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CLOSED-LOOP AMPLITUDE-MODULATED TRANSCRANIAL ALTERNATING CURRENT STIMULATION FOR STRESS MANAGEMENT: A *PROOF-OF-MECHANISM* STUDY

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SUMMARY

1. ABSTRACT

Introduction

Stress is the physiological response to dangerous and challenging situations, whose imbalance results in pathological conditions, particularly relevant in psychiatry. Furthermore, chronic stress can affect different systems and is widely recognised as a risk factor for a great variety of diseases. The genesis of stress involves distinct patterns of brain activity. EEG systems adopt affordable and well-established portable devices capturing the cortical electrophysiological signals in the form of brain waves and providing high temporal resolution for real-time monitoring. Frontal midline theta (FMT) waves have been detected under meditation, creativity and mental task performance, and have been reported to respond to self-regulation under real-time monitoring in neurofeedback procedures. Alpha waves relate to relax states and have been stimulated in patients with anxiety, showing positive effects on stress management. Neurostimulation can rely on magnetic or electric brain-machine interaction and has been proven to modulate brain activity and enhance neuroplasticity for the sake of a long-lasting effect. Unlike neurofeedback, it does not require high levels of compliance, making it a more suitable option for psychiatric patients. EEG systems can be employed for real-time monitoring of brain activity to pursue adaptive, brain state-dependent stimulation. Amplitude-modulated transcranial alternating current stimulation (AM-tACS) and EEG can be combined to achieve closed-loop amplitude-modulated transcranial alternating current stimulation (CLAM-tACS). The term ‘closed-loop’ refers to the cyclical process alternating brain activity detection and state-dependent stimulation. Little is known about the feasibility of CLAM-tACS for phase-dependent stimulation and the role of tACS in stress management.

Study purpose

This study aims to investigate the role of tACS in managing stress by enhancing FMT waves and to evaluate the suitability of CLAM-tACS in modulating brain activity, in order to compare open-loop and closed-loop stimulation.

Materials and methods

The CLAM-tACS system developed in the Berlin Clinical Neurotechnology Lab at the Charité (Berlin, DE) was employed for applying phase-dependent neuromodulation on healthy participants performing the Stroop test, designed to induce mental stress through cognitive load. Stress was monitored via alpha wave amplitude, heartrate, heartrate variability and skin conductance. Performance and electrophysiological measures were analysed to assess modulation and compare closed-loop stimulation, open-loop stimulation, no stimulation, and no cognitive load conditions.

Results

We finally analysed 16 subjects. Open-loop stimulation was found to significantly improve performance ($p = 0.0385$). Phase-dependent neuromodulation was not found significant. Closed-loop stimulation did not improve performance. Against our previsions based on the literature, Stroop test seemed to be suboptimal to elicit stress responses.

Discussion

The significant performance enhancement under open-loop stimulation suggests that FMT wave tACS and its potential role in deep brain stimulation warrant further investigation. Findings under closed-loop stimulation suggest that CLAM-tACS might not represent a valuable tool for stress management. However, improvements to the stimulation system and methodology to address their limitations, particularly by studying within-subject variability, could potentially change the future perspectives of this novel technique. We suggest that further investigation should also carefully consider the differences in stress responses between men and women.

Conclusions

We believe that this study, despite its critical aspects, can provide valuable insights for the future development of neurotechnologies for BCI and neuromodulation.

2. INTRODUCTION

This study aims to explore a novel **neurostimulation** technique based **on brain-computer interfaces** to modulate brain activity and improve **stress management**.

2.1. Stress: physiology, pathology and clinical implications

Stress is a complex phenomenon that can be observed in both physiological and pathological conditions. Its clinical implications are particularly relevant in psychiatry, but it is also recognised as a crucial factor in the development of many different diseases affecting different systems.

2.1.1. Stress physiology

Stress is a physiological **response** to a potentially **dangerous or challenging situation** and is part of a well-balanced **emotional asset**. From an evolutionary perspective, stress should be seen as a conserved mechanism in the animal kingdom that prepares the body to **react to danger**, i.e. to ***fight or flight***. The central nervous system recognises environmental stimuli or patterns of stimuli that are associated with danger through an **innate or in-life-learned response**. A **brain-to-body coupling** enables the body to respond to danger, primarily through two mediators: a neural signal and a hormonal signal. The former is mainly determined by the **orthosympathetic system**, the latter mainly by hormones from the adrenal glands, precisely by **glucocorticoids** produced in the *zona fasciculata* (also known as the middle zone) of the adrenal cortex and by **catecholamines** produced in the *medulla*.

The **orthosympathetic system** is part of the **autonomic system**, i.e. the part of the neural system that is not controlled by the subject's will. For the sake of brevity, we will not discuss the ontological meaning of 'will': in this context, we define as 'will' the complex cognitive process, mainly acting in the frontal cortex, that generates an action for the sake of the **organism's conservation** from the computation of data coming to the brain from the sensory system. The **sensory system** carries information coming from outside the body via **exteroceptors**, inside the body via **interceptors**, about the mutual position of the body's components relative to each

other via **proprioceptors** and of the body as a whole in the Earth's gravitational field via the proprioceptors of the **vestibular system**. This information is then processed in the **cortex** to respond to changes in the environment and in the body that may positively or negatively affect the organism. Charles Scott Sherrington, one of the main founders of modern neurophysiology, used to say: '*Life's aim is an act*'. In fact, it is difficult to say whether *life*'s aim is an **act**, but we can easily affirm that the *nervous system*'s indeed is. In the animal kingdom, sessile animals, i.e. animal which never moves but rather stay steady on a surface such as sponges or some molluscs, do not actually have a proper nervous system at all. What is even more noticeable is that some species of the phylum Cnidaria have a complex biological cycle with both sessile and motile (meaning moving) forms, and the nervous system happens to be more complex when they are in their motile form. Every organism is a biological system that tries to avoid death, and sometimes this means having to move to find food or other resources necessary for life, or to escape from danger. To coordinate this movement, the nervous system complexity raises: **the more complex the movement, the more complex the nervous system becomes**. A complex nervous system is very energy consuming, requiring a huge mass of neural tissue which requires a huge amount of resources. **To avoid unnecessary energy consumption, the nervous system is compartmentalized** so that some computations are done just by certain components. Computations that lead to **movement** involve the **conscious nervous system**, which as a whole gives raise to the idea of unity and individuality leading to the apperception of the 'I'. On the other hand, computations that are not directly related to movement are elaborated independently of consciousness, thus constituting the autonomic nervous system. However, the two system, meaning the conscious and autonomic, cannot be completely separated, and they communicate directly or indirectly through hormones. The autonomic nervous system is divided into two main subsystems: the **orthosympathetic**, also known just as sympathetic, and the **parasympathetic**, sometimes acting with opposite effects. They can act directly on the organs or cause some organs to release hormones as 'messengers' of their commands. In both cases, among other activities, the autonomic nervous system can contribute to the **modulation of the cortex**, in ways which are still not completely understood. The **perception** of this modulation involves **psychological states** such as **emotions** and **stress**.

The **release of corticosteroids** is mainly controlled by a system called the **HPA axis**, i.e. the hypothalamus, pituitary gland and adrenal glands. The cognitive evaluation of a 'critical situation' leads the hypothalamus to release the corticotropin-releasing hormone (CRH), which in the pituitary gland triggers the release of adrenocorticotropic hormone (ACTH), which acts at the level of the adrenal glands, prompting them to release glucocorticoids. Fibres from the **amygdaloid nuclei** mediate responses to **emotional stress**, such as fear, anxiety and apprehension, by increasing ACTH secretion which raises the level of circulating cortisol (Barret et al., 2016). **Cortisol** plays the main role in the fight-or-flight response, making **blood and salivary cortisol** among the most reliable **markers of stress in research and clinical practice**, even though stress should be considered as a complex phenomenon that does not allow to establish a true *gold standard*.

Both neural and hormonal signalling lead to some key physiological changes, all of which tend to make the body ready to ***fight or flight***. Although both situations imply a complex sequence of movements, the **lack of time** plays in favour of a **fast response**, that is why an already learned sequence of movements is preferred. *Complex and slow thoughts* are suppressed, while *immediate (but not necessarily simple) and fast thoughts* are enhanced. From a neurophysiological perspective, that means the pre-frontal cortex activity, which is related to complex thoughts, is suppressed in favour of a more **basal activity**. Stress increases **heart and respiratory rate** to increase the flow of oxygen and metabolites. To maximalize the locomotor activity, a **vasodilation** occurs **in striated muscles** to enhance the flow of oxygen and nutrients into the muscles and the elimination of catabolites. **Energy metabolism** is enhanced in muscle fibres. The sympathetic system leads to **mydriasis**, i.e. pupil's dilation, probably for the sake of improving the vision. Stress reduces the activity of the digestive system: the balance in favour of the sympathetic activity over the parasympathetic leads to a **reduction of the peristaltic movements and the closure of the sphincters**. A **vasoconstriction of the splanchnic vessels** reduces the blood flow, in favour of a greater capacity of the striated muscles. The urinary system is also similarly affected: the **bladder's detrusor relaxes** and the **urethral sphincter contracts**. Chronic stress may lead to urinary retention even in presence of urinary urgency by **increasing tension in the pelvic floor muscles**, making it difficult for the bladder to fully empty and

increasing the risk for **urinary tract infections** (UTIs). However, chronic psychological stress can lead to an increase in the frequency of bladder contractions and a release of the urethral sphincter affecting urinary function and exacerbating lower urinary tract (LUT) dysfunction (LUTD), particularly in patients with overactive bladder (OAB) or interstitial cystitis–bladder pain syndrome (IC/BPS). An increasing amount of evidence has highlighted the close relationship between chronic stress and LUTD. This effect may be due to an imbalanced stress response, while the exact mechanisms underlying it remain unknown. (Gao and Rodriguez 2022)

2.1.2. Stress pathology

Stress becomes pathological when it is **quantitative or qualitative imbalanced**.

- **Quantitative imbalanced**

  Stress can be quantitative unbalance if **the response is excessively severe**. This is the case in some kind of **affective disorders**, especially in **anxiety**, but the exact causes are not yet fully understood. (Even though an excessively weak stress response may be also considered pathological, in this chapter we are going to focus only on the excessively strong stress response, thus being perceived as part of the 'stress-related disorders' in the clinical practice. Nevertheless, the reduction in stress response as part of a pathological issue will be part of our discussion on the results of the study (chapter 6.4.). On the other hand, an **excessively frequent stress factor** can lead to a **cumulative pathological effect**, e.g. in **post-traumatic stress disorder (PTSD)**, such as after **prolonged exposure to war or other dangerous situations**. Stress can also become pathological when it is elicited by a **non-dangerous factor which is mistakenly interpreted as dangerous** by the subject, for example in **obsessive-compulsive disorder (OCD), generalize anxiety disorders (GAD) or panic attacks (PA)**.

- **Qualitative imbalanced**

  Stress can also come from the feeling of **being overwhelmed by duties and not being able to perform properly due to lack of time or scarcity of resources**. More precisely, that is the kind of stress *occupational medicine* usually deals with. In this case, there is no such a thing as a dangerous factor, but rather stress is a more complex response, deeply involving the pre-frontal cortex, i.e. involving more *complex and slow thoughts*, where the subject is *aware* of being in a situation where they have to highly perform in order to carry their duties out. This is the kind of stress that almost everyone experiences at least once in their life and should be considered as a ***social disorder*** and therefore fought not only at the single subject level, but rather from a social perspective in the contest of a specific community (in a specific company, in education, in the care system, etc.) or of a general change in the society's habits.

2.1.3. Stress-related disorders

An enhanced stress response is responsible for a sundry group of psychiatric disorders.

- **Acute stress disorder (ASD)**

  To meet the DSM-5-TR criteria for the diagnosis of acute stress disorder, patients must have been directly or indirectly exposed to a traumatic event, and ≥ 9 of the following symptoms from any of the 5 categories (intrusion, negative mood, dissociation, avoidance, and arousal) must be present for a period of 3 days up to 1 month. ASD and post-traumatic stress disorders (PTSD) are similar, except that ASD typically begins immediately after the trauma and lasts from 3 days to 1 month, whereas PTSD may start either as a continuation of ASD or as a separate occurrence that begins a month or more after the trauma. It may also begin with delayed expression 6 months or more after the trauma. (Carpenter et al., 2018)

- **Chronic stress disorder (CSD)**

  CSD occurs when the subject is constantly oppressed by stress. Stress is physiological just as an acute response and when prolonged can lead to pathological alteration of the homeostasis of the organism. In the *psychoanalytic school* (Freud, Lacan, Galimberti) and the *continental European existential and phenomenological school* (Kierkegaard, Freud, Sartre, Heidegger) the *fear* ('Furcht, 'paura', 'peur') is distinguished from the *anguish* ('Angst', 'angoscia', 'angoisse') because the former prompts to find a solution to a dangerous situation, while the latter implies the conceptualization of the absence of a solution.

- **Post-traumatic stress disorder (PTDS)**

  PTDS is a disabling disorder that develops after exposure to a traumatic event. It is characterized by intrusive thoughts, nightmares, and flashbacks; avoidance of reminders of the trauma; negative cognitions and mood; hypervigilance and sleep disturbance. The diagnosis is based on clinical criteria. Treatment includes psychotherapy and sometimes adjunctive pharmacological therapy. (Goldstein et al., 2016)

- **Anxiety disorders**

Anxiety disorders are characterized by persistent and excessive fear and anxiety and the dysfunctional behavioural changes that a patient may use to mitigate these feelings. Anxiety disorders are differentiated from one another based on the specific objects or situations that induce fear, anxiety, and associated behavioural changes. (Pennix et al., 2021)

Generalized anxiety disorder (GAD) is characterized by excessive anxiety and worry about a number of activities or events that are present more days than not for ≥ 6 months. It usually goes with panic disorders and may be associated with other psychiatric disorders such as AUD (alchol use disorders) and major depression. The diagnosis is based on clinical criteria. Treatment includes behavioural interventions, psychotherapy, pharmacotherapy, or a combination of these. The prevalence is twice as high in women as in men. (Wittchen et al. 1994)

Panic disorder is the recurrence over time of **panic attacks**, i.e. suddenly onset of a distress psychological discomfort that reveals itself psychosomatically in symptoms such as dizziness, shortness of breath, palpitations or nausea. (Kessler et al., 2006; Ehlers, 1993)

- **Sleep disorders**

  Sleep disorders are conditions that affect the quality, amount and timing of sleep. Common sleep disorders include insomnia, restless legs syndrome, narcolepsy and sleep apnoea. Sleep disorders can affect your mental health and physical health. The link between insomnia, i.e. sleep deprivation (that may be voluntary, necessary or not unexpected by the subject), is worthy much of attention, especially when dealing with stress disorders in young adults coping with their academic and/or job duties. (Gardani et al., 2021)

2.1.4. Stress as a risk factor

Stress not only plays the main role in many psychiatric disorders but is also a key **risk factor** for the development of a great variety of diseases, such as **cardiovascular diseases**, **immunosuppression**, **gastrointestinal disorders**, **neurological diseases**, **obesity and metabolic disorders**. The mechanism by which stress increases the risk for such a wide range of different pathologies probably relies on the complexity of its own nature, i.e. stress is not a single event, but rather a series of events mainly driven by glucocorticoids, which act themselves on many pathways, but also on catecholamines and the autonomic nervous system, resulting in multiple effects on many different scenarios. Indeed, the **glucocorticoid receptor (GR)** is an evolutionarily conserved nuclear receptor superfamily protein that acts as a **ligand-dependent transcription factor**. Acting at the nuclear level, glucocorticoids can potentially ‘communicate’ with all kinds of cells in all kinds of tissues though cell- and tissue-specific GR with cell- and tissue-specific transcriptions giving cell- and tissue-specific responses (Nicolaide, Chrousos, Kino, 2023). **Epigenetics** also seems to play a role in stress by enhancing the final complexity of the system. For example, childhood abuse alters HPA stress responses and increases the risk of suicide and experimental findings in rats suggest

an effect of parental care on the epigenetic regulation of hippocampal glucocorticoid receptor expression (McGowan et al., 2009).

2.2. Electroencephalogram

Electroencephalogram (EEG) is a technique used for **measuring the electrical activity of the brain from the scalp** that was developed in Germany (precisely at the University of Jena) by the physician Hans Berger who described it for the first time in 1929. (*Über das Electrencephalogramm des Menschen*, in Archiv für Psychiatrie und Nervenkrankheiten, vol. 87)

EEG shows the activity of the brain as a **function of voltage over time**:

$$\frac{dV}{dt}$$

where:

- *d* represents the **first derivative**,
- *V* is the **voltage**,
- *t* is the **time**.

Voltage is the **difference between the electrical potential of two points**. Usually, when more than one point is measured, as **in the case of EEG**, one point is used as the **reference** for all the others.

EEG signal can be plotted on a Cartesian plane with voltage on the y-axis and time on the x-axis. Because the amplitude of the electrical potential varies over time, sometimes getting stronger and sometimes weaker, the resulting figure is a **wave**. Waves can have many characteristics, but the most important in EEG analysis are frequency, amplitude and power:

- The **frequency** is the **number of complete cycles per unit of time** and is measured in Hz (Hertz).
- **Amplitude** is the **maximal absolute value** reached by the wave in the cycle. In the case of brain waves, it is a measure of **voltage** and is measured in μV.

- **Power** is the quantity of **energy** carried out by the wave over time and is directly proportional to the squared amplitude. In the case of brain waves, it is measured in μV^2 .

In modern EEG, **electrodes, called 'channels'**, are placed on the scalp to measure the **voltage**. The measurement is made possible thanks to two special electrodes called ground and reference. The **ground electrode** establishes a common electrical baseline for the EEG system, helping to stabilize the electrical potential across all electrodes thus reducing noise. The **reference electrode** is used as the baseline point against which the electrical activity recorded by the other electrodes is compared. Different setups allow for different numbers of channels, from 8 to 32 to 64 or more, and for different locations of ground and reference. Higher channel density leads to a better cortical mapping but also increases the computational and analytical effort to decode the signal as a whole.

The **first law of Ohm** states that the electric current through a conductor between two points is directly proportional to the voltage across the two points. The ratio of the voltage to the current is the resistance that the current offers as it 'travels' between the two points.

$$V=RI$$

where:

- V is the **voltage**,
- R is the **resistance**,
- I is the **electrical current**.

The first law of Ohm is true in the case of direct current (DC). In the case of **alternating current** (AC), the actual opposition the current offers while it flows back and forth from electric pole to electric pole is a combination of resistance and **reactance**. The latter is caused by inductors and capacitors in a circuit and, unlike resistance, does not dissipate energy as heat.

The reactance caused by **inductors** is directly proportional to the AC frequency:

$$X_L = 2\pi fL$$

where:

- X_L is the **inductive reactance**,
- f is the **frequency of the AC signal**,
- L is the **inductance**.

The reactance caused by **capacitors** is inversely proportional to the AC frequency:

$$X_C = (2\pi fC)^{-1}$$

where:

- X_C is the **capacitive reactance**,
- f is the **frequency of the AC signal**,
- C is the **capacitance**.

The combination of resistance and reactance results in **impedance**, which is a complex quantity whose real part is the resistance and the imaginary part is the reactance:

$$Z=R+jX$$

where:

- Z is the **impedance**,
- R is the **resistance**,
- X is the **reactance**.

(Alexander, Sadiku, 2016)

EEG systems, especially when used in research or in clinical practice, rarely leave the ‘bare’ electrode touching the skin. The overall impedance is reduced acting at the **electrode-scalp interaction** by means of **interfaces**. Some setups take advantage of a spongy material soaked with water, sometimes salt or electrolyte rich. However, when high sensitivity is needed, an electrolyte gel is used, together with peeling. **Electrolyte gel** consists in a gel enriched with electrolytes, molecules that enhance electrical conductivity. The gel forms a bridge between the electrode and the scalp. When a lot of channels are used, gel might not be an optimal solution and must be used carefully, because the closer the channels are, the higher the

probability is for gel bridges to form, mixing the signal coming from two or more channels. **Peeling** is the removal of the corneal layer using abrasive substances and scratching, for example with a blunt needle.

It is important to notice that the electrodes do not really measure the electrical activity of the brain, but rather the **electrical activity of the scalp**. The signal reaching the scalp comes not only from the cortex, nor the brain or the encephalon, but potentially from the whole body. Really, external sources may also partially contribute to the measure activity. However, the electrical potential dissipates as it passes through the tissue, meaning that the voltage from tissues closer to the electrode is higher than the voltage from tissues farther away. This conveys that electrical signals lose strength as they travel through the body, resulting in stronger signals from tissues near the electrode and weaker signals from tissues farther away. In conclusion, the signal contribution is directly proportional to the voltage and indirectly proportional to the distance. **The cortex is responsible for the stronger contribution to the signal**, being the closest and more represented (i.e. with the largest mass) excitable tissue to the scalp. A huge noise in EEG signal comes from the electromyoactivity resulting from **eye blinking**. That is one of the reasons why eye should preferentially be closed during the measures and the signal must undergo **pre-processing and filtering** methods before being used for research. Nevertheless, with the benefit of approximation, we can say EEG signal to measure *mostly* the brain activity. For the sake of clarity, we can avoid repeating this concept and think of EEG as a way for measuring brain activity.

EEG may be seen as an extraordinary tool to ‘read’ brain activity, but the fact that the source of the signal is so grossly identified reduces much of its applications. The measured signal is not the bijective function of the exact brain activity. In fact, it is not even a function, because each signal does not have one and only one brain activity that may originate it. In ‘plain English’, this means that you cannot tell which was the exact source of the signal, because different brain activities can lead to the same EEG signal. However, each brain activity should lead to a specific signal, thus the brain activity being the surjective function of EEG signal. In other words, you cannot tell what’s happening in the brain from EEG signal, even though you could be potentially able to learn how to ‘tell the machine’ what to do by learning how to ‘produce’ a certain signal. That is the case of **brain-computer interface (BCI) devices** that rely on **two different states**, i.e. *on and off*, for

example for closing (on) and opening (off) an exoskeleton hand, to report which you may learn how to enhance **concentration (on) and relaxation states (off)**.

Despite the poor localisation of the signal source, **EEG is optimal to measure signal's variation during time**, that making it the *gold standard* in terms of **time resolution** of the signal and the key tool to build up systems that require real-time monitoring, such as BCI devices, including those for neuromodulation.

Furthermore, when considering the use of EEG to develop tools for 'reading' brain activity, we should always remember that it may be combined with **magnetoencephalogram (MEG)**. Different kinds of MEG systems are being developed, but the **high cost** of the machines is a huge limiting factor for its clinical, i.e. not just research-oriented, application, unless a certain use of MEG is clearly identified as much more effective than EEG, in order to justify the much higher price of the therapy. MEG is much better in terms of **spatial resolution**, but less precise in terms of temporal resolution. For this reason, EEG and MEG are, to some extent, complementary. The combination of EEG and MEG may lead to new and unexpected tools for dealing with brain activity.

2.3. Brain waves

EEG signal can then be broken down into **frequency bands**, for each of which the amplitude can be measured. Frequency bands have been **associated with specific physiological and pathological states**. The most common banding used in research and clinical practice is to classify brain waves into delta, theta, alpha, beta and gamma, in increasing order of frequency.

- **Delta waves**

  Delta waves oscillate in the frequency window of **0.5–4 Hz** and are most prominent during deep sleep **(slow-wave sleep**) and are associated with restorative sleep processes. They also occur during **deep relaxation** and **meditative states**. Increased delta activity in awake adults can indicate brain dysfunction or damage, such as in cases of traumatic brain injury,

encephalopathy, or brain tumours. Decreased delta activity during sleep can suggest disturbances in sleep quality, or neurological dysfunction.

- **Theta waves**

  Theta waves oscillate in the frequency range of **4–8 Hz** and are linked with light sleep, drowsiness, and the **early stages of sleep**. They are also present during **relaxed states** such as **meditation** and in states od **creativeness**. Increased theta activity in awake individuals can indicate cognitive impairment, attention deficits, or emotional disturbances, often seen in ADHD or certain forms of dementia. Decreased theta activity may be associated with impaired memory function or difficulty in achieving restful sleep.

- **Alpha waves**

  Alpha waves oscillate in the frequency range of **8–12 Hz** and are prominent during **relaxed awake states with closed eyes**. They are associated with calmness, relaxation and a state of alert but relaxed wakefulness. They play a role in mental coordination and overall brain health. Increased alpha amplitude often signifies relaxation and a well-functioning nervous system and are typically observed during calm and relaxed states. Decreased alpha amplitude may be linked to mental fatigue, anxiety or depression and can also be reduced in neurological conditions such as epilepsy or brain lesions.

- **Beta waves**

  Beta waves oscillate in the frequency range of **12–30 Hz** are associated with **active thinking, problem-solving and focused attention**. They are prevalent during alertness and active mental processes. Increased beta activity can be associated with increased stress or anxiety, as well as increased cognitive activity or alertness. Decreased beta activity might indicate cognitive decline, mental fatigue, or certain neurological conditions.

- **Gamma waves**

  Gamma waves oscillate in the frequency range of **30–100 Hz** and are linked with high-level cognitive functioning, such as **information processing, perception and problem-solving**. They are observed during tasks requiring intense focus and sensory processing. Increased gamma amplitude often signifies heightened cognitive activity, improved sensory processing or intense focus. Decreased gamma amplitude may be related to cognitive deficits, neurological disorders or reduced sensory processing capabilities.

2.4. Neuromodulation

Neuromodulation is the practice of modifying the neural activity. This can be done in many ways for the understanding of which we first need to discuss the nature of the neural tissue.

2.4.1. Neural tissue physiology

The neural tissue is excitable and mainly composed by **neurones**, which are composed by soma, axon and dendrites. The **soma** is the part of the cell containing the nucleus and the major organelles, which are the components of the cells necessary for the cellular activities. Both the **axon** and the **dendrites** are appendages of the soma that allow the connection with excitable cells, i.e. other neurons or muscles, or glandular cells triggering the releasing of molecules. Dendrites are usually branch-like formations (from the ancient Greek *dendron*, meaning ‘tree’) that receive stimulations from other cells. The axon is a branch used by the neuron to stimulate other cells, sometimes very far from the soma. In humans, some neurons can be over a metre long, resulting in the longest cells in the human body. The connection between neurons is done through the **synapse** (from ancient Greek, meaning ‘which connects’), while the connection between a neuron and a muscle fibre through the neuromuscular plaque, which is a specialized structure resulting from the interaction of the two cells. In both cases, the communication is mediated by molecules and membrane proteins acting in both sides of the interface (being it the synapse or the neuromuscular plaque). Synapses

can be chemical or electrical: chemicals involve a larger number of components, while electricals are simpler and composed by gap junctions but are less represented in the adult nervus tissue. For the sake of clarity, we will now only look at what happens when a neuron is excited by another neuron.

Neurons are excitable cells because they are specialized to change their **voltage** by largely modifying the **ions concentrations** in the cytosol. Ions are exchanged with the extracellular matrix through **ion channels**. In the synapse, the stimulating neuron is represented by the **pre-synaptic zone** and the stimulated neuron by the **post-synaptic zone**, divided by the **synaptic cleft**. In the pre-synaptic zone, a change in the voltage mediated by **calcium channels** triggers **vesicles** containing **neurotransmitters** to fuse with the cellular membrane by action of specific **membrane proteins** (sinapsine, synaptobrevin, SNAP, SNARE, etc.) releasing the neurotransmitters into the synaptic cleft. In the stimulated neuron membrane calcium and sodium channels open when activated by the neurotransmitters. In both membranes, **neurotransmitter transporters** recycle the neurotransmitters. In electrical synapses, gap junctions work as ionic ‘bridges’ from one cell to the other.

For the voltage change to run through the cell, **voltage-gated ion channels** are densely populating the cell membrane, so that their opening is much quicker than what would happen if only molecule-modulated channels would be present, that means waiting for a huge amount of molecules being recycled or produced and spread in the cytosol. Speed is increased by the presence of **Schwann cells** which wrap their bodies around an axon segment many times creating a **myelin sheath**, which electrically isolate that portion of the neuron, leaving just some segment ‘bare’ and excitable. This is a ‘trick’ found by evolution to ‘shorten’ the effective length of the axon. Because some segments are covered in the myelin sheet, the electric current can ‘pass though’ them and ‘jump’ directly to the new naked segment. This mechanism is known as **saltatory conduction**. There is a certain equilibrium between the length of the axon and the length of the sum of the excitable segments: if the ratio is too high, meaning too less segments are left bare, the electrical current dissipates and no excitation is possible downwards; if the ratio is too low, meaning too many segments are left bare, the excitation slows down.

The role of **sensory neurons is** to perceive changes in the environment and transmit them to the upper neural stations of data processing, such as the brain, the spinal

cord or the plexuses. The **change in the environment** can be interpreted by changes in the concentration of certain ions or molecules, as in the case of temperature, pain, olfactory and gustatory sensations, or rather by changes inside the cells mediated by physical stimulation, as in the case of tactile and visual sensations - more precisely, tactile sensation is mediated by pressure or movement, while visual sensation is mediated by a complex system of molecules sensitive to the electromagnetic field in the range of visual light, i.e. 400-700 nm in wavelength.

2.4.2. Neuroplasticity

Neuroplasticity is the ability to **structurally or functionally change the interaction between neurons**. Various mechanism of neuroplasticity have been described:

- **Synaptic plasticity**
  Synaptic plasticity is crucial for learning and memory and consist of long-term potentiation (LTP) and long-term depression (LTD), respectively strengthening and weakening the signal transmission by respectively increasing and decreasing the voltage.
- **Neurogenesis**
  Neurogenesis (as deducible from the ancient Greek etymology) is the creation of new neurons, particularly in the hippocampus, a region of the brain associated with learning and memory. Once thought to be an ability lost in adulthood, the discover of adult neurogenesis in human brain has been recently proved, opening new possibility for research and clinical advances (Boldrini et al 2018; Kumar et al 2019).
- **Dendritic and synaptic remodelling**
  Dendritic and synaptic remodelling is the process that changes the connectivity of the neurons by altering the number and location of dendrites and synapses.

The aim of certain **neuromodulation** technique is to take advantage of neuroplasticity for a **long-lasting effect** on the brain activity.

2.4.3. Neuromodulation classification

We can now understand the mechanisms underlying neuromodulation techniques: neuromodulation can be sensory, chemical or electromagnetic.

- **Sensory neuromodulation**

  Sensory neuromodulation involves the use of physical stimulation to excite neural tissue. Sometimes, the term sensory neuromodulation is used to refer to techniques involving electrical stimulation such as *vagus nerve stimulation* (VNS), that is why the more precise term **somatosensory neuromodulation** is usually preferred. This kind of stimulation have been widely used in empirical and traditional medicine, such as massage, acupuncture (which is a technique to stimulate skin sensors) and therapies involving heat, or sometimes cold, such as thermal medicine. A modern approach to sensory neuromodulation is **optogenetics.** Opsonins are photosensitive ion channels derived from algae that open when stimulated by electromagnetic waves at specific wavelength. Thanks to gene therapy, *in vivo* cells can be engineered and programmed to express these proteins on their membranes and be excited by specific wavelengths. This novel approach has been tested in animal models in different neural tissue, with particularly promising results in the **retina** (Gauvain et al., 2021). Furthermore, the optogenetic way to establish a **neural bypass after spinal cord injuries** have been proven possible (Kathe et al., 2021).

- **Chemical neuromodulation**

  Chemical neuromodulation is the **pharmacological approach**, that can be divided into two main groups: the neurological and the psychiatric medications, both regarding different classes of drugs.

  - ❖ **Neurological medication**

  Antiepileptics, also known as **anticonvulsants**, are medications used to prevent and control seizures. They stabilize neuronal activity in the brain, reducing the likelihood and severity of seizures. They can be classified in **broad-spectrum** antiepileptics, effective against a wide range of seizure

types, and **narrow-spectrum antiepileptics** which target specific types of seizures.

Parkinson's disease medications are used to manage symptoms of Parkinson's disease, a neurodegenerative disorder characterized by tremor, rigidity, and bradykinesia. **Dopamine agonists** mimic the effects of dopamine, a neurotransmitter deficient in Parkinson's. **Levodopa and carbidopa** are used in combination: the former converts to dopamine in the brain and the latter prevents levodopa from converting prematurely outside the brain. **MAO-B inhibitors** inhibit the breakdown of dopamine in the brain, thereby increasing its availability.

Muscle relaxants are medications that relieve muscle spasticity or pain by acting on the central nervous system (central muscle relaxans) or directly on muscle tissues (peripherally acting muscle relaxans).

Cognitive enhancers are medications designed to improve cognitive function, particularly in conditions like Alzheimer's disease. **Cholinesterase inhibitors** increase levels of acetylcholine, a neurotransmitter important for memory and learning. **NMDA receptor antagonists** regulate glutamate activity, which is involved in learning and memory.

Analgesics are medications used to relieve pain, including neuropathic and chronic pain conditions. **Opioids** bind to opioid receptors in the brain to reduce the perception of pain and can be classified in **strong** and **weak** depending on the existence of a threshold dose (only in weak opioids). **Non-opioid analgesics** includes acetaminophen and NSAIDs; used for mild to moderate pain and inflammation. **Adiuvants** are drugs not classified as analgesics that can nevertheless help for neuropathic pain such as chronic pain, trigeminal and postherpetic neuralgy, diabetic neuropathy; the most used are duloxetine, an antidepressant, and the anticonvulsants carbamazepine, gabapentin and pregabalin.

- **Psychiatric medications**

Antidepressants are medications used to treat depression and some anxiety disorders by affecting neurotransmitter levels in the brain. **Selective serotonin reuptake inhibitors (SSRIs) i**ncrease serotonin levels by inhibiting its reuptake. **Serotonin-norepinephrine reuptake inhibitors (SNRIs)** increase both serotonin and norepinephrine levels. **Tricyclic antidepressants (TCAs)** consist in an older class of antidepressants that affects multiple neurotransmitters. **Monoamine Oxidase Inhibitors (MAOIs)** inhibit the breakdown of neurotransmitters like serotonin and norepinephrine.

Anxiolytics are medications used to alleviate symptoms of anxiety and panic disorders. They can be classified in can be classified in benzodiazepines, which enhance the effect of the neurotransmitter GABA, leading to sedation and anxiety reduction; and **non-benzodiazepine**, which does not have the sedative effects.

Antipsychotics are medications used to manage symptoms of psychosis, such as hallucinations and delusions. Dopamine receptor antagonists are the oldest class and for that reason these medications are known as **typical antipsychotics**. **Atypical antipsychotics** is the newest class with a broader mechanism of action and fewer side effects.

Mood stabilizers are medications used to stabilize mood swings, particularly in bipolar disorder. **Lithium** is the most known stabilizer used to prevent manic and depressive episodes. **Anticonvulsants** are also used as mood stabilizers, especially in bipolar disorder, such as valproate, lamotrigine and carbamazepine.

- **Other medications affecting the nervous system**

Some medication may not necessary act directly on the neural tissue, but still act on its activity by enhancing or suppressing the neural system signals.

Adrenergic agonists are medications that enhance the effects of the sympathetic nervous system by stimulating adrenergic receptors, leading to

effects such as increased heart rate, blood pressure, bronchodilation, and vasoconstriction. **Alpha-1 agonists** stimulate alpha-1 adrenergic receptors, leading to vasoconstriction and increased blood pressure. **Alpha-2 Agonists** stimulate alpha-2 adrenergic receptors, primarily in the central nervous system, leading to reduced sympathetic outflow. **Beta-1 Agonists** stimulate beta-1 adrenergic receptors, mainly in the heart. **Beta-2 Agonists** stimulate beta-2 adrenergic receptors, primarily in the bronchial smooth muscle.

Anticholinergics, as known as **antimuscarinic**, are medications that block the action of acetylcholine at muscarinic receptors in the body. Acetylcholine is a neurotransmitter involved in various functions of the parasympathetic nervous system, which controls many involuntary bodily functions. By inhibiting acetylcholine's action, anticholinergic drugs reduce parasympathetic nervous system activity and have a range of effects depending on the specific receptors they target and the system in which they act. **Atropine** increases heart rate, dilates pupils, reduces salivation, and is used in preoperative medication to reduce secretions, bradycardia treatment, pupil dilation for eye exams, or as an antidote for organophosphate poisoning. **Scopolamine** prevents travel and postoperative nausea. **Ipratropium** and **tiotropium** provide bronchodilation, reduces mucus secretion, and are used in the management of chronic obstructive pulmonary disease (COPD) and asthma. **Benztropine** reduces tremors and rigidity and is used in the treatment of the Parkinson’s disease to alleviation of extrapyramidal symptoms from antipsychotic drugs. **Oxybutynin**, **darifenacin** and **solifenacin** relieve bladder spasms, reduce urinary urgency and frequency, and are used in the management of overactive bladder and urinary incontinence. **Propantheline** reduces gastrointestinal motility and decreases gastric acid secretion and is used for the treatment of peptic ulcers and the management of irritable bowel syndrome (IBS).

- **Electromagnetic neuromodulation**

  Electromagnetic neuromodulation consists in stimulation using electrical or magnetic fields. It is commonly referred to as **neurostimulation**. It has been applied to different parts of the neural system, such as the brain, the spinal cord, the nerves and the neural plexuses. Neurostimulation can be **invasive**

or **non-invasive**. The stimulation's target can be a small group of cells, ideally even one single motoneuron or muscle fibre, or rather a more complex system such as the brain as a whole. The target can vary based on the goal of the modulation and the level of invasiveness accepted.

Generally, to stimulate a **small number of cells**, the invasive approach is preferred, leading to promising way to establish **neural bypasses** (Bouton, 2017) and **artificial retinas** (Weiland and Humayud, 2014). **Deep brain stimulation** (DBS) for **Parkinson disease** probably represents the most important clinical result in the field of neurostimulation, having radically changed the approach to the morbus. DBS of the subthalamic nucleus or globus pallidus internus is often recommended for patients with levodopa-induced dyskinesias or significant motor fluctuations; this procedure can modulate overactivity in the basal ganglia and thus decrease parkinsonian symptoms (Fox, Katzenschlager, Lim et al., 2018). An invasive non-specific stimulation is used in plexuses stimulation where, instead of acting in a specific neural path, you try to suppress or enhance the neural activity to avoid functional atypia. This is the case of **neurostimulation of the spinal cord or the sacral plexus** as an empirical treatment for **orchialgia**, i.e. chronic testicular pain (Mamaril-Davis et al., 2022). **Non-invasive stimulation** is preferred when you want **to enhance or suppress certain brain activity measured through EEG**. This is a well-established method in neurology and psychiatry. However, the real mechanism of action is still unknown. Because of the high temporal precision of the EEG, the main advantage of an EEG-dependent non-invasive stimulation is that it can follow brain activity over time. However, this is not so simple, as discussed later. The main neurostimulation techniques can be classified into electrical, magnetic and ultrasound-based.

Electrical neurostimulation can be performed within an invasive or non-invasive method. **Non-invasive** neurostimulations are performed releasing electric current at the skin level to stimulate the neural tissue located at deeper body's districts. **Transcranial direct current stimulation (tDCS)** and the **transcranial alternating current stimulation (tACS)** can

modulate the **brain activity** by releasing electric current on the scalp (a detailed description is found in the following chapter). The vagus nerve stimulation can also be non-invasive by applying electric current on certain area of the ear or the neck **(nVNS)**. **Invasive** neurostimulations are performed with implantable devices in the neural tissue, such as the **deep brain stimulation (DBS)**, whose main role has been played as a drug-resistant Parkinson's disease's treatment; the **vagus nerve stimulation (VNS)**, used for epilepsy and depression; the **spinal cord stimulation (SCS)**, used to relieve chronic pain; and **peripheral nerve stimulation (PNS)**, to manage pain or restore motor function.

Magnetic neurostimulation can be applied to brain activity through **transcranial magnetic stimulation (TMS)**, a non-invasive method that uses magnetic fields to induce electrical currents in the nervous system. TMS devices are still mainly operator-dependent, because the stimulator has to be manually located. However, different approaches are under development

Transcranial focused ultrasound (tFUS) is a novel technique still in the experimental phase that uses an **ultrasound-based neurostimulation** to modulate **brain activity**. The main advantages of using ultrasound instead of electric current are the feasibility of a combination with ultrasound imaging and the ease of its usage.

2.4.4. Transcranial alternating current stimulation

tACS is a form of **non-invasive brain neurostimulation** that uses low-intensity (1 – 2 mA) electric currents to modulate brain waves. As the name suggests, the stimulation occurs via **alternating current (AC)**, a type of electrical current in which the direction of the electrons' flow switches back and forth at **regular cycles** (or intervals), moving from one electrode to another. In direct current (DC), the cathode and the anode have different polarity, with the cathode being the negative pole and the anode the positive one. In AC, the polarity cyclically switches, so that the electrical current cyclically changes verse, even though the direction (i.e. the

'path' in which the currents flows) remains the same. This alternating nature is essential in AC stimulation to **avoid prolonged polarization of the tissue** beneath the electrodes, which can occur in DC stimulation. The electrodes are located on the scalp, the exact position depending on the area to stimulate: the spatial reciprocal relation of the electrodes determines the flow direction, as charges tend to move towards the shortest path. An electrolyte-rich gel is usually used as interface between the electrodes and the scalp to attach the electrodes and enhance the conductivity. On flat surfaces, the current tends to spread uniformly between electrodes, but on a curved surface like the head, the current lines may bend, curve, or concentrate more in certain regions and create more complex electric field patterns, which could affect the stimulation of the inner brain. Some electric current flows through the upper levels, i.e. the soft tissues above the skull, and may lead to excitation of muscles, such as eyelid muscles resulting in **blinking** to **blepharospasm**, or **activation of skin receptors** resulting in sensation that may also be unpleasant or disturbing. A good system's setup should affect mostly the cortex. However, interpersonal variations may occur depending on the subject own sensitivity. tACS is also known to affect brain activity by stimulating the retina (Schutter, 2016) and the cranial and peripheral nerves (Asamoah, Khatoun, Mc Laughlin, 2019).

Generally, tACS should not be taken into consideration for deep brain stimulation, both for lack of control on the flow at brain's deeper level and for safety reasons: the electric current dissipates as it flows, so that the demanded current to affect deeper regions would be too high at the cortex level. **TMS** is more promising for this purpose. A magnetic field produces a perpendicular electrical field, that means a magnetic wave produces an electric wave perpendicular to itself. Two magnetic waves, as all waves, can interfere with each other, producing a third wave which is the combination of the two original ones. Interference can be constructive or disruptive: the former means the amplitude of the resulting wave is higher than the single ones, the latter means the amplitude of the resulting wave is smaller than the single ones. You can use **two magnetic waves that are too weak to produce alone a stimulation** and combine them so that they **interfere constructively** in certain regions of the brain producing an electric wave strong enough to stimulate the neural tissue.

tACS can modulate cognitive processes by entraining brain oscillations and inducing **long-term synaptic plasticity**. Therefore, tACS has been investigated in **cognitive neuroscience** and, more recently, in **neurology** and **psychiatry**. Results show the ability of tACS to reset disturbed brain oscillations, thereby improving behavioural outcomes. In addition to its **potential therapeutic role**, the reactivity of the brain circuits to tACS could serve as **a tool for the diagnosis, classification or prognosis of psychiatric disorders**. Many diseases have been approached with tACS, among other dementia, mind-cognitive impairment (MCI), alcohol used disorder (AUD), schizophrenia, obsessive compulsive disorders (OCD), attention-deficit/hyperactivity disorder (ADHD), insomnia, major depression and bipolar disorders. These neuropsychiatric conditions are all characterized by an altered oscillation pattern, that tACS seeks to restore. tACS can be used to alter brain plasticity for a long-lasting effect, so that a continuous neurostimulation is not needed to get a **long-lasting effect.** However, **portable devices** are being tested for specific purposes: one example is the BCI in development at the Swiss Federal Institute of Technology Zurich (ETH-Zurich) with the financial support of the European Space Agency (ESA) to enhance sleep quality during long-lasting spaceflights.

2.4.5. Closed-loop amplitude-modulated transcranial alternating current stimulation

Amlitude-modulated transcranial alternating current stimulation (**AM-tACS**) is a variation of tACS where the amplitude of the alternating current is modulated over time. Closed-loop amplitude-modulated transcranial alternating current stimulation (**CLAM-tACS**) is a **novel kind of AM-tACS** in which stimulation is delivered in a **brain state-dependent** modality. To reduce tACS artefacts in EEG or MEG signal at the frequency of the targeted brain waves, the use of amplitude-modulated stimulation waveforms (AM-tACS) was introduced (Chander et al. 2016, Kasten et al. 2018, Witkowski et al. 2016). As previously mentioned, brain waves alterations are associated with specific diseases. CLAM-tACS provides a tool to enhance or suppress brain wave by, respectively, constructive or disruptive **interference between brain waves and the given current**. **EEG signal** is used to **monitor the brain activity** enabling changes in the stimulation to maintain the target. The EEG

system and the stimulator are embedded in a **brain-machine system** that cyclically reads the brain activity and release the electrical stimulation. In order to be considered in real-time, the stimulation must occur in range of milliseconds if the stimulus timing depends on oscillatory phase or seconds if the stimulus amplitude depends on spectral power (Zrenner and Ziemann, 2024). To distinguish the natural brain activity signal from that coming from the stimulation, a **computational filter** is used. In our study, we used the CLAM-tACS system developed in the **Berlin Clinical Neurotechnology Lab** at the **Charité** (Berlin, DE).

2.5. Alpha and theta waves in stress and neuromodulation

Alterations in alpha and theta waves have been reported to **associated with stress**:

- **Alpha waves** are generally linked to a **relax and calm state** and they usually decrease with stress arousal. **Alpha waves' amplitude** has already been tested as a **measure for stress monitoring**, together with heart rate variability and skin conductance (Attar et al., 2021). Alpha wave **tACS** has proven effective in the treatment of **anxiety** (Clancy et al., 2018) and **other neuropsychiatric disorders** (Haslacher et al, 2024; De Koninck et al., 2023).

- **Theta waves** at the midline of the frontal area can be observed in normal subjects during **mental task performance**, **meditation**, **rest and sleep**. **Frontal midline theta (FMT) rhythm** is a train of rhythmic waves at the frequency of 6 Hz and can be induced by various mental tasks (Inanaga 1998). Neurofeedback has already been positively tested in FMT (Enriquez-Geppert et al., 2014).

Neurofeedback is a psychophysiological procedure which employs real-time monitoring of brain activity to provide feedback of neural activation for the purpose of **self-regulation**. Learning control over specific neural substrates has been shown to change specific behaviours. As a progenitor of BCI, neurofeedback has provided a novel way to investigate brain function and neuroplasticity (Sitaram et al., 2019).

In this study, we investigate the feasibility of CLAM-tACS for the neuromodulation of FMT waves and assess its physiological implications in a clinical perspective.

3. STUDY PURPOSE

This study investigates the role of **FMT wave tACS** in managing stress and evaluates the suitability of **FMT wave CLAM-tACS** in modulating brain activity, in order to **compare** open-loop and closed-loop stimulation. Neurostimulation was applied to **healthy participants** under **mental stress** induced by **cognitive load**.

This study can be divided into three steps:

1. First, testing **FMT wave tACS** for **stress management**.
2. Second, assessing **FMT wave CLAM-tACS** for **phase-dependent neuromodulation**.
3. Finally, **comparing conditions** under closed-loop stimulation, open-loop stimulation, no stimulation and no cognitive load.

According to **our hypotheses**, **open-loop stimulation** should **improve stress management**. Additionally, we expect **closed-loop stimulation** to **modulate brain activity** and lead to **better outcomes** compared to open-loop stimulation.

We employed the novel **CLAM-tACS system** from the **Berlin Clinical Neurotechnology Lab** (Charite, Berlin, DE). **Electrophysiological signals** from the **brain**, **heart** and **skin** have been recorded and analysed for a ***proof-of-mechanism*** of this novel technique.

The results should **support or refute clinical trials** in patients with stress-related disorders and individuals with high comorbidity, aiming to reduce the effects of stress as a risk factor.

By working on **neural plasticity**, we further aim to establish a **clinical paradigm** based on **multiple neurostimulation sessions** to **stably alter brain activity** and significantly improve the patients' ability to **cope with stress** in their **daily life**.

4. MATERIALS AND METHODS

The **CLAM-tACS system** was employed during a task designed to induce stress responses through cognitive load. **Electrophysiological data** from the **brain**, **heart**, and **skin** were recorded and analysed, alongside other data collected prior to the session.

4.1. CLAM-tACS

The closed-loop amplitude modulated transcranial alternating current stimulation (CLAM-tACS) was composed by the **EEG system**, the **stimulation device** and the informatic system for the **real time EEG processing**.

4.1.1. EEG system

The recordings were conducted using a **64-channel EEG system** based on an interface platform (NeurOne® by Bittium Corp., Oulu, Finland). Electrodes were located according to the **international 10-20 system**, a standardized method used to ensure consistent and reproducible placement across different recordings and individuals (Acharya, Acharya, 2019; Jasper, 1958).

The **amplifier** was configured in **DC-mode** to capture a **wide range of brain signals**, even very slow electrical changes. AC-mode (alternating current) captures fast changes as brain waves but is not optimal for capturing slow baseline shifts or direct current potentials.

The amplifier had a **dynamic range** of ±430 mV. The dynamic range is the range of signal strengths the amplifier can record without distortion. By being able to detect brain signals from -430 mV to +430 mV., the amplifier was able to capture both weak and strong signals.

The amplifier had a **resolution** of 51 nV/bit and a range of **24 bits** to digitize the signal. Thanks to such characteristics, the system was highly sensitive to tiny electrical changes, providing very detailed data about the brain’s electrical activity.

Electrode impedances were kept **below 10 kOhm** for the sake of data quality.

Oscillatory phenomena, such as brain waves, are characterized by the cyclical assumption of certain values. **Samples of the assumed values are taken to find out which oscillation is assuming those specific values**. Because two different oscillations can cyclically assume the same values, meaning they can overlap at some point in time, the number of samples must be enough to be able to distinguish a certain oscillation from all the others. In other case, a distorted representation of the recorded oscillation may occur, giving rise to a phenomenon called aliasing. As the Latin word *alias* suggests, the real oscillation appears as 'in disguise' and cannot be properly recognize. The **Nyquist–Shannon sampling theorem** states the sampling frequency, also known as the Nyquist frequency or the folding frequency, must at least twice the highest frequency present in the signal (Por, van Kooten, Sarkovic, 2019).

In this experiment, **sampling was performed at 2 kHz**, meaning the EEG system took 2000 samples per second of the brain's electrical activity. Such a high-rate sampling allows us to capture fast changes in the brain's electrical signals. An **anti-aliasing low-pass filter with a 500 Hz cut-off** was applied to remove unwanted high-frequency signals above 500 Hz, helping to focus on the frequencies relevant for brain activity and avoiding a distorted representation of the oscillations.

The brainwave data collected by the EEG system was transmitted in **real-time** using a communication method called **UDP** (User Datagram Protocol). The data were sent quickly to the computer and **immediately processed**.

To **optimize the signal**, both peeling and a gel interface have been applied on the scalp. The peeling gels (Nuprep® by Weaver & Co., Aurora, CO, USA and Everi® by Spes Medica, Genova, Italy) were applied to the subject's skin with a cotton swab. Blunt needles have also been used sometimes to dislodge hairs and help the gel reaching the skin. An electrolyte-rich gel (Electro-Gel® by ECI) was and applied with syringes and used as interface between the scalp and the electrodes.

4.1.2. Stimulating device

Amplitude-modulated transcranial alternating current stimulation (AM-tACS) was applied to the scalp using a **commercial stimulator** (Digitimer DS5®, Digitimer Ltd, UK) controlled by a **signal generator** (SDG 2042X® by Siglent, Shenzhen, China), which applied amplitude-modulation to the carrier signal.

Two circular rubber electrodes of 34 mm diameter and 2 mm thickness were positioned on the scalp and attached with a conductive and adhesive past (Ten 20 Conductive® by Weaver & Co., Aurora, CO, USA). One electrode was allocated on the forehead and the other on the head's top, circa Fpz and Cpz. The measuring electrodes above the rubber electrodes have been excluded from the recording.

The scalp was delivered with a **current** in the range of 400-1000 mA. The **carrier signal frequency**, which is the frequency of a waveform that is used to carry information in communication systems, was set at 8kHz.

4.1.3. Real-time EEG processing

The current realisation was controlled by the signal generator depending on an input voltage signal received from a **computing device** (Speedgoat Performance Real-Time Target Machine®, Speedgoat GmbH, Liebefeld, Switzerland) used to execute the real-time signal processing pipeline.

In computer science and informatics, a **pipeline** refers to a sequence of software and computational steps to be applied in a certain order. Altogether, they compose a **chain-process** to perform certain operations and get a particular outcome. Pipelines are often used in real-time data processing for a **step-by-step handling of complex data**.

The EEG real-time processing pipeline consisted in a **Simulink Real-Time R2022a multi-rate model** using **MATLAB** as coding language (MATLAB® and Simulink® by Mathworks Ltd, Natick, MA, USA).

4.1.3.1. Linearly-constrained minimum-variance beamforming

To extract the target oscillation and suppress stimulation artifacts in EEG signals recorded in the presence of AM-tACS, the linearly-constrained minimum-variance beamforming **adaptive spatial filtering technique** was employed.

The **LCMV beamformer** is used in EEG and MEG analysis to **isolate neural sources of interest** by **enhancing signals from a target location** while **minimizing interference** from other sources. It works by applying **spatial weights** to the recorded signals, allowing the filter to **focus on activity from a specific direction or location** in the brain. By optimizing these weights to reduce noise and unrelated signals, the LCMV beamformer provides a clearer picture of the neural dynamics at the target site. This approach is particularly useful for studying localized brain activity in complex, noisy environments (Van Veen et al., 1997).

The objective is to minimize the output variance, i.e. the power, of the beamformer, a process known as **output power minimization**:

$$\boldsymbol{P_{out} = w^T R w}$$

where:

- P_{out} is the **output power** of the beamformer,
- w is the **weight vector** for the beamformer and w^T is the **transpose** of the weight vector w, changing from row to column and *vice versa*.
- R is the **covariance matrix** of the received signals.

The beamformer must satisfy **constraints** on the desired signal direction:

$$\boldsymbol{Aw = \beta}$$

where:

- A is the **direction** vector express as a **matrix**,
- β is the **scalar normalization factor**, which is usually **set to 1** to ensure that the beamformer maintains a **unit gain** in the desired direction, but can potentially assume different real number values.

The LCMV beamformer **takes as input of the covariance matrix the EEG data**, as well as a **forward model**, mapping from the target source to the sensors. To obtain the forward model, we applied the **spatio-spectral decomposition** (Nikulin

et al., 2011) to EEG data recorded **in absence of CLAM-tACS to extract the strongest 4-8 Hz oscillations** from broadband activity.

4.1.3.2. Signal processing pipeline

The EEG data were initially processed at 1 Hz, i.e. once per second, by computing the LCMV beamformer weights using the **covariance matrix** of the data recorded during the **last 25 seconds of a CLAM-tACS stimulation session**. A **finite impulse response (FIR) filter** was used to **select the theta waves**, which oscillate in the frequency range 4-8 Hz. Subsequently, data were processed at 500 Hz, i.e. 500 times per second. First, a **virtual electrode** was computed by applying the LCMV beamformer weights to the **latest 500 ms of EEG data**. Finally, an **endpoint-corrected Hilbert transform** was applied (Schreglmann et al., 2021) to reduce broadband noise and obtain the real-time phase estimate of 4-8 Hz target oscillations.

The **Hilbert transform** is a pure mathematical tool used in signal processing to extract **magnitude** and **phase**. It works by converting the real signal to a **complex signal** composed by a real part consisting in the real signal and an **imaginary part** which is usually referred to as the Hilbert transform of the real signal.

In the context of the Hilbert transform, the **magnitude** refers to the **instantaneous size** of the signal at each point in time and is calculated as the **absolute value of the complex signal**, also known as Euclidean norm.

Given the complex number:

$$z = a + bi,$$

the **Euclidean norm** is:

$$|z| = \sqrt{a^2 + b^2}.$$

On the contrary, the **amplitude** refers to **maximum value** (peak) of the signal, which is often **constant** for periodic signals.

While the magnitude can provide the instantaneous amplitude, it is more comprehensive, as it accounts for both the real and imaginary parts of the analytic

signal, and it varies over time, while the amplitude accounts just for the real part and is usually constant.

The Hilbert transform has to be applied only to specific oscillations, in this case to theta waves in range 4-8 Hz. However, because the function operates by means of approximations, selecting values which are ± the value on which it is operating, it cannot act on the limiting values without guessing the values which are outside the threshold, thus distorting the results. To avoid these 'ripple effects' on the data, a **transient zone** is created, usually of the size of 10% the limiting values, resulting in two transient zone: -10% of the minimum frequency and +10% of the maximum frequency (Cohen, 2014). The **endpoint-corrected** Hilbert Transform is the result of this implementation.

4.1.4. Recording procedure

The CLAM-tACS system had to be calibrated for each participant.

First a **calibration recording without stimulation** was performed for **5 minutes with eyes closed**. The brain's electrical activity was mathematically reduced to multiple dipoles, representing direction and verse of the electrical current flow. The most suitable **dipole** for representing **frontal mid-line theta activity** was manually selected by the recording operator based on plots representing the current's location and intensity by mean of figures and colors. The selected dipole was used as the recorded signal to be executed in the Simulink pipeline.

Subsequently, a second **calibration recording with stimulation** was performed for **5 minutes with open eyes** to find out the **phase delay** specific to each participant.

Once the phase delay was properly processed, the system was ready to apply the CLAM-tACS, the task could be run and the recording could start.

4.2. Task description

To test the closed-loop system and compare it to the open-loop and the no-stimulation conditions, we built up a within subject study based on the ***Stroop color and word test*** as cognitive task.

4.2.1 The Stroop color and word test

The **Stroop test** is a well-established tool to **elicit cognitive stress**. Different varieties of the test have been developed since its first appearance in 1935 in a work by the American psychologist John Ridley Stroop. (*Studies of interference in serial verbal reactions*, Journal of Experimental Psychology, vol. 18)

In our study, the subject was shown **three aligned words**. The **central word** meant a color and was written in a different ink than the color meant by the word itself. In other words, there was **incongruence between the meaning and the ink**. For example, the word 'blue' was written in red:

BLUE

The **other two words meant, respectively, the color meant by the central word and the ink and were written in neutral ink**, in this case white on black board:

BLUE BLUE RED

The subject was asked to indicate the color of the ink of the central word by pressing on the PC's keyboard the letter 'F' for the word in their left side and the letter 'J' for the word in their right side.

The subject had **800 ms to answer**. **The next stimulus was presented 100 ms after a response was given**. These time windows were chosen because they seem to be optimal for stress arousal. (Renaud and Blondin, 1997)

A right answer was shown the subject as '**correct'**, a wrong or not given answer as '**incorrect'** and a right answer given after 800 ms as '**too late'**. For accuracy analysis, we counted both 'incorrect' and 'too late' answers as wrong.

Before starting the task, the subject was offered to rehearse.

4.2.2. Stimulations conditions

Each subject was exposed to the task in **seven different stimulation conditions**.

1) No stimulation
2) Open-loop stimulation
3) Closed-loop stimulation at 0°

4) Closed-loop stimulation at 90°
5) Closed-loop stimulation at 180°
6) Closed-loop stimulation at 270°
7) No stimulation congruence

Condition number 2) *Open-loop stimulation* consists in AM-tACS. Conditions number 3, 4, 5, 6) *Closed-loop stimulation at 0°, 90°, 180°, 270°* consist in CLAM-tACS at different phases. Condition number 7) *No stimulation congruence* consists in a situation without the Stroop test, i.e. with color-word congruence.

Each condition lasted **5 minutes** and a **one-minute pause** was programmed in between. 5 minutes have been used according to standard procedures for the measurement of heart rate variability (HRV) (Cygankiewicz and Zareba, 2013). For the pause, 1 minute has been chosen to keep the overall experiment duration shorter than 45 minutes, commonly recognized as a good time window to keep concentration. The Pomodoro Technique, named after the usage of a common kitchen timer in the shape of a tomato, in Italian "pomodoro", points out that a good timing for keeping productivity high should be working for 25 minutes and then have a 5-minutes pause. However, you may extend the working phase to 45 minutes and have 15 minutes pause (Wang, Gobbo and Lane, 2010).

The conditions have been **permuted** through the subjects.

4.3. Performance and stress monitoring

We monitor both performance and stress:

- **Performance** was monitored based on the **accuracy** of the given answers.

- **Stress** was detected monitoring **brain activity** and the **electrophysiological activity of heart and skin**.

    - Brain activity was monitored analysing alpha and theta waves. **Alpha waves** have already been used to monitor stress (Attar et al., 2021), and we tried to do the same for **theta waves**, too.
    - Heart electrophysiological activity was monitored analysing **heart rate (HR)** and **heart rate variability (HRV)**. Both were monitored with a **2-**

points electrocardiogram (2-P ECG), and the 2 electrodes have been positioned on the *subclavicular region* on the *medioclavicular line*.

- Skin electrophysiological activity was monitored analysing **electro-dermal activity (EDA)**, particularly *skin conductance*, using 2 electrodes, positioned on the palm surface of the second phalanges of the right hand second and third finger, and connected to a biosignal sensor (g-sensor® by g.tec, Graz, AU).

Heartrate variability refers to the fluctuations in heartrate around the average and has been proven a valuable tool for assessing the interplay between sympathetic and parasympathetic functions of the autonomic nervous system (van Ravenswaaij-Arts et al., 1993). **Heartrate** and **heartrate variability** can measure and monitor stress and (Kim et al., 2018) have been specifically employed for **mental stress** (Taelman et al., 2008). They have already been employed as biomarker in various **psychiatric disease** (Berntson and Cacioppo, 2004). **Skin conductance** has also been employed as psychophysiological parameter for mental stress (Lazarus et al., 1963; Ogorevc et al., 2011).

As a final consideration, we would like to point out that **we excluded salivary cortisol** as a monitoring parameter because we would have had to take at least eight samples for each participant, i.e. one at the beginning, one during each break and one at the end, which would have been uneconomical and even chaotic. Moreover, we would like to underly the advantage of using **only parameters that can be read in real time by a computer**, in order to set up a stimulation system that should be as independent as possible from external activities.

4.4. Recruitment criteria

We have been looking for **healthy participants**. We ensured the participants **did not have active neurological or psychiatric disorders,** were not taking **neurological or psychiatric drugs** and that they were not **colourblind**. One subject reported to have been diagnosed with panic disorders (F41) and one more with migraine. Both did use medications in the past but were not assuming any at the time of the experiment.

4.5. Subjects' variables

We collected the following information about the subjects:

- **Immutable conditions**

  We took note about the **sex** and **age**.

- **Perceived Stress Scale**

  Each subject was asked to fill Perceived Stress Scale for a **self-evaluation of their perception of stress in the previous month**. This scale was developed in 1983 by the psychologist Sheldon Cohen to assess resilience to stressful situations (Cohen et al., 1983). We submitted the **10-items PSS** from the **Department of Psychology at the Carnegie Mellon University**. Given the multilingualism of the sample, we submitted everyone the **English** version. The 10-items PSS is composed of **10 questions** each of them asking how **often did a certain situation happen in the previous month**. The subject must choose one out of **five possible answers indicating the frequency** as *never, almost never, sometimes, fairly often, very often*. Each answer was assigned a score in *crescendo* order: 0, 1, 2, 3, 4. For questions number 4, 5, 7, and 8 the score must be inverted. The score sum is the assigned PSS. The questionnaire is found at the end of the chapter.

- **Health status**

  We took note of factors that relate general health to stress: **sleep**, **sport activity**, **relaxation practices**, **smoke**. More precisely, we asked about how many hours a day they sleep, how many times per week they perform physical activity, if they regularly practice relaxation techniques such as mindfulness, meditation, yoga, respiratory physiotherapy; and if they smoke on a regular basis. **Short sleep** and **insomnia** are linked with stress, both as **risk factors** and as **consequences** of chronic stress, somehow embedded in a vicious circle (Floam et al., 2014; Basta et al., 2007). On the contrary, **regular sport activity** and **regular relaxation** are **protecting factors** for stress (van der Zwan et al., 2015). **Smoke** relates with **stress** and **negative affect** (the experience of poor existence- and self-concepts) (Kassel, Stroud,

Paronis, 2003) and it seems that the **perturbation of the HPA axis** during **early smoking abstinence** exacerbate **withdrawal symptoms** and may contribute to the **rapid relapse** observed in the majority of smokers.

- **Day-specific informations**
  We took note of the **time of the day** when we performed the experiment, if they had **slept badly or way to short the night before**, of the amount of **coffees or energetic drinks** consumed in the day of the experiment and **how much time before** the experiment had they eaten, to take the effect of digestion into account.

- **Current**
  In our study, the **target current** was **1000 mA**. However, some people are disturbed by such a voltage, particularly reporting unpleasant sensation at the skin, blepharospasm or head pressure. For that reason, **we agreed the current for the experiment with each subject after a test** in which we released alternating current in 100 mA increments starting at 200 mA.

4.6. Analysis

A statistical analysis of the subjects' variables was conducted to characterize the sample and compare the electrophysiological findings with the subjects' characteristics. Electrophysiological data have been analysed both before and after sample manipulation.

4.6.1. Statistical analysis of the subjects' variables

The **dichotomous variables** *sex*, *relaxation practices*, *smoke*, *last sleep*, and the **dichotomized variable** *time of the day* were reported.

The **continuous variables** *PSS*, *age*, *sleep*, *sport*, *meals*, *coffees or energetic drinks* and *current* and have been analysed in terms of mean, median, mode, standard deviation, range, minimum and maximal values, quartiles and interquartile range.

4.6.2. Analysis of the electrophysiological data

Python was used as coding language for statistical analysis. The **MNE package** was used for analysing electrophysiological data (Gramfort et al., 2013) and was implemented with the **CLAM-NIBS package** for the analysis of closed-loop amplitude-modulated non-invasive brain stimulation (Haslacher, 2024). **Visual Studio Code®** (by Mycrosoft, Redomnd, WA, USA) was used as code editor.

We analysed the answers' accuracy as binary accuracy, the amplitude of alpha and theta waves, the heartrate and the heartrate variability and the skin conductance.

We tested whether the **CLAM-tACS** system was able to **modulate the brain's activity** by analysing the **closed-loop conditions**, precisely condition number 3) *Closed-loop stimulation at 0°*, condition number 4) *Closed-loop stimulation at 90°*, condition number 5) *Closed-loop stimulation at 180°*, and condition number 6) *Closed-loop stimulation at 270°*.

Then **each condition was compared** to condition number 1) *No stimulation*.

We further apply outlier removal methods to reevaluate the data after the outliers' exclusion.

4.6.3. Phase-dependent modulation

Theta amplitude was primarily analysed (Donoghue et al., 2020) to test the effect of closed-loop stimulation for phase-dependent modulation of FMT waves. We later looked for a modulating effect on answers' **accuracy** and the stress monitoring measures **alpha amplitude**, **heartrate**, **heartrate variability** and **skin conductance**. Modulation has been tested at the **group level** comparing the stimulation over the four target phases 0°, 90°, 180° and 270° by calculating the **p-value** with a **significance threshold of 0.05 (5%)**. Modulation at the subject level was not considered because the results could have been biased by the conditions' order. Indeed, we suspected that the subject might have benefit from the learning process and better perform as the task progressed. However, the subject's performance could also have worsened after some time due to fatigue. To avoid bias at the group level, the conditions have been permuted.

4.6.4. Conditions' comparisons

We compare answers' **accuracy** and the stress monitoring measure **heartrate**, **heartrate variability** and **skin conductance** under the various conditions to no stimulation. We could not compare alpha amplitude due to technical issue avoiding amplitude comparisons between conditions with and without stimulation (Westner et al., 2022; FieldTrip). The **difference** between each condition and the condition without stimulation was analysed by calculating the **t-test** and its significance by calculating the **p-value** with a **significance threshold of 0.05 (5%)**.

To avoid type 1 errors, i.e. false positives, resulting from multiple t testing, the **Bonferroni correction** (Bonferroni, 1935; Bonferroni, 1936) has been applied by adjusting the significance level α:

$$\boldsymbol{\alpha_B = \frac{\alpha}{n}}$$

where:

- α_B is the significance level adjusted by the Bonferroni correction
- α is the significance level
- n is the number of comparisons.

4.6.5. Outliers' exclusion

We tested two different methods to exclude outliers for the **group level analyses**:

- **Tukey's fences**

  Tukey's method (Yukey, 1977) excludes those values which are lower than a minimum, the *lower fence*, or higher than a maximum, the *upper fence*. These fences are calculated by respectively subtracting a certain value *K* from the first quartile (Q1) and adding the same value *K* to the third quartile (Q3). *K* is calculated by multiplying the interquartile range (IQR) by a factor *f*. For milder exclusion criteria, *outer fences* are used, where *f* = 3; whereas for stricter exclusion criteria *inner fences* are preferred, where *f* = 1.5.

  - Outer fences:
    - lower_fence = Q1 - 3 * IQR

- upper_fence = Q3 + 3 * IQR

- Inner fences:
  - lower_fence = Q1 - 1.5 * IQR
  - upper_fence = Q3 + 1.5 * IQR

In this study, Tukey's **inner fences** have been applied to both **modulation** at the group level and **conditions' comparisons**.

- **Yuen t-test**

Yuen t-test (Yuen, 1974) is an **improved version of the Student's t-test** used to **compare two groups** while **minimizing the influence of outliers**. The upper and lower tails of both distributions are 'trimmed' by an arbitrary fraction which is usually the 20%. This means the 20% of the higher values and the 20% of the lowest values for both distributions are excluded. Compared to the standard Student's t-test, the Yuen t-test is more robust when applied to **samples which are not normally distributed**.

In this study, Yuen t-test has been used **for comparing the stimulation conditions.**

4.6.6. Biasing

We biased **modulation** and **conditions' comparisons** for the dichotomic variables *sex* and regular *relaxation practice* and for the variable *time of the day* dichotomized as *morning and afternoon* sessions (noon was count in the morning group). The sample was not biased on the dichotomic variable regular *smoke* or bad *last sleep* because for both variables the size of one of the resulting group was not significant (respectively 4 and 5).

5. RESULTS

We recruited 38 subjects. Due to participants' and laboratory's schedule-related issues, we had to cancel 10 appointments and finally performed 28 sessions. We had to interrupt one session due to the subject's incompatibility with the system. As a result, **we recorded data from 27 subjects**.

5.1. Data exclusion

While analysing the data, we had to exclude some subjects due to technical issues. We excluded 3 subjects because some conditions were missing. A systematic error encoded in the informatic system which could not be solved was responsible for bad phase tracking at 0° and 180°. For this reason, **we had to exclude condition number 3) *Closed-loop stimulation at 0°* and condition number 5) *Closed-loop stimulation at 180°* from the conditions' comparisons**. We had to exclude 8 subjects because of bad phase tracking at 90° and 270°. We kept those subject whose actual phase was in range ± 45° from the target phase, edges included.

Finally, **we performed a complete statistical analysis on 16 subjects**. From now on, we are going to refer to this number as the **total number of subjects** for all statistical analysis, **unless otherwise specify**.

A systematic error encoded in the informatic system which could not be solved was responsible for bad phase tracking at 0° and 180°. For this reason, **we had to exclude condition number 3) *Closed-loop stimulation at 0°* and condition number 5) *Closed-loop stimulation at 180°* from the conditions' comparisons**.

In the analysis of **heartrate and heartrate variability**, one subject had to be excluded because of bad ECG measure, resulting in **15 subjects** analysed.

5.2. Descriptive statistics of the subjects' variables

- **Sex**

  5 subjects were female, 11 subjects were male.

- **Age**

  Our sample was mostly composed by **young adults**, most of them being university students, i.e. bachelor, master and doctoral students. This should also leverage the 'intelligence level' of the subjects.

  Mean: 24.13 years. SD: 2.55. Range: 22-31 years.

Quartiles and interquartile range IRQ = Q3 – Q1 (years)	
Q1	22
Q2	23
Q3	25.5
IRQ	3.5

- **Perceived Stress Scale (PSS)**

  We had to exclude one subject because one answer in the questionnaire could not be resolved. The following result come from a sample of 15 subjects.
  The subject reported **an average PSS of around 14 points**. According to literature (Cohen and Janicki-Deverts, 2012), the average PSS should be around 17 points. This may suggest our participants were on average more **resilient** to stress than the general population.

  Mean: 14.2 points. SD: 5.93. Range 3-24 points.

Quartiles and interquartile range IRQ = Q3 – Q1 (points)	
Q1	10
Q2	14
Q3	19
IRQ	9

- **Sleep**

  The subjects reported to sleep on average **around 7.5 hours per night**.

  Means: 7.47 hours. SD: 0.74. Range: 6.5-9 hours.

Quartiles and interquartile range IRQ = Q3 – Q1 (hours)	
Q1	7
Q2	7.5
Q3	8
IRQ	1

- **Sport**

  The subjects reported to practice sport on average **around 3 times per week**.

  Mean: 2.94 times. SD: 2.00. Range: 0-6 times.

Quartiles and interquartile range IRQ = Q3 – Q1 (times)	
Q1	1.5
Q2	2.75
Q3	4.25
IRQ	2.75

- **Relaxation practices**

  8 subjects, i.e. almost half of the sample, reported to do relaxation practices **regularly on a weekly basis**. Among the 11 subjects who do not, one subject reported to do it occasionally.

- **Smoke**

  3 subjects reported to **smoke regularly**. 13 subjects reported to do not smoke regularly or do not smoke at all.

- **Time of the day**

  6 sessions took place in the morning. 10 sessions took place in the afternoon.

- **Last sleep**

  6 subjects reported to have slept badly or too short and being affected by it, i.e. feeling tired. One subject reported a too short sleep but did not feel tired.

- **Last meal**

  The subjects got into session on average **around 2 and a half hours from their last consistent meal**.

  NB: We counted only full hours. Less than an hour before was counted as 0.

  Mean: 2.59 hours. SD: 2.87. Range: 0-12 hours.

Quartiles and interquartile range IRQ = Q3 – Q1 (hours)	
Q1	1
Q2	2
Q3	3.5
IRQ	2.5

- **Coffees or energetic drinks**

  The subjects drunk on average **around 1 coffee or energetic drink** on the session's day and before the session.

  Mean: 1 drink. SD: 0.97. Range: 0-3 drinks.

Quartiles and interquartile range IRQ = Q3 – Q1 (drinks)	
Q1	0
Q2	1
Q3	2
IRQ	2

- **Current**

  The subjects received on average **alternating current stimulation at 800 mA**, but **most** of them received the **target current at 1000 mA**.

  Mean: 800 mA. SD: 220.39. Range: 400-1000 mA.

Quartiles and interquartile range IRQ = Q3 – Q1 (mA)	
Q1	600
Q2	900
Q3	1000
IRQ	400

5.3. Phase-dependent modulation

Phase-dependent modulation **was not significant** for theta amplitude (Fig. 1) nor for answers' accuracy and the stress monitoring measures alpha amplitude, heartrate, heartrate variability and skin conductance (Fig. 2). No improvement in the significance of the p-value could be observed after the application of Tukey's inner fences. No improvement in the significance of the p-value could be observed after biasing for sex, time of the day or regular relaxation practice.

5.4. Conditions' comparisons

Performance under **open-loop stimulation** was **significantly enhanced** compared to no stimulation with a significantly higher answers' accuracy ($p = 0.0385$) (Fig. 3). Significance did not survive the Bonferroni correction nor the Yuen t-test (Fig. 4). **Closed-loop stimulation** led to **worse performance** than open-loop and was **not found significant** compared to no stimulation.

Stress monitoring measures were **not found** to be **significantly different** under different conditions (Fig. 5, 6). Consequently, none of the p-value survive the Bonferroni correction for multiple comparisons. No improvement in the significance of the p-value could be observed after the application of Tukey's inner fences. No improvement in the significance of the p-value could be observed after biasing for sex, time of the day or regular relaxation practice.

Fig. 1) Theta amplitude phase-dependent modulation

Theta wave modulation was not significant (p = 0.4365).

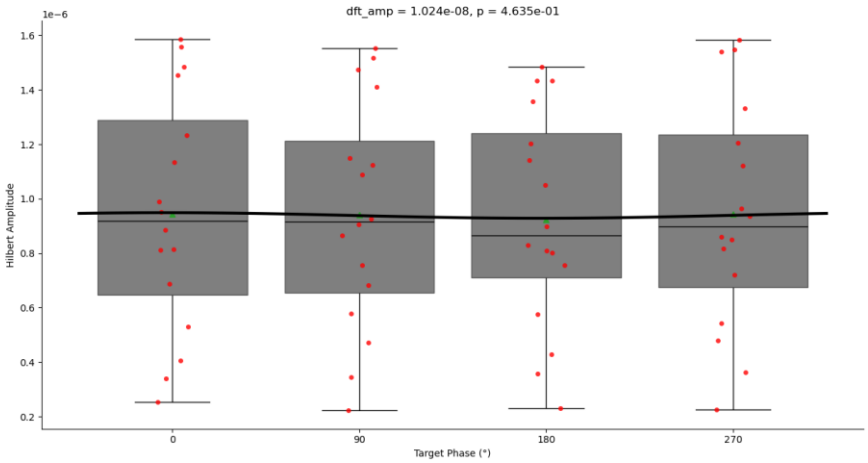
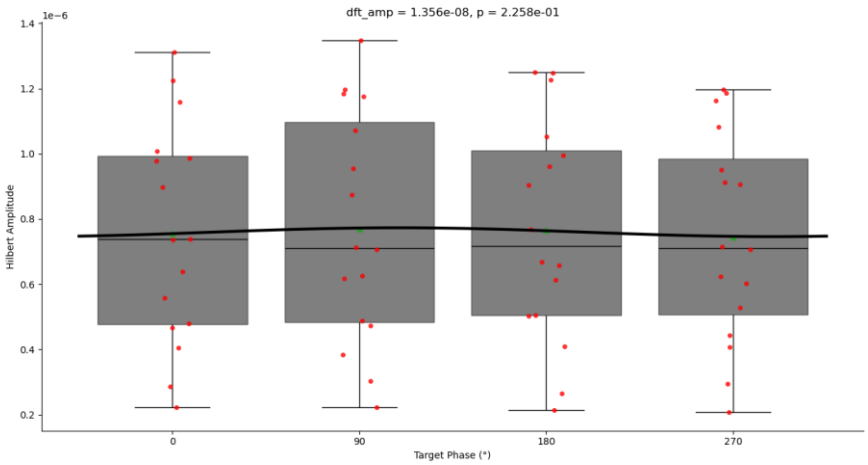


Fig. 2) Alpha amplitude phase-dependent modulation

Alpha wave modulation was not significant (p = 0.2258).



Similar results were found for accuracy, heartrate, heartrate variability and skin conductance: modulation was not significant for any of these monitoring measures.

Fig. 3) Accuracy conditions' comparisons

Binary accuracy was significantly higher under no stimulation with color-word congruence (p_4 = 0.0016) and under open-loop stimulation (p_1 = 0.0385) compared to no stimulation with color-word conflict.

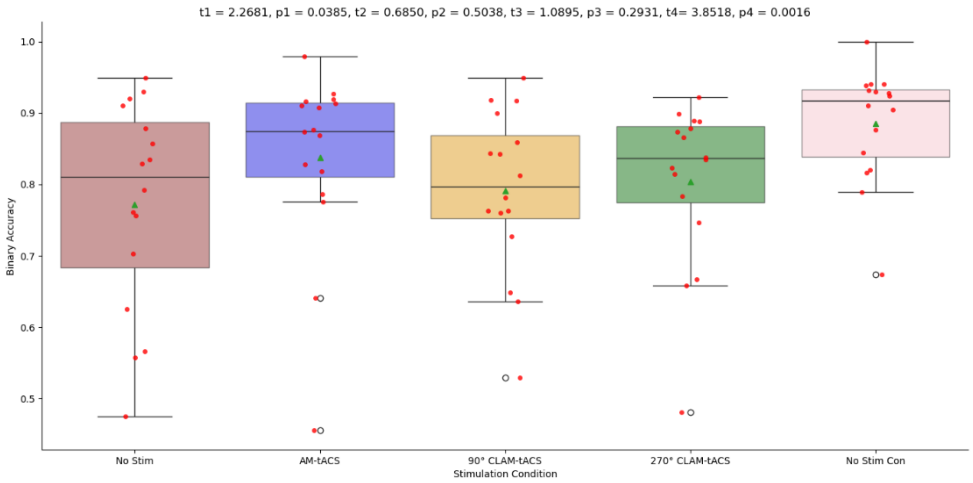


Fig. 4) Accuracy conditions' comparisons – Yuen t-test

Binary accuracy was still significantly higher under no stimulation with color-word congruence (p_4 = 0.0156) but not under open-loop stimulation (p_1 = 0.1247) compared to no stimulation with color-word conflict.

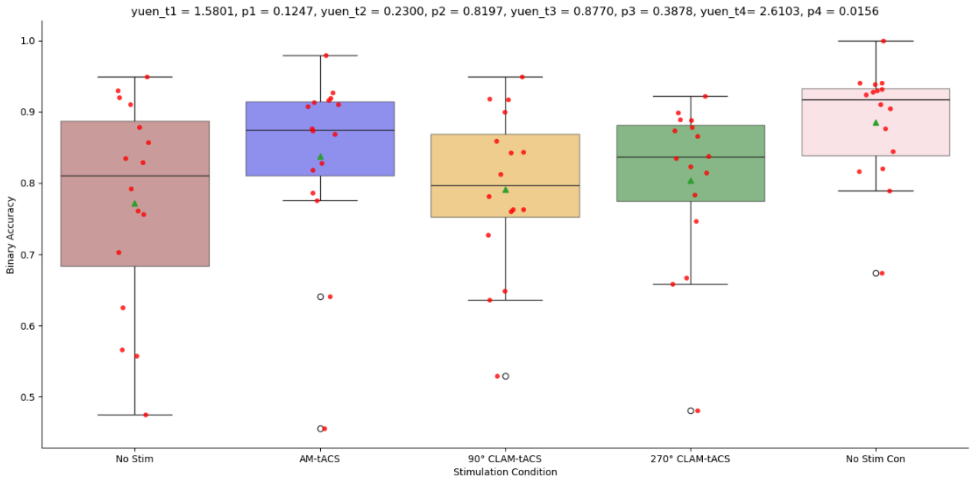


Fig. 5) Heartrate conditions' comparisons

Average RR interval was not significantly higher under no stimulation with color-word congruence ($p_4 = 0.7609$) compared to no stimulation with color-word conflict.

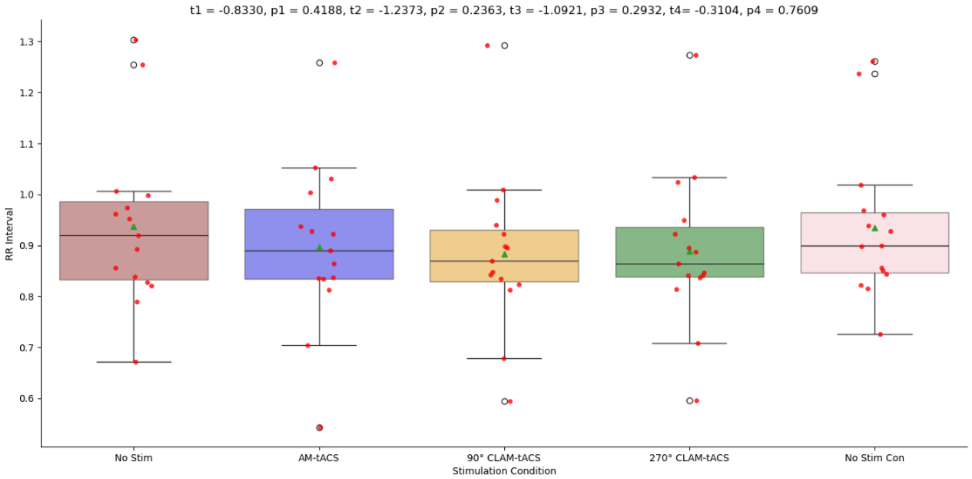
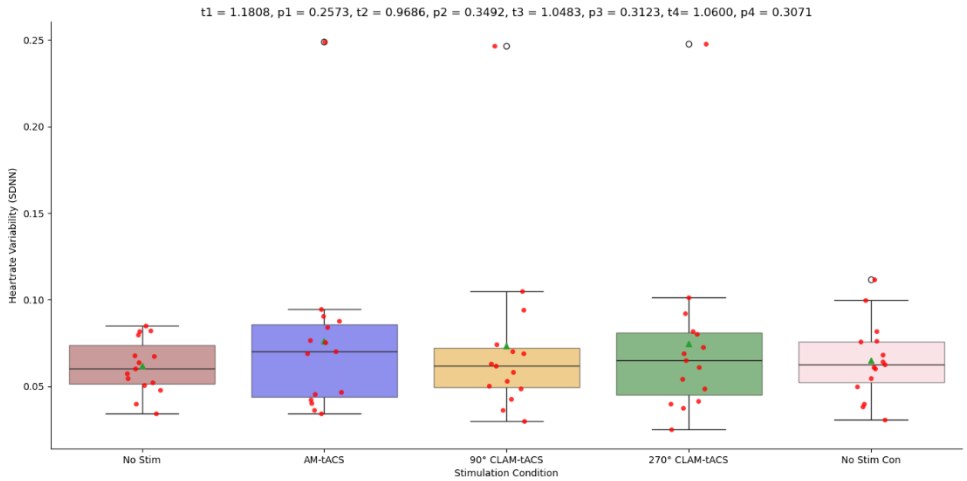


Fig.6) Heartrate variability conditions' comparisons

Average SDNN was not significantly higher under no stimulation with color-word congruence ($p_4 = 0.3071$) compared to no stimulation with color-word conflict.



6. DISCUSSION

The results of this study show that **FMT wave tACS** can **enhance performances** under **cognitive load** on **healthy participants**. However, our hypotheses that closed-loop stimulation was suitable for phase-dependent modulation of brain activity and led to better management of stress compared to open-loop stimulation, no stimulation and no cognitive load, are not supported by the result of this study**. Our findings do not provide evidence that FMT wave CLAM-tACS can modulate brain activity**, either directly through brain wave alterations or indirectly via electrophysiological changes in the heart and skin. Our findings also suggest that the **cognitive load** induced by the Stroop test was **suboptimal** for eliciting stress responses, avoiding consideration on stress management. However, these results warrant further discussion.

6.1. Effectiveness of the Stroop test

The answers' accuracy without color-word conflict was found to be highly significant compared to no stimulation ($p << 0.05$; Fig. 3, 4), suggesting that **the Stroop test** was **effective** in inducing **cognitive load**.

However, heartrate, heartrate variability and skin conductance were not found to be significantly enhanced with color-word conflict ($p >> 0.05$; Fig. 5, 6), suggesting that the **cognitive load** was **suboptimal** to elicit **significant stress responses**. This finding contrasts with our expectations, based on the literature regarding the efficacy of the Stroop test in inducing stress (Renaud and Blondin, 1997; Scarpina and Tagini, 2017). This might be influenced by the subjects' resilience, which appeared to be higher compared to the general population, as suggested by lower PSS scores (Chapter 5.2.), with an average of 14 points against an average of 17 points reported for the general population (Cohen and Janicki-Deverts, 2012). It is worth noting that our sample was primarily composed of university students (bachelor's, master's, MD, PhD), who might have benefited from their training in coping with mental and occupational stress to fulfil their academic duties.

6.2. Considerations on findings under AM-tACS

The answers' accuracy under open-loop stimulation was found to be significantly higher compared to no stimulation ($p = 0.0385$; Fig. 3), while heartrate, heartrate variability and skin conductance between the same conditions were not found to be significantly different. While the performance significance under open-loop stimulation did not survive the Yuen t-test, a trend for significance was still observed ($p = 0.1247$; Fig. 4). We believe these findings warrant further investigation. We hypothesise the following explanations:

- Considering that **in healthy participant stress is a physiological response**, i.e. a bequeathal of evolution linked to the *fight-or-flight* mechanism, AM-tACS might induce a **disrupting effect** on brain activity, making the brain less effective in eliciting stress responses. This effect is not seen under closed-loop conditions, likely because the actual stimulation phase is close to the target phase.
- AM-tACS might induce an **adaptive brain response**, i.e. the brain tries to adapt to the unspecific stimulation, resulting in better outcomes.
- AM-tACS might elicit brain activity by **power realisation**.
- Being target unspecific, AM-tACS might have a higher influence on **deep brain activity** than CLAM-tACS. Evidence of deep brain stimulation through tACS have recently been reported (Shan et al., 2023).

tACS of alpha waves has been successfully tested for the clinical improvement in anxiety patients (Clancy et al., 2018). 'Given the limited research on the therapeutic effects of tACS for anxiety, - and considering our findings, we agree with some authors who suggest that - systematic investigations into effective tACS – and AM-tACS - parameters and stimulation targets across healthy and anxious populations are essential' (Chen, Gao and You, 2024).

CLAM-tACS has recently been shown to enhance working memory, opening a new frontier in assisting patients with mild cognitive impairment (MCI) (Haslacher et al., 2024). We hypothesize that closed-loop stimulation might be more effective in improving cognitive functions which are mostly limited to the cortex, such as working memory. rather than addressing complex phenomena largely involving the

deep brain circuits, such as stress responses, where open-loop stimulation seems to be more promising.

6.3. Limitations of the study

This study is affected by three main limitations. First, due to methodological constraints**, brain wave amplitudes could not be compared between conditions with and without stimulations**. Second, the study's paradigm did not permit analysis of **within-subject variability**. Indeed, to test heartrate variability under certain stimulation conditions, each condition must last at least 5 min, as discuss in the method section (chapter 4.2.2.). In studies where heartrate variability is not measured, multiple stimulation conditions are approached differently to overcome the order effect, for example changing condition after less than a minute and without breaks (Haslacher et al., 2023). Finally, **challenges with phase tracking at 0° and 180°** remained unresolved, reducing the study's power to test and compare closed-loop stimulation at different phases.

6.3.1. Considerations and hypotheses about within-subject variability

We biased our subjects for sex, time of the day and regular relaxation practice without observing any improvement in the significance of the p-value. Nevertheless, other bias might be taken into account. In fact, a within-subject analysis reveals significant outcomes for some of the subjects. Looking at the subjects with significant results for modulation, we can observe how different subjects have **different preference of target phase**. Within-subject analysis was not included in our study because, as stated in chapter 4.5.1., task confidence and fatigue might have played a role, making permutation a choice of necessity for the analysis at the group level. However, we cannot exclude the possibility that the difference in target phase preference is due to a real within-subject variability, which should be further investigated.

- **Accuracy**

  Analysing accuracy at the subject level, modulation is significant for 4 subjects. These subjects do not differ much from the general population, but, interestingly, 3 of them are female. The probability that this happens randomly, calculated with the *hypergeometric probability formula*, is only 6%. Even if such a small sample has a scarce statistical representation of the general female population, these findings suggest deflecting attention on **female subjects** for further investigation**.** However, these findings also suggest that female subjects might be more likely to learn by doing or might have a lower tendency to become tired while performing the task.

- **Amplitude**

  Alpha amplitude modulation at the subject level was significant for 7 subjects, 4 of which were female. Theta amplitude modulation at the subject level was significant for 7 subjects, 5 of which were female. The probability to have these numbers of female subject randomly, calculated with the hypergeometric probability formula, is 7.2% for alpha amplitude and 0.48% for theta amplitude. Again, even if such a small sample has a scarce statistical representation of the general female population, these findings suggest deflecting attention on **female subjects** for further investigation.

- **Heartrate and heartrate variability**

  Heartrate modulation at the subject level was significant for all subjects but one, i.e. for 15 over 16 subjects. Heartrate variability modulation at the subject level was significant for 13 over 16 subjects. These findings suggest that the within-subject **target phase preference** might really play a role and should be further investigated.

- **Skin conductance**

  No subject had a significant modulation for skin conductance, suggesting there might have been a **systemic error** in the setup.

6.3.1.1. Sex differences in stress responses

According to the literature, men and women respond differently to stress stimuli: while men behave according to the *fight-or-flight* mechanism, **women** seem to behave according to the ***tend-and-befriend*** mechanism, typical in **female mammals** and apparently driven by **oxytocin** (Carter and Altemus, 1997; Taylor et al., 2000). Women also exhibit a **higher self-perception of stress**, which is reflected in higher Perceived Stress Scale scores (Lavoie and Douglas, 2012). Moving toward the clinics, the **prevalence of stress-related and affective disorders**, particularly depression and generalised anxiety and fear, is higher in women (McLean and Anderson, 2009; Verma et al., 2011). On the contrary, the prevalence of behavioural disorders like substance abuse and antisocial personality is higher in men. Finally, **women are more likely to respond to stressful conditions with affective disorders** while men are more likely to respond to stressful conditions with behavioural disorders (Hill and Needham, 2013).

While biasing for sex did not change the significance of our findings, results on accuracy and amplitude suggest that gender differences may be relevant for neuromodulation, underlying the need for different approaches for men and women. The conflict among these findings might derive from the **suboptimal size of the sample for sex biased analyses**.

6.3.2. Significance of the exclusion of conditions with closed-loop stimulation at 0° and 180° from the conditions' comparisons

Conditions under closed-loop stimulation at 0° and 180° had to be excluded from the conditions' comparisons due to a systemic error. The implications of such losses are evident. However, **prior studies** (McNamara et al., 2022; Zhang et al., 2021) made us hypothesize that the **best neuromodulation** would occur **at 90° or 270°**. Therefore, we can affirm that **the findings are still relevant**.

6.4. Future perspectives

Our findings support the effort to understand the role of neurostimulation for stress management from a clinical perspective, both for patients with **stress-related disorders** and for mitigating the **negative effects of stress on overall health**. We suggest that further investigations on the employment of **closed-loop stimulation** should firmly consider **within-subject variability** and **differences in stress response between men and women**. We also suggest to further investigate the role of **FMT wave open-loop stimulation** and its effect on **deep brain activity**.

7. CONCLUSIONS

This study contributes to the understanding of **tACS applications for stress-related disorders** and invite further investigation of the potential of **non-invasive stimulation to modulate deep brain activity**.

Our findings suggest that **CLAM-tACS** might not currently serve as a valuable tool for FMT waves phase-dependent neuromodulation and stress management. However, **improvements to the stimulation system and to the methodology** to address the limitations of this study could potentially alter the actual results. We underscore the need to inspect the **within-subject variability** and carefully consider the **differences in stress responses between men and women**.

We believe that this study, despite its critical aspects, can provide valuable insights for the **future development** of neurotechnology for **BCI** and **neuromodulation**.

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