholders in Thync, Inc

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