A STUDY ON THE FUNCTIONAL ROLE OF THE LOCUS COERULEUS IN MEMORY FORMATION AND ALZHEIMER'S DISEASE

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THESIS

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DECLARATION

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SUMMARY

Over the past decade, the locus coeruleus (LC) has fast become a key instrument of study, resulting in a growing amount of evidence signifying its prominence and influence within the central nervous system. Moreover, the breakdown of the locus coeruleus-noradrenergic mechanism has been implicated in neurodegenerative and psychiatric disorders and may play a punitive role in the manifestations of these disorders. Therefore, to contribute to a deeper understanding of the locus coeruleus-noradrenaline (LC-NA) system, this thesis has two primary objectives of research: (1) to investigate the role of the LC in memory – via validating the mechanistic evaluation of how (i.e., direct vs. indirect) and determining when (i.e., immediate vs. offline) non-invasive electrical stimulation affects neural circuits, and (2) to examine the role of the LC in Alzheimer's disease (AD) – by way of putting forth a new hypothesis that has identified and integrated characteristics in the LC-NA system in females that distinctly spotlight their precarious nature to disproportionately developing AD.

Recent investigations have begun to assess the clinical significance of therapeutic non-invasive brain stimulation techniques to modify neuroplasticity and upregulate neuronal excitability in different neurological conditions, including memory deficits. The current thesis was built upon an existing proposal that employs non-invasive transcutaneous electrical stimulation of the greater occipital nerve (NITESGON) to activate the LC-NA pathway to enhance memory performance. Findings presented here provide evidence for the involvement of NITESGON using alternating current (AC) to generate an immediate effect during memory encoding presumptively via increased attention, whereas NITESGON utilizing direct current (DC) elicits an offline effect transpiring during the consolidation of information in both healthy, young and older adult populations. Additionally, further exploration regarding NITESGON's effect during the consolidation

phase resulted in findings that lend support to the behavioral tagging (BT) hypothesis, such that a learning event within a short time period of an additional independent novel event or stimuli will be more likely to convert to long-term memory (LTM), and therefore suggests that the application of NITESGON can be administered within a window of opportunity to promote memory stabilization. Taken together, the results presented demonstrate the LC's prominent role in providing favorable effects on strengthening memory when targeted by NITESGON and offers substantial evidence to further propel the interest and growing body of non-invasive stimulation research forward.

Research on enhancing and preserving human memory, such as the research performed above, has increased primarily due to AD's prevalence and inexorable conditions. Upon review of AD literature, it was apparent that the involvement of the LC-NA system and sex differences are two of the most rapidly emerging topics. To date, current literature either investigates the LC, due to it being one of the first brain areas to develop AD pathology, or acknowledges the neuroprotective effects of estrogens and how the loss of these female hormones have the capacity to contribute to the sex differences seen in AD; however, existing research has neglected to examine these two rationales concurrently. Reflecting upon previous literature led to a reevaluation of the approach taken in regards to female vulnerability to AD and put forth a new hypothesis considering how sex differences in the LC-NA structure and function could account for why females are more likely to develop AD, specifically, LC morphology, the paucity of estrogens, neuroinflammation, blood-brain barrier (BBB) permeability, apolipoprotein ε4 polymorphism (APOE ε4), and cognitive reserve. Collectively, the theoretical perspective presented aims to assist in alleviating current challenges surrounding female AD by providing thought-provoking connections into the interrelationship between the disruption of the female LC-NA system, the decline of estrogens, and AD vulnerability.

LIST OF PUBLICATIONS

This thesis incorporates material previously published in the following manuscripts:

2022 **Luckey, A. M.**, McLeod, L., Haung, Y., Mohan, A., & Vanneste, S. Making memories last: The peripheral effect of direct current stimulation on strengthening memories. *Elife (under review)*

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LIST OF ABBREVIATIONS

Aβ	Amyloid beta
AC	Alternate current
AD	Alzheimer's disease
ALF	Accelerated long-term forgetting
ANOVA	Analysis of variance
APOE ε4	Apolipoprotein ε4
AR	Adrenergic receptors
ATN	Amyloid/tau/neurodegeneration
BB	Braak and Braak
BBB	Blood-brain barrier
BT	Behavioral tagging
cAMP	Cyclic adenosine monophosphate
CCS	Cache County study
CEE	Conjugated equine estrogens
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
DBH	Dopamine β-hydroxylase
DC	Direct current
DOPEGAL	3, 4-dihydroxyphenylglycollaldehyde
E1	Estrone
E2	Estradiol or 17β-estradiol
E3	Estrone
EC	Endothelial cells
EEG	Electroencephalography
ELITE	Early versus later intervention trial with estradiol
ER	Estrogen receptors
ERP	Event-related potential
fMRI	Functional magnetic resonance imaging
HRT	Hormone replacement therapy
KEEPS	Kronos early estrogen prevention study
LC	Locus coeruleus
LC-NA	Locus coeruleus-noradrenaline

LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MANOVA	Multivariate analysis of variance
MCI	Mild cognitive impairment
NA	Noradrenaline
NFT	Neurofibrillary tangles
NIBS	Non-invasive brain stimulation
NITESGON	Non-invasive transcutaneous electrical stimulation of the greater occipital nerve
NTS	Nucleus tractus solitarius
ON	Greater occipital nerve
ON-tACS	Occipital nerve- transcutaneous alternate current stimulation
ON-tDCS	Occipital nerve- transcutaneous direct current stimulation
ONS	Occipital nerve stimulation
ROI	Region of interest
rsEEG	Resting-state Electroencephalography
sAA	Salivary α-amylase
sLORETA	Standardized low resolution brain electromagnetic tomography
STC	Synaptic tag-and-capture
STM	Short-term memory
STRAW+10	Stages of reproductive aging workshop
tACS	Transcranial/transcutaneous alternate current stimulation
tDCS	Transcranial/transcutaneous direct current stimulation
tES	Transcranial electrical stimulation
TH	Tyrosine hydroxylase
TJs	Tight junctions
TMS	Transcranial magnetic stimulation
VAS	Visual analog scale
VTA	Ventral tegmental area
WHI	Women's health initiative

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1 General Introduction

1.1 Importance of Subject

The incurable condition of dementia is arguably one of the greatest challenges of the 21st century. According to the World Health Organization, AD accounts for 60-70% of the estimated 55 million cases of dementia worldwide, a figure projected to rise to 78 million in 2030 and 139 million by 2050. Despite billions of dollars being invested in research, the pharmaceutical industry has been largely unsuccessful at developing disease-modifying treatments for AD (Yiannopoulou et al., 2019). From a global-societal perspective, cognitive decline, one of the earliest signs of AD, threatens older adults' independence and quality of life and presents treatment-related challenges to the healthcare system (Wallin et al., 2018). Therefore, maintaining cognitive abilities into old age and postponing or preventing pathologies leading to late-life dementia are key aims for science and society (Wallin et al., 2018). Additionally, various cognitive and lifestyle interventions (prescribed physical or brain exercises and dietary modifications) have shown some efficacy in delaying cognitive decline. However, these interventions have only shown promise in early disease stages, and expecting them to counteract age-related cognitive decline successfully is inadvisable (Schneider et al., 2020). However, it has been recognized that non-invasive brain stimulation, a device-based regimen that aims to stimulate the release of the brain's "natural drugs," holds merit as a potential remedy (Yavari et al., 2018). Nonetheless, neurostimulation's potential for cognitive maintenance and plasticity remains uncharted, particularly when identifying specific, plausible brain regions to target.

Until now, the LC's small anatomical size and location in the brainstem have made it nearly impractical to examine. However, progressive technologies have facilitated unparalleled access to the LC that has never been seen before and introduced tools that

can experimentally and non-invasively manipulate neural systems (van Boekholdt et al., 2021). These tools can be utilized to make neuroscientific inferences regarding the neural processes underlying specific behaviors, including those tied to learning and memory (Chase et al., 2020). Experimentation focusing on LC activation via electrical stimulation to enhance physiological and cognitive processes may be vital in preventing cognitive deficits.

1.2 Locus Coeruleus

Unequivocally, the LC is responsible for the far-reaching noradrenergic neurotransmitter network heavily engaged in behavioral and physiological processes (Samuels & Szabadi, 2008). Despite decades of vigorous efforts, the scientific community has just begun to conceptualize how diversly and significantly the LC influences brain function and behavior throughout the human lifespan (Mather & Harley, 2016). Prior investigations were met with challenges, such as procuring methods to test hypotheses pertaining to LC structure and function; however, recent technological improvements have positioned us to be at the threshold of innovative discoveries that will assist in new perspectives and avenues of research on the multifarious functions of the LC-NA system (Foote & Berridge, 2019; Poe et al., 2020).

1.2.1 Anatomy

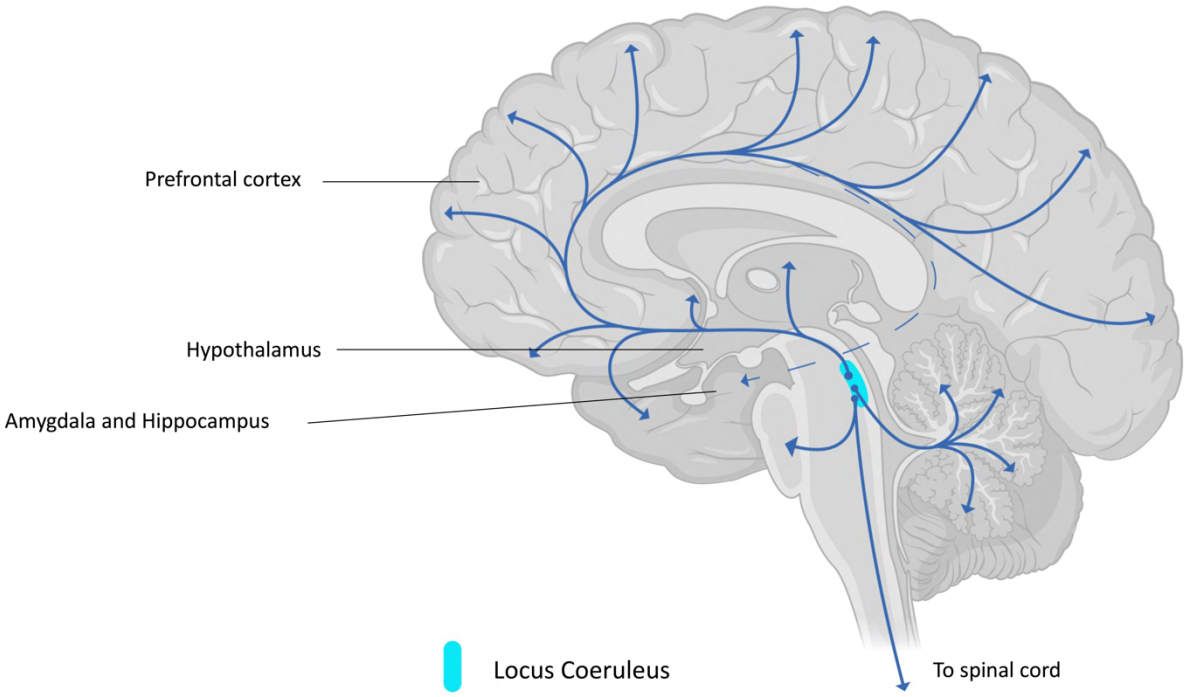
The adult human LC is estimated to contain 40,000-60,000 medium-sized neurons distributed bilaterally and have a remarkable axonal diffusion throughout the brain (Samuels & Szabadi, 2008; Sharma et al., 2010). The LC's efferent projections consist of two ascending fiber systems innervating (1) the cortex and (2) cerebellar forebrain as well as a descending pathway in the periventricular bundle projecting to the spinal cord

(see figure 1.1) (Counts & Mufson, 2010; Jones & Friedman, 1983; Szabadi, 2013). Moreover, the LC receives afferent projections from reportedly up to 111 distinct brain regions, including brainstem structures in addition to inputs from the forebrain (Schwarz & Luo, 2015). These connections allow the LC to integrate low-level autonomic stimuli with high-level cognitive information and transfer this signal throughout the brain (Avery & Krichmar, 2017).

However, it must be noted that recent topical reviews discussing the organization of the efferent projections have reported that the axons of LC neurons are less extensive than previously determined, and instead propose that LC neuron populations may discharge via a pattern intended for a specific target (Breton-Provencher et al., 2021; Poe et al., 2020). This has led researchers to retract the initial concept of the LC uniformly releasing NA and reassess their previous assumption denoting the LC as a homogenous nucleus to now consider the LC as a heterogeneous nucleus capable of innervating a preferred anatomical and functional terminal field, enabling neurons to modulate distinct behaviors and cognitive functions (Breton-Provencher et al., 2021; Poe et al., 2020).

Figure 1.1

Location of the Locus Coeruleus and Distribution of NA



Note. The locus coeruleus (LC, Latin for "blue spot" *(shown in bright blue))* is located on the lateral floor of the fourth ventricle and upper dorsolateral pons. Created with BioRender.com

1.2.2 LC-NA Activity

Upon activation of the LC, noradrenaline (NA) release is broadcasted throughout major cortical and subcortical regions (Berridge & Waterhouse, 2003), thus generating excitatory or inhibitory effects on neural activity (Salgado et al., 2016). Modulatory effects vary across brain regions and are heavily dependent upon levels of NA concentration and adrenergic receptor (AR) type, whereby α_1 ARs are suggested to inhibit LC activity, α_2 ARs enhance LC activity, and β ARs mediate either inhibitory or excitatory LC activity (McBurney-Lin et al., 2019; Salgado et al., 2016).

The LC-NA system was previously thought to solely regulate arousal and autonomic functions, such as consciousness, wakefulness, and attentiveness (Berridge & Waterhouse, 2003). However, a greater understanding of organizational afferent projections to the LC has led to a widespread agreement that the LC-NA system is one of the four principle neuromodulatory systems responsible for responding to novel or otherwise salient stimuli and sensory signal processing; this can either be a result from bottom-up processes, characterized by stimuli driven activation of the LC, or top-down inputs from the prefrontal cortex whereby the LC activity is modulated by the brain state in order to regulate behavior (Avery & Krichmar, 2017; McBurney-Lin et al., 2019; Waterhouse & Navarra, 2019). Regardless of direction, the noradrenergic mechanism underlies behaviors that require both focused or flexible awareness.

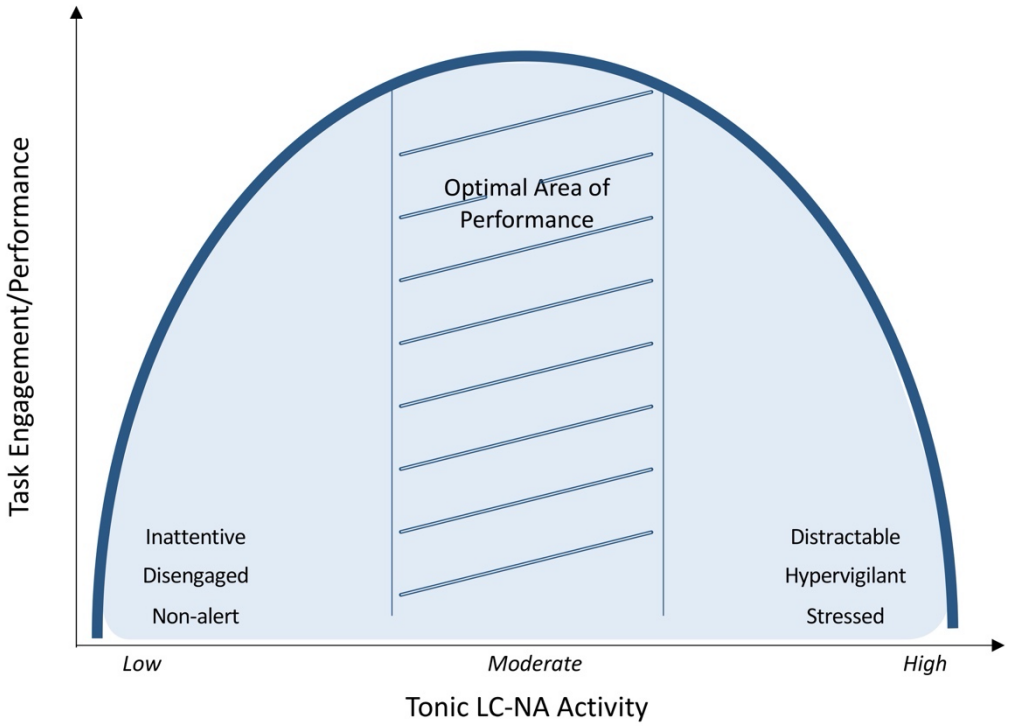
Two prominent theoretical models, adaptive gain theory and network reset theory, have shaped the understanding of the relationship between LC-NA activation and brain function. Adaptive gain theory illustrates the transition between tonic and phasic LC activity modes via an inverted-U shaped model (i.e., Yerkes-Dodson relationship; see figure 1.2) to explain the LC alternating between exploration and exploitation behaviors, respectively (Aston-Jones & Cohen, 2005a, 2005b). The inverted-U represents three respective states of LC-NA activation. Low levels of tonic NA activity result in disengaged or explorative behavior, whereas an excess of tonic NA activity promotes distractibility and hyperarousal; these represent two opposite ends of the model (Aston-Jones & Cohen, 2005a, 2005b). Excess tonic activity is associated with more exploratory behavior and disconnection from the general context due to NA facilitating the processing of multifarious events, including potentially irrelevant events (Jepma et al., 2010). Conversely, moderate levels of phasic NA activity, representing the midpoint of the inverted-U, optimally engage exploitative behavior, where task engagement is enhanced,

and performance of the task is optimized due to the release of NA being more selective in response to specific events associated with the task (Aston-Jones & Cohen, 2005a, 2005b; Jepma et al., 2010). LC phasic activity, or high frequency, rapid bursts of activity in response to novel stimuli, is thought to disrupt the constant discharge of LC tonic activity in response to a sensory stimulus. This enables the LC to filter out extraneous information and focus on a specific task, thus resulting in optimal behavioral response (Waterhouse & Navarra, 2019). Additionally, phasic activity has been recognized as a key contributor in network reset theories as LC phasic discharge enables "network resets" or sends a "neural interrupt signal" that prompts or prepares for a reorganization of neuronal activity throughout the brain in order to permit behavioral and cognitive flexibility (Bouret & Sara, 2005; Yu & Dayan, 2005).

Contrary to the aforementioned models, a more recent model, known as context-dependent modular coding, has considered that the LC acts more heterogeneously and thus is able to deliberately target regions depending upon the stimuli (Likhtik & Johansen, 2019). While questions regarding the organizational and versatility of the LC-NA system remain, it is known that once NA is released, it is capable of eliciting an array of neuromodulatory actions. These actions not only extend beyond the processing of incoming stimuli and arousal but creates a framework enabling a myriad of higher cognitive functions, ranging from attention and goal-directed behavior to influencing episodic and working memory (Arnsten & Li, 2005; Cahill & McGaugh, 1996; McGaugh & Roozendaal, 2009; Robbins & Roberts, 2007; Sara, 2009b).

Figure 1.2

Inverted-U LC-NA activity



Note. This figure illustrates how individual task engagement is low when NA tonic activity is decreased (left), an association that is often seen with disengagement and being inattentive. Optimal levels of tonic LC-NA discharge (middle) represent a high response rate to task engagement and performance (phasic) and is associated with focused attention. Tonic activity in excess of optimal levels (right) produces exploratory behavior and is associated with distractibility and stress. Modified from Aston-Jones & Cohen, 2005.

1.2.3 LC-NA Activity in Attention, Learning, and Memory

Prior to today's understanding, Seymour Kety hypothesized that the LC-NA mechanism played a role in learning and memory via enhancing neural circuit activity (Kety, 1970). Kety's hypothesis is now recognized considering the LC-NA system is

known to be a significant contributor to the signal transduction and synaptic plasticity required for the LC's dual involvement in behaviors and cognition, primarily attention, learning, and memory. Recent studies investigating the dynamics of the LC-NA system on attention indicated a causal link between stimulus-activation of the LC and attention modulation, resulting in decreased distractibility and increased goal-directed attention, respectively (Bari et al., 2020). Additionally, it established that the LC could mediate attention and arousal via two separate mechanisms upon activation (Bari et al., 2020). The LC's capability to modify these behaviors is critical to modulating memory, particularly when considering that memory and attention are highly dependent on one another to operate.

Memory encoding and consolidation are both time-dependent processes where information is captured by the brain (i.e., encoding) and physiologically organized to become permanent LTM (i.e., consolidation) that is then able to be recalled at a later date (i.e., retrieval) (Crowley et al., 2019). LC-NA modulatory actions within regions throughout the medial temporal lobe may permit learning by enhancing encoding due to environmental arousal and/or novel experiences. Both encoding and retrieval require the LC-NA system to exert modulatory actions needed to divert one's attention and respond to relevant stimuli while simultaneously suppressing irrelevant stimuli (Berridge & Waterhouse, 2003; Foote et al., 1983). Moreover, a recent functional magnetic resonance imaging (fMRI) study spotlights the dynamic behavior of the LC during arousal-related memory processing stages whereby emotionally arousing stimuli triggered engagement from the LC and the amygdala during encoding; however, during consolidation and recollection stages, activity shifted to hippocampal involvement (Jacobs et al., 2020). Recent findings have shown that activation of the LC not only promotes NA release throughout the cortex but also co-releases dopamine in the hippocampus, further

suggesting a strong modulatory influence on synaptic plasticity (Kempadoo et al., 2016; Takeuchi et al., 2016), thereby playing a crucial role in episodic memory formation (Wagatsuma et al., 2018). Within seconds of a learned experience, a continuous consolidation process that involves LC-reliant structural and chemical changes in neural ensembles is initiated and lasts anywhere from minutes to years (Dudai et al., 2015; Sara, 2009b). Interventions that aim to enhance and/or exploit the neural mechanisms involved in memory, as well as strengthen it, have been under substantial investigation in order to advance memory research (Eschenko et al., 2017).

1.3 Non-invasive Brain Stimulation

Research employing electrical stimulation to the brain has a long-standing history in science and medicine. Today, experimental research exploits various non-invasive brain stimulation (NIBS) technologies and techniques, also known as *electroceuticals*, as therapeutic interventions. A majority of NIBS techniques, such as transcranial magnetic stimulation (TMS) and transcranial electrical stimulation (tES), modify long-lasting neuroplasticity in key brain regions that contribute to relevant cognitive processes or brain regions that are impacted by neurological disease (Sanches et al., 2020). A notable difference between tES and TMS is exhibited in the modality of each stimulation technique. TES is employed via scalp electrodes to modify cellular membrane polarization to boost or suppress neuronal excitability (Bocci et al., 2020). In contrast, TMS generates an interim magnetic field to directly induce a vast discharge of synchronized action potentials in the stimulated region (Di Lazzaro & Falato, 2020).

It is important to note that the bulk of tES and TMS studies operate under the assumption that the utilized stimulation method primarily reaches and affects the outermost layers of the cortex. For instance, NIBS studies intending to improve working

memory frequently depict increased memory performance when targeting readily accessible brain regions, such as the prefrontal cortex, whereas cognitive functions that rely upon deep brain structures, such as the hippocampus, may be nonrespondent to NIBS (Begemann et al., 2020). Current research examining which of the two techniques is most effective and suitable for clinical use is currently underway (Begemann et al., 2020; Inukai et al., 2016), as well as studies pursuing a universal protocol for both tES and TMS, respectively, in order to clarify optimal procedures.

TES has seen momentous inclines in evolution and utilization over the past decade to the extent that various forms are quickly becoming key instruments of chronic therapy (Lefaucheur et al., 2017; Struber & Herrmann, 2020). Amongst these, transcranial direct current stimulation (tDCS) and transcranial alternating current stimulation (tACS) have risen to the forefront of neuromodulatory interventions given the advantageous characteristics they share, such as their non-invasive, highly tolerable approach, convenience (e.g., portable, rechargeable battery system), low cost, detection of cognitive enhancement, and lack of serious adverse effects (Hoy & Fitzgerald, 2010; Matsumoto & Ugawa, 2017). Irrespective of their promising attributes, an explicit mechanistic understanding and the efficacy of both tES techniques are extensively being explored.

Current evidence suggests that the mechanism of action of tDCS differs from that of tACS (Reed & Cohen Kadosh, 2018), such that tDCS has long been antiquated with altering the threshold for neuronal discharge (Stagg & Nitsche, 2011). The present understanding of tDCS proposes that it employs a unidirectional, direct current at a low or weak intensity (1- 2 mA; see figure 1.3A) via two or more strategically placed electrodes (at least one anode and one cathode) in order to transmit the current from the device to the scalp in an effort to polarize the region (Nitsche & Paulus, 2000). In doing so, tDCS aims to modulate cortical excitability and spontaneous activity through

subthreshold alterations on neuronal resting membrane potentials, thus increasing or decreasing the neuron's likelihood to fire an action potential (Nitsche & Paulus, 2000; Reed & Cohen Kadosh, 2018; Woods et al., 2016). Based on the desired outcome, experimental research may exploit one of two primary strategies of tDCS. When looking to increase the probability of generating an action potential, research may utilize anodal stimulation, which typically depolarizes the membrane potential, thus shifting the resting potential towards its firing threshold (Nitsche et al., 2005; Radman et al., 2009; Rahman et al., 2013). Conversely, studies will most likely choose cathodal stimulation when aiming to decrease the probability of generating an action potential seeing that cathodal stimulation generally hyperpolarizes the membrane potential (Nitsche et al., 2005; Radman et al., 2009; Rahman et al., 2013). Prior to the usage of tDCS, it is necessary to consider all components of the methodological design, such as the size and placement of the electrodes and selection of a tolerable stimulus protocol (i.e., current intensity) (Woods et al., 2016), given that they are key indicators of current density and the distribution of the electrical field (Jackson et al., 2016). Further attention is advised on current intensity and stimulation duration, owing to their potential to substantially impact short- and long-term effects produced by tDCS (Nitsche & Paulus, 2000).

Effects generated by tDCS have been deemed similar to that of long-term potentiation (LTP) and long-term depression (LTD) (Kronberg et al., 2017). The ability to produce such outcomes has accelerated tDCS' usage as a treatment option for various neurocognitive disorders associated with plasticity deficits (Jahshan et al., 2017) and tDCS' capability to modify plasticity for memory stabilization (Zhao & Woodman, 2021). Moreover, a relatively recent model, known as activity-selectivity, suggests that it is preferential to target neuronal populations that are simultaneously active when applying tDCS while also taking into account the state of the brain when stimulation is

administered, thereby generating stimulus-specific brain state effects via tDCS (Boroda et al., 2020). It is of note that this has been emphasized in prior research, whereby it was suggested that tDCS be employed with the behavior it strives to modulate (Fritsch et al., 2010), therefore, gaining momentum as an explanation of cognitive enhancement when tasks are aided by t/DCS (Kronberg et al., 2020; Podda et al., 2016).

Distinguishing itself from tDCS, tACS omits the unidirectional voltage component and influences the polarization of neurons via sinusoidal fluctuations of the membrane potential (Salehinejad et al., 2021). tACS exerts a sine wave current between the anode and cathode electrodes in a half-cycle fashion in order to modify brain oscillations via neural entrainment or synchronized firing of neurons through external stimuli (see figure 1.3B) (Tavakoli & Yun, 2017). tACS has the capability to either enhance and/or impose oscillatory synchronization in the spike-timing activity of individual neurons (Krause et al., 2019), as well as the ability to extend its reach across brain regions in the interest of entraining oscillations across more extensive neural networks (Reinhart & Nguyen, 2019). To entrain endogenous oscillations, tACS delivers an exogeneous oscillation at an equal or increased frequency to enhance the existing oscillation's power (i.e., amplitude), whereas transmitting a signal below the intrinsic frequency will reduce the intrinsic oscillation (Klink et al., 2020). Frequency, intensity, and phase are three parameters of tACS oscillations that each play a significant role in defining the direction and cyclical pattern of the delivered stimulation (Antal & Paulus, 2013).

Cortical oscillations are fundamental processes underlying cognition and behavior (Buzsaki & Draguhn, 2004). Moreover, alterations in neuronal synchrony have been identified during early stages of neurodegenerative disorders (Ahnaou et al., 2017), resulting in an increase in studies utilizing tACS to intervene with and modulate synchrony to restore oscillatory activity. tACS may be applied across a wide array of

frequency ranges, typically within conventional electroencephalography (EEG) frequencies (.01 to 80 Hz), and is generally considered to be brain-state dependent, such that the effect of tACS is influenced by the state the brain is in at the time of application. Accordingly, tACS requires a more concrete stimulation paradigm when preparing experimental study designs or intervention protocols (Woods et al., 2016). For example, researchers need to predetermine the sought-after cognitive or behavioral processes in order to identify the underlying native oscillatory properties and frequencies of these processes to entrain the associated oscillations (Woods et al., 2016). Entraining oscillations at a fixed frequency in specific brain areas enables the researchers to extract causal inferences between brain modulation and obtained effects, such as improved communication leading to improved cognition and/or behavior (Herrmann et al., 2013). Although neural entrainment transpiring is yet to be unanimously supported (Beliaeva et al., 2021), the rationale that tACS' frequency may be applied at the exact frequency rhythm fundamental for optimal brain functioning results in favoring frequency-specific synchrony effect via tACS (Reinhart & Nguyen, 2019).

Frequency-specific effects of tACS (Antonenko et al., 2016; Klink et al., 2020) and effects of anodal tDCS (Chase et al., 2020; Summers et al., 2016) modulation have been identified in various cognitive domains, including visual and auditory perception, attention, motor learning, decision making, and memory performance, thereby leading to implications for usage to subside cognitive dysfunction in neuropsychiatric disorders, such as attention-deficit/hyperactivity disorder (attention), Parkinson's disease (motor), and AD (memory). Despite the popularity and promising effects of non-invasive stimulation, there is an ongoing debate concerning whether the electrical current modulating the excitability of neurons is provided directly and in a regionally constrained manner, or if the current flow is transmitted indirectly via activation of other non-

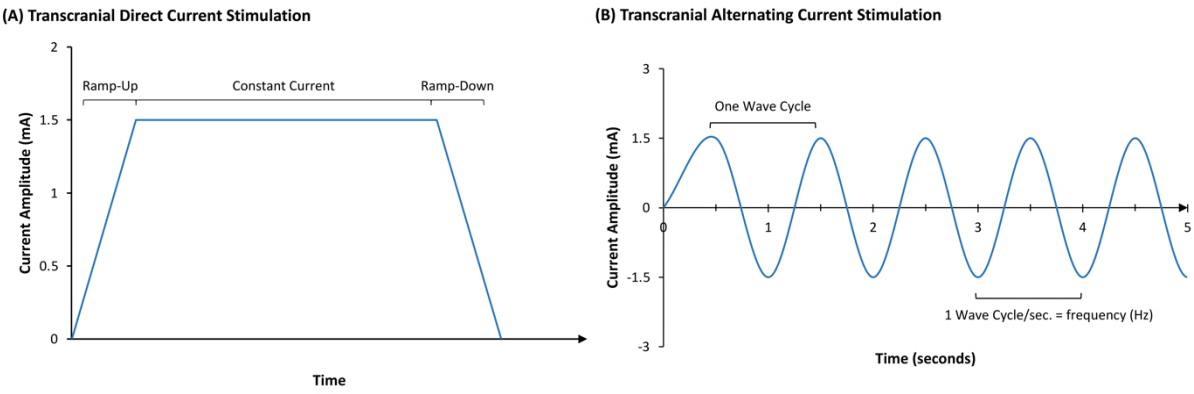
neuronal elements, including peripheral nerves. An additional, highly inquired element of tES is whether effects take place online so that they occur immediately or simultaneously during stimulation, or if they occur offline, whereby the effects outlast the period of stimulation (Liu et al., 2018; Voroslakos et al., 2018). These aspects of tES protocols remain inconclusive in large part due to the absence of an adequate mechanistic understanding, which has also led to variability in the technique, difficulty in replication, and potentially deleterious effects.

Calculations on a realistic head model and validation studies in both animal and human experiments have indicated that only 25 (up to 50) percent of the applied current reaches the brain due to the high electrical resistance of the skull while the remaining current is shunted through extracranial soft tissues (Liu et al., 2018). Consequently, an alternate explanation suggests that tES primarily affects neural circuits indirectly, i.e., via peripheral nerves. In line with this concept, evidence from a recent series of experiments in rats and humans demonstrated that the effects of non-invasive electrical stimulation were mainly caused by transcutaneous stimulation of peripheral nerves (Asamoah et al., 2019). Similarly, prior studies have demonstrated that nerve stimulation paired with an auditory or motor task can induce task-specific plasticity, signified by cortical changes in either auditory or motor networks in animals that received vagus nerve stimulation (Engineer et al., 2011; Porter et al., 2012). Seeing that there is a myriad of variables, including individual differences in neuroanatomy (e.g., scalp and skull thickness), it is more likely that non-invasive electrical stimulation is delivered via peripheral nerves. However, future studies are warranted, considering that the field remains in dispute regarding the aforementioned elements of tES. Experiments geared towards a more accurate understanding of these tES techniques will offer greater insight into the neural

correlates of cognition and assist in developing a protocol that will yield consistent, clinically meaningful effects to combat cognitive deficits in patient populations.

Figure 1.3

Non-invasive Brain Stimulation Techniques



Note. This figure illustrates examples of stimulation waveforms for **(A)** transcranial direct (anodal) current stimulation and **(B)** transcranial alternating current stimulation. Transcranial direct current is delivered at a constant current for a specified amount of time. An initial ramp-up period is used to reach the desired current amplitude, and a ramp-down period is used to gradually descend to 0 after stimulation. Transcranial alternating current is represented by a sinusoidal waveform. The number of wave cycles a sine wave completed within one second is referred to as its frequency. Frequencies are measured in hertz (Hz).

1.4 The Peripheral Effect of Direct Current Stimulation

Experts in the field today have extensively debated whether tDCS of the scalp directly modulates brain activity in a regionally constrained manner (Asamoah et al., 2019; Voroslakos et al., 2018). However, recent work produced by our laboratory, divided amongst three separate sets of experiments, provides an alternative explanation of tDCS'

capability of producing significant effects utilizing NITESGON to (1) upregulate the LC-NA system, (2) confirm that the occipital nerve was sufficient to induce these changes, and (3) suggest an augmentation of memory performance (Vanneste et al., 2020).

These findings align with previous research that has shown that the LC-NA system can be activated by stimulation of peripheral nerves such as the vagus nerve (McIntyre et al., 2012). The current studies utilized the greater occipital nerve (ON), a lesser-known peripheral pathway capable of having similar effects to those of the vagus nerve (Plazier et al., 2011). Both peripheral pathways have been found to influence bottom-up regulations of cortical gain, psychological arousal, and neurobiological responses to environmental stimuli and stressors via the LC-NA system (Sara, 2009b; Tyler et al., 2015). More importantly, what distinguishes the greater occipital nerve from the vagus nerve is that the ON includes several branches innervating the posterior occiput up to the vertex of the scalp (Weiner & Alo, 2018), thus can be more easily accessible via non-invasive stimulation of the scalp (Kano et al., 1976). In contrast, targeting the non-superficial or deep vagus nerve can be more challenging. The ON arises from the C2 spinal nerve, with specific afferent fibers that travel to the brainstem and project to the nucleus tractus solitarius (NTS) (van Boekholdt et al., 2021). Information is then linked across the brainstem amongst networks within the reticular formation, whereby sensory information is processed via the thalamus and somatosensory cortex. Additionally, activation of the LC promotes NA release in the cortex. Of particular interest to the current thesis are the LC-NA projections to brain regions associated with learning and memory formation, such as the prefrontal cortex, amygdala, and hippocampus (Couto et al., 2006; van Boekholdt et al., 2021).

The first set of experiments were designed to establish NITESGON's capability to upregulate the LC-NA system. In order to demonstrate DC stimulation's ability to cause

activation of the greater occipital nerve, target the LC, and promote NA release, the first study of this series examined the effect of NITESGON on LC-NA activity via the three most common associated proxy measures: pupillometry, salivary α-amylase (sAA), and neurophysiology [event-related potentials (ERPs)]. Evident from P3b ERP, a strong cortical electrophysiological correlate of the LC-NA response which peaks at 300 to 600 ms after a task-relevant stimulus (Nieuwenhuis et al., 2005; Polich, 2007), electroencephalogram data collected during a standard P3b evoking auditory oddball task revealed that NITESGON led to an increased peak and mean amplitude seen over the left parietal electrode side.

In addition, utilization of light stimulation revealed that NITESGON increased mean pupil diameter. Pupillometry is a pupil diameter measurement that assesses LC activation. LC-NA activity has been highlighted as a neuromodulatory pathway that controls pupil constriction and pupil dilation via inhibition/excitation of the autonomic nervous system (Joshi & Gold, 2020). Pupillometry is a promising proxy measure used to evaluate the association between task engagement and LC activity in humans (Murphy et al., 2014) that has been confirmed via intracranial recording and pharmacological challenge studies (Hou et al., 2005).

Lastly, sAA levels, the last proxy tested for, increased during and immediately following NITESGON. Salivary alpha-amylase is an enzyme that begins the digestion of carbohydrates and is provoked by both central parasympathetic and sympathetic nervous system activation (Nater & Rohleder, 2009). Salivary alpha-amylase is often a non-invasive yet valid indirect indicator for endogenous NA activity (Nater & Rohleder, 2009). Prior evidence using functional brain imaging while participants view emotionally arousing slides revealed increases in amygdala activity and sAA; these increases were

then significantly reduced by the beta-adrenergic antagonist propranolol (van Stegeren et al., 2007).

Utilizing these proxy measures enabled the tracking of LC-NA activity changes from baseline measurements; however, caution is advised when interpreting results considering these proxy measures are affiliated with limitations that must be acknowledged, such as being substantially variable, as well as the potential of other brain regions and monoamine neurotransmitters being associated with changes seen in pupil dilation (Joshi, 2021; Joshi & Gold, 2020) and sAA concentration levels (Jones et al., 2020). Upon analysis, significant intercorrelations were found amongst the three commonly utilized LC-NA activity proxy measures, suggesting that NITESGON can induce changes in LC activity, thus promoting NA release.

Evidence of NITESGON prompting NA release from the first study led to a subsequent study utilizing 5-minute resting-state EEG recordings immediately before and after a 20-minute stimulation session to demonstrate how NITESGON could modulate the medial temporal cortex through the LC-NA pathway. A source reconstructed analysis of the EEG data exhibited increased synchronization for the theta (3 to 8 Hz) frequency band at the medial temporal cortex as well as increased theta-gamma (30 to 50 Hz) phase-amplitude coupling in the right medial temporal cortex after NITESGON. These findings are significant, seeing that previous research has suggested that neural oscillations in the theta frequency and gamma frequency, respectively, as well as both frequencies interacting together (i.e., phase-amplitude coupling), in the corticohippocampal network, are essential for human memory formation (Nyhus & Curran, 2010). Moreover, animal studies have demonstrated the essentiality of LC activation for inducing theta rhythm changes in the hippocampus (Broncel et al., 2020) as well as increasing gamma power in the prefrontal cortex, thus allowing for the mediation of incoming information (Neves et

al., 2018). Combined with the aforementioned literature, these findings suggest NITESGON targeting the LC can generate the necessary LC activation needed for memory encoding and formation.

In order to show the relationship between LC-NA activity changes and the hippocampus, a third study using resting-state functional connectivity MRI was conducted. The magnetic resonance scanning session was divided into three consecutive blocks: before, during, and after 20-minutes of NITESGON. Using the LC as a seed region, a correlational analysis measuring functional connectivity indicated that NITESGON increased the correlation strength between the LC and the dorsal anterior cingulate cortex and temporoparietal junction during stimulation as well as the LC and the left and right hippocampus, dorsolateral prefrontal cortex, precuneus, and right angular cortex after stimulation, all of which are areas that are known to contribute to bottom-up attention in memory and memory consolidation (Cabeza et al., 2008; Huang et al., 2019). Additionally, a region of interest (ROI)-to-ROI analysis indicated that NITESGON increased the connectivity between the right amygdala and the right hippocampus with the LC during stimulation as well as the right hippocampus and the LC after stimulation. Furthermore, a second ROI-to-ROI analysis depicted an increase in connectivity between the LC and the left hippocampus after stimulation. The findings of the ROI-to-ROI analyses are significant considering how the increased connectivity between the three brain regions shares similarities with connections that transpire in the presence of novel or salient stimuli, which has previously been identified to increase memory consolidation (McReynolds et al., 2014), thus implying NITESGON may potentially produce similar effects.

The second set of experiments in the series of studies were designed to substantiate that the ON is the transcutaneous mechanism that drives the effect of NITESGON via the

utilization of memory tasks. In order to verify stimulation was being directed towards the ON, a study consisting of male Sprague rats with biocompatible microerenathane cuffs implanted around their isolated left ON was conducted. Occipital nerve stimulation (ONS) was delivered via wire electrodes immediately following inhibitory avoidance and object recognition training, respectively. Rats that received ONS immediately after object recognition training demonstrated increased objective recognition when tested 24-hours later, in addition to rats that received ONS immediately after footshock training displayed increased inhibitory avoidance upon testing 24-hours later, thus providing evidence that invasive ONS targeting the LC induced a memory effect.

Identification of ONS targeting the LC inducing a memory effect in rodents led to a second study demonstrating that the induced memory effect is associated with the occipital nerve in humans. This was done by applying stimulation during study blocks of a word association memory task with two NITESGON application groups, one applied via the left anode and right cathode and one applied via the left cathode and right anode, and two active control conditions whereby stimulation was administered to the trigeminal nerve or neck via the cervical nerves five and six. To evaluate the effects of NITESGON, Day 1 measurements included learning rate during the word association task, resting-state EEG data, sAA concentration levels, and a visual analog scale measuring alertness immediately before and after NITESGON. To assess LTM effects, word recall was measured on Day 7. No difference was observed in the number of words learned on Day 1 amongst the four groups; however, both NITESGON groups exhibited increased memory recollection compared to both control groups on Day 7. This correlated with sAA levels taken on Day 1, as well as increased gamma power in the medial temporal cortex, the precuneus, and the dorsal lateral prefrontal cortex immediately after stimulation. These areas are similar to the aforementioned resting-state fMRI data and

have been associated with memory before (Cabeza et al., 2008; Huang et al., 2019). The increase in gamma power was of particular interest, especially when taking into account how previous research has identified gamma oscillations as key role players in LTM and potential subsequent recall predictors (Osipova et al., 2006; Sederberg et al., 2003). Moreover, it was confirmed that the significant effect of NITESGON was not a sensation effect seeing that no group difference was obtained in a visual analog scale for alertness that was taken before and after stimulation.

In order to show that the memory effect of NITESGON was caused by transcutaneous stimulation of peripheral nerves as opposed to transcranial stimulation of cortical neurons, a follow-up study utilizing a topical skin anesthetic (lidocaine/prilocaine) cream was conducted. The cream reduced the contribution from transcutaneous stimulation via blocking the sodium channels in peripheral nerves in the skin, thus stabilizing the membrane potential and increasing the threshold for firing an action potential (Kumar et al., 2015). NITESGON was applied to two groups during a word association memory task, one with the anesthesia cream applied under the tDCS electrodes and one with no anesthesia cream. Upon being tested 7-days after initial learning, the group that had no anesthesia cream under the tDCS electrodes exhibited an increase in words recalled correctly, suggesting that NITESGON affects neural circuits indirectly; this is in accordance with recent research which demonstrated that tACS targeting the motor system is mainly driven by transcutaneous nerve stimulation (Asamoah et al., 2019).

Although the evidence presented highly supports stimulation is being delivered indirectly through peripheral nerves, it is noteworthy that recent research suggests a small portion of the current reaches the brain directly, thus potentially working in tandem with the current that is delivered indirectly (Asamoah et al., 2019; Voroslakos et al., 2018). Findings from these studies support the rationale behind this concept, seeing how

participants with the anesthesia condition, representing transcranial stimulation, showed a significantly lower effect than non-anesthesia condition participants, representing transcutaneous stimulation, yet had a greater effect than the participants who received sham stimulation, representing no stimulation, in prior studies. Additionally, it must be recognized that there is a substantial likelihood of stimulating other neural pathways in addition to the LC-NA pathway. Previous animal research indicates that peripheral nerve stimulation such as vagus nerve stimulation also activates the dopaminergic (Perez et al., 2014), serotonergic (Hulsey et al., 2019), and cholinergic (Hulsey et al., 2016) pathways which release dopamine (Duszkiewicz et al., 2019) and acetylcholine (Maurer & Williams, 2017) both of which play an important role in inducing long-term plasticity changes related to memory consolidation. However, as demonstrated in prior studies above, it was affirmed that the ON and LC-NA pathway were the targets being stimulated/activated.

Based on the findings in the first and second series of experiments, which indicated NITESGON modulated the hippocampal LC-NA pathway, the third set of experiments were designed to investigate if NITESGON applied during memory encoding of two distinct association tasks could enhance memory formation. To show that NITESGON applied during encoding can modulate the memory performance of a face-name association memory task that was based on the task of Jacobs and colleagues (Jacobs et al., 2015), a study was conducted where participants received active or sham stimulation 5-minutes during the encoding phase and 10-minutes of the consolidation phase in a face-name association task. Those who received NITESGON during encoding and consolidation showed marginally increased accuracy in face recognition as well as the name related to the face. To further demonstrate the LTM effects of NITESGON, a study was conducted whereby NITESGON was applied during learning of a well-known

Swahili-English word association memory task that was based on recall rather than recognition (Karpicke & Roediger, 2008), thus making the task more challenging (Mandler, 1980) along with requiring more hippocampal activity (Holdstock et al., 2002). No difference was seen between the active or sham NITESGON groups on Day 1; however, the NITESGON group recalled significantly more words on Day 7. These findings suggest that the application of DC NITESGON during training enhances memory performance via peripheral stimulation and is conceivably mediated through the activation of brainstem nuclei, including the LC-NA pathway.

Overall, the findings of the three separate sets of experiments propose that a majority of electrical stimulation effects are transmitted via ascending fibers of the ON that synapse with neurons in the NTS, which in turn facilitates NA release, thus augmenting functional connectivity with the hippocampus to strengthen memory formation. Contrary to previous pharmaceutical approaches, NITESGON has exhibited the capability of providing persistent, stimulus-specific modifications to neural circuits with minimal side effects. These findings contribute considerable value to research today, particularly when taking into account how NA not only has biological effects on brain structure and function but that it may also facilitate networks for arousal, novelty, attention, and memory processes.

1.5 Specific Aims of the Research

Based on the evidence presented in Chapter 1, there is a unique opportunity to expand the field's knowledge by highlighting the LC's significant influence over brain functions and behaviors. Collectively, this thesis aims to achieve two primary objectives by reviewing opposite ends of the spectrum, that is, the LC-NA mechanism. On one end, this thesis aims to (1) investigate the role of the LC in memory – by examining if

upregulating the LC system via non-invasive stimulation generates substantial, positive effects on memory formation and storage. Conversely, on the other end of the spectrum, this thesis aims to (2) evaluate the role of the LC in AD – by exploring if sex differences in the LC mechanism lead to considerable, negative repercussions in female AD.

1.5.1 Investigating the Role of the LC in Memory

The significant surge in popularity of non-invasive stimulation techniques has generated a rise in studies attempting to discover a non-pharmacological approach to modify the brain; however, pre-existing research has not yet determined a distinguished protocol for the use of tES. Evidence detailed to this point has established that it is conceivable to utilize a non-invasive approach to artificially regulate LC arousal while entraining the memory pathway.

Studies comprising Chapters 2 through 4 were designed to distinguish whether effects of specific NITESGON techniques occur simultaneously or offline and to validate if stimulation is transmitted indirectly. In conjunction with the overall design, Chapters 2 through 4 of the present thesis aimed to improve memory performance and pinpoint the phenomena underlying tES effects in order to obtain adequate evidence to endorse the proposed procedures for NITESGON. This, in turn, should facilitate establishing a reproducible paradigm for a tES method capable of augmenting memory formation via modulation of the LC through peripheral nerve stimulation.

In order to evaluate the effects of active and sham NITESGON during a verbal associative memory task and/or a spatial navigation memory task, each experiment measured the cumulative percentage learned word pairs/object-locations on Day 1 as well as the percentage of correctly recalled word pairs/object-locations on Day 7; measuring correctly recalled words/locations permitted the assessment of possible long-term effects.

If successful, future research would be able to make inferences regarding the neural processes underlying behaviors tied to learning and memory.

To elaborate on the first aim of the present thesis, **Chapter 2** aimed to reproduce the behavioral and biomarker results of Chapter 1 Section 1.4 in an older (i.e., between the ages of 55 and 70 years), healthy, adult population. Changes in sAA concentration levels were also analyzed to determine if NITESGON resulted in LC pathway activation in healthy older persons. This experiment allowed for any potential limitations of NITESGON to be eliminated and supported its use to improve memory performance via activation of the LC-NA pathway.

Chapter 3 aimed to identify the stage of memory that was best suited for NITESGON application to induce memory improvement. Five independent experiments were utilized to determine if NITESGON possessed the capability to boost the retention of memories when applied shortly before, during, or shortly after encoding or instead, increase recall when applied during retrieval. Taken together, these experiments would indicate if a window of opportunity for stimulation to enhance memory recall and learned experiences existed.

Chapter 4 aimed to comparatively investigate the effects of AC and DC NITESGON on associative memory performance. An insufficient number of studies directly comparing the effects of tACS and tDCS in cognitive performance have caused optimal parameters and procedural applications to remain undefined. Here, comparative analyses were utilized to evaluate the behavioral effects of these different neuromodulation techniques while utilizing an identical experimental design.

1.5.2 Evaluating the Role of the LC in AD

Current literature either investigates the LC due to it being one of the first brain areas to develop AD pathology or acknowledges the neuroprotective effects of estrogens and how the loss of these female hormones have the capacity to contribute to the sex differences seen in AD; however, existing research has disregarded concurrently examining these two rationales, thus highlighting a major gap in the current understanding of risk and prevention of AD. Therefore, the present thesis aimed to reevaluate the approach taken when examining female AD susceptibility and how treatment for this heterogeneous disease may need to be distinctly developed for females and males separately. **Chapter 6** presents a novel hypothesis endeavoring to explain how female AD vulnerability is based upon the differential and detrimental effects of depleted estrogens and degradation of the LC-NA system in females, and further (1) brings awareness to the need for in-depth investigations into the loss of estrogens and diminished production of the LC-NA system to combat AD sex differences; and (2) lays the groundwork for future studies to delineate the sex differences in AD.

1.5.3 Translating Aims to Research

It is widely recognized that the LC-NA system plays a crucial role in determining late-life cognitive abilities and that memory tends to decline as adults age (Mather & Harley, 2016). Furthermore, the LC has been identified as one of the earliest hosts of tau pathology (Braak & Del Tredici, 2011), and dysregulation of the LC-NA system is one of the neuromodulatory systems associated with memory impairment in AD (David Weinshenker, 2008). The breakdown of the LC noradrenergic mechanism has not only been implicated in AD but may also play a role in the manifestations of this disorder (Weinshenker, 2018). Given the substantially increasing population affected by age-

induced cognitive disabilities combined with the lack of effective neurological interventions, developing a novel therapeutic remedy is paramount. Therefore, exploiting NITESGON's potential to improve and strengthen memory during favorable, intact conditions may prove essential for the development of preventative and therapeutic strategies.

2 Modulation of Associative Memory: A Randomized Trial in Older Adults

2.1 Introduction

Progressions in medicine and public health, improved standards of living, and advancements in education and nutrition have lengthened the human lifespan (Lindenberger, 2014). In turn, this increase in longevity has exhibited the variability and malleable nature of cognitive development in older individuals (Lindenberger, 2014) and is anticipated to increase the prevalence of cognitive impairment and neurocognitive disorders (McDonald, 2017). Therefore, it is crucial to harness the human brain's potential for neuroplasticity in order to maintain the viability of neural structures and postpone the onset of cognitive decline. Current research is investigating a diverse multitude of intervention techniques to achieve this, including cognitive training and lifestyle modifications (Schneider et al., 2020) and the use of tES (Balconi et al., 2021).

Of these three, tES has emerged as a popular intervention technique deemed to potentially possess the means to enhance cognitive function as well as potentially delay cognitive decline (Yavari et al., 2018). TES aims to stimulate the brain in ways that avoid the complications of trying to upregulate neurotransmitters artificially by using pharmacological methods. TES applies a weak current to modify cortical excitability, inducing long-lasting changes with no significant adverse effects and low levels of discomfort for participants (Lefaucheur et al., 2017; Matsumoto & Ugawa, 2017). Moreover, at a cellular level, tES has been deemed to potentially modify the LTP essential for memory stabilization, thus gaining momentum as an explanation for augmenting learning when combining tES with behavioral training (Kronberg et al., 2020; Zhao & Woodman, 2021). NITESGON, a tES technique utilizing DC, has recently demonstrated the ability to upregulate the LC-NA system, confirm that the occipital nerve was

sufficient to induce these changes, and suggest an enhancement of memory performance in a healthy, young adult (18-35 years old) population – NITESGON improved memory recall that ensued up to 7-days after learning (Vanneste et al., 2020). Intriguingly, NITESGON yielded an LTM effect but did not trigger an immediate effect on learning, suggesting that the effect is generated offline during the consolidation of memories (Vanneste et al., 2020). However, the effect of NITESGON was not assessed on an older population; therefore, the current study was designed to discover if prior laboratory findings pertaining to improvements in memory recall performance using NITESGON could be replicated in healthy, older individuals.

It is widely recognized that memory, a primary function of human cognition, tends to decline as adults continue to age. Empirical findings ranging from the early 20th century to today have based multiple respective models of age-related memory decline on multifarious mechanisms, including decreases in processing speed and attention, an increase in memory interference, and a deficiency to encode information (Park & Festini, 2017). Regardless of what mechanism(s) contribute to memory decline in aging, laboratory research has demonstrated that age-related memory effects become more evident as task difficulty increases (Park & Festini, 2017). For example, greater deficits in word recall in comparison to word recognition occur in older adults due to the increased mental effort required in addition to imagery and verbal mnemonics being less likely to be utilized in comparison to younger populations (Luo & Craik, 2008). Although the root causes of age-related memory decline are not yet entirely understood, evidence suggests that the downregulation of neuromodulatory processes is an influence (Avery & Krichmar, 2017; Lindenberger, 2014), thus making tES a viable candidate to utilize given that the goal of tES is to modulate brain activity and trigger the release of

neuromodulators selectively. However, tES has received relatively little attention in aging research.

Considering the numerous factors that contribute to individual variability, such as head anatomy, local circuitry, and particularly age, it cannot be assumed that a "one-size-fits-all" tES technique may be utilized or exist (Li et al., 2015; Ridding & Ziemann, 2010; Yavari et al., 2018). Therefore, examining the effects of NITESGON in older individuals was a prerequisite for continuing to substantiate the use of NITESGON to enhance memory performance. If results failed to replicate previous findings, thus signifying a deficiency in improving memory recall, the current study would provide crucial information by indicating NITESGON targeting the LC-NA system via DC should not be pursued as an intervention technique. Moreover, if the evidence produced by the study was able to replicate findings from the previous work, further studies could be designed to better understand the beneficial characteristics of NITESGON and how it may be implemented towards cognitive improvement or delaying cognitive decline in older adults. Given the results produced by the previous experiment, it was hypothesized that NITESGON could upregulate memory in healthy older persons via peripheral nerve stimulation targeting the LC-NA pathway.

2.2 Methods

2.2.1 Design

This experiment was designed as a double-blind, sham-controlled, randomized, three-visit, parallel-group study aimed to directly compare the effects of DC NITESGON on associative memory recall performance using an older adult population. The study employed a mixed factorial design with time (Day 1 vs. Day 7 vs. Day 28) as the within-subjects variable and group (active NITESGON vs. sham NITESGON) as the between-

subjects variable. This study was in accordance with the ethical standards of the Helsinki declaration (1964). All participants provided written informed consent.

2.2.2 Power Analysis

G*Power (Faul et al., 2007) was used to perform an a priori power analysis for an omnibus one-way ANOVA. The estimated effect size for the current study was based on a prior study published in *Science Advances* (Vanneste et al., 2020). This experiment utilized two groups (active and sham NITESGON) to investigate the long-term effect of NITESGON on memory performance when NITESGON was administered during a word association task. A significant difference was observed between the active and sham NITESGON groups 7-days after initial learning, whereby active NITESGON recalled more word pairs than those who received sham NITESGON; this study indicated that the best estimate of the true population standardized mean difference (Cohen's f) was 1. This large effect size estimate was entered into the power analysis with the following input parameters $\alpha = 0.05$ and power (1-β err prob) = 0.8 with two groups. The power analysis indicated that a total sample size of at least N = 12 was required to detect a difference between the two groups with at least 80% power.

2.2.3 Participants

Participants included 30 healthy, native-English speaking adults (22 females, 8 males; mean age of 64.6 years, *SD* = 5.86 years). Participants were screened (e.g., tES contraindications, neurological impairments, not participated in a tES study, normal or corrected to normal vision) prior to enrolling into the study. None of the participants had a history of major psychiatric or neurological disorders or any tES contraindications, including previous history of brain injuries or epileptic insults, cardiovascular

abnormalities, implanted devices, taking neuropsychiatric medications or blood pressure medication use, prescribed stimulants use, or chronic use of illicit drugs (i.e., marijuana and cocaine).

Participants were excluded from the study if screening discovered they were familiar with Swahili/Arabic language or Swahili culture due to the nature of the stimuli. Furthermore, participants were asked to abstain from alcoholic beverages for 24-hours and caffeinated beverages for 16-hours prior to the scheduled study session. Additionally, participants were asked to withhold from using any hair styling products the day of the study. To assure the highest levels of accuracy for saliva collection, participants were asked to refrain from the following products or activities for the associated time window prior to saliva collection: dental work for at least 48-hours, major meals for 60-minutes, brushing their teeth for 45-minutes, as well as water or rinsing their mouth for 10-minutes in order to avoid any risk of lowering pH levels and influencing bacterial growth. Participants were also asked to refrain from taking any nonapproved prescription drugs, steroidal/anti-inflammatory drugs and were also asked to avoid foods high in sugar content or acidity, and nicotine consumption. Lastly, if participants were scheduled for a study in the afternoon, they were requested to avoid taking a nap during the day to account for the amylase awakening response (Ali & Nater, 2020). Salivary alpha-amylase has been shown to have a distinct diurnal profile whereby sAA levels are low within 30-minutes of awakening and rise throughout the day (Nater et al., 2007).

Participants performed a wide range of tests covering mood, executive function, and memory, including Beck Depression Inventory (BDI) (Beck et al., 1996), Beck Anxiety Inventory (BAI) (Beck & Steer, 1993), Mini-Mental State Examination (MMSE) (Folstein et al., 1975), California Verbal Learning Test (CVLT-II) (Delis, 2000), Trail Making Test A (TMT-A) and B (TMT-B) (Reitan, 1958), Wechsler Adult Intelligence

Scale (WAIS-IV) Digit Span and Coding (Wechsler, 2008), Controlled Oral Word Association Test (COWAT) (Benton et al., 1994), and Delis-Kaplan Executive Function System (D-KEFS) (Delis et al., 2001). All participants in the study scored above the single cutoff score, indicating they had no cognitive impairment and that their memory performance was within the normal range for their age and education level.

2.2.4 Materials

2.2.4.1 Word Association Task.

All participants underwent a computerized, word association task consisting of Swahili-English vocabulary learning that was adapted from a well-established study design published in *Science* (Karpicke & Roediger, 2008). The 50 Swahili-English word pairs were taken from the Nelson and Dunlosky study (Nelson & Dunlosky, 1994). The task was programmed in Visual Studio software using C# and shown on a computer with a ~27-inch screen positioned at eye level.

Participants had six alternating study and test periods to learn the list of 50 Swahili-English (e.g., Swahili: tumbili, English: monkey) vocabulary word pairs made up of common day-to-day words. The verbal paired-association memory task was divided into 3 blocks with each block consisting of a study period (S), followed by a 30-second rest period, and a test period (T). Participants studied and were tested on the exhaustive list of 50 word pairs in block 1. Whereas in blocks 2 and 3, the comprehensive list of 50 word pairs were studied in each study period, but only items that had not yet been recalled were tested in the test periods (denoted SDT_N, where T_N indicates that only the non-recalled pairs were repeatedly tested). Therefore, in blocks 2 and 3, the number of word pairs tested diminished across the blocks and varied according to the testing period. This

paradigm was used to ensure that all participants would avoid a ceiling effect (Karpicke & Roediger, 2008).

During the study period, each word pair (black words on white background) was presented one below the other in the middle of the screen for 5-seconds to provide adequate time for encoding. Participants were instructed to learn as many word pairs as they could, so they may recall the English word when given the Swahili word. During the cued-recall test period, participants were instructed to type in the correct English translation of the Swahili word that was presented for 16-seconds using a computer keyboard. If participants failed to recall a word pair during testing, they were not given any feedback. Once the 16-seconds expired, the computer program would automatically advance to the next Swahili word regardless of whether the participant had entered a response. Participants' responses were recorded by the computer program. The word pairs sequence was randomized between participants, blocks, and periods. Refer to figure 2.1A for experimental task design.

2.2.4.2 NITESGON.

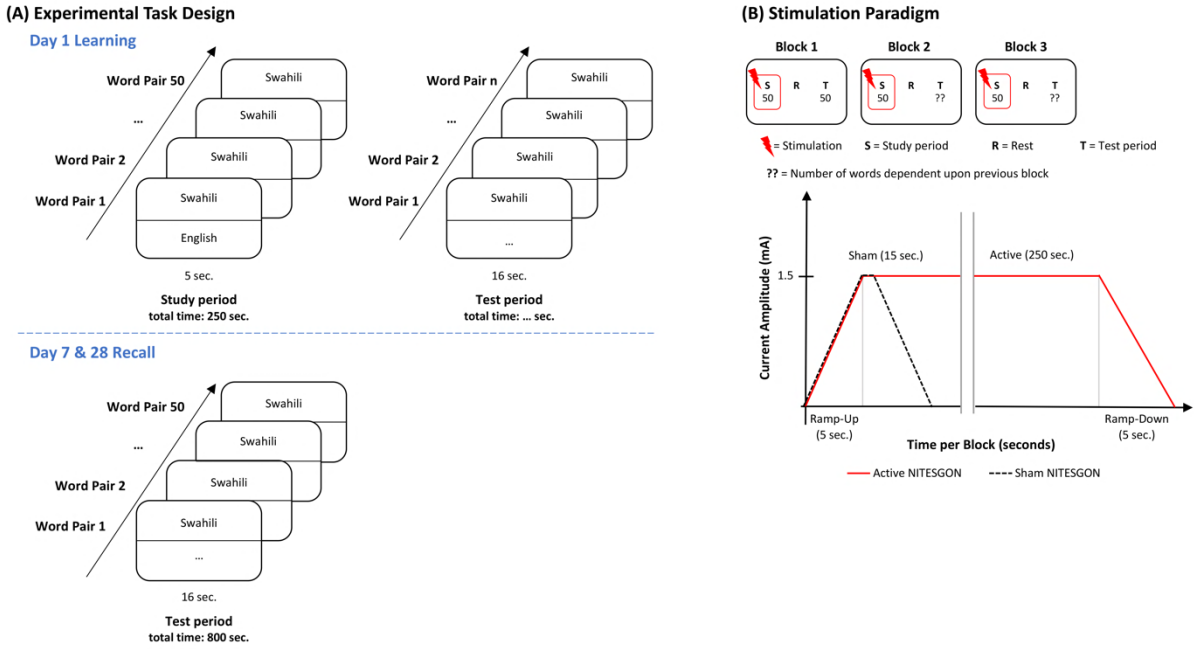
NITESGON utilizing DC was transmitted via a saline-soaked (1.3% saline) pair of synthetic sponges (5 cm x 7 cm) and was delivered by a specially developed, battery-driven, constant current stimulator with a maximum output of 10 mA (Eldith©; http://www.neuroconn.de). For each participant receiving NITESGON, the anodal electrode was placed over the left C2 nerve dermatome, and the cathodal electrode was placed over the right C2 dermatome. To maintain consistency across all participants, research assistants were trained to map out the placement according to the length of the participant's head.

To minimize skin sensations and to acclimate participants to the stimulation, the current intensity was ramped up (gradually increasing) until it reached its programmed maximum output (1.5 mA). After stimulating for the desired duration per the group (active or sham), the current was ramped down (gradually decreased), denoting the end of the stimulation. The impedance under each electrode was maintained under 10 kΩ. The stimulation times differed depending on the condition (active vs. sham).

There were two groups – active and sham NITESGON – with 15 participants each. Active NITESGON consisted of a ramp-up time of 5-seconds, followed by a constant current of 1.5 mA (current density 0.4285 A/m^2) for 250-seconds (5-seconds x 50 word pairs) during each of the 3 study periods, and ended with a ramp-down time of 5-seconds, resulting in a total stimulation time of 12.5-minutes (i.e., 250-seconds × 3 study periods). For the sham NITESGON group, the current intensity was ramped-up to 1.5 mA over 5-seconds, followed by a constant current of 1.5 mA for 15-seconds, and ended with a ramp-down time of 5-seconds, resulting in a total stimulation time of 45-seconds (i.e., 15-seconds x 3 study periods) (refer to figure 2.1B for stimulation paradigm). The rationale behind the sham procedure was to mimic the transient skin sensation at the beginning of active NITESGON without producing any conditioning effects on the brain. All participants received NITESGON for an additional 15-seconds prior to the first study period to help participants habituate to the sensation and ensure they were comfortable with the sensation.

Figure 2.1

Schematic of Study Design During the Word Association Task



Note. The above schematic provides an illustrative depiction of the **(A)** experimental task design whereby participants partook in a total of 3 alternating study and test periods during Day 1 learning and returned on Day 7 and Day 28 for recall testing. **(B)** The stimulation paradigm provides the parameters used for active and sham NITESGON delivered during the study periods in Blocks 1-3 on Day 1 learning.

2.2.4.3 Salivary α-Amylase and Cortisol.

Participants' saliva was collected four times during the experiment: before stimulation, immediately after stimulation, 7-days after stimulation, and 28-days after stimulation. To collect saliva, participants were requested to gently tip their head backward and collect saliva on the floor of their mouth. When ready, participants passively drooled into the collection aid mouthpiece provided by Salimetrics laboratory (Salimetrics, LLC, USA; https://salimetrics.com). The participants were requested to collect 2 ml of saliva in one straight flow and avoid breaks between drool as much as

possible. The length of time to collect 2 ml of saliva was noted, and the timer was started only when participants began to passively drool into the tube. All saliva samples were stored in 2 ml cryovials and immediately stored in an -80° C laboratory freezer. Upon completion of the collection procedures, a total of 120 saliva samples were packed in dry ice and sent to the Salimetrics laboratory for analysis. The Salimetrics analysis protocols and determination techniques for the targeted biomarkers are described below.

The flow rate was calculated using the formula given by Salimetrics: $Flow\ rate\ \left(\frac{ml}{min}\right) = \frac{amount\ of\ saliva\ (ml)}{time\ (min)}$. This flow rate correction was used to calculate the concentration of sAA, which was used as a biomarker for NA as it provided a non-invasive yet valid indicator of central sympathetic nervous system activation (Nater & Rohleder, 2009). Furthermore, the tubes were also weighed; the weight of the saliva was determined as the difference between the weights of the full tube and the empty tube. The amount of sAA in the sample is directly proportional to the increase in absorbance at 405 nm. Ten microliters (μL) of the sample were diluted and well mixed. Eight μL of the diluted samples were then pipetted into individual wells of a 96-well microtiter plate. A volume of 320 μL of preheated chromogenic substrate solution was added to each well, and the plate was rotated at 500 to 600 RPM at 37° C for 3-minutes. The optical density of the sample was determined at the 1-minute mark and again at the 3-minute mark.

To determine cortisol levels, a known proxy for corticotrophin-releasing factor neurons, a highly sensitive enzyme immunoassay was used. This specific test uses 25 μL of saliva per determination and has a lower sensitivity of 0.007 μg/dL, a standard curve ranges from 0.012 to 3.0 μg/dL, an average intra-assay coefficient of variation of 4.6% and an average inter-assay coefficient of variation of 5.9%. Given that the LC receives corticotrophin-releasing factor neurons from the hypothalamus in response to

environmental stressors (Takai et al., 2004), salivary cortisol was utilized as a control measure for stress-related LC-activation.

2.2.4.4 tES Exit Questionnaire and Stimulation Blinding.

All participants were asked to complete the tES exit questionnaire that was taken from the Brunoni et al. study (Brunoni et al., 2011) after they had concluded the NITESGON procedure in order to assess for possible side effects of NITESGON. The tES exit questionnaire study and measures symptoms covering headache, neck pain, scalp pain, tingling, itching, sleepiness, trouble concentrating, and acute mood changes on a 4-point scale, ranging from 1 = 'absent' to 4 = 'severe.' If the participant had indicated any symptoms present, they were then asked to specify on a 5-point scale, ranging from 1 = 'none' to 5 = 'definitely,' to determine if they thought this symptom was related to the application of tES. Lastly, to determine if the stimulation was well blinded, the participants were asked to guess if they thought they were placed in the "active" or "non-active" group.

2.2.5 Procedures

Eligible participants were scheduled for three visits to complete the study. Participants were randomly assigned to one of two groups during the study period on Day 1: 15 participants received active NITESGON, and 15 participants received sham NITESGON. Day 1 consisted of pre-assessments (i.e., a battery of tests covering mood, executive function, and memory) and pre-NITESGON saliva intake, followed by the word association task and the administration of NITESGON. The researcher responsible for controlling the NITESGON device was not involved in instructing the participant; instead, this was performed by a second researcher who was blind to the stimulation

protocol. Day 1 concluded with a post-NITESGON questionnaire (i.e., tES exit questionnaire) and saliva intake. Participants were asked to refrain from studying or searching for the learned word pairs throughout the week. Participants returned 7- and 28-days after their first visit for memory testing to measure possible long-term effects on associative memory performance but did not receive NITESGON, nor were they able to review word pairs. A saliva intake was also collected from each participant before taking part in the recall memory testing on Day 7 and Day 28. A third researcher who was not responsible for the task or NITESGON on Day 1 conducted the visit on Day 7 and Day 28.

2.2.6 Statistical Analysis

2.2.6.1 Participants.

Chi-square (X^2) tests were calculated to compare differences between the active and sham NITESGON groups for sex, Hispanic background, race, and handedness. In addition, one-way analysis of variance (ANOVA) were computed to detect differences between the active and sham NITESGON groups for BDI, BAI, MMSE, CVLT-II, TMT-A, TMT-B, WAIS-IV Digit Span, WAIS-IV Coding, COWAT, or the D-KEFS.

2.2.6.2 Word Association Task.

Two measurements were taken from the word association task to evaluate the effects of NITESGON: (1) the cumulative percentage of learned Swahili-English word pairs on Day 1, and (2) the percentage of correctly recalled Swahili-English word pairs on Day 7.

A repeated measures ANOVA was utilized to compare the effect of NITESGON (active NITESGON vs. sham NITESGON) on the percentage of correctly recalled Swahili-English word pairs on Day 1, Day 7, and Day 28. In this analysis, group served

as the between-subjects variable, and the percentage of correctly recalled word pairs on Day 1, Day 7, and Day 28 served as the within-subjects variable. If significance was obtained, a simple contrast analysis was applied to detect the effect of correctly recalled words at Day 7 and Day 28, corrected for the number of words learned on Day 1. Additionally, a one-way ANOVA was run to assess the percentage of forgotten words from Day 7 to Day 28, whereby groups were the between-subjects variable and the subtraction of forgotten words was used as the dependent variable.

To compare the effect of NITESGON (active NITESGON vs. sham NITESGON) on the speed of processing per session, two repeated measures ANOVA were run with group as the between-subjects variable and processing speed during block 1, block 2, and block 3 (on Day 1) as well as processing speed on Day 1 (averaged across blocks), Day 7, and Day 28 as the within-subjects variable. If significance was obtained, a simple contrast analysis was applied to detect the effect of processing speed, respectively, for active versus sham NITESGON groups.

2.2.6.3 Salivary α-Amylase and Cortisol.

Salivary α-amylase levels were measured by using the saliva collected via the passive drool method. A multivariate analysis of variance (MANOVA) was utilized to test the effect of NITESGON on the LC-NA pathway. In this analysis, group (active NITESGON vs. sham NITESGON) served as the between-subjects variable, and sAA levels post-stimulation on Day 1, Day 7, and Day 28 served as the within-subjects variable, with sAA levels at baseline as the covariate. If significance was obtained, three separate univariate ANOVAs were applied with group as the between-subjects variable and sAA levels immediately post-stimulation on Day 1, Day 7, and Day 28 as the between-subjects variables, respectively. A similar analysis was applied for cortisol concentration levels.

2.2.6.4 tES Exit Questionnaire and Stimulation Blinding.

A MANOVA was used to assess the differences between scores on side effects marked on the tES exit questionnaire between active and sham NITESGON groups, with group as the between-subjects variable and the different side effects as the dependent variable.

A χ^2 analysis was run to assess if participants in the two stimulation groups were well blinded during the stimulation session (i.e., what stimulation participants received compared to what participants expected). Statistical analysis was performed using IBM SPSS (version 26) software.

2.3 Results

2.3.1 Participants

Thirty participants between the ages of 55 and 70 years old were enrolled. No differences were obtained for sex ($\chi^2(1, N = 30) = 1.53$, $p = .22$), Hispanic background ($\chi^2(1, N = 30) = 0.00$, $p = 1.00$), race ($\chi^2(3, N = 30) = 4.00$, $p = .26$) or handedness ($\chi^2(1, N = 30) = 0.00$, $p = 1.00$) between the active and sham NITESGON groups. Furthermore, no difference was demonstrated for age ($F_{1,28} = 0.004$, $p = .95$), years of education ($F_{1,28} = 0.30$, $p = .59$), BDI ($F_{1,28} = 0.24$, $p = .63$), BAI ($F_{1,28} = 0.01$, $p = .93$), MMSE ($F_{1,28} = 0.51$, $p = .48$), CVLT ($F_{1,28} = 2.19$, $p = .15$), TMT-A ($F_{1,28} = 1.01$, $p = .32$), TMT-B ($F_{1,28} < 0.001$, $p = .99$), WAIS-Digit Span ($F_{1,28} = 0.25$, $p = .62$), WAIS-Coding ($F_{1,28} = 2.02$, $p = .17$), COWAT ($F_{1,28} = 0.43$, $p = .52$), and D-KEFS ($F_{1,28} = 1.10$, $p = .30$) between the active and sham NITESGON groups. Refer to Figure 1 in Section 9.1 of the Appendix for participant overview.

2.3.2 Word Association Task

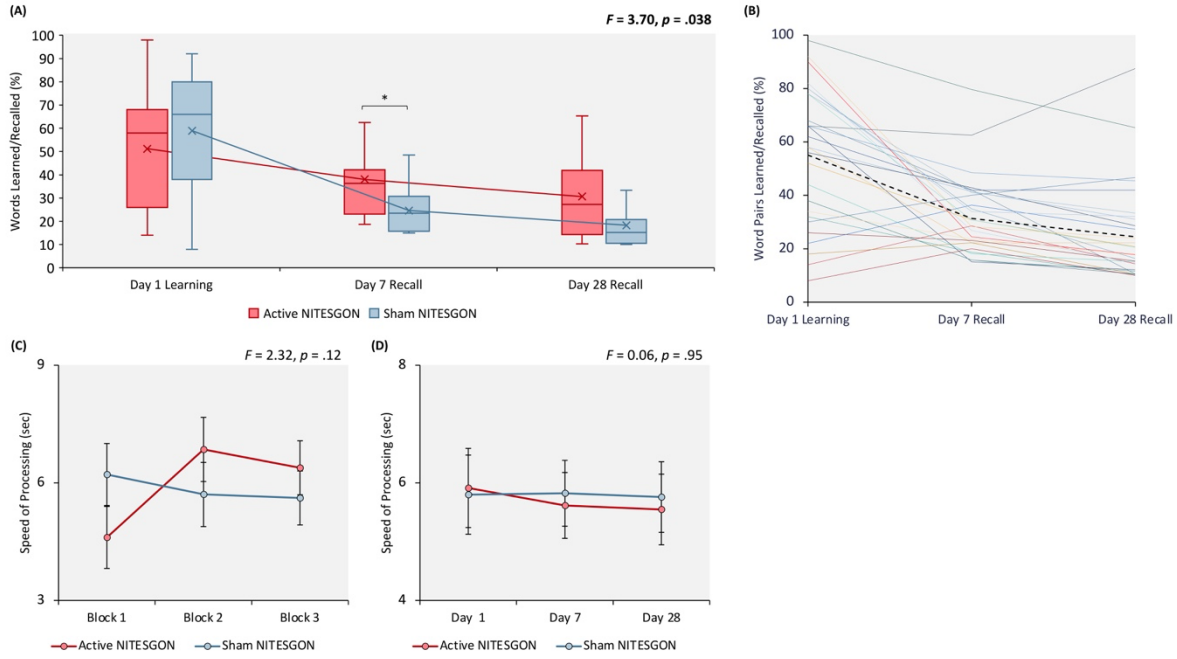
During the word association task on Day 1, participants could be presented with a maximum of 150 trials. On average, participants in the active NITESGON group underwent 127 trials (*SD* = 14, min = 97, max = 147), whereas participants in the sham NITESGON group underwent an average of 123 trials (*SD* = 13, min = 105, max = 145). No participant learned all 50-word pairs across the three study periods.

A repeated-measures ANOVA with NITESGON groups (active vs. sham) as the between-subjects variable and the percentage of correctly recalled word pairs on Day 1, Day 7, and Day 28 as the within-subjects variable detected a statistically significant interaction effect ($F_{2,27} = 3.70$, $p = .038$, $\eta^2 = .22$; see figure 2.2A). A simple contrast analysis revealed no significant effect on Day 1 ($F_{1,28} = 0.70$, $p = .41$) between the active (*M* = 51.20, *SD* = 25.88; 25.6 words out of 50) and sham NITESGON groups (*M* = 58.93, *SD* = 24.88; 29.47 words out of 50). However, at Day 7, a significant effect was obtained ($F_{1,28} = 7.49$, $p = .01$, $\eta^2 = .21$) indicating that the active NITESGON group correctly recalled more word pairs (*M* = 38.04, *SD* = 16.06, 19.02 words out of 50) than the sham NITESGON group (*M* = 24.65, *SD* = 10.04, 12.33 words out of 50). On Day 28, no significant effect was obtained ($F_{1,28} = 3.93$, $p = .057$, $\eta^2 = .12$), indicating that the active (*M* = 30.66, *SD* = 22.10, 15.33 words out of 50) and sham NITESGON groups (*M* = 18.26, *SD* = 9.90, 9.13 words out of 50) did not differ in the percentage of correctly recalled word pairs. However, given the medium-to-large effect size, this null finding can be interpreted with caution and suggest that there was low statistical power that may have resulted in a high risk of type II error. Moreover, no significant difference was found between the active NITESGON group (*M* = 7.38, *SD* = 12.74) and sham NITESGON group (*M* = 6.39, *SD* = 2.98) in terms of how many word pairs participants forgot between Day 7 and Day 28 ($F_{1,28} = 0.09$, $p = .77$). NITESGON did not have an effect on speed of

processing when comparing active and sham NITESGON groups on Day 1 learning within each block ($F_{2,27} = 2.32$, $p = .12$; see figure 2.2C) or overall, on Day 1, Day 7, and Day 28 ($F_{2,27} = 0.06$, $p = .95$; see figure 2.2D).

Figure 2.2

Behavior Effects of NITESGON



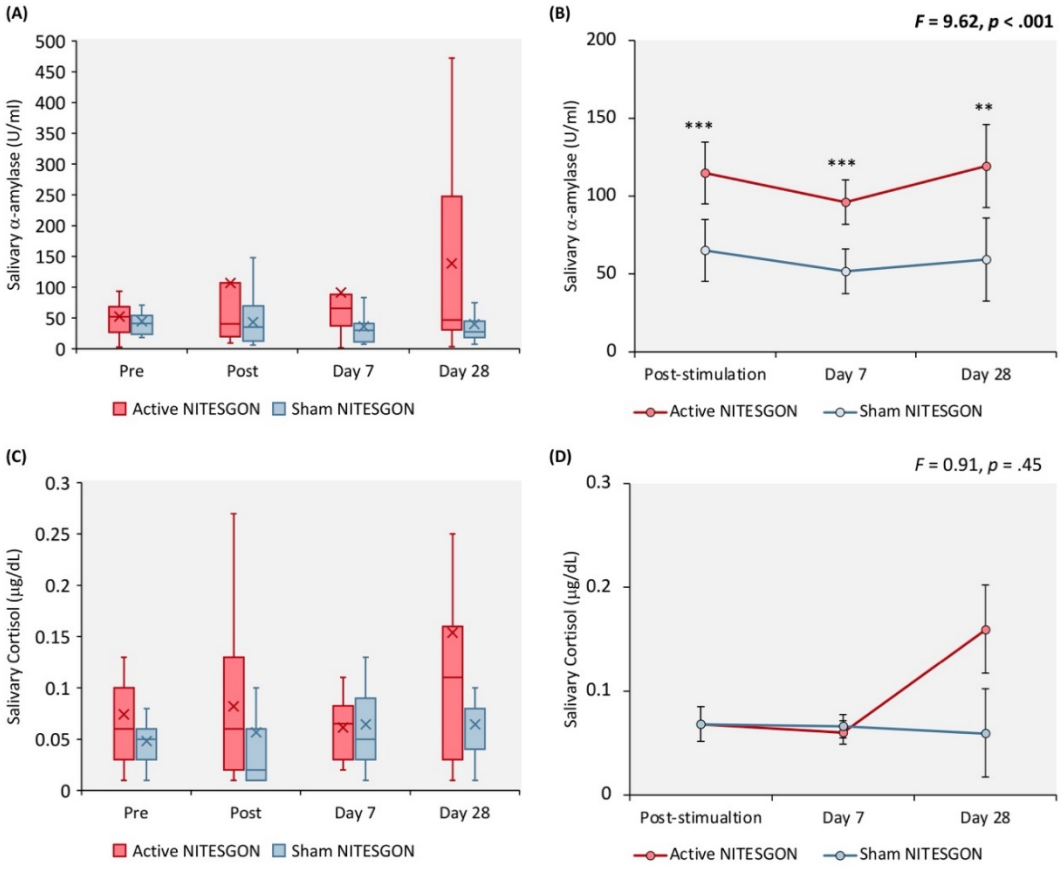
Note. **(A)** A significant effect was detected between the active and sham NITESGON groups for correctly recalled word pairs on Day 7. **(B)** Visual representation of performance of all participants across the three time points. **(C, D)** No significant difference was detected between the active and sham NITESGON groups for processing speed on Day 1 within each block or overall, on Day 1, Day 7, and Day 28. Error bars represent standard errors of the mean. Asterisks represent significant differences (* $p <$ .05).

2.3.3 Salivary α-Amylase and Cortisol

A MANOVA was performed to compare the effect of NITESGON on sAA concentration levels post-stimulation on Day 1, Day 7, and Day 28, corrected for baseline. Results yielded a statistically significant effect ($F_{3,25} = 9.62$, $p < .001$, $\eta^2 = .53$ see figure 2.3A). Separate univariate ANOVAs revealed a significant increase in sAA concentration immediately post-stimulation on Day 1 ($F_{1,27} = 19.14$, $p < .001$, $\eta^2 = .41$); Day 7 ($F_{1,27} = 18.56$, $p < .001$, $\eta^2 = .41$); and Day 28 ($F_{1,27} = 8.44$, $p = .007$, $\eta^2 = .23$) for the active NITESGON group in comparison to the sham NITESGON group. No effect was obtained for cortisol levels post-stimulation on Day 1, Day 7, and Day 28, corrected for baseline ($F_{3,25} = 0.91$, $p = .45$; see figure 2.3B).

Figure 2.3

Salivary α-Amylase & Cortisol



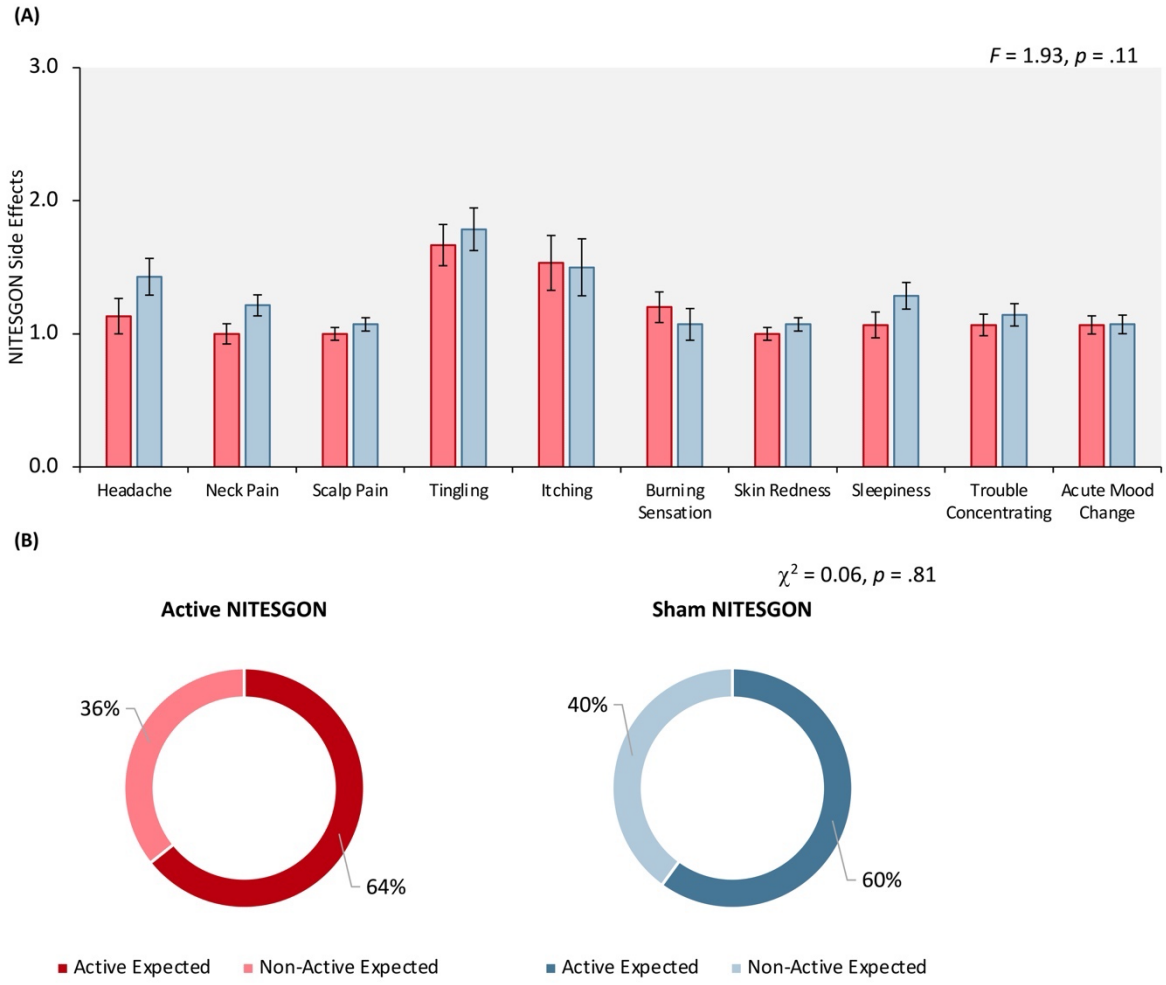
Note. **(A)** Distribution of sAA taken pre-stimulation, post-stimulation, on Day 7, and Day 28. **(B)** A significant effect was detected between the active and sham NITESGON groups for sAA concentration levels post-stimulation on Day 1, Day 7, and Day 28, corrected for baseline. **(C)** Distribution of salivary cortisol taken pre-stimulation, post-stimulation, on Day 7, and Day 28. **(D)** No significant difference was detected between the active and sham NITESGON groups for salivary cortisol concentration levels. Error bars represent standard errors of the mean. Asterisks represent significant differences (** $p < .01$; *** $p < .001$).

2.3.4 tES Exit Questionnaire & Stimulation Blinding

No significant difference was demonstrated between the active and sham NITESGON groups for side effects marked on the tES exit questionnaire ($F_{10,18} = 1.93$, $p = .11$; see figure 2.4A). All stimulation sessions were well tolerated, and no major adverse events were reported throughout the study. Additionally, participants were unable to tell if they were assigned to the active or sham NITESGON group ($\chi^2(1, N = 29) = 0.06$, $p = .81$; see figure 2.4B). In the active NITESGON group, 36% of the participants anticipated perceiving non-active stimulation, while 64% expected active stimulation. For the sham NITESGON group, 40% of the participants anticipated perceiving non-active stimulation, while 60% expected active stimulation.

Figure 2.4

NITESGON Related Side Effects & Blinding



Note. No significant difference was detected between the active and sham NITESGON groups **(A)** in tES related side effects experienced or **(B)** perceived group assignment. Error bars represent standard errors of the mean.

2.4 Discussion

The objective of the present study was to determine if NITESGON utilizing DC to upregulate the LC-NA pathway during an associative memory task would improve memory recall performance in healthy, older adults. This experiment was designed to replicate previous laboratory findings that established NITESGON's ability to enhance

memory performance in healthy young adults (Vanneste et al., 2020) to determine whether future research should continue to investigate NITESGON as a modulator of memory.

Consistent with prior results, the current study revealed that NITESGON can enhance memory in older individuals after one session, with an effect that may last up to 28-days after stimulation. Additionally, NITESGON did not exhibit an online effect during the learning period, given that no difference was observed between the active and sham NITESGON groups; however, a differences did emerge 7- and 28-days after learning the word association task, indicating NITESGON induced a memory effect that occurred offline during memory consolidation. Furthermore, these findings suggest that NITESGON modulates the memory system and aligns with the concept that neural processes underlying memory become consolidated after learning (McGaugh, 2015). Moreover, no changes were seen in speed of processing between the active and sham NITESGON groups during the learning period on Day 1 or during the test period on Day 7 or Day 28, thus validating that the effect of NITESGON is a product of altering the neurobiological mechanisms fundamental for memory processing as opposed to an immediate, general attention/concentration effect. NITESGON inducing enhanced memory performance in older participants is of particular intrigue considering memory decline is associated with increased age. In addition, the word association task participants completed required more mental effort than alternative tasks such as word recognition (Luo & Craik, 2008), indicating that NITESGON was effective on a task that is characterized as more difficult for older persons.

As previously mentioned, it cannot be assumed that tES techniques are universally applicable due to various individual variability factors such as age (Li et al., 2015; Ridding & Ziemann, 2010; Yavari et al., 2018). Therefore, it is noteworthy that

alterations in sAA concentration levels, a known biomarker for NA release denoting LC activation (Nater & Rohleder, 2009), were analogous to prior results, given that they increased immediately after NITESGON application (Vanneste et al., 2020). This suggests that the neural mechanism underlying NITESGON modulation in older individuals corresponds to that of a younger population, such that specific afferent activity can modulate noradrenergic neurons via direct projections from the ON to the NTS (Couto et al., 2006). In response, NTS neurons influence central NA activity, a key contributor of neuroplasticity and memory processes (Hansen, 2017; Sara, 2015), through direct synapses on neurons in the LC to the hippocampus (Couto et al., 2006).

Moreover, the increased sAA concentration levels ensued 7- and 28-days after NITESGON. Although it was assumed sAA concentration levels would increase immediately after NITESGON, the change enduring 7- and 28-days after NITESGON was unexpected. Recent research has demonstrated a generalized effect to other stimuli associated with a specific experience (Noble et al., 2019), as well as how the arousal associated with a context can be modulated using vagus nerve stimulation (Genheimer et al., 2017), comparable to the pathway upon ON activation. Based on these findings, it is possible that NITESGON also generated a more generalized effect related to the context, thus bringing participants back to the context associated with the learning task elevated NA levels, which was in turn reflected by an increase in sAA. Moreover, cortisol levels, a known proxy for corticotrophin-releasing factor neurons, were utilized as a control measure for stress-related LC-activation. Given that the LC receives corticotrophin-releasing factor neurons from the hypothalamus in response to environmental stressors (Takai et al., 2004), a rather notable and further corroborating finding was the absence of alterations in cortisol concentration levels, thus suggesting that the memory enhancement

induced via NITESGON was a result of an LC-arousal response to novelty as opposed to stress.

It is well known that the LC-NA system exerts powerful effects on neural processing and is highly responsible for responding to novelty-induced stimuli. Furthermore, recent animal research has emphasized that the LC system distinctly responds to novelty-induced activation via the co-release of dopamine to the hippocampus, a process required for memory consolidation (Duszkiewicz et al., 2019). These LC hippocampal projections have a significant influence on synaptic plasticity and have been found to be more abundant than that of the ventral tegmental area (VTA) (Kempadoo et al., 2016; McNamara et al., 2014; Takeuchi et al., 2016), signifying the LC provides more significant contributions in memory persistence than previously thought. Taking this into account, it is plausible that the upregulation of the LC via NITESGON during the word association task led to the present findings. Additionally, these findings indicate that the LC-NA pathway is involved in NITESGON's memory modulation effect in both younger and older populations, which coincides with the concept of the LC-NA system operating at the synaptic, cellular, and network levels in addition to playing an essential role in facilitating cognitive functions such as learning and memory (Sara, 2009b, 2015).

In conclusion, the results of this study provide evidence that NITESGON can enhance associative memory performance in healthy, older individuals up to 28-days after one session via stimulation of the LC-NA pathway. Taken together with prior laboratory findings, the current research has established that NITESGON's ability to modulate memory performance applies to healthy young individuals and is effective in healthy older adults. Furthermore, a strength of the current study was its consistency in collecting data on older individuals during early morning and early afternoon hours, considering this limited the variability in age-related cognitive deficits due to these testing times

aligning with the common chronotype of older adults (Maylor & Badham, 2018). Moreover, no significant or long-lasting adverse side effects were observed during stimulation, thus suggesting the utilization of NITESGON holds merit as a potential intervention technique to be used in clinical settings. Given the increased longevity of the human lifespan, age-related memory decline, and the rise in the prevalence of neurocognitive disorders, the underlying mechanism of NITESGON should be further investigated, considering it may possess the ability to alter neuroplasticity and potentially enhance cognitive functions and postpone memory decline.

3 The Peripheral Effect of NITESGON on Strengthening Memories

3.1 Introduction

Research on enhancing and preserving human memory has substantially increased in the last few decades, due in large part to the prevalence and inexorable condition of AD ("2021 Alzheimer's disease facts and figures," 2021). Early behavioral indicators of AD include a decline in an individual's ability to retain learned information and remember events, situations, and objects, alluding to synaptic connections losing strength as AD emerges (Reza-Zaldivar et al., 2020). As a result, recent investigations have begun to assess the prospective clinical significance of therapeutic non-invasive brain stimulation techniques to modify neuroplasticity and upregulate neuronal excitability in different neurological conditions including memory deficits (Lefaucheur et al., 2017). Despite gaining substantial consideration as a potential therapy, professionals remain indecisive whether non-invasive brain stimulation is delivered directly despite previous rodent and human studies providing evidence that observed effects were primarily induced via peripheral nerve stimulation (Asamoah et al., 2019; Liu et al., 2018; Voroslakos et al., 2018). In addition, original work by our laboratory demonstrated that NITESGON using DC to peripherally target the LC-NA system during learning induces improvements in memory recall in younger (18–25 years) and older (>55 years) adults up to 28-days after learning (Luckey et al., 2020; Vanneste et al., 2020). Notably, NITESGON yielded an LTM effect, but did not trigger an immediate effect on learning, suggesting that the effect is generated offline during the consolidation of memories as opposed to during learning or encoding of new memories (Luckey et al., 2020; Vanneste et al., 2020).

These findings are intriguing, given that most episodic-like memories that are formed are forgotten, while others are retained for longer periods of time and are subject to memory stabilization (Squire, 1992; Squire et al., 1992; Tonegawa et al., 2018). This is

referred to as synaptic consolidation, a process which stabilizes new information into memory over a timespan of minutes to hours (Squire et al., 2015). To illustrate the neurobiological account of synaptic consolidation, Frey and Morris introduced a plasticity model known as the synaptic tag-and-capture (STC) hypothesis that proposed a cellular mechanism explaining how early-LTP can transform into late-LTP resulting in a more consolidated memory trace (Frey & Morris, 1997; Moncada et al., 2015; Morris & Frey, 1997). More specifically, new experiences or memories resulting in early-LTP set a tag on weak, unstable memory traces which in turn are capable of capturing plasticity-related proteins formed by a novel experience which are required for transforming early- to late-LTP. An interesting phenomenon regarding the STC mechanism is that the strength of synaptic plasticity is dependent on the stimuli that sets the tag as well as events that occur prior to or following the stimuli (Frey & Morris, 1997; Moncada et al., 2015; Morris & Frey, 1997).

STC has since been translated into a learning and memory paradigm referred to as BT (Moncada et al., 2015; Viola et al., 2014). BT proposes weak training that typically generates short-term memory (STM) can utilize the tag and capture process to consolidate into a stabilized LTM when a weak event is preceded or followed by a strong event within a limited time window. It has been indicated that these three aspects must be accounted for to portray BT in experimental settings (Moncada et al., 2015; Viola et al., 2014). By doing so, animal models have provided exceptional and consistent evidence of BT by demonstrating how rodents' who undergo a weak learning or training task (i.e., inhibitory avoidance, spatial object recognition, contextual fear conditioning) associated with a novel task (i.e., exploration of an open field) before or after the weak task resulted in long-lasting memory formation (Ballarini et al., 2009; Cassini et al., 2013; Moncada et al., 2011; Moncada & Viola, 2007).

Although evidence of BT in humans is limited, findings have demonstrated that novelty, acting as a strong event, enables the promotion or improvement of LTM formation of associated weak events when provided within a restricted time window – highly analogous to the characteristics of STC (Ballarini et al., 2013; Dunsmoor et al., 2015; Kalbe & Schwabe, 2021; Oyarzun et al., 2016; Patil et al., 2017; Ramirez Butavand et al., 2020). A commonality between the aforementioned rodent and human studies is the requirement of a novel experience to transform the STM to LTM. Interestingly, the neural mechanism that controls this novelty response is the LC-NA pathway (Takeuchi et al., 2016; Vankov et al., 1995). Moreover, recent research has identified a link between the LC and BT, attributable to the LC's pivotal role during the presentation of a salient or arousing event (i.e., strong stimulus) (Sara, 2009b, 2015) as well as being at the helm of regulating the synthesis of new proteins required for memory consolidation in the hippocampus (Moncada, 2017).

Although BT is yet to be confirmed in humans, given that the LC-NA system is one of the primary neuronal systems responsible for attending to novel stimuli, it is hypothesized that NITESGON upregulating the LC-NA system induces LTM effects by modulating synaptic consolidation in the hippocampus via the mechanism of BT. In order to assess the effects of NITESGON, five experiments were split into two sets of studies: the first two studies were used to confirm which phase of memory effects were observed, and the final three studies were used to confirm BT as the underlying mechanism of NITESGON.

3.2 NITESGON During or Immediately After Encoding and During Retrieval

A primary interest in non-invasive brain stimulation that is currently under speculation is whether effects take place online so that they occur immediately or simultaneously

during stimulation, or if they occur offline, whereby the effects outlast the period of stimulation (Liu et al., 2018, Voroslakos et al., 2018). In the interest of the current debate, Experiment 1 was designed as a double-blind, sham-controlled, randomized parallel-group study to compare the effects of NITESGON on associative memory recall performance when applied either during the study period of a word association task or applied immediately following the memory task. Two measurements were taken from the word association task to evaluate the effects of NITESGON: (1) the cumulative percentage of learned Swahili-English word pairs on Day 1, and (2) the percentage of correctly recalled Swahili-English word pairs on Day 7. This would directly test whether NITESGON plays a more central role during encoding or consolidation. It was hypothesized that participants would be able to establish long-term memories upon modulating the LC both during learning (i.e., encoding), as denoted previously, and immediately after learning (i.e., consolidation).

Additionally, sAA and resting-state EEG were collected pre-and-post-NITESGON to confirm and replicate previous findings (Vanneste et al., 2020). Existing research recognizes sAA as an indirect measure of parasympathetic and sympathetic nervous system activation that can be utilized as a proxy measure of endogenous NA activity (Nater & Rohleder, 2009). Moreover, previous research has established the critical role gamma frequency has in each stage of memory for successful LTM formation, encoding (Jun et al., 2021), consolidation (Kanta et al., 2019), and retrieval (Lin et al., 2019), and that increased gamma power during encoding is a predictor of subsequent memory recall (Osipova et al., 2006; Sederberg et al., 2003). Therefore, it is hypothesized that if NITESGON activates the LC-NA pathway to influence memory formation, then post-NITESGON changes will show an increase in sAA concentration levels and induce electrophysiological changes within the gamma range in the medial temporal cortex.

A separate follow-up study sought to examine the effect of NITESGON during memory retrieval. This study was designed to detect whether NITESGON had an immediate effect during recall and served as a confirmatory study as to whether the effect of NITESGON is specific to the consolidation of information. To do so, Experiment 2 was designed as a double-blind, sham-controlled, randomized parallel-group study to compare the effects of NITESGON when applied during the retrieval phase 7-days after initial learning of the Swahili-English word associations. It was hypothesized that no difference would be seen between the active and sham NITESGON groups. Overall, Experiments 1 and 2 confirm which phase of memory NITESGON's effects occur and lay the groundwork for delineating the mechanism of NITESGON.

3.3 Methods

3.3.1 Power Analysis

G*Power (Faul et al., 2007) was used to perform an a priori power analysis for an omnibus one-way ANOVA. The estimated effect size for the current study was based on prior studies published in *Science Advances* (Vanneste et al., 2020). This experiment utilized two groups (active and sham NITESGON) to investigate the long-term effect of NITESGON on memory performance when NITESGON was administered during a word association task. A significant difference was observed 7-days after initial learning; this study indicated that the best estimate of the true population standardized mean difference was 0.5, meaning that individuals who received active NITESGON will have a higher percentage of words recalled compared to those who received sham NITESGON. This large effect size estimate (partial eta square) was entered into the power analysis with the following input parameters $\alpha = 0.05$ and power (1-β err prob) = 0.8 with two groups. The

power analysis indicated that a total sample size of at least N = 12 was required to detect a difference between the two groups with at least 80% probability.

3.3.2 Participants

All participants in Experiment 1 and 2 were healthy, native-English speaking adults with a similar educational background (i.e., enrolled as undergraduate students). Experiment 1 included 45 adults (21 males, 24 females; mean age of 19.82 years, *SD* = 2.53 years), and Experiment 2 included 20 adults (12 males, 8 females; mean age of 21.03 years, *SD* = 2.63 years). Participants had normal to corrected vision, and all had the maximum score on the Mini-Mental State Examination. Participants were screened (e.g., tES contraindications, neurological impairments, not participated in a tES study) prior to enrolling in the study. None of the participants had a history of major psychiatric or neurological disorders or any tES contraindications, including previous history of brain injuries or epileptic insults, cardiovascular abnormalities, implanted devices, taking neuropsychiatric medications, prescribed stimulants use, or chronic use of illicit drugs (i.e., marijuana and cocaine).

Participants were excluded from the study if screening discovered they were familiar with Swahili/Arabic language or Swahili culture due to the nature of the stimuli. Furthermore, participants were asked to abstain from alcoholic beverages for 24-hours and caffeinated beverages for 16-hours prior to the EEG recording to avoid alcohol- or caffeine-induced changes in the EEG stream. Additionally, participants were asked to withhold from using any hair styling products the day of the study. To assure the highest levels of accuracy of saliva collection, participants were asked to refrain from the following products or activities for the associated time window prior to saliva collection: dental work for at least 48-hours, main meals for 60-minutes, brushing their teeth for 45-

minutes, as well as water or rinsing their mouth for 10-minutes in order to avoid any risk of lowering pH levels and influencing bacterial growth. Participants were also asked to refrain from taking any nonapproved prescription drugs, steroidal/anti-inflammatory drugs and were also asked to avoid foods high in sugar content or acidity, and nicotine consumption. Lastly, if participants were scheduled for a study in the afternoon, they were requested to avoid taking a nap during the day to account for the amylase awakening response (Ali & Nater, 2020). Salivary alpha-amylase has been shown to have a distinct diurnal profile whereby sAA levels are low within 30-minutes of awakening and rise throughout the day (Nater et al., 2007).

Experiments 1 and 2 were in accordance with the ethical standards of the Helsinki declaration (1964). All participants provided written informed consent.

3.3.3 Materials

3.3.3.1 Word Association Memory Task.

For Experiments 1 and 2, associative memory performance was measured using a computerized Swahili-English verbal paired-associative learning task. This task was adapted from a well-established study design published in *Science* (Karpicke & Roediger, 2008). The 75 Swahili-English word pairs were taken from the Nelson and Dunlosky study (Nelson & Dunlosky, 1994), excluding the word pair Rafiki-friend, as this word is the name of a character in The Lion King and therefore could have been greatly familiar to participants. The task was programmed in Visual Studio software using C# and shown on a computer with a ~27-inch screen positioned at eye level.

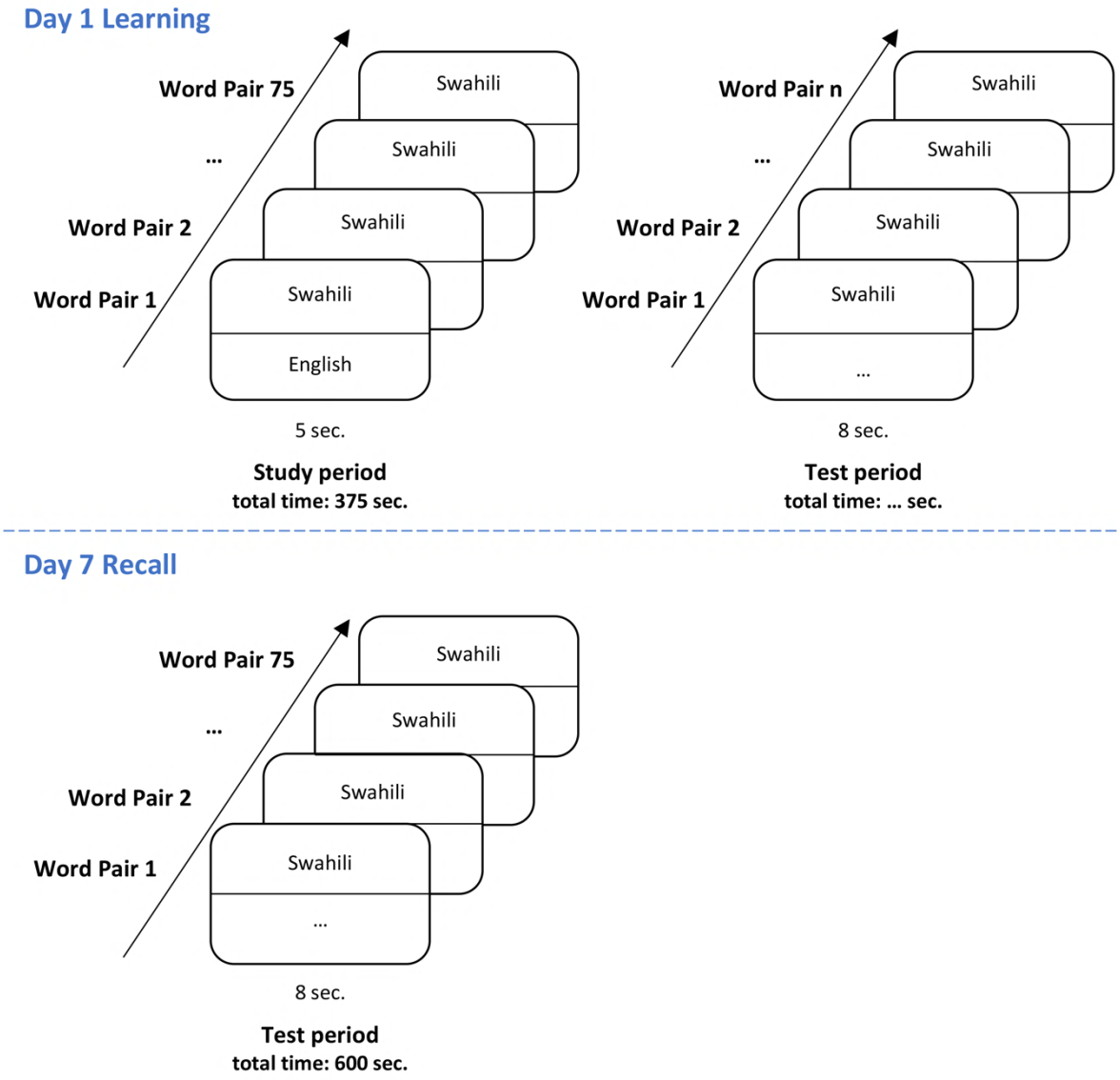
Participants had eight alternating study and test periods to learn the list of 75 Swahili-English (e.g., Swahili: tumbili, English: monkey) vocabulary word pairs made up of common day-to-day words. The verbal paired-association memory task was divided into

4 blocks with each block consisting of a study period (S), followed by a 30-second rest period, and a test period (T). Participants studied and were tested on the exhaustive list of 75 word pairs in block 1. Whereas in blocks 2 through 4, the comprehensive list of 75 word pairs were studied in each study period, but only items that had not yet been recalled were tested in the test periods (denoted SDT_N, where T_N indicates that only the non-recalled pairs were repeatedly tested). Therefore, in blocks 2 through 4, the number of word pairs tested diminished across the blocks and varied according to the testing period. This paradigm was used to ensure that all participants would avoid a ceiling effect (Karpicke & Roediger, 2008).

During the study period, each word pair (black words on white background) was presented one below the other in the middle of the screen for 5-seconds to provide adequate time for encoding. Participants were instructed to learn as many word pairs as they could, so they may recall the English word when given the Swahili word. During the cued-recall test period, participants were instructed to type in the correct English translation of the Swahili word that was presented for 8-seconds using a computer keyboard. If participants failed to recall a word pair during testing, they were not given any feedback. Once the 8-seconds expired, the computer program would automatically advance to the next Swahili word regardless of whether the participant had entered a response. Participants' responses were recorded by the computer program. The word pairs sequence was randomized between participants, blocks, and periods. Refer to figure 3.1 for experimental task design.

Figure 3.1

Experiment 1 and 2: Schematic of Experimental Task Design



Note. The above schematic provides an illustrative depiction of the experimental task design whereby participants partook in a total of 8 alternating study and test periods during Day 1 learning, and returned on Day 7 for recall testing.

3.3.3.2 NITESGON.

NITESGON utilizing DC was transmitted via a saline-soaked (1.3% saline) pair of synthetic sponges (5 cm x 7 cm) and was delivered by a specially developed, battery-

driven, constant current stimulator with a maximum output of 10 mA (Eldith©; http://www.neuroconn.de). For each participant receiving NITESGON, the anodal electrode was placed over the left C2 nerve dermatome and the cathodal electrode was placed over the right C2 nerve dermatome. To maintain consistency across all participants, research assistants were trained to map out the placement according to the length of the participant's head.

To minimize skin sensations and to acclimate participants to the stimulation types, the current intensity was ramped-up (gradually increasing) until it reached its programmed maximum output (1.5 mA). After stimulating for the desired duration per the group (active or sham), the current was ramped-down (gradually decreased) denoting the end of the stimulation. The impedance under each electrode was maintained under 10 kΩ. The timing of NITESGON application varied amongst experiments as well as the respective group participants were randomly assigned to (refer to figure 3.2 for the breakdown of NITESGON application). The ramp-up, ramp-down and stimulation times were different depending on condition (active vs. sham) and experimental needs.

There were three groups in Experiment 1 – *group 1:* active NITESGON during learning (i.e., study periods of the word association task) and sham NITESGON immediately after learning; *group 2:* sham NITESGON during learning and active NITESGON immediately after learning during the memory consolidation period, and *group 3:* sham NITESGON both during and after learning of the word association task – with 15 participants each. Active NITESGON during learning consisted of the following parameters: a 30-second ramp-up period, followed by a constant current of 1.5 mA (current density 0.4285 A/m^2) for 375-seconds (5-seconds x 75 word pairs) during each of the 4 study periods, and ended with a ramp-down period of 30-seconds, resulting in a total stimulation time of 25-minutes (i.e., 375-seconds × 4 study periods). Sham

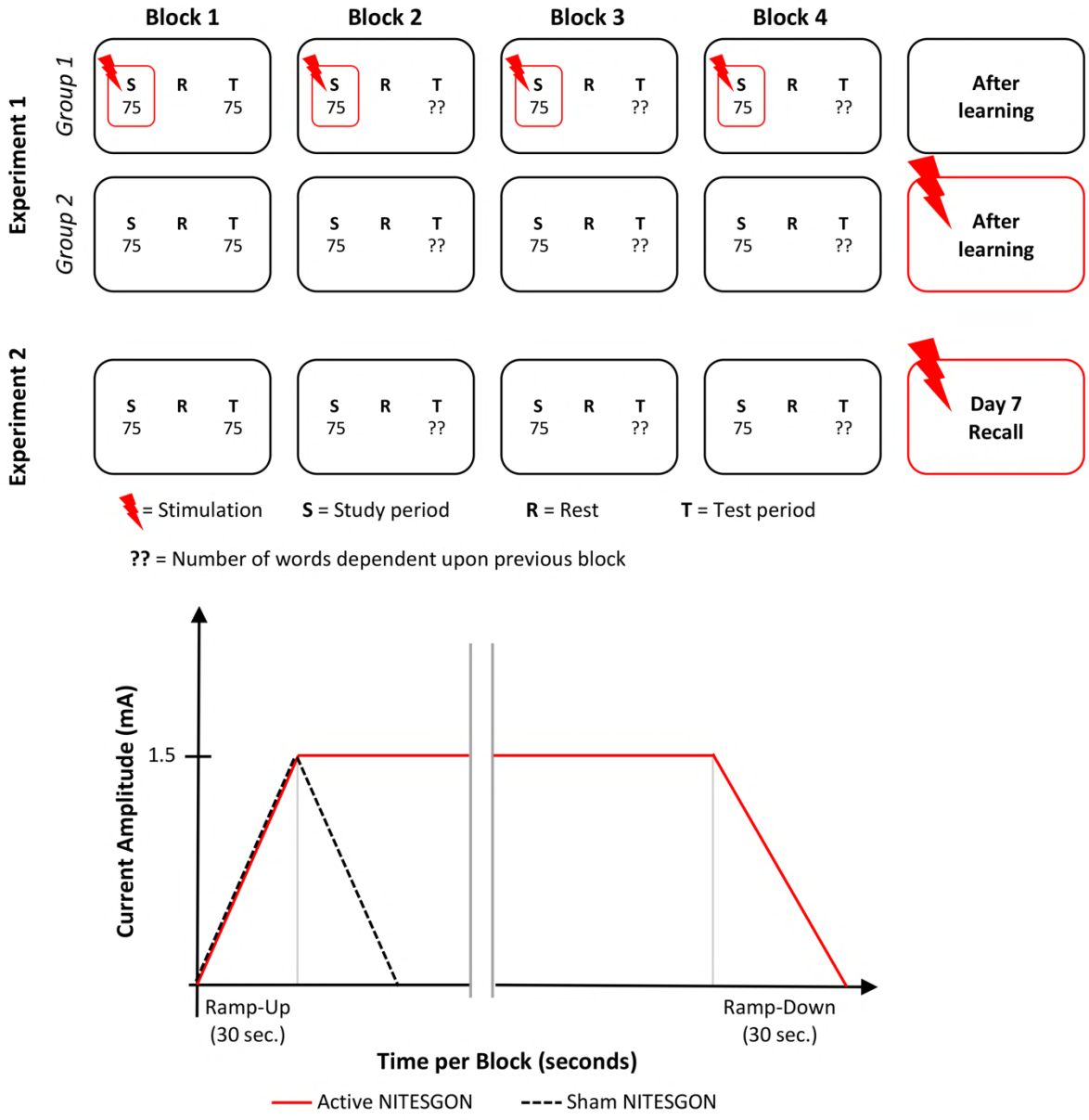
NITESGON delivered immediately after learning consisted of a 30-second ramp-up and ramp-down period, resulting in 60-seconds of mimicked stimulation. Whereas, sham NITESGON during learning consisted of the same stimulation paradigm however, was delivered and lasted 60-seconds per study period, resulting in a total time of 4-minutes (60-seconds x 4 study periods) of mimicked stimulation. Active NITESGON that was delivered immediately after learning consisted of the following parameters: a 30-second ramp-up period, followed by 25-minutes of constant current stimulation at 1.5 mA, and ended with a 30-second ramp-down period. Lastly, sham NITESGON during and immediately after learning resulted in 5-minutes of mimicked stimulation (i.e., 4-minutes during learning and 1-minute of stimulation immediately after learning).

There were two groups in Experiment 2 – active or sham NITESGON during retrieval – with 10 participants each. Active NITESGON consisted of a ramp-up period of 30-seconds followed by a constant current of 1.5 mA (current density 0.4285 A/m^2) for 600-seconds (10-minutes; 8-seconds x 75 word pairs) during the test period, and ended with a ramp-down period of 30-seconds. For the sham NITESGON group, the current intensity was ramped-up to 1.5 mA over 30-seconds and immediately ramped-down over 30-seconds. Hence, sham NITESGON provided only 60-seconds of mimicked stimulation when delivered during the test period.

The rationale behind the sham procedure for both Experiments 1 and 2 was to emulate the transient skin sensation at the beginning of active NITESGON without producing any conditioning effects on the brain. All participants received NITESGON for an additional 15-seconds prior to the first period as a means to help participants habituate to the sensation and ensure they were comfortable with the sensation.

Figure 3.2

Experiment 1 and 2: Timing of NITESGON Application



Note. Distinctions in the timing of NITESGON application based on experiment and individuals' group are illustrated in the figure above.

3.3.3.3 Resting-state EEG.

In Experiment 1, continuous EEG data was collected from each participant pre- and post-NITESGON procedures. The data was collected using a 64-channel Neuroscan

Synamps2 Quick Cap configured per the International 10-20 placement system with the midline reference located at the vertex and the ground electrode located at AFZ using the Neuroscan Scan 4.5 software (Neuroscan, http://compumedicsneuroscan.com). The impedance on each electrode was maintained at less than 5 kΩ. The data were sampled using the Neuroscan Synamps2 amplifier at 500 Hz with online band-pass filtering at .1–100 Hz.

Eyes-closed recordings (sampling rate = 1 kHz, band passed DC–200 Hz) were obtained in a dark room which was dimly lit with a small lamp with each participant sitting upright in a comfortable chair; data collection lasted approximately 5-minutes. The alertness of participants was checked by monitoring both slowing of the alpha rhythm and appearance of spindles in the EEG stream to prevent possible enhancement of the theta power due to drowsiness during recording (Moazami-Goudarzi et al., 2010). No participants included in the current study showed such EEG changes during measurements.

3.3.3.4 Salivary α-Amylase.

In Experiment 1, saliva was collected twice: once immediately prior to NITESGON stimulation and once immediately after NITESGON stimulation. When the participants were ready to collect saliva, they were requested to gently tip their head backwards and collect saliva on the floor of their mouth and when ready, passively drool into the collection aid mouthpiece provided by Salimetrics laboratory (Salimetrics, LLC, USA; https://salimetrics.com).The participants were requested to collect 2 ml of saliva in one straight flow and to avoid breaks between drool as much as possible. The length of time to collect 2 ml of saliva was noted, and the timer was started only when participants began to passively drool into the tube. All saliva samples were stored in 2 ml cryovials, and

immediately stored in an -80° C laboratory freezer. Upon completion of the collection procedures, all saliva samples were packed in dry ice and sent to the Salimetrics laboratory for analysis. The Salimetrics analysis protocols and determination techniques for the targeted biomarker is described below.

The flow rate was calculated using the formula given by Salimetrics: $Flow\ rate\ \left(\frac{ml}{min}\right) = \frac{amount\ of\ saliva\ (ml)}{time\ (min)}$. This flow rate correction was used in the calculation of concentration of sAA, which was used as a biomarker for NA as it provided a noninvasive yet valid indicator of central sympathetic nervous system activation (Nater & Rohleder, 2009). Furthermore, the tubes were also weighed; the weight of the saliva was determined as the difference between the weights of the full tube and the empty tube. The amount of sAA in the sample is directly proportional to the increase in absorbance at 405 nm. Ten μL of the sample were diluted and well mixed. Eight μL of the diluted samples were then pipetted into individual wells of 96-well microtiter plate. A volume of 320 μL of preheated chromogenic substrate solution was added to each well and the plate was rotated at 500 to 600 RPM at 37° C for 3-minutes. The optical density of the sample was determined at the 1-minute mark and again at the 3-minute mark.

3.3.4 Procedures

Eligible participants were scheduled for two visits to complete the study, with each experiment using a different cohort. In Experiment 1, Day 1 consisted of learning the word association task and the administration of NITESGON. Participants were randomly assigned to one of three groups during the study period. The researcher responsible for controlling the NITESGON device was not involved in instructing the participant; instead, this was performed by a second researcher who was blind to the stimulation protocol. Resting-state EEG (rsEEG) and saliva were collected twice for all participants,

once immediately before and once immediately after the NITESGON application. Participants were asked to refrain from studying or searching for the learned word pairs throughout the week. Participants returned 7-days after initial learning for memory testing; however, they did not receive NITESGON, nor were they able to review word pairs. A third researcher who was not responsible for the task or NITESGON on Day 1 conducted the visit on Day 7.

In Experiment 2, Day 1 consisted of learning the word association task with no stimulation. Participants were asked to refrain from studying or searching for the learned word pairs throughout the week. Participants returned 7-days after initial learning for memory testing and were randomly assigned to receive either active or sham NITESGON during the recall test period (i.e., retrieval phase). A second researcher who was not responsible for the task on Day 1 conducted the visit on Day 7.

3.3.5 EEG Preprocessing

For the EEG preprocessing, the data were resampled to 128 Hz, band-pass filtered (Finite Impulse Response filter) to 2–44 Hz, and re-referenced to the average reference using EEGLAB (Delorme & Makeig, 2004). The EEG data were then plotted for a careful inspection of artifacts. All episodic artifacts suggestive of eye blinks, eye movements, jaw tension, teeth clenching, or body movements were manually removed from the EEG stream. In addition, an independent component analysis was conducted to verify further whether all artifacts were excluded.

3.3.6 EEG Source Localization

Standardized low resolution brain electromagnetic tomography (sLORETA) was used to estimate the intracerebral electrical sources that generated the scalp-recorded activity

in each of the gamma frequency bands (30.5– 44 Hz) (Pascual-Marqui, 2002). SLORETA computes neuronal activity as current density (A/m^2) without assuming a predefined number of active sources. The sLORETA solution space consists of 6,239 voxels (voxel size: 5 × 5 × 5 mm) and is restricted to cortical gray matter and hippocampi, as defined by the digitized Montreal Neurological Institute (MNI) 152 template (Fuchs et al., 2002). Scalp electrode coordinates on the MNI brain are derived from the international 10–20 system (Jurcak et al., 2007).

The tomography of sLORETA has received considerable validation from studies combining sLORETA with other more established spatial localization methods such as fMRI (Mulert et al., 2004; Vitacco et al., 2002), structural MRI (Worrell et al., 2000), and positron emission tomography (PET) (Dierks et al., 2000; Pizzagalli et al., 2004; Zumsteg et al., 2005). Further sLORETA validation is based on accepting that the localization findings obtained from invasive, implanted depth electrodes, of which there are several studies in epilepsy (Zumsteg, Lozano, & Wennberg, 2006; Zumsteg, Lozano, Wieser, et al., 2006) and cognitive ERPs (Volpe et al., 2007) as ground truth.

3.3.7 Statistical Analysis

3.3.7.1 Word Association Task.

Two measurements were taken from the word association task to evaluate the effects of NITESGON: (1) the cumulative percentage of learned Swahili-English word pairs on Day 1, and (2) the percentage of correctly recalled Swahili-English word pairs on Day 7.

On Day 1, a Kruskal-Wallis H test was utilized to compare the cumulative percentage of learned word pairs (i.e., after Block 4) between the three stimulation groups (*group 1*: active NITESGON during learning and sham NITESGON after learning vs. *group 2:* sham NITESGON during learning and active NITESGON after learning vs. *group 3:*

sham NITESGON during and after learning). In this analysis, group served as the between-subjects variable, and the cumulative learning percentage served as the dependent variable.

To assess for possible long-term memory effects on Day 7, a Kruskal-Wallis H test was utilized to compare the percentage of correctly recalled word pairs (corrected for how many words they learned on Day 1) between the three stimulation groups. In this analysis, group served as the between-subjects variable, and the percentage of correctly recalled word pairs served as the dependent variable. For both analyses, if significance was obtained, post hoc Mann-Whitney tests were run to detect group differences. The Holm-Bonferroni method was applied to correct for multiple comparisons.

3.3.7.2 Salivary α-Amylase.

Salivary α-amylase levels were measured by using the saliva collected via the passive drool method. A repeated measures ANOVA was utilized to test the effect of NITESGON on the LC-NA pathway. In this analysis, group (*group 1*: active NITESGON during learning and sham NITESGON after learning vs. *group 2:* sham NITESGON during learning and active NITESGON after learning vs. *group 3:* sham NITESGON during and after learning) served as the between-subjects variable and sAA concentration levels (pre- and post-NITESGON) served as the within-subjects variable. If significance was obtained, a simple contrast analysis was applied to compare the different conditions using a Bonferroni correction.

A Pearson correlation coefficient was computed to assess the linear relationship between the difference in sAA concentration levels (post-pre) and the percentage of correctly recalled Swahili-English word pairs on Day 7.

3.3.7.3 EEG – Whole Brain Analysis.

A whole brain analysis was used to compare gamma activity pre- and post-NITESGON. These activity changes were then correlated with the percentage of correctly recalled Swahili-English word pairs on Day 7 using a Pearson correlation. Non-parametric statistical analyses of functional sLORETA images (statistical nonparametric mapping) were performed for each contrast employing a t-statistic for paired groups and corrected for multiple comparisons ($p < 0.05$). The significance threshold for all tests was based on a permutation test with 5000 permutations and corrected for multiple comparisons (Nichols & Holmes, 2002).

3.3.8 Experiment 2 Statistical Analysis

3.3.8.1 Word Association Task.

Two measurements were taken from the word association task to evaluate the effects of NITESGON: (1) the cumulative percentage of learned Swahili-English word pairs on Day 1, and (2) the percentage of correctly recalled Swahili-English word pairs on Day 7.

On Day 1, a Kruskal-Wallis H test was utilized to compare the cumulative percentage of learned word pairs (i.e., after Block 4) between the two stimulation groups (active NITESGON vs. sham NITESGON). In this analysis, group served as the between-subjects variable, and the cumulative learning percentage served as the dependent variable.

To assess for possible immediate memory effects during retrieval, a Kruskal-Wallis H test was utilized to compare the percentage of correctly recalled word pairs between the two stimulation groups on Day 7. In this analysis, group served as the between-subjects variable, and the percentage of correctly recalled word pairs served as the dependent variable.

3.4 Results

3.4.1 Experiment 1

During the word association task on Day 1, participants could be presented with a maximum of 300 trials. On average, participants in the active NITESGON during learning group underwent 128 trials (*SD* = 27, min = 83, max = 165), whereas the active NITESGON immediately after learning group underwent an average of 134 trials (*SD* = 22, min = 97, max = 169), and the sham NITESGON group underwent an average of 137 trials (*SD* = 20, min = 105, max = 171). Five participants (3 in the active NITESGON during learning group and 2 in the sham NITESGON group) learned all 75-word pairs across the four study periods.

On Day 1, a Kruskal-Wallis H test was utilized to compare the cumulative percentage of learned word pairs between the three stimulation groups. Results yielded no significant difference (H_2 = 0.44, p = .80; see figure 3.3A) between how many word pairs were learned between the active NITESGON during learning (*Mdn* = 88.00, IQR = 64-96.67; 66 words out of 75), active NITESGON immediately after learning during consolidation, (*Mdn* = 78.67, IQR = 74.67-92.67; 59 words out of 75) and sham NITESGON during and after learning groups (*Mdn* = 82.67, IQR = 65.34-94; 62 words out of 75), thus indicating that NITESGON had no effect during initial learning of the word association task.

To assess for possible long-term memory effects on Day 7, a Kruskal-Wallis H test was utilized to compare the percentage of correctly recalled word pairs between the three stimulation groups. Results indicated a statistically significant difference in memory recall (H_2= 8.15, p = .02, η^2 = .19; see figure 3.3B). Post hoc analyses using Mann-Whitney tests were applied for closer inspection. Results revealed that participants correctly recalled more word pairs when NITESGON was applied either during learning

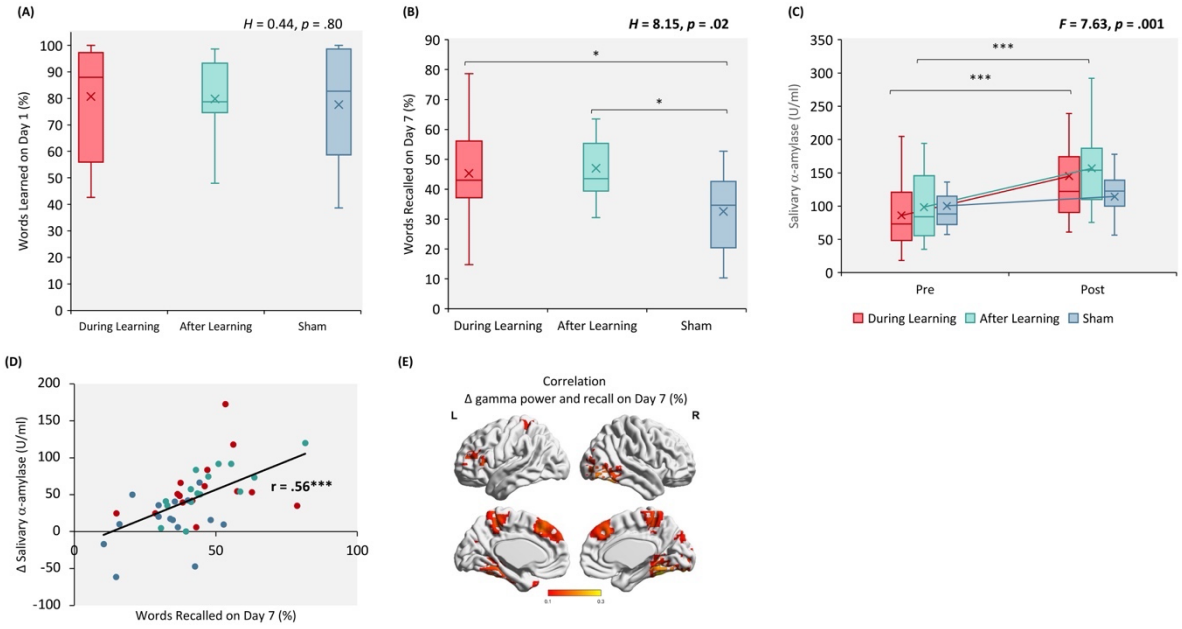
(*Mdn* = 43.06, IQR = 37.32-54.72; 32 words out of 75, $p = .02$) or after learning the task (*Mdn* = 43.48, IQR = 40.23-53.12; 33 words out of 75, $p = .01$) relative to participants who received sham NITESGON both during and immediately after learning the task (*Mdn* = 34.67, IQR = 25.08-41.30; 26 words out of 75). However, no significant difference was attained on Day 7 recall between the groups that received active NITESGON either during learning the word association task or immediately after learning the word association task ($p = 0.78$). Observed increases in memory recall remained significant after Holm-Bonferroni correction.

Moreover, a repeated measures ANOVA was performed to compare the effect of NITESGON on sAA concentration levels pre- and post-stimulation. Results yielded a statistically significant interaction effect ($F_{2,42} = 7.63$, $p = .001$, $\eta^2 = .27$; see figure 3.3C). A simple contrast analysis revealed a significant increase in sAA concentration post-stimulation for the active NITESGON during learning group (pre: $M = 85.94$, $SD = 49.89$ vs. post: $M = 144.99$, $SD = 81.59$; $p < .001$), as well as, for the group who received active NITESGON immediately after learning (pre: $M = 98.37$, $SD = 50.93$ vs. post: $M = 156.71$, $SD = 58.66$; $p < .001$). However, no significant difference was seen between pre- and post-stimulation in the sham NITESGON group (pre: $M = 100.28$, $SD = 41.95$ vs. post: $M = 114.30$, $SD = 41.02$; $p = .14$). Furthermore, the overall difference in sAA (post-pre) concentration levels correlated with memory recall on Day 7 ($r = .56$, $p < .001$; see figure 3.3D). In addition, memory recollection on Day 7 was associated with increased gamma power in the medial temporal cortex as well as the precuneus and dorsal lateral prefrontal cortex immediately after stimulation ($r = .11$, $p = .011$; see figure 3.3E). Together, these results indicate that no online or immediate effect occurred when NITESGON was applied during the learning (i.e., encoding) period of the word association task. However,

NITESGON during or immediately after learning improved memory recall on Day 7, likely mediated by an offline effect occurring during consolidation.

Figure 3.3

Effects of NITESGON During and Immediately After Learning



Note. **(A)** No significant difference was observed in the cumulative percentage of learned word pairs between active and sham NITESGON during or immediately after the study period of the word association task. **(B)** Active NITESGON during or immediately after the word association task improved the percentage of correctly recalled word pairs on Day 7. **(C)** After NITESGON, sAA concentration levels increased for both active NITESGON groups, but not for the sham NITESGON group. **(D)** The percentage of correctly recalled word pairs on Day 7 correlated with the difference in sAA levels during Day 1. **(E)** Improved memory recall on Day 7 was associated with increased activity in the medial temporal lobe as well as anterior and posterior cingulate cortex immediately after NITESGON for the gamma frequency band. Asterisks represent significant differences (* $p < .05$; ** $p < .01$; *** $p < .001$).

3.4.2 Experiment 2

To test whether the effect of NITESGON is specific to the consolidation of information, memory recall was assessed on Day 7 whereby NITESGON was employed during retrieval in Experiment 2. One participant was removed from the sham NITESGON group due to unusually low learning and recall performances; the following behavioral analyses are comprised of 19 participants.

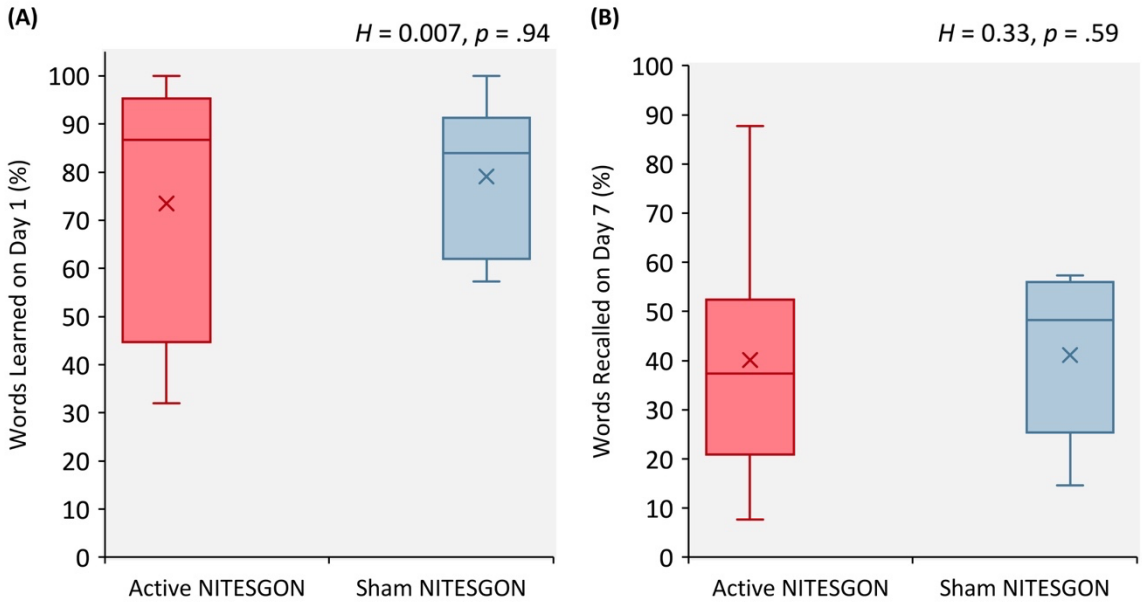
During the word association task on Day 1, participants could be presented with a maximum of 300 trials. On average, participants in the active NITESGON group underwent 133 trials (SD = 26, min = 100, max = 180) and the sham NITESGON group underwent an average of 139 trials (SD = 17, min = 114, max = 167). Two participants in the sham NITESGON group learned all 75-word pairs across the four study periods.

On Day 1, a Kruskal-Wallis H test was utilized to compare the cumulative percentage of learned word pairs to rule out initial learning differences amongst the two stimulation groups. No statistically significant learning difference (H_1 = 0.007, p = .94; see figure 3.4A) was observed between participants in the active NITESGON group (*Mdn* = 86.67, IQR = 51.33-94.34; 65 words out of 75) and sham NITESGON group (*Mdn* = 84, IQR = 64-88; 63 words out of 75).

To assess for possible immediate memory effects during retrieval, a Kruskal-Wallis H test was utilized to compare the percentage of correctly recalled word pairs between the two stimulation groups on Day 7. No statistically significant difference (H_1 = 0.33, p = .59; see figure 3.4B) was observed between the active (*Mdn* = 37.33, IQR = 23.28-47.66; 28 words out of 75) and sham NITESGON groups (*Mdn* =48.28, IQR = 30.77-55.81; 36 words out of 75). These findings confirm that NITESGON does not induce an online effect that immediately improves memory retrieval. However, this finding must be interpreted with caution given the small sample size.

Figure 3.4

NITESGON During Retrieval



Note. For Experiment 2, NITESGON was administered on Day 7 during retrieval. **(A)** No significant difference was observed in the cumulative percentage of learned word pairs between participants in the active and sham NITESGON groups on Day 1. **(B)** No significant difference was observed in the percentage of correctly recalled word pairs between active and sham NITESGON during the test period (retrieval) on Day 7.

3.5 Using Behavioral Tagging to Explore the Mechanism of NITESGON

Collectively, Experiment 1 and 2 allude to the effect of NITESGON occurring offline during the consolidation phase as opposed to the learning-encoding or retrieval phase. From the perspective of the BT hypothesis, the evidence put forth by Experiment 1 suggests NITESGON can be regarded as the novel event that induces LC-mediated arousal that strengthens the weak superficially encoded training (i.e., the word association task). Moreover, for long-lasting memories to transpire, the BT hypothesis proposes that the novel experience must coincide with a crucial time window that exists in association

to the weak encoding training (Moncada et al., 2015; Viola et al., 2014). Evidence of such a window has previously been investigated in both rodent and human BT paradigms, whereby rodents undergoing an inhibitory avoidance task (i.e., weak stimulus) resulted in inhibitory avoidance-LTM when followed by an exploration of a novel open field (i.e., strong stimulus) within a subsequent 1-hour time window (Moncada & Viola, 2007). Similarly, humans had a retroactive memory effect on items learned (i.e., weak stimulus) approximately 5-minutes before receiving an aversive electric shock (i.e., strong stimulus) (Dunsmoor et al., 2015).

In addition to the retroactive timeframe, studies have demonstrated that the temporal period whereby a novel experience must occur to develop long-lasting memories may also precede the weak encoding session (Moncada et al., 2015; Viola et al., 2014). Evidence indicated that rodents exploring a novel open field (i.e., strong stimulus) 1-hour prior to a weak spatial object recognition training produced spatial object recognition-LTM (Ballarini et al., 2009), whereas humans receiving an aversive electric shock (i.e., strong stimulus) approximately 3-minutes before learning items produced a proactive memory enhancement effect (Dunsmoor et al., 2015). Collectively, these previous studies provide evidence of both retroactive and proactive memory enhancement whereby experiences can be retroactively or proactively credited, respectively, resulting in a more stabilized memory and thus significantly remembered (Ballarini et al., 2009; Dunsmoor et al., 2015; Moncada & Viola, 2007).

Despite this, the BT hypothesis indicates that the learning tag set by weak training is still susceptible to being hindered or weakened due to two learning experiences occurring too close in proximity, or learning tags being set in the same brain structure (i.e., hippocampus), thus leading to tags being positioned against one another to compete for available synthesized proteins (Moncada et al., 2015; Viola et al., 2014). It has been

suggested that competition amongst learning tags is one of the primary factors to cause memory interference, resulting in information not being stored in LTM or becoming confused or combined with further information learned, thus distorting or disrupting their future retrieval (Moncada et al., 2015; Viola et al., 2014).

To explore the plausibility of BT functioning as the underlying mechanism of the LC modulating hippocampal consolidation, three experiments were conducted to explore potential retroactive and proactive effects of NITESGON and its influence on memory interference. Experiment 3 first sought to verify if NITESGON (i.e., strong event) applied during a second task would result in a significant retroactive memory effect on a non-stimulated first task (i.e., weak event). To test the hypothesis, Experiment 3 was designed as a double-blind, sham-controlled, randomized parallel-group study aimed to compare the effects of NITESGON on associative memory recall performance of both a word association memory task as well as a second spatial navigation object-location task while receiving either active or sham NITESGON.

Conversely, Experiment 4 sought to verify if NITESGON (i.e., strong event) applied during a first task would result in a significant proactive memory effect on a non-stimulated second task (i.e., weak event). To test this hypothesis, Experiment 4 was designed as a double-blind, sham-controlled, randomized parallel-group study aimed to compare the effects of NITESGON on associative memory recall performance of both a word association memory task while receiving either active or sham NITESGON as well as a second spatial navigation object-location task. The word association and spatial navigation object-location memory tasks were selected because they would not interfere with one another, seeing that both require different episodic information.

Lastly, in an attempt to evaluate if interference impacts NITESGON as the strong stimulus, Experiment 5 was designed as a double-blind, sham-controlled, randomized

parallel-group study aimed to compare the effects of NITESGON on associative memory recall performance of both a word association memory task as well as a second task of the same domain (i.e., Japanese-English word association task).

Considering how previous experiments of the present study suggest the effect obtained by NITESGON improves the consolidation of information via BT, it is hypothesized that information learned preceding and subsequently following NITESGON will be retroactively and proactively credited, thus exhibiting an increase in the number of correctly recalled items in the first and second tasks. Additionally, it is hypothesized that NITESGON employed during the first task may reduce the overall interference effect on the second task.

As outlined in Experiment 1, sAA and resting-state EEG were also collected pre-and-post-NITESGON in Experiments 3-5 to confirm and replicate previous findings (Vanneste et al., 2020). Once more, it was hypothesized that NITESGON will increase sAA concentration levels and induce electrophysiological changes within the gamma range in the medial temporal cortex.

3.6 Methods

3.6.1 Power Analysis

G*Power (Faul et al., 2007) was used to perform an a priori power analysis for an omnibus one-way ANOVA. The estimated effect size for the current study was based on a prior study published in *Science Advances* (Vanneste et al., 2020). This experiment utilized two groups (active and sham NITESGON) to investigate the long-term effect of NITESGON on memory performance when NITESGON was administered during a word association task. A significant difference was observed between the active and sham NITESGON groups 7-days after initial learning, whereby active NITESGON recalled

more word pairs than those who received sham NITESGON; this study indicated that the best estimate of the true population standardized mean difference (Cohen's f) was 1. This large effect size estimate was entered into the power analysis with the following input parameters $\alpha = 0.05$ and power (1-β err prob) = 0.8 with two groups. The power analysis indicated that a total sample size of at least N = 12 was required to detect a difference between the two groups with at least 80% power.

3.6.2 Participants

All participants in the studies were healthy, native-English speaking adults with a similar educational background. Experiment 3 included 20 adults (9 males, 11 females; mean age of 21.11 years, *SD* = 2.03 years), Experiment 4 included 24 adults (13 males, 11 females; mean age of 20.83 years, *SD* = 2.21 years), and Experiment 5 included 31 adults (15 males, 16 females; mean age of 21.36 years, *SD* = 2.42 years).

Participants were screened and enrolled as described in section 3.3.1. Experiment 5 added familiarity of Japanese language or culture to the participant screening procedure; if indicated, the participant was excluded from the study due to the nature of the stimuli.

3.6.3 Materials

3.6.3.1 Word Association Task.

Associative memory performance was measured using a computerized verbal learning task. *Experiments 3, 4, and 5 utilized the same Swahili-English word association task design described in section 3.3.2.1.* However, participants had the opportunity to learn a list of 50 word pairs repetitively across a total of three alternating study and test periods each.

In Experiment 5, associative memory performance was measured using two computerized verbal paired-associate learning tasks. One task comprised of the Swahili-English vocabulary learning, and the other task consisted of a newly introduced Japanese-English (e.g., Japanese: Kumo, English: cloud) word association task. The Japanese-English word association task used the same Swahili-English word pairs, however, the Swahili words were replaced by Japanese vocabulary words modified from Kornmeier et al. (Kornmeier et al., 2014). Refer to figure 3.5B for experimental task design.

3.6.3.2 Object-location Task.

In Experiments 3 and 4, participants partook in a second memory performance task immediately following the word association task. The second memory task consisted of a spatial navigation object-location association task that was based on previous research (Nilakantan et al., 2017). Using the same SDT_N paradigm, participants were instructed to view and remember 50 sequentially presented object locations on a blue-red-gray background grid with an eye-to-screen distance of ~24-inches during three separate study-test blocks.

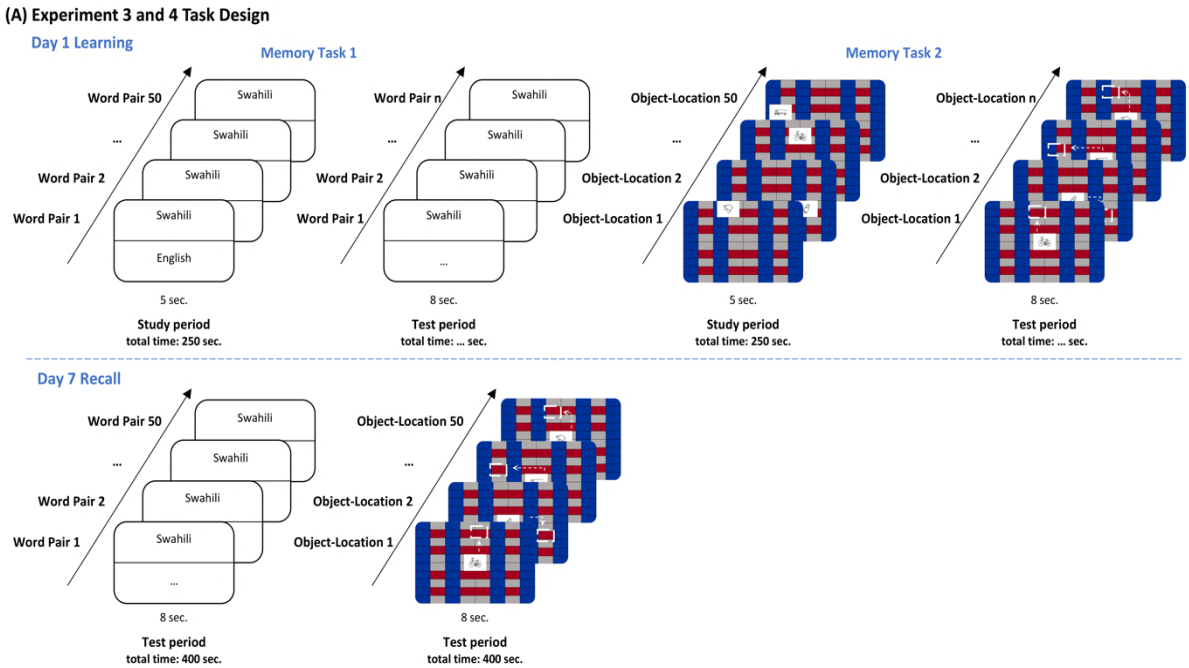
The objects consisted of black and white line drawings from the Boston Naming Test; 10 objects from each of the following categories were used: animals, foods, modes of transportation, tools, and household objects (Kaplan et al., 1978; 1983). Each object was presented one at a time in a randomized order at a randomized location for 5-seconds each (1-second ISI). Objects were presented within a white-box background (4.88 cm x 4.88 cm) and had a red dot superimposed at the object center to mark the precise location. Participants were instructed to study and remember the objects' locations as accurately and precisely as possible. Upon the completion of each study period, a cued-recall test was administered. During the test period, the studied objects were presented one at a time

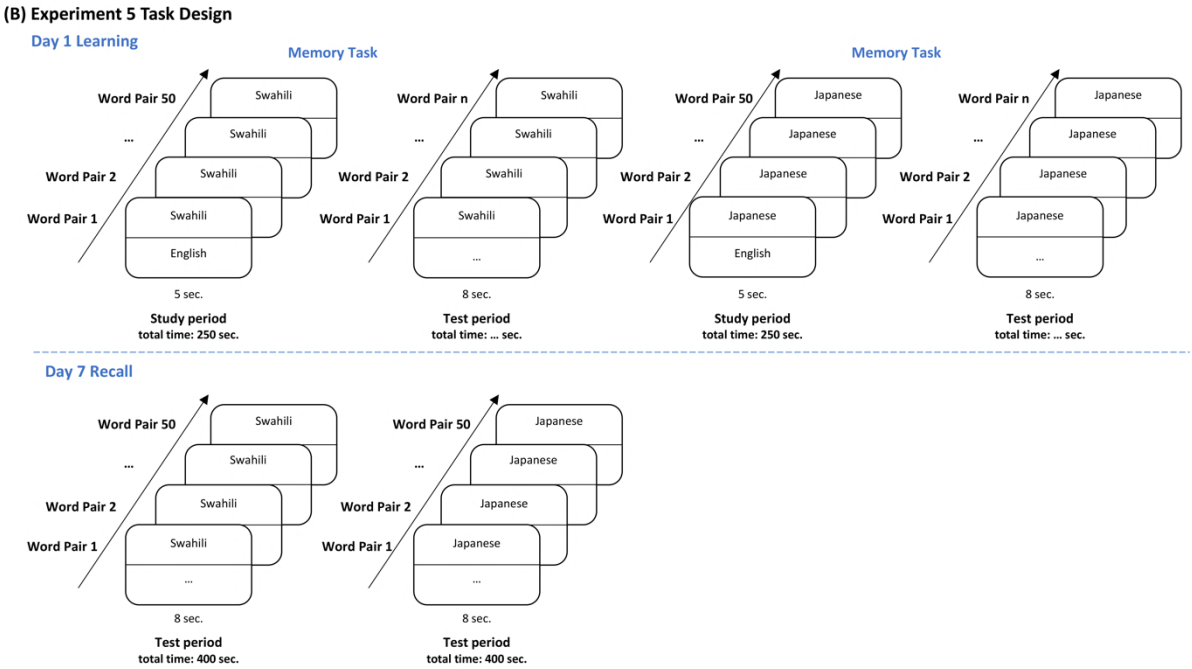
in the center of the screen in a randomized order. Participants were then required to recall the studied locations of the present object. At the beginning of every trial, a fixation cross was presented at the center of the screen for 2-seconds. After this 2-second period, participants were able to use the mouse to click on and move the object from the center of the screen to its recalled location (i.e., in a drag and drop manner). A location was deemed to be correctly identified if the object was placed within a 2.5 cm circumference surrounding the center of the object's precise location.

Experiments 3 and 4 invariably utilized the Swahili-English verbal associative task as the first task and the spatial navigation object-location task as the second task for all participants Refer to figure 3.5A for experimental task design.

Figure 3.5

Experiment 3/4 and 5: Schematic of Experimental Task Design





Note. The above schematic provides an illustrative depiction of **(A)** Experiment 3 and 4's task design whereby participants partook in a total of 6 alternating study and test periods for each memory task during Day 1 learning, and returned on Day 7 for recall testing. **(B)** Experiment 5 followed a similar design, however, Experiment 5 introduced a second word association task to examine interference effects.

3.6.3.3 NITESGON.

NITESGON *utilizing DC was administered using the same device and electrode placement described in section 3.3.2.2.*

In Experiment 3, all participants received sham NITESGON during each study period of the first task using the following parameters: a 5-second ramp up period, followed by a constant current of 1.5 mA (current density 0.4285 A/m^2) for 15-seconds, and ended with a ramp-down period of 5-seconds, resulting in a total time of 45-seconds (15-seconds x 3 study periods) of mimicked stimulation. However, 10 participants received active NITESGON and 10 participants received sham NITESGON during the second task. Sham stimulation parameters were the same as used in the first task and stayed

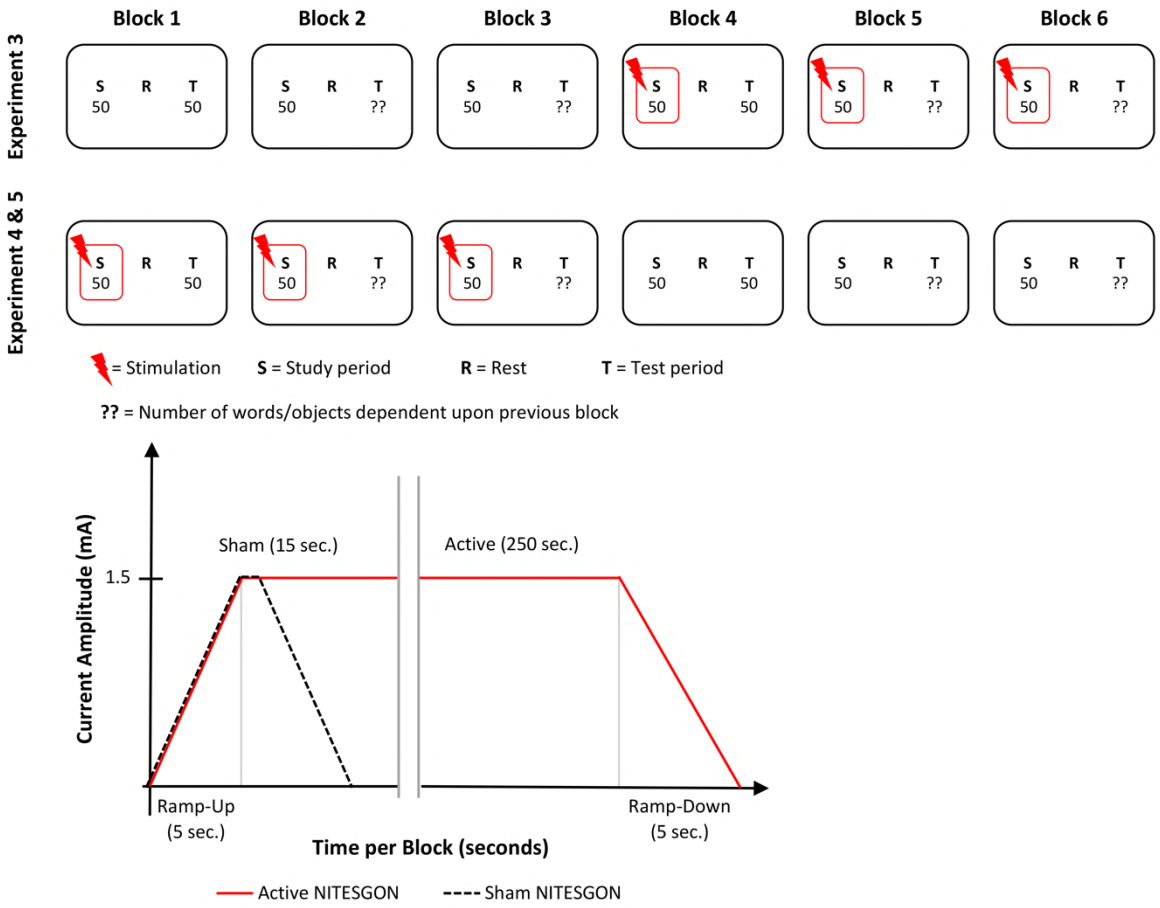
consistent in each of the three study periods for the second task. Participants given active NITESGON received a 5-second ramp-up period, followed by a constant current of 1.5 mA for 250-seconds (5-seconds x 50 word pairs), and ended with a 5-second ramp-down period during each of the 3 study periods of the second task on Day 1, resulting in a total stimulation time of 12.5-minutes (250-seconds x 3 study periods). In addition, all participants received NITESGON for 15-seconds prior to the first study period as a means to help participants habituate to the sensation and ensure they were comfortable with the sensation.

Differing from Experiment 3, Experiment 4 had all participants receive sham NITESGON during each study period of the second task as opposed to the first task. Eleven participants received active NITESGON and 13 participants received sham NITESGON during the first task.

In Experiment 5, 15 participants received active NITESGON and 16 participants received sham NITESGON during the first task, whereby all participants received sham NITESGON during the second task. Sham and active NITESGON parameters remained consistent across Experiments 3 through 5. Refer to figure 3.6 for a breakdown of NITESGON paradigms used.

Figure 3.6

Experiment 3/4 and 5: NITESGON Paradigm



Note. The schematic above exhibits the variations in NITESGON application paradigms utilized during the study periods of Blocks 1-6 on Day 1 learning in Experiments 3, 4, & 5.

3.6.3.4 Resting-state EEG.

Continuous EEG data was collected from each participant pre- and post-NITESGON. *Experiment 3 through 5 utilized rsEEG protocols and procedures consistent to those described in section 3.3.2.3.*

3.6.3.5 Salivary α-Amylase.

Saliva was collected twice: once immediately prior to NITESGON stimulation and once immediately following NITESGON stimulation. *Experiment 3 through 5 utilized sAA protocols and procedures consistent to those described in section 3.3.2.4.*

3.6.4 Procedures

Eligible participants were scheduled for two visits to complete the study, with each experiment using a different cohort. In Experiment 3, Day 1 consisted of learning the word association task (i.e., task 1) and the spatial navigation task (i.e., task 2), and the administration of NITESGON. All participants received sham NITESGON during task 1 study periods, and were then randomly assigned to one of two groups (active or sham) during task 2 study periods. Participants returned 7-days after initial learning for memory testing on both task 1 and task 2; however, they did not receive NITESGON, nor were they able to review word pairs or objects' locations.

In Experiment 4, Day 1 consisted of learning the word association task (i.e., task 1) and the spatial navigation task (i.e., task 2), and the administration of NITESGON. All participants were randomly assigned to one of two groups (active or sham) during task 1 study periods, and then all received sham NITESGON during task 2 study periods. Participants returned 7-days after initial learning for memory testing on both task 1 and task 2; however, they did not receive NITESGON, nor were they able to review word pairs or objects' locations.

In Experiment 5, Day 1 consisted of learning two word association tasks, made up of Swahili-English word associations and Japanese-English word associations, and the administration of NITESGON. All participants were randomly assigned to one of two groups (active or sham) during the study period of the first word association task (i.e.,

task 1), and then all received sham NITESGON during the study period of the second word association task (i.e., task 2). The order of the two word association tasks was randomized over the participants in a 1:1 ratio to remove a possible order effect. Participants returned 7-days after initial learning for memory testing on both task 1 and task 2; however, they did not receive NITESGON, nor were they able to review word pairs.

The researcher responsible for controlling the NITESGON device was not involved in instructing the participant; instead, this was performed by a second researcher who was blind to the stimulation protocol. Resting-state EEG and sAA were collected twice for all participants, once immediately before and once immediately after the NITESGON application. Participants were asked to refrain from studying or searching for the learned word pairs throughout the week. A third researcher who was not responsible for the task or NITESGON on Day 1 conducted the visit on Day 7.

3.6.5 EEG Preprocessing and Source Localization

The continuous rsEEG data collected in Experiments 3 through 5 were preprocessed and the source-level gamma activity pre- and post-NITESGON procedures for the two groups were determined as described in sections 3.3.4 and 3.3.5.

3.6.6 Experiments 3-5 Statistical Analysis

3.6.6.1 Experiments 3 & 4 - Word Association and Spatial-Navigation Task.

Four measurements, two from the word association task and two from the spatial-navigation task, were taken to evaluate the effects of NITESGON: (1) the cumulative percentage of learned Swahili-English word pairs and (2) objects-locations on Day 1, and

(3) the percentage of correctly recalled Swahili-English word pairs and (4) objects-locations on Day 7.

On Day 1, a MANOVA was utilized to compare the cumulative percentage of learned word pairs during the first task and the cumulative percentage of learned locations during the second task between the two stimulation groups (active NITESGON vs. sham NITESGON). In this analysis, group served as the between-subjects variable, and the cumulative learning percentage for both tasks served as the dependent variables.

To assess for possible long-term memory effects on Day 7, a MANOVA was utilized to compare the percentage of correctly recalled word pairs (corrected for how many words they learned on Day 1) and correctly recalled locations (corrected for how many locations they learned on Day 1) between the two stimulation groups. In this analysis, group served as the between-subjects variable, and the percentage of correctly recalled word pairs and correctly recalled locations served as the dependent variables. For both analyses, if significance was obtained, two separate univariate ANOVAs were applied with group as the between-subjects variable and correctly recalled words and recalled locations as dependent variables for task 1 or task 2, respectively.

3.6.6.2 Experiment 5 - Word Association Tasks.

Four measurements, two from the Swahili-English word association task and two from the Japanese-English word association task, were taken to evaluate the effects of NITESGON: (1) the cumulative percentage of learned Swahili-English word pairs and (2) Japanese-English word pairs on Day 1, and (3) the percentage of correctly recalled Swahili-English word pairs and (4) Japanese-English word pairs on Day 7.

On Day 1, a repeated measures ANOVA was utilized to compare the effect of NITESGON on the cumulative percentage of learned Swahili-English and Japanese-

English word pairs. In this analysis, group served as the between-subjects variable, and the cumulative learning percentage for both word association tasks were the within-subjects variable.

To assess for possible long-term memory effects on Day 7, a repeated measures ANOVA was utilized to compare the effect of NITESGON on the percentage of correctly recalled Swahili-English and Japanese-English word pairs (corrected for how many words they learned on Day 1). In this analysis, group served as the between-subjects variable, and the cumulative percentage of correctly recalled word pairs for both word association tasks were the within-subjects variables. If significance was obtained, a simple contrast analysis was applied to compare the different conditions using a Bonferroni correction. A one-tailed test of significance was used given the a priori significant differences observed in active NITESGON groups on word recall.

Additionally, interference was calculated by subtracting the percentage of correctly recalled word pairs from the second task on Day 7 from the percentage of correctly recalled word pairs from the first task on Day 7. This number gave a proxy of interference. A one-way ANOVA was utilized to compare the percentage of interference between the two stimulation groups. In this analysis, group served as the between-subjects variable, and the calculated proxy of interference served as the dependent variable.

3.6.6.3 Salivary α-Amylase.

Salivary α-amylase levels were measured pre-and post-NITESGON by using the saliva collected via the passive drool method. *The effect of NITESGON on sAA concentration levels pre-and post-NITESGON were compared as described in section 3.3.6.2.*

3.6.6.4 EEG – Whole Brain Analysis.

A whole brain analysis was used to compare gamma activity pre- and post-NITESGON. *This activity was correlated with the number of correctly recalled items (words/locations) on Day 7 as described in section 3.3.6.3.*

3.6.7 Experiments 1-5 – Stimulation Blinding

For Experiments 1 through 5, χ^2 analyses were run to assess if participants in their respective stimulation groups were well blinded during the stimulation sessions (i.e., what stimulation participants received compared to what participants expected). Statistical analyses were performed using IBM SPSS (version 26) software.

3.7 Results

3.7.1 Experiment 3

On Day 1, participants underwent two learning association tasks. During the word association task, participants could be presented with a maximum of 150 trials. On average, participants in the active NITESGON group underwent 111 trials (*SD* = 14, min = 93, max = 128), whereas the sham NITESGON group underwent an average of 107 trials (*SD* = 15, min = 93, max = 132). During the spatial navigation object-location task, participants could be presented with a maximum of 150 trials. On average, participants in the active NITESGON group underwent 83 trials (*SD* = 8, min = 69, max = 95), whereas the sham NITESGON group underwent an average of 74 trials (*SD* = 10, min = 59, max = 91). Two participants in the sham NITESGON group learned all 50 object-locations across the three study periods.

Experiment 3 sought to explore the retroactive effects of NITESGON application. On Day 1, a MANOVA was utilized to compare the cumulative percentage of learned word

pairs and learned locations between the two stimulation groups. Results yielded no significant difference in learning ($\lambda = 0.96$, $F_{2,17} = 0.32$, $p = .73$; see figure 3.7A) for the first task (i.e., word association task) and second task (i.e., object-location task) between the active (task 1: $M = 80.20$, $SD = 14.69$; 40.10 words out of 50; task 2: $M = 92.40$, $SD = 6.54$; 46.20 locations out of 50) and sham NITESGON groups (task 1: $M = 80.30$, $SD = 14.88$; 40.15 words out of 50; task 2: $M = 94.40$, $SD = 4.40$; 47.20 locations out of 50).

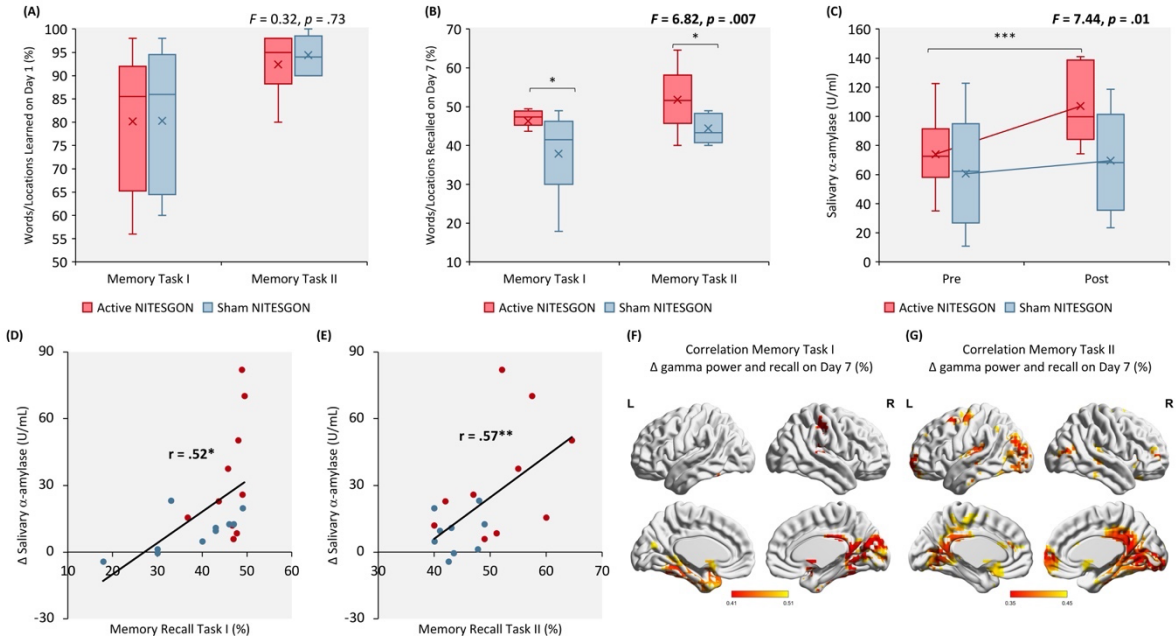
To assess for possible long-term memory effects on Day 7, a MANOVA was utilized to compare the percentage of correctly recalled word pairs and correctly recalled locations between the two stimulation groups. Results indicated a statistically significant effect was obtained for recall ($\lambda = 0.56$, $F_{2,17} = 6.82$, $p = .007$, $\eta^2 = .45$; see figure 3.7B) for both the first ($F_{1,18} = 6.28$ $p = .02$, $\eta^2 = .26$) and second tasks ($F_{1,18} = 7.51$, $p = .01$, $\eta^2 = .29$), revealing an increase for the active NITESGON group in word recall (task 1: $M = 46.26$, $SD = 3.76$; 23.13 words out of 50), as well as object-location recall (task 2: $M = 51.82$, $SD = 7.75$; 25.91 locations out of 50) in comparison to the sham NITESGON group (task 1: $M = 37.89$, $SD = 9.88$; 18.95 words out of 50; task 2: $M = 44.39$, $SD = 3.68$; 22.20 locations out of 50).

Moreover, a repeated measures ANOVA was performed to compare the effect of NITESGON on sAA concentration levels pre- and post-stimulation. Results yielded a statistically significant interaction effect ($F_{1,18} = 7.44$, $p = .01$, $\eta^2 = .29$; see figure 3.7C). A simple contrast analysis revealed a significant increase in sAA concentration post-stimulation for the active NITESGON group (pre: $M = 74.01$, $SD = 26.58$ vs. post: $M = 107.11$, $SD = 25.91$; $p < .001$). However, no significant difference was seen between pre- and post-stimulation in the sham NITESGON group (pre: $M = 60.61$, $SD = 37.93$vs. post: $M = 107.11$, $SD = 25.91$; $p = .17$). Furthermore, the overall difference in sAA (post-pre) concentration levels correlated with memory recall on Day 7 for both the word

association task (r = .52 p = .019; see figure 3.7D) and the object-location task (r = .57, p = .008; see figure 3.7E). Memory recollection on Day 7 was associated with increased gamma power in the medial temporal cortex immediately after stimulation for both the first (r = .41, p = .009; see figure 3.7F) and second memory tasks (r = .35, p = .018; see figure 3.7G).

Figure 3.7

Effects of NITESGON During the Second Task - Retroactive Effect



Note. **(A)** No significant difference was observed in the cumulative percentage of learned word pairs between active and sham NITESGON after the study period for the first task (i.e., word association task) or second task (i.e., object-location task). **(B)** NITESGON improved the percentage of correctly recalled items on Day 7 for the active NITESGON group relative to the sham NITESGON group for both the first and second tasks. **(C)** After NITESGON, sAA concentration levels increased for the active NITESGON group, but not for the sham NITESGON group. **(D, E)** The percentage of correctly recalled items on Day 7 correlated with the difference in sAA levels during Day 1 for the first and

second tasks. **(F, G)** Improved memory recall on Day 7 was associated with increased activity in the medial temporal lobe immediately after NITESGON for the gamma frequency band. Asterisks represent significant differences (* $p < .05$; ** $p < .01$; *** $p < .001$).

3.7.2 Experiment 4

Next, the proactive effects of NITESGON were assessed in Experiment 4. On Day 1, participants underwent two learning association tasks. During the word association task, participants could be presented with a maximum of 150 trials. On average, participants in the active NITESGON group underwent 111 trials (*SD* = 6, min = 100, max = 119), whereas the sham NITESGON group underwent an average of 110 trials (*SD* = 5, min = 102, max = 120). During the spatial navigation object-location task, participants could be presented with a maximum of 150 trials. On average, participants in the active NITESGON group underwent 58 trials (*SD* = 5, min = 52, max = 70), whereas the sham NITESGON group underwent an average of 60 trials (*SD* = 8, min = 52, max = 80). Two participants (one in the active NITESGON group and one in the sham NITESGON group) learned all 50 object-locations across the three study periods.

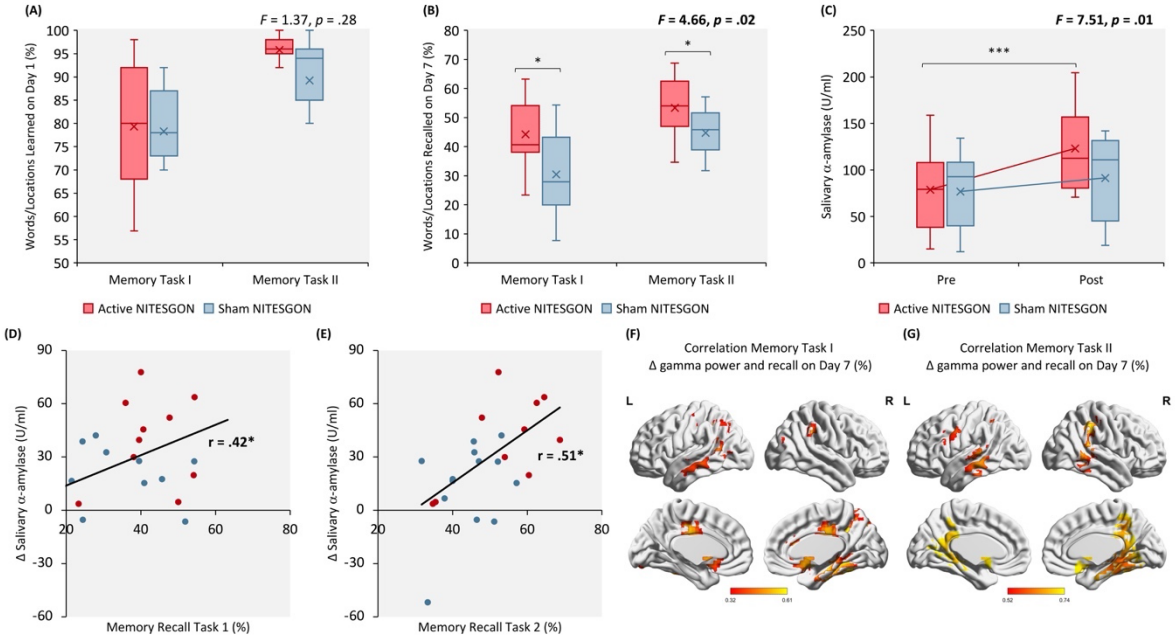
On Day 1, a MANOVA was utilized to compare the cumulative percentage of learned word pairs and learned locations between the two stimulation groups. Results yielded no significant difference in learning ($\lambda = 0.89$, $F_{2,21} = 1.37$, $p = .28$; see figure 3.8A) for the first task (i.e., word association task) and second task (i.e., object-location task) between the active (task 1: *M* = 88.45, *SD* = 25.32; 44.23 words out of 50; task 2: *M* = 87.28, *SD* = 28.44; 43.64 locations out of 50) and sham NITESGON groups (task 1: *M* = 78.31, *SD* = 12.00; 39.16 words out of 50; task 2: *M* = 89.23, *SD* = 11.59; 44.62 locations out of 50).

To assess for possible long-term memory effects on Day 7, a MANOVA was utilized to compare the percentage of correctly recalled word pairs and correctly recalled locations between the two stimulation groups. Results indicated a statistically significant effect was obtained for recall ($\lambda = 0.69$, $F_{2,21} = 4.66$, $p = .02$, $\eta^2 = .31$; see figure 3.8B) for both the first ($F_{1,22} = 6.32$, $p = .02$, $\eta^2 = .22$) and second tasks ($F_{1,22} = 4.87$, $p = .038$, $\eta^2 = .18$), revealing an increase in word recall (task 1: $M = 44.26$, $SD = 10.98$; 22.13 words out of 50), as well as object-location recall (task 2: $M = 53.33$, $SD = 11.29$; 26.67 locations out of 50) for the active NITESGON group in comparison to the sham NITESGON group (task 1: $M = 30.46$, $SD = 15.13$; 15.23 words out of 50; task 2: $M = 44.72$, $SD = 7.75$; 22.36 locations out of 50).

Moreover, a repeated measures ANOVA was performed to compare the effect of NITESGON on sAA concentration levels pre- and post-stimulation. Results yielded a statistically significant interaction effect ($F_{1,22} = 7.51$, $p = .01$, $\eta^2 = .25$; see figure 3.8C). A simple contrast analysis revealed a significant increase in sAA concentration post-stimulation for the active NITESGON group (pre: $M = 78.73$, $SD = 47.67$ vs. post: $M = 123.10$, $SD = 43.64$; $p < .001$). However, no significant difference was seen between pre- and post-stimulation in the sham NITESGON group (pre: $M = 76.76$, $SD = 39.87$ vs. post: $M = 91.38$, $SD = 44.68$; $p = .06$). Furthermore, the overall difference in sAA (post-pre) concentration levels correlated with memory recall on Day 7 for both the word association task ($r = .42$ $p = .04$; see figure 3.8D) and the object-location task ($r = .51$, $p = .01$; see figure 3.8E). Memory recollection on Day 7 was associated with increased gamma power in the medial temporal cortex immediately after stimulation for both the first ($r = .32$, $p = .037$; see figure 3.8F) and second memory tasks ($r = .52$, $p = .012$; see figure 3.8G).

Figure 3.8

Effects of NITESGON During the First Task - Proactive Effect



Note. **(A)** No significant difference was observed in the cumulative percentage of learned items between active and sham NITESGON after the study period for the first task (i.e., word association task) or second task (i.e., object-location task). **(B)** NITESGON improved the percentage of correctly recalled items on Day 7 for the active NITESGON group relative to the sham NITESGON group for both the first and second tasks. **(C)** After NITESGON, sAA concentration levels increased for the active NITESGON group, but not for the sham NITESGON group. **(D, E)** The percentage of correctly recalled items on Day 7 correlated with the difference in sAA levels during Day 1 for the first and second tasks. **(F, G)** Improved memory recall on Day 7 was associated with increased activity in the medial temporal lobe immediately after NITESGON for the gamma frequency band. Asterisks represent significant differences (* $p < .05$; ** $p < .01$; *** $p < .001$).

3.7.3 Experiment 5

The findings of Experiment 3 and 4 revealed that NITESGON enabled both a retroactive and proactive memory effect 7-days after initial learning of two tasks of a dissimilar domain. However, an additional investigation was needed to understand the effects of NITESGON acting as a strong stimulus when two tasks of a similar domain are subsequently learned in order to assess another aspect of the BT process. Experiment 5 sought to explore if NITESGON could reduce memory interference. On Day 1, participants underwent two learning word association tasks. During the word association task I, participants could be presented with a maximum of 150 trials. On average, participants in the active NITESGON group underwent 116 trials (*SD* = 15, min = 77, max = 139), whereas the sham NITESGON group underwent an average of 120 trials (*SD* = 12, min = 95, max = 136). One participant in the sham NITESGON group learned all 50-word pairs of word association task I across the three study periods. During the word association task II, participants could be presented with a maximum of 150 trials. On average, participants in the active NITESGON group underwent 109 trials (*SD* = 14, min = 84, max = 136), whereas the sham NITESGON group underwent an average of 109 trials (*SD* = 10, min = 85, max = 130).

On Day 1, a repeated measures ANOVA was utilized to compare the cumulative percentage of learned Swahili-English and Japanese-English word pairs between the two stimulation groups. Results yielded no significant difference ($F_{1,29}$ = 0.84, p = .37; see figure 3.9A) between the active (task 1: M = 64.80, SD = 17.08; 32.40 words out of 50; task 2: M = 70.40, SD = 22.71; 35.20 words out of 50) and sham (task 1: M = 64.50, SD = 19.09; 32.25 words out of 50; task 2: M = 75.25, SD = 10.68; 37.63 words out of 50) NITESGON group for learning during the first ($F_{1,29}$ = 0.002, p = .96) and second task ($F_{1,29}$ = 0.59, p = .45).

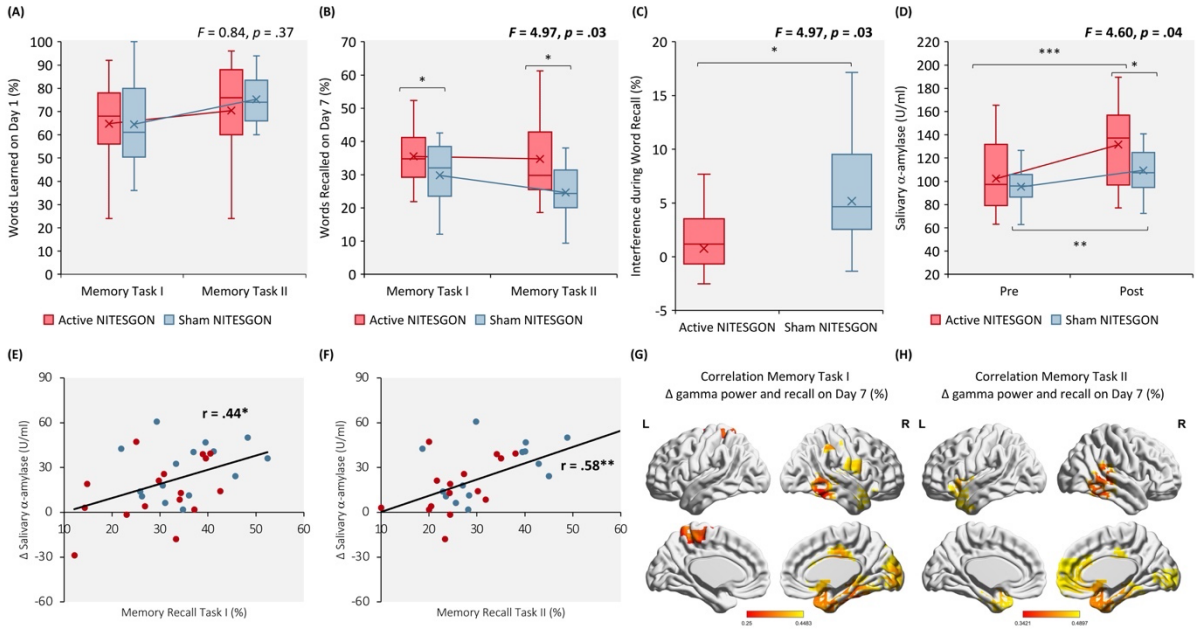
To assess for possible long-term memory effects on Day 7, a repeated measures ANOVA was utilized to compare the percentage of correctly recalled Swahili-English and Japanese-English word pairs between the two stimulation groups. Results yielded a statistically significant interaction effect ($F_{1,29} = 4.97$, $p = .034$, $\eta^2 = .15$; see figure 3.9B). A simple contrast analysis detected a significant difference in the percentage of correctly recalled word pairs for the first task ($F_{1,29} = 2.96$, $p = .048$ (one-tailed); $\eta^2 = .09$) when comparing the active (task 1: $M = 35.52$, $SD = 8.69$; 17.76 words out of 50) and sham NITESGON groups (task 1: $M = 29.81$, $SD = 9.72$; 14.91 words out of 50). Additionally, a significant increase in the percentage of correctly recalled word pairs for the second task ($F_{1,29} = 7.89$, $p = .005$ (one-tailed), $\eta^2 = .21$) was observed for the active NITESGON group (task 2: $M = 34.76$, $SD = 11.74$; 17.38 words out of 50) when compared to the sham NITESGON group (task 2: $M = 24.64$, $SD = 8.10$; 12.32 words out of 50). Furthermore, to assess for a potential interference effect, a one-way ANOVA was utilized to compare the amount of interference between the two stimulation groups. Results detected a significant difference ($F_{1,29} = 4.96$, $p = .03$, $\eta^2 = .15$; see figure 3.9C) in the amount of interference between the active (difference: $M = 0.76$, $SD = 4.93$) and sham NITESGON groups (difference: $M = 5.17$, $SD = 5.99$), thus the sham NITESGON group experienced greater memory interference.

Moreover, a repeated measures ANOVA was performed to compare the effect of NITESGON on sAA concentration levels pre- and post-stimulation. Results yielded a statistically significant interaction effect ($F_{1,29} = 4.60$, $p = .04$, $\eta^2 = .14$; see figure 3.9D). A simple contrast analysis revealed a significant increase in sAA concentration post-stimulation for the active NITESGON group (pre: $M = 102.27$, $SD = 30.91$ vs. post: $M = 131.54$, $SD = 34.49$; $p < .001$) as well as in the sham NITESGON group (pre: $M = 95.29$, $SD = 17.55$ vs. post: $M = 109.46$, $SD = 29.52$; $p = .01$). However, a significant increase

in sAA concentration was observed for the active NITESGON group in comparison to the sham NITESGON group post-stimulation ($F_{1,29}$ = 4.89, p = .035, η^2 = .14). No significant difference was obtained in sAA between the active NITESGON group in comparison to the sham NITESGON group before learning the word association tasks ($F_{1,29}$ = 0.61, p = .44). Furthermore, the overall difference in sAA (post-pre) concentration levels correlated with memory recall on Day 7 for both the first word association task (r = .44 p = .01; see figure 3.9E) and the second word association task (r = .58, p = .001; see figure 3.9F). Memory recollection on Day 7 was also associated with increased gamma power in the medial temporal cortex immediately after stimulation for both the first (r = .25, p = .042; see figure 3.9G) and second memory tasks (r = .34, p = .032; see figure 3.9H).

Figure 3.9

Effects of NITESGON on Memory Interference



Note. **(A)** No significant difference was observed in the cumulative percentage of learned word pairs between active and sham NITESGON after the study period for the first task

(i.e., word association task) or second task (i.e., word association task). **(B)** NITESGON improved the percentage of correctly recalled word pairs on Day 7 for the active NITESGON group relative to the sham NITESGON group for both the first and second tasks. **(C)** A significant difference was observed in the amount of interference, revealing that the interference effect was reduced for the active NITESGON group relative to the sham NITESGON group. **(D)** After NITESGON, sAA concentration levels increased for both the active and sham NITESGON groups, however, a significant difference was observed between the post active NITESGON group in comparison to the post sham NITESGON group. **(E, F)** The percentage of correctly recalled word pairs on Day 7 correlated with sAA levels during Day 1 for the first and second tasks. **(G, H)** Improved memory recall on Day 7 was associated with increased activity in the medial temporal lobe immediately after NITESGON for the gamma frequency band. Asterisks represent significant differences (* $p < .05$; ** $p < .01$; *** $p < .001$).

3.7.4 Stimulation Blinding

For all experiments, participants were unable to accurately guess whether they were assigned to the active or sham NITESGON group, thus suggesting that the sham NITESGON protocol was reliable (see figure 3.10).

Figure 3.10

Stimulation Blinding

Note. Results of the participants' assumption as to which stimulation group they belonged suggested that sham NITESGON protocol was reliable.

3.8 Discussion

The aim of the present research was to examine the previous hypothesis suggesting that the tag-and-capture mechanism exists within plasticity (i.e., STC) and/or memory models (i.e., BT) (Moncada et al., 2015; Viola et al., 2014). Collectively, the reported findings support the hypothesis that NITESGON targeting the LC induces LTM in hippocampal-dependent behavioral tasks via BT as the underlying mechanism. Here, NITESGON was utilized as the necessary novel experience within close temporal-proximity of the transient encoding of a learning task to induce general, long-lasting memory formation.

Evidence presented by Experiments 1 and 2 provided significant indications to which phase of memory the effect of NITESGON occurs in as well as worthwhile insight into

the aforementioned online-offline debate (Liu et al., 2018; Voroslakos et al., 2018). NITESGON did not generate an immediate, online memory effect during learning or during retrieval processes, but rather elicited a favorable, LTM-inducing behavioral effect seen 7-days after initial learning. These findings are indicative of NITESGON's effect occurring offline during consolidation, and thus led to the assessment of NITESGON within the framework of the STC/BT hypotheses in order to acquire a better understanding of the potential underlying mechanisms of initial memory consolidation.

Typically, a weak learning task creates transient STM that diminishes before reaching consolidation (Moncada & Viola, 2007). However, utilizing NITESGON as the novel experience within an applicable temporal-proximity of the weak learning experience in the dual-task BT paradigm in Experiments 3-5 produced significant evidence that endorsed the conceptual premise of the STC-BT hypotheses by which effects of NITESGON strengthened memories during consolidation. Experiments 3 and 4 revealed NITESGON improved memory performance for both tasks of the dual-task paradigm in addition to emphasizing the efficacy of NITESGON to generate retroactive and proactive memory effects – NITESGON retroactively credited the word associations learned prior to the object-locations as well as proactively credited the object-locations learned subsequently to the word associations. These findings are analogous to previous rodent (Cassini et al., 2013; Moncada & Viola, 2007) and human studies (Ballarini et al., 2013; Ramirez Butavand et al., 2020) which consistently reported at least a 2-hour time-dependent window in which a strong stimulus must occur. The observation of NITESGON-induced retroactive and proactive memory enhancement validates the essential disposition of a temporal-proximity associated with the weak training event when considering modulation of late-LTP needed for memory formation in the BT hypothesis (Moncada et al., 2015; Viola et al., 2014). Although previous research has

suggested that millisecond pairing between nerve stimulation and auditory or motor learning is essential to induce targeted plasticity (Conlon et al., 2020; Engineer et al., 2011; Marks et al., 2018), the present data indicates that this design may not be as crucial as previously thought.

The present study also observed contradictory findings indicating the effects of NITESGON are not task specific. Advantageous effects were seen for both a spatial-navigation task as well as a word association task, suggesting that the effect of NITESGON is more general in nature given that these tasks rely on episodic and semantic memory, respectively. Prominent BT studies proposed that retroactive and proactive effects of the BT mechanism are explicitly restricted to strengthening weak category-specific memories (Dunsmoor et al., 2015; Oyarzun et al., 2016; Patil et al., 2017), however, a more recent study aimed to replicate these findings identified that such effects are not category specific (Kalbe & Schwabe, 2021). Considering the contrast in findings amongst the current research and the aforementioned studies, it is important to note that each study utilized a different associative novel experience including aversion shock (to either the wrist or leg) or reward motivation (Dunsmoor et al., 2015; Kalbe & Schwabe, 2021; Oyarzun et al., 2016; Patil et al., 2017), thus distinguishing the current research as the only study to use brain stimulation. Therefore, novelty-triggered effects of NITESGON may override or surpass restrictions that are met via alternative novelty-inducing methods and enable NITESGON to produce general proactive and retroactive memory effects rather than being limited to category-specific memories.

Experiment 5 provided additional evidence that effects of NITESGON are capable of overcoming previously acknowledged limitations to memory, particularly interference (Crossley et al., 2019; Robertson, 2012). Given that memory is initially fragile when acquired (McGaugh, 1966), the susceptibility to interference is enhanced the sooner the

interfering event occurs to the learning experience and can depend heavily on the similitude between the learned and the intervening stimuli (Varma et al., 2017), it is of particular interest that no interference effect was observed upon utilization of NITESGON. Intriguingly, evidence showed that NITESGON as the novel experience diminished interference effects that were presumed to occur and enhanced memory recall performance for the two word association tasks made up of identical English translations. With respect to the BT hypothesis, it is postulated that the interference suppression effect exhibited by NITESGON may be a result of increasing the availability of synthesized proteins, which in turn resulted in less competition amongst the learning tags (Moncada et al., 2015; Viola et al., 2014). Although the present results reflect the putative tag-and-capture mechanism, the molecular evidence of plasticity-related protein dependency for LTM formation in humans is currently under investigation (Okuda et al., 2020). Apart from this, the present methodology and results provide evidence that all other characteristics of the BT mechanism necessary for initial memory consolidation occurred: a weak event setting a learning tag, a strong event occurring within a critical temporal-proximity, and the strong event strengthening the weak event.

An additional requirement for the BT mechanism to occur is for dopamine to be released to the hippocampus for the synthetization of proteins, thus enabling their capture by behavioral tags (Okuda et al., 2020). Recent animal research has shifted from a prevailing hypothesis alluding to hippocampal-dependent memory being mediated by dopamine via the VTA (Rossato et al., 2009) to the LC due to hippocampal-projections being more abundant, as well as the LC's possible corelease of NA and dopamine needed for the post-encoding enhancement of memory persistence (Kempadoo et al., 2016; McNamara et al., 2014; Takeuchi et al., 2016). However, it could be argued that the VTA indirectly contributes to the formation of memories via other brain areas, given that recent

animal research has suggested that VTA dopamine neurons project to the amygdala and may contribute to emotional memory in conjunction with the LC (Giustino et al., 2020; Tang et al., 2020), as well as contribute to synaptic consolidation independently and complementary to the LC (Moncada, 2017). The role of the LC-NA system in synaptic plasticity and molecular memory consolidation has been well established over the past decades (Sara, 2009a, 2015), however, further experimental investigations are needed in order to establish its role in the STC/BT mechanisms.

In conclusion, the collective findings identify that the most prominent influence of NITESGON-induced LC-arousal transpires during initial memory consolidation of information, and thus contributes to the limited amount of BT evidence in human participants. Moreover, the evidence of the present study adds to the growing body of research endorsing NIBS as a potential therapeutic approach to mitigate deficits in episodic memory specifically related to memory consolidation, which is one of the earliest detectable cognitive abnormalities in neurocognitive disorders (Wearn et al., 2020; Weston et al., 2018; Yau et al., 2015). Findings produced by these experiments are substantial given that NITESGON may have the potential of improving memory recall by hampering the disruption of memory consolidation.

4 Learning and Long-Term Memory: A Comparison of Alternating and Direct Current Stimulation

4.1 Introduction

Associative learning and memory refer to the ability to associate two items, thus establishing a link which in turn constructs a declarative memory – a long-term memory that associates experiences, objects, and words through spatial, temporal, or other context relationships (Squire, 2004; Suzuki, 2007). Daily life is highly dependent upon associative memory, such that it enables remembering a name to a face or a word to its meaning. Therefore, deficits in associative memory, such as those seen in patients of AD, can have critical implications that substantially affect routine, normal life tasks (Reitz et al., 2011). In an effort to delay or prevent such deficits, experimental and clinical fields have reintroduced tDCS and tACS as a tool to modify neuroplasticity to induce neurocognitive enrichment (Antonenko et al., 2016; Chase et al., 2020; Klink et al., 2020; Summers et al., 2016).

tDCS and tACS have risen to the forefront of neuromodulatory interventions given their advantageous characteristics, such as their non-invasive, highly tolerable approach, convenience, low cost, detection of cognitive enhancement, and lack of serious adverse events (Hoy & Fitzgerald, 2010; Matsumoto & Ugawa, 2017). The current understanding of tDCS' and tACS' mechanisms of action suggest that technical differences exist in their delivery and modulation of the brain (for mechanistic overview, see Chapter 1 - Section 1.3); nonetheless, they both facilitate the extraction of causal links between brain modulation via tES and the obtained behavioral effects (Reed & Cohen Kadosh, 2018). However, a lack of direct comparison studies of the two techniques has hindered our understanding of which tES technique is most effective in generating beneficial improvement for associative memory.

Declarative (associative) memory is measured in experimental settings via tasks assessing recognition/familiarity or recall stimuli such as faces, locations, and words. Only one prior study has directly compared the effects of high definition (HD)-tDCS and theta band HD-tACS applied to the right fusiform cortex in healthy individuals during a visual memory task to assess which technique best enhanced associative memory performance (Lang et al., 2019). Theta band HD-tACS generated improved associative memory performance in identifying correct picture-pairs immediately after learning, yet prolonged effects of stimulation were not seen in either condition 24-hours post testing (Lang et al., 2019). However, it should be noted that the study explicitly included HD-tACS to more focally target the fusiform cortex due to its well-recognized function in facial encoding (Kuskowski & Pardo, 1999), as well as theta band frequency for its known improvement in visual memory task performance (Polania et al., 2012), suggesting the effects may be specific to facial associative memory.

Alternatively, the present study incorporated a highly divergent study design regarding stimulation protocol, electrode location, and task type, to directly compare the behavioral effects generated by tDCS and tACS (an active and control for each: anodal tDCS, sham tDCS, 40 Hz tACS, 1 Hz tACS) when applied to the ON targeting the LC while learning an associative memory task – replicating previous laboratory parameters strategic placement of electrodes on the left and right C2 nerve dermatomes in an effort to polarize the region. All stimulation techniques used in this experiment are characterized as NITESGON, however, to enable a simpler interpretation and distinction amongst the individual techniques used, they have been entitled in association with the type and power of current.

In a series of experiments, our laboratory demonstrated ON-tDCS' viability to upregulate the LC-NA pathway to increase LC and hippocampus connectivity and

improve associative memory recall in young and older healthy participants (Luckey et al., 2020; Vanneste et al., 2020). These experiments highlight the current proposal of how specific afferent activity can modulate NA neurons via direct projections from the ON to the NTS (Couto et al., 2006). In response, NTS neurons influence central NA activity through direct synapses on neurons in the LC (Couto et al., 2006). Additionally, the LC-NA system is known to be a significant contributor to the signal transduction and synaptic plasticity required for the LC's dual involvement in behaviors and cognition, primarily attention, learning, and memory (Sara, 2009b), such that NA release throughout the cortex, in addition to increased levels of dopamine in both the prefrontal cortex and hippocampus (Devoto et al., 2001; Poe et al., 2020), further suggests a strong modulatory influence on synaptic plasticity (Kempadoo et al., 2016; Takeuchi et al., 2016), thereby playing a crucial role in memory formation (Wagatsuma et al., 2018).

Although electrical stimulation of the scalp modulating the excitability of neurons directly is currently debated, there is a growing body of evidence that recognizes the potential of neurophysiological effects of tES primarily being caused by transcutaneous stimulation of peripheral nerves (Asamoah et al., 2019; Liu et al., 2018; Voroslakos et al., 2018). Moreover, a substantial finding indicated that only 25 (up to 50) percent of applied current reaches the brain due to the skull's high electrical resistance (Voroslakos et al., 2018), thus suggesting tES affecting neural circuits indirectly via peripheral nerves holds merit and should be further explored in experimental designs.

Modulation of associative memory may benefit from an alternative stimulation from tDCS, such that current tACS paradigms hypothesize that tACS' frequency may be applied to amplify power and entrain endogenous brain rhythms to improve communication and process incoming information and thus elicit advantageous effects (Antal & Herrmann, 2016; Beliaeva et al., 2021). More specifically, gamma band tACS

is most commonly employed due to its involvement in attention and LTM processes (Fries, 2009; Jensen et al., 2007), as well as it being the frequency band that corresponds to AD progression (Babiloni et al., 2016). This rationale is broadly supported by recent research exhibiting significant effects of tACS when gamma tACS (>40 Hz) applied to the left prefrontal cortex during both encoding and recognition/retrieval enhanced declarative memory performance of a word learning task (Javadi et al., 2017; Nomura et al., 2019), analogous to our previous work demonstrating ON-tDCS improving memory of an associative word learning task.

Given that tACS and tDCS protocols have demonstrated successful improvements of associative memory, and both techniques have the potential to modulate LC-NA activity, a key contributor of neuroplasticity and memory processes (Hansen, 2017; Sara, 2015), via the ON, this chapter directly compares which stimulation technique generated greater associative memory performance enhancements. To investigate if behavioral outcome differences exist between ON-tACS and ON-tDCS, two measurements were taken from a word association task: (1) the cumulative percentage of learned Swahili-English word pairs on Day 1 and, (2) the percentage of correctly recalled Swahili-English word pairs on Day 7. The present study hypothesized 40 Hz ON-tACS and active ON-tDCS would enhance memory recall in comparison to the control stimulations on Day 7. Moreover, it was hypothesized that 40 Hz ON-tACS would have an online effect during the encoding of new information while active ON-tDCS would have an offline effect during the consolidation process. This study provides valuable evidence for subsequent paradigm construction to enhance learning and memory.

4.2 Methods

4.2.1 Design

The study was designed as a randomized, double-blinded, two-visit, active-controlled study aimed to directly compare the effects of ON-tACS and ON-tDCS on associative memory recall performance. The study employed a mixed factorial design with time (Day 1 vs. Day 7) as the within-subjects variable and group (active ON-tDCS vs. sham ON-tDCS vs. 40 Hz ON-tACS vs. 1 Hz ON-tACS) as the between-subjects variable. This study was in accordance with the ethical standards of the Helsinki declaration (1964). All participants provided written informed consent.

4.2.2 Power Analysis

G*Power (Faul et al., 2007) was used to perform an a priori power analysis for an omnibus one-way ANOVA. The estimated effect size for the current study was based on a prior study published in *Science Advances* (Vanneste et al., 2020). This experiment utilized four groups (two active NITESGON groups and two active-control groups) to investigate the long-term effect of NITESGON on memory performance when NITESGON was administered during a word association task. A significant difference was observed between the NITESGON groups and active-control groups 7-days after initial learning, whereby the NITESGON groups recalled more word pairs compared to those who received the active-control stimulations; this study indicated that the best estimate of the true population standardized mean difference (Cohen's f) was 0.47. This large effect size estimate was entered into the power analysis with the following input parameters $\alpha = 0.05$ and power (1-β err prob) = 0.8 with four groups. The power analysis indicated that a total sample size of at least N = 56 was required to detect a difference between the two groups with at least 80% power. However, this study introduced a

second stimulation paradigm (i.e., 40 Hz AC NITESGON) to be used as pilot research for future investigations; therefore, a sample size larger than the power analysis was utilized.

4.2.3 Participants

Participants were 85 healthy, native-English speaking adults (49 females, 36 males; mean age of 21.56 years, *SD* = 2.11 years) with a similar undergraduate background in education who all had the maximum score on the Mini-Mental State Examination (Folstein et al., 1975) as well as normal or corrected-to-normal vision. Participants were screened over the phone (e.g., handedness, tES contraindications, neurological impairments, not participated in a tES study) prior to enrolling into the study. None of the participants had a history of major psychiatric or neurological disorders, or any tES contraindications, including previous history of brain injuries or epileptic insults, cardiovascular abnormalities, implanted devices, taking neuropsychiatric medications, prescribed stimulants use, or chronic use of illicit drugs.

If screening discovered that participants were familiar with Swahili/Arabic language or Swahili culture, then the participant was excluded from the study due to the nature of the stimuli. Furthermore, study instructions were emailed to the participants to ensure that they abstained from alcoholic beverages, and energy or caffeinated beverages for 24-hours prior to the scheduled study session. Additionally, participants were asked to withhold from using any hair styling products (e.g., hair gel, hair spray...) the day of the study. To assure the highest levels of accuracy for saliva collection, participants were asked to refrain from the following products or activities for the associated time window prior to saliva collection: dental work for at least 48-hours, major meals for 60-minutes, brushing their teeth for 45-minutes, as well as water or rinsing their mouth for 10-minutes

in order to avoid any risk of lowering pH levels and influencing bacterial growth. Participants were also asked to refrain from taking any nonapproved prescription drugs, steroidal/anti-inflammatory drugs and were also asked to avoid foods high in sugar content or acidity, and nicotine consumption. Lastly, if participants were scheduled for a study in the afternoon, they were requested to avoid taking a nap during the day to account for the amylase awakening response (Ali & Nater, 2020). Salivary alpha-amylase has been shown to have a distinct diurnal profile whereby sAA levels are low within 30-minutes of awakening and rise throughout the day (Nater et al., 2007).

4.2.4 Materials

4.2.4.1 Word Association Memory Task.

All participants underwent a computerized, word association task consisting of Swahili-English vocabulary learning that was adapted from a well-established study design published in *Science* (Karpicke & Roediger, 2008). The 50 Swahili-English word pairs were taken from the Nelson and Dunlosky study (Nelson & Dunlosky, 1994), excluding the word pair rafiki-friend, as this word is also the name of a character in The Lion King and therefore could have been greatly familiar to participants. The task was programmed in Visual Studio software using C# and shown on a computer with a ~27-inch screen positioned at eye level.

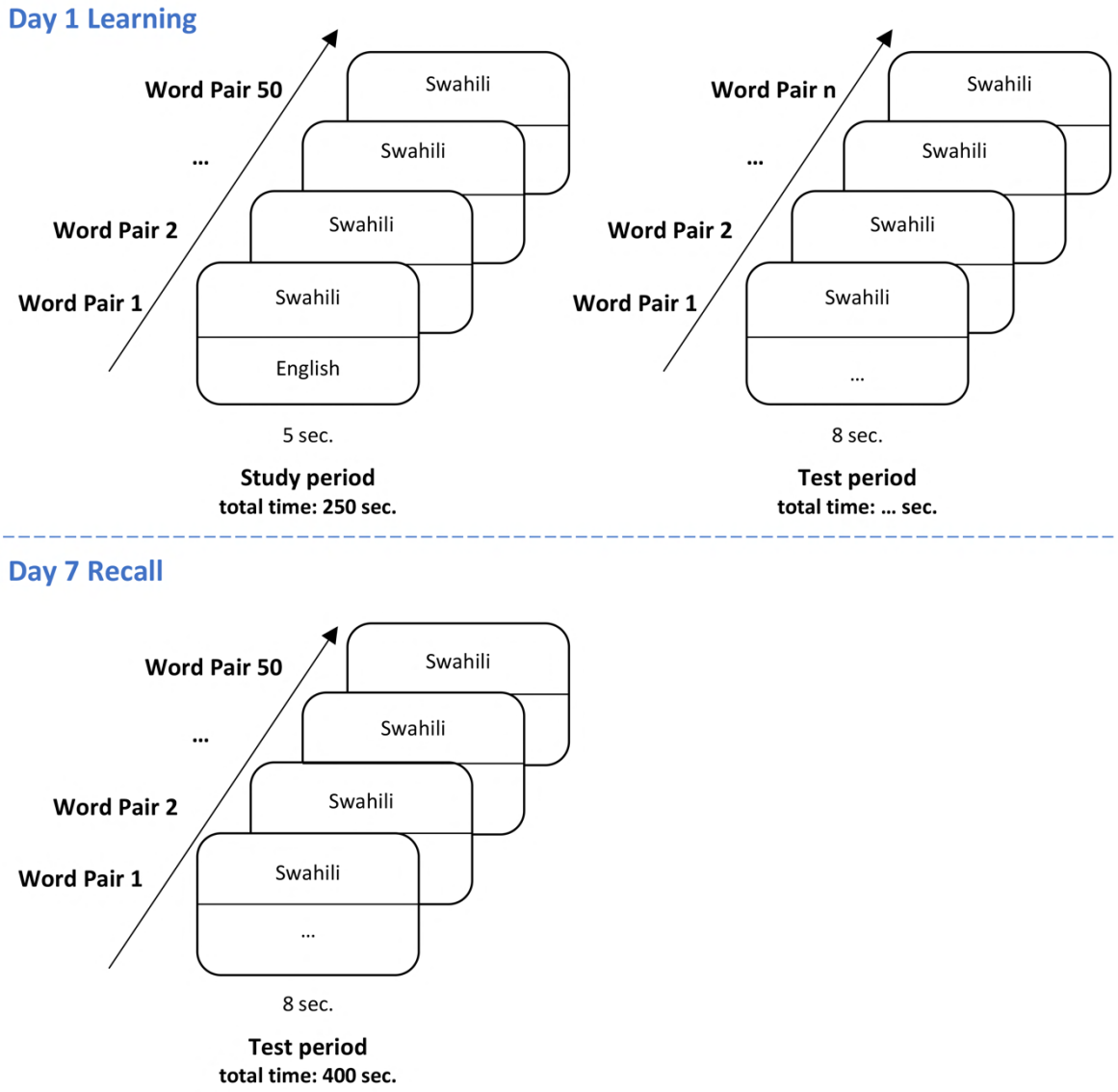
Participants had six alternating study and test periods to learn the list of 50 Swahili-English (e.g., Swahili: tumbili, English: monkey) vocabulary word pairs made up of common day-to-day words. The verbal paired-association memory task was divided into 3 blocks with each block consisting of a study period (S), followed by a 30-second rest period, and a test period (T). Participants studied and were tested on the exhaustive list of 50 word pairs in block 1. Whereas in blocks 2 and 3, the comprehensive list of 50 word

pairs were studied in each study period, but only items that had not yet been recalled were tested in the test periods (denoted SDT_N, where T_N indicates that only the non-recalled pairs were repeatedly tested). Therefore, in blocks 2 and 3, the number of word pairs tested diminished across the blocks and varied according to the testing period. This paradigm was used to ensure that all participants would avoid a ceiling effect (Karpicke & Roediger, 2008).

During the study period, each word pair (black words on white background) was presented one below the other in the middle of the screen for 5-seconds to provide adequate time for encoding. Participants were instructed to learn as many word pairs as they could, so they may recall the English word when given the Swahili word. During the cued-recalled test period, participants were instructed to type in the correct English translation of the Swahili word that was presented for 8-seconds using a computer keyboard. If participants failed to recall a word pair during testing, they were not given any feedback. Once the 8-seconds expired, the computer program would automatically advance to the next Swahili word regardless of whether the participant had entered a response. Participants' responses were recorded by the computer program. The word pairs sequence was randomized between participants, blocks, and periods. Refer to figure 4.1 for design overview.

Figure 4.1

Schematic of Study Design During the Word Association Task



Note. The above schematic provides an illustrative depiction of the **(A)** experimental task design whereby participants partook in a total of 6 alternating study and test periods during Day 1 learning, and returned on Day 7 for recall testing.

4.2.4.2 ON-tDCS and ON-tACS.

Alternate current (AC) and direct current (DC) were transmitted via a saline-soaked (1.3% saline) pair of synthetic sponges (5 cm x 7 cm) and were delivered by a specially

developed, battery-driven, constant or alternating current stimulator with a maximum output of 10 mA (Eldith©; http://www.neuroconn.de). Based upon previous laboratory evidence, each participant receiving ON-tDCS had the anodal electrode, acting as the target stimulation, placed over the left C2 nerve dermatome and the cathodal electrode, acting as the reference, placed over the right C2 nerve dermatome. The same electrode placement was used for participants receiving ON-tACS, however, due to its nature of stimulation each electrode functioned as the anode/cathode electrode, respectively (Tavakoli & Yun, 2017). In order to maintain consistency across all participants, research assistants were trained to map out the placement according to the length of the participant's head. To minimize skin sensations and to acclimate participants to the stimulation types, the current intensity was ramped-up (gradually increasing) until it reached its programed maximum output for the duration of each study period, followed by a ramp-down period (gradually decreasing) denoting the end of the study period. The impedance under each electrode was maintained under 10 kΩ.

Active ON-tDCS was applied to the left C2 dermatome at a constant current of 1.5 mA (current density 0.4285 A/m^2) during each of the 3 study periods, resulting in a total stimulation time of 12.5-minutes (i.e., 250-seconds x 3 study periods). Sham ON-tDCS was applied to the left C2 dermatome at a constant current of 1.5 mA during each of the 3 study periods, resulting in a total stimulation time of 45-seconds (i.e., 15-seconds x 3 study periods). The rationale behind the sham procedure was to mimic the transient skin sensation at the beginning of active ON-tDCS without producing any conditioning effects on the brain. ON-tACS was applied using a current of ± 1.5 mA peak intensity during each of the 3 study blocks (3 mA peak to peak; current density 0.8571 A/m^2). The two groups receiving AC stimulation differed in the frequency (Hz) of tACS. The stimulation was phasic with a sinusoidal waveform. One group received stimulation delivered at a

frequency of 40 Hz with 0° phase difference. The second group, active-control, received stimulation delivered at a frequency of 1 Hz with 0° phase difference. The frequency was fixed for the duration of 12.5-minutes in both groups (250-seconds x 3 study periods). Twenty-five participants received active ON-tDCS, 25 participants received sham ON-tDCS, 24 participants received 40 Hz ON-tACS, and 11 participants received 1 Hz ON-tACS.

4.2.4.3 Salivary α-Amylase.

Salivary α-amylase, a marker of endogenous NA activity, was used to test the effect of ON-tES on the LC-NA pathway. Participants' saliva was collected two times during the experiment: before and after stimulation. When the participants were ready to collect saliva, they were requested to gently tip their head backwards and collect saliva on the floor of their mouth and when ready, passively drool into the mouthpiece of the tube provided by Salimetrics (Salimetrics, LLC, USA; https://salimetrics.com). The participants were requested to collect 2 ml of saliva in one straight flow and avoid breaks between drool as much as possible. The length of time to collect 2 ml of saliva was noted and the timer was started only when participants began to passively drool into the tube. All saliva samples were stored in 2 ml cryovials and immediately stored in an -80° C laboratory freezer. Due to a technique problem, a batch was lost. Upon completion of the collection procedures, a total of 132 saliva samples from 66 participants (24 active ON-tDCS, 21 sham ON-tDCS, 10 40 Hz ON-tACS, and 11 1 Hz ON-tACS) were packed in dry ice and sent to the Salimetrics laboratory for analysis. The Salimetrics analysis protocols and determination techniques for the targeted biomarker are described below.

The flow rate was calculated using the formula given by Salimetrics: $rate\left(\frac{ml}{min}\right) = \frac{amount\ of\ saliva\ (ml)}{time\ (min)}$. This flow rate correction was used in the calculation of

concentration of sAA. Furthermore, the tubes were also weighed; the weight of the saliva was determined as the difference between the weights of the full tube and the empty tube. The amount of sAA in the sample was directly proportional to the increase in absorbance at 405 nm. Ten μL of the sample was diluted and well mixed. Eight μL of the diluted samples were then pipetted into individual wells of 96-well microtiter plate. A volume of 320 μL of preheated chromogenic substrate solution was added to each well and the plate was rotated at 500–600 RPM at 37° C for 3-minutes. The optical density of the sample was determined at the 1-minute mark and again at the 3-minute mark.

4.2.4.4 Visual Analog Scale - Alertness.

To measure the alertness of the participant, a visual analog scale (VAS) was used before and after the ON-tES procedure. The VAS measured alertness using a subjective, continuous 10-inch line whereby participants marked a response to the question "How alert are you at this moment?".

4.2.4.5 tES Exit Questionnaire and Stimulation Blinding.

All participants were asked to complete the tES exit questionnaire that was taken from the Brunoni et al. study (Brunoni et al., 2011) after they had concluded the NITESGON procedure in order to assess for possible side effects of NITESGON. The tES exit questionnaire measured symptoms covering headache, neck pain, scalp pain, tingling, itching, sleepiness, trouble concentrating, and acute mood changes on a 4-point scale, ranging from 1 = 'absent' to 4 = 'severe.' If the participant had indicated any symptoms present, they were then asked to specify on a 5-point scale, ranging from 1 = 'none' to 5 = 'definitely', to determine if they thought this symptom was related to the application of tES. Lastly, to determine if the stimulation was well blinded, the

participants were asked to guess if they thought they were placed in the “active” or “non-active” group.

4.2.5 Procedures

Eligible participants were scheduled for two visits to complete the study. Day 1 consisted of pre-assessments (i.e., MMSE, VAS, saliva collection) followed by the word association task and the administration of ON-tES. Participants were informed of the randomized allocation into one of four groups consisting of either active or control protocols during the study period: participants either received active ON-tDCS, sham ON-tDCS, 40 Hz ON-tACS, or 1 Hz ON-tACS. The researcher who controlled the tES device was not involved in instructing the participant; this was performed by a second researcher who was blind to the stimulation protocol and not in the room during the stimulation. Day 1 concluded with post ON-tES questionnaires (saliva collection, VAS, tES Exit Questionnaire). Participants were asked to refrain from studying or searching for the word pairs learned throughout the week. Participants returned 7-days after their first visit for memory testing to measure possible long-term effects on associative memory performance, but did not receive ON-tES, nor were they able to review word pairs. A third researcher who was not responsible for the task or ON-tES on Day 1 conducted the visit on Day 7.

4.2.6 Statistical Analysis

4.2.6.1 Word Association Task.

Two measurements were taken from the word association task to evaluate the effects of ON-tACS and ON-tDCS: (1) the cumulative percentage of learned Swahili-English

word pairs on Day 1, and (2) the percentage of correctly recalled Swahili-English word pairs on Day 7.

On Day 1, a Kruskal-Wallis H test was utilized to compare the cumulative percentage of learned word pairs (i.e., after Block 3) between the four stimulation groups (active ON-tDCS vs. sham ON-tDCS vs. 40 Hz ON-tACS vs. 1 Hz ON-tACS). In this analysis, group served as the between-subjects variable, and the cumulative learning percentage served as the dependent variable.

To assess for possible long-term memory effects on Day 7, a Kruskal-Wallis H test was utilized to compare the percentage of correctly recalled word pairs (corrected for how many words they learned on Day 1) between the four stimulation groups. In this analysis, group served as the between-subjects variable, and the percentage of correctly recalled word pairs served as the dependent variable.

Additionally, the percentage of word pairs forgotten was calculated by subtracting the cumulative percentage of word pairs learned on Day 1 from the percentage of word pairs recalled on Day 7. A Kruskal-Wallis H test was utilized to compare the percentage of forgotten word pairs between the four stimulation groups. In this analysis, group served as the between-subjects variable, and the percentage of forgotten word pairs served as the dependent variable. For all analyses, if significance was obtained, post hoc Mann-Whitney tests were run to detect group differences. The Holm-Bonferroni method was applied to correct for multiple comparisons.

4.2.6.2 Salivary α-Amylase.

Salivary α-amylase levels were measured by using the saliva collected via the passive drool method. A repeated measures ANOVA was utilized to test the effect of ON-tES on the LC-NA pathway. In this analysis, group (active ON-tDCS vs. sham ON-tDCS vs. 40

Hz ON-tACS vs. 1 Hz ON-tACS) served as the between-subjects variable and sAA concentration levels (pre-and post-ON-tES) served as the within-subjects variable. If significance was obtained, a simple contrast analysis was applied to compare the different conditions using a Bonferroni correction.

4.2.6.3 Visual Analog Scale - Alertness.

A repeated measures ANOVA was used to analyze the effect of ON-tES on alertness (VAS) with group (active ON-tDCS vs. sham ON-tDCS vs. 40 Hz ON-tACS vs. 1 Hz ON-tACS) as the between-subjects variable and VAS (pre- vs. post-measurement) as the within-subjects variable. If significance was obtained, a simple contrast analysis was applied to compare the different conditions using a Bonferroni correction.

4.2.6.4 tES Exit Questionnaire and Stimulation Blinding.

A MANOVA was used to assess the differences between scores on side effects marked on the tES exit questionnaire, with group (active ON-tDCS vs. sham ON-tDCS vs. 40 Hz ON-tACS vs. 1 Hz ON-tACS) as the between-subjects variable and the different side effects as the dependent variable.

A χ^2 analysis was run to assess if participants in the two stimulation groups were well blinded during the stimulation session (i.e., what stimulation participants received compared to what participants expected). Statistical analysis was performed using IBM SPSS (version 26) software.

4.3 Results

4.3.1 Word Association Task

During the word association task on Day 1, participants could be presented with a maximum of 150 trials. On average, participants in the DC NITESGON group underwent 118 trials (SD = 16, min = 83, max = 143), 40 Hz AC NITESGON underwent an average of 106 trials (SD = 16, min = 69, max = 134), 1 Hz AC NITESGON underwent an average of 124 trials (SD = 12, min = 100, max = 140), and DC sham NITESGON underwent an average of 118 trials (SD = 12, min = 95, max = 142). Three participants in the 40 Hz ON-tACS group learned all 50-word pairs across the three study periods.

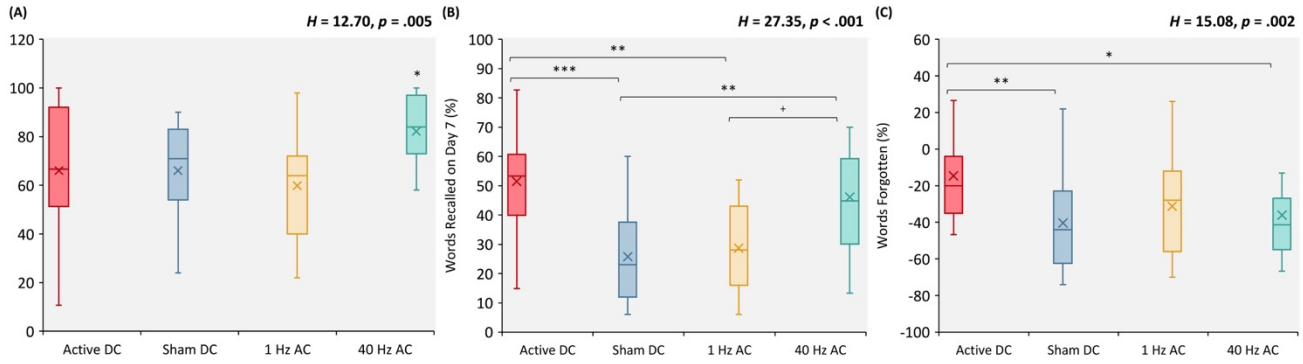
On Day 1, a Kruskal-Wallis H test was utilized to compare the cumulative percentage of learned word pairs between the four stimulation groups. Results yielded a significant difference in the cumulative percentage of learned word pairs ($H_3 = 12.70$, $p = .005$, $\eta^2 = .15$; see figure 4.2A). Post hoc analyses using Mann-Whitney tests were applied for closer inspection. Results revealed that participants who received 40 Hz ON-tACS learned more word pairs (*Mdn* = 84, IQR = 74-96; 42 words out of 50) in comparison to participants who received 1 Hz ON-tACS (*Mdn* = 64, IQR = 44-70; 32 words out of 50), active ON-tDCS (*Mdn* = 66.67, IQR = 52-90.67; 33 words out of 50), and sham ON-tDCS (*Mdn* = 71, IQR = 54-81; 34 words out of 50). Accordingly, 40 Hz ON-tACS was highly statistically significantly different than 1 Hz ON-tACS ($p = .004$), active ON-tDCS ($p = .008$), and sham ON-tDCS ($p = .005$). The cumulative percentage of learned word pairs during the study periods in the 1 Hz ON-tACS, active ON-tDCS, and sham ON-tDCS groups did not differ significantly. Observed increase in learning for the 40 Hz group remained significant after Holm-Bonferroni correction.

To assess for possible long-term memory effects on Day 7, a Kruskal-Wallis H test was utilized to compare the percentage of correctly recalled word pairs between the four

stimulation groups. Results indicated that the effect of stimulation group significantly influenced the percentage of recalled word pairs ($H_3 = 27.35$, $p < .001$, $\eta^2 = .33$; see figure 4.2B). Post hoc analyses using Mann-Whitney tests further revealed active ON-tDCS (*Mdn* = 53.33, IQR = 41.67-60; 27 words out of 50) was highly significantly superior to sham ON-tDCS (*Mdn* = 23, IQR = 12-36.5; 12 words out of 50) ($p < .001$) and 1 Hz ON-tACS (*Mdn* = 28, IQR = 18-39.5; 14words out of 50) ($p = .001$), but not significantly different from 40 Hz ON-tACS (*Mdn* = 44.83, IQR = 30.95-58; 23 words out of 50) ($p = 0.189$). Observed increase in memory recall remained significant after Holm-Bonferroni correction. In addition, 40 Hz ON-tACS was statistically significantly more effective than sham ON-tDCS ($p = .001$) after Holm-Bonferroni correction, however, 1 Hz ON-tACS ($p = .023$) did not survive multiple corrections. Moreover, a Kruskal-Wallis H test reported a significant difference when comparing stimulation groups and the percentages of word pairs forgotten from Day 1 to Day 7 ($H_3 = 15.08$, $p = .002$, $\eta^2 = .18$; see figure 4.2C). Mann-Whitney post hoc analyses revealed participants who received active ON-tDCS (*Mdn* = -20, IQR = -34.2-(-4); 10 words out of 50) forgot significantly fewer word pairs than those who received 40 Hz ON-tACS (*Mdn* = -41.43, IQR = -53.05-(-26.86); 20 words out of 50) ($p = .001$) and sham ON-tDCS (*Mdn* = -44, IQR = -59.5-(-25); 22 words out of 50) ($p < .001$), however, not significantly different from 1 Hz ON-tACS (*Mdn* = -28, IQR = -55-(-16); 14 words out of 50) ($p = .07$). Observed decrease in forgetfulness remained significant after Holm-Bonferroni correction.

Figure 4.2

Behavior Effects of ON-tDCS and ON-tACS



Note. **(A)** A significant difference was observed in the cumulative percentage of learned word pairs between the stimulation groups, 40 Hz ON-tACS learned more word pairs on Day 1. **(B)** Active ON-tDCS and 40 Hz ON-tACS improved the percentage of correctly recalled word pairs on Day 7. **(C)** A significant difference was observed in the amount of forgotten word pairs, revealing that the active ON-tDCS group had the lowest percentage of forgotten word pairs. Asterisks represent significant differences (* $p < .05$; ** $p < .01$; *** $p < .001$; + did not remain significant after Holm-Bonferroni correction).

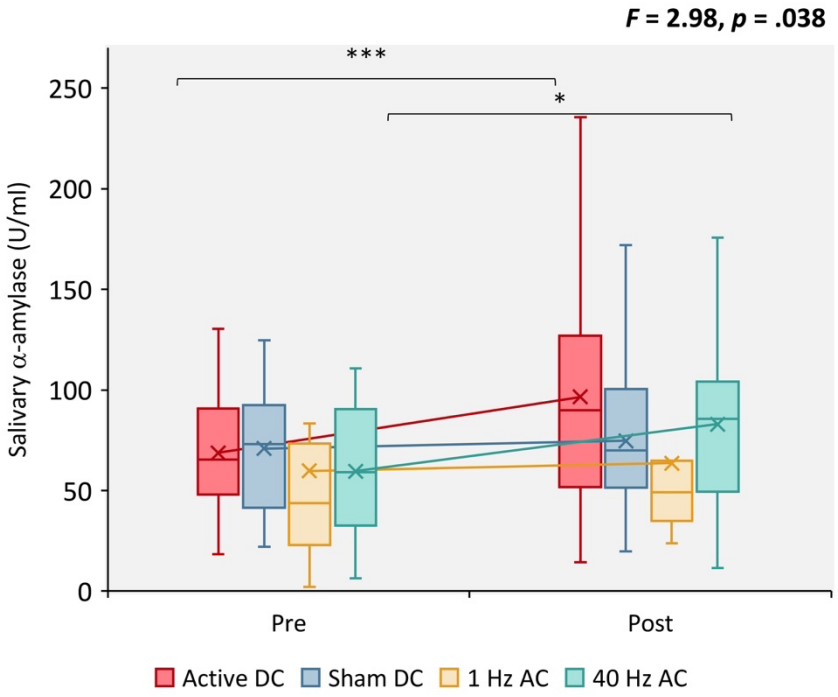
4.3.2 Salivary α-Amylase

Moreover, a repeated measures ANOVA was performed to compare the effect of NITESGON on sAA concentration levels pre- and post-stimulation. Results yielded a statistically significant interaction effect for sAA ($F_{3,62}$= 2.98, p = .038, η^2 = .13; see figure 4.3). A simple contrast analysis revealed a significant effect for both active ON-tDCS ($F_{1,62}$= 19.32, $p < .001$, η^2 = .23) and 40 Hz ON-tACS ($F_{1,62}$= 5.71, p = .02, η^2 = .08), indicating an increase in sAA after stimulation in comparison to before stimulation. No effect was obtained for sham ON-tDCS ($F_{1,62}$= 0.71, p = .55) and 1 Hz ON-tACS

($F_{1,62}$= 0.17, p = .68). A direct comparison between active ON-tDCS and 40 Hz ON-tACS revealed no effect ($F_{1,32}$= 0.11, p = .75).

Figure 4.3

Salivary α-Amylase



Note. After NITESGON, sAA concentration levels increased for the active ON-tDCS and 40 Hz ON-tACS groups, but not for the sham ON-tDCS and 1 Hz ON-tACS group. Asterisks represent significant differences (* p < .05; ** p < .01; *** p < .001).

4.3.3 Visual Analog Scale - Alertness

A repeated-measures ANOVA detected a non-significant interaction effect between VAS – Alert (pre- and post-ON-tES) and stimulation group ($F_{3,81}$= 0.59, p = .62; see figure 4.4A), indicative of participants' alertness not changing because of stimulation application.

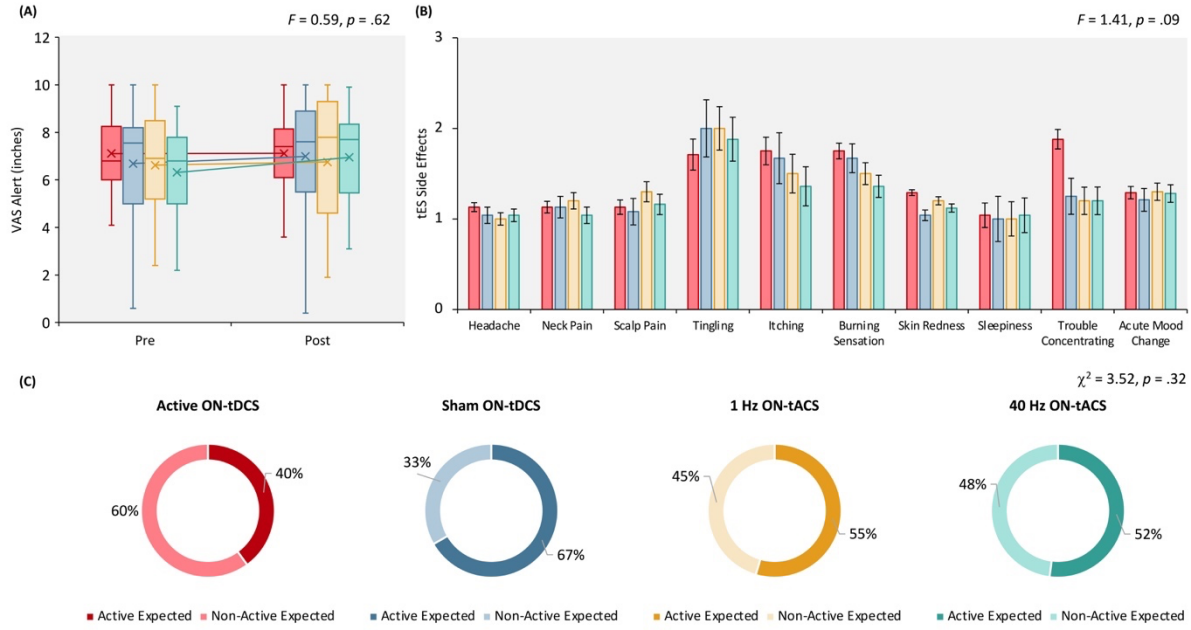
4.3.4 tES Exit Questionnaire & Stimulation Blindness

No statistically significant difference was observed between the four stimulation groups for side effects marked on the tES exit questionnaire ($F_{30,216} = 1.41$, $p = .09$; see figure 4.4B*)*. All stimulation sessions were well tolerated, and no major adverse events were reported throughout the study. However, after closer inspection, a one-way ANOVA revealed a significant difference amongst the participant-reported levels of trouble concentrating between stimulation groups ($F_{3,81} = 5.04$, $p = .003$, $\eta^2 = .16$). A Tukey's HSD post hoc analysis revealed participants who received active ON-tDCS ($M = 1.92$, $SD = 0.86$) reported greater difficulty concentrating compared to those who received 40 Hz ON-tACS ($M = 1.20$, $SD = 0.50$) ($p = .004$) and sham ON-tDCS ($M = 1.25$, $SD = 0.68$) ($p = .01$), but not those who received 1Hz ON-tACS ($M = 1.45$, $SD = 0.93$) ($p = .29$).

A χ^2 analysis performed found no significant relation between the stimulation administered and the participant's assumed stimulation they received ($\chi^2(3, N = 85) = 3.52$, $p = .32$; see figure 4.4C), thus suggesting that participants were well-blinded.

Figure 4.4

ON-tES Related Side Effects & Blinding



Note. No significant difference was detected between the four stimulation groups **(A)** in the visual analog scale for alertness or **(B)** in tES related side effects experienced. **(C)** The pie charts illustrate no difference in perceived group assignment. Error bars represent standard errors of the mean.

4.4 Discussion

The present study aimed to compare the effects of 40 Hz ON-tACS, active ON-tDCS, and their control counterparts during a verbal associative memory task. Interestingly, only 40 Hz ON-tACS enhanced participants' cumulative percentage of words learned on Day 1 in comparison to the other three groups. Contrarily, the cumulative learning percentages for 1 Hz ON-tACS, active ON-tDCS, and sham ON-tDCS were similar. Furthermore, participants who received active ON-tDCS or 40 Hz ON-tACS exhibited increased memory recall on Day 7 in comparison to those who received sham ON-tDCS and 1 Hz ON-tACS. Although the percentage of correctly recalled word pairs did not differ

between the active ON-tDCS and 40 Hz ON-tACS groups, it was observed that participants assigned to the 40 Hz ON-tACS group forgot significantly more word pairs in comparison to the active ON-tDCS group. These findings provide support for the hypothesis that active ON-tDCS and 40 Hz ON-tACS induce distinct behavioral effects, whereby 40 Hz ON-tACS manifested the greater effect on Day 1, online during encoding with a lesser effect seen offline on Day 7, whereas active ON-tDCS elicited an effect offline during consolidation thereby exhibiting a more influential impact on Day 7 during recall.

The present study provides a third replication of ON-tDCS having no direct effect on learning, but does have an effect on memory being observed on Day 7, further depicting active ON-tDCS' viability for upregulating the LC-NA pathway to modulate memory, and corroborating that the effect transpires offline, during consolidation (Luckey et al., 2020; Vanneste et al., 2020). Therefore, these findings suggest that the effects of active ON-tDCS occur and influence neuronal plasticity beyond the stimulation timeframe, thus potentially giving rise to a novel channel of stimulation via the LC which may be exploited to foster initial (cellular) memory consolidation.

At a cellular level, tDCS has been deemed to potentially modify the LTP essential for memory stabilization, thus gaining momentum as an explanation of cognitive enhancement when tasks are aided by t/DCS (Kronberg et al., 2017; Podda et al., 2016). It is hypothesized that active ON-tDCS' neurophysiological mechanism reflects the STC hypothesis, thus behavioral effects of active ON-tDCS during an associative memory task mimics the BT hypothesis, such that a learning event within a short time period of an additional independent novel event or stimuli will be more likely to convert to LTM (Okuda et al., 2020; Viola et al., 2014). Recent investigations of STC/BT hypotheses revealed evidence for LC-driven discharge of NA and dopamine being fundamental for

the formation of novelty-induced memories (Okuda et al., 2020), ascribable to the non-invasive approach utilized here to elevate the novelty response of the LC. Moreover, transforming early-LTP to late-LTP via similar effects of electrical stimulation has been identified in other brain regions, such as the basolateral amygdala (Frey et al., 2001; Straube & Frey, 2003), thus supporting future investigations continue to employ tDCS as a tool to ameliorate impaired synaptic plasticity to improve learning and memory in neuropsychiatric populations (Forrest et al., 2018; Reza-Zaldivar et al., 2020).

In comparison to ON-tDCS' exclusive memory improvement on Day 7, 40 Hz ON-tACS exhibited memory improvement on Day 1 and Day 7. In contrast, the 1 Hz ON-tACS active-control group produced no memory enhancement on either day, thus suggesting the difference of effects is most likely caused by the significant variance in the administered frequencies. Moreover, lower-intensity frequencies have failed to reliably entrain neural oscillations, thus proposing greater intensities of stimulation are required for immediate and long-term effects (Lafon et al., 2017). The memory performance effects of 40 Hz ON-tACS seen on Day 7 attest to gamma frequencies' association with long-term memory processes (Fries, 2009; Jensen et al., 2007). The observed increase in memory recall could be attributed to the STC-BT hypothesis given the similarities in timing of stimulation that resulted in post-encoding consolidation seen on Day 7. However, the two noteworthy findings of (1) 40 Hz ON-tACS learning significantly more word pairs on Day 1 and (2) ON-tACS forgetting significantly more word pairs from Day 1 to Day 7 when comparing the effects of the two techniques suggest that the effects of ON-tACS are a result of immediate memory benefits owing to a potential enhancement of enhanced attentional mechanisms.

Existing literature has linked gamma oscillations with focused attention, a requisite for processing incoming stimuli and memorization (Fries, 2015; Tallon-Baudry et al.,

2005; Vidal et al., 2021). Furthermore, a more specific study has associated gamma tACS with voluntary disengagement and reorienting of attention in young, healthy adults (Hopfinger et al., 2017), further endorsing the proposal that 40 Hz ON-tACS may induce increased attention. The precise mechanisms of AC NITESGON and tACS remain to be elucidated; however, it is hypothesized that a stimulation pathway upregulating the LC via rhythmic activity may shift the LC into a phasic state. Given that LC phasic activity has shown to facilitate filtering out irrelevant information and increasing attention, reflective of behaviors amid focused attention tasks (Aston-Jones & Cohen, 2005b; Benarroch, 2018; Berridge & Waterhouse, 2003) makes the LC a vital component for alterations in attention and cognitive flexibility needed for learning (Aston-Jones & Cohen, 2005b; Bouret & Sara, 2004; Vazey & Aston-Jones, 2012).

Increasing the plausibility of the present proposal, recent rodent research has indicated that modifications in goal-directed behavior, such as increasing focused attention and decreasing distractibility, are largely LC driven (Bari et al., 2020), similar to our designed stimulation; however, replication is needed in human studies. Therefore, it can be speculated that participants learned more words on Day 1 in comparison to Active ON-tDCS and control groups due to 40 Hz ON-tACS producing enhanced attention when completing the task, thus inferring that improving attention will improve learning and memory, and thereby offering an alternative approach to boost memory performance.

These findings and proposed rationales are substantial, seeing that this area of research is currently being pursued due to prior evidence linking brain plasticity alterations, an early marker for AD, with gamma activity decrements (Babiloni et al., 2016). However, current research is limited and methods vary, particularly regarding tACS administration. Therefore, it is suggested that future research be continued to pursue tACS application to modulate gamma activity to improve cognitive performance.

5 General Discussion– Role of LC in Memory

5.1 Summary

Today's global society has been confronted with a rapidly growing proportion of people over the age of 60 (World Health Organization, 2021) that goes together with the increasing prevalence of age-related cognitive decline and neurocognitive disorders (Wallin et al., 2018). Accordingly, neuroscientific research has shifted its focus towards brain health, and considerable interest has been invested in discovering preventative strategies that maintain and strengthen physiological and cognitive proficiency as well as intervention strategies aimed to postpone or control adverse pathologies.

The first objective was to investigate the LC's role in memory – at the end of the spectrum in which the LC-NA mechanism functions as an essential modulator in core brain functions and behaviors. To achieve this, the role of the LC in memory was assessed by validating the mechanistic evaluation of how (i.e., indirect) and determining when (i.e., online vs. offline) non-invasive electrical stimulation of the LC affects neural circuits to induce substantial, positive effects on memory formation and storage.

The evidence detailed in Chapter 1 Section 1.4 established that utilizing a non-invasive approach to artificially upregulate LC activity while entraining the memory pathway in a young, healthy adult population is conceivable. Building upon these findings, research conducted in Chapters 2 through 4 intended to bolster the understanding of the previously observed NITESGON phenomena by critically examining memory performance to provide a coherent framework for the LC system's influential impact across various neural mechanisms and processes.

Results of **Chapter 2** replicated the behavioral and biomarker results of previous NITESGON studies in an older (i.e., between the ages of 55 and 70 years), healthy, adult population whereby DC NITESGON demonstrated significantly improved memory

recall 7-days and after learning. Although not significant, DC NITESGON increased the percentage of words recalled 28-days after learning. Given the medium-to-large effect size, this null finding can be interpreted with caution and suggest that low statistical power may have resulted in a high risk of type II error. Furthermore, sAA and cortisol concentration measures confirmed the favorable implication of NITESGON was prompted via LC-induced arousal, as opposed to LC-induced stress, to upregulate memory, thus establishing that one session of NITESGON facilitates memory improvement in two distinct age populations via LC arousal – eliminating potential age limitations of NITESGON application.

Furthermore, **Chapter 3** advanced the overall knowledge of NITESGON by identifying consolidation as the phase of memory in which DC NITESGON effects transpire, exhibited by enhanced recall on Day 7. More distinctly, NITESGON generated retroactive and proactive memory improvement effects, signifying the existence of a temporal window in which LC-mediated arousal induces memory enhancement offline, presumably during initial memory consolidation. Additionally, when a second word association task (e.g., Japanese-English) was introduced to interfere with that of the other word association task (e.g., Swahili-English), it was found that NITESGON diminished the interference effect. Evaluation of these findings proposes that the effects of DC NITESGON targeting the LC result from the BT hypothesis' neurobiological model.

Moreover, Chapters 2 and 3 exclusively utilized DC NITESGON; however, it remained unknown if observed modulatory effects translated to other NITESGON applications targeting the LC. Direct comparison of DC and AC NITESGON memory effects in **Chapter 4** revealed two notable findings: (1) 40 Hz AC increased the cumulative percentage of word pairs learned on Day 1, and (2) 40 Hz AC and DC increased the percentage of correctly recalled word pairs on Day 7, suggesting both

induce distinct behavioral effects. This evidence identified 40 Hz AC NITESGON had more impact when administered during encoding by inducing an immediate, online effect owing to an increase in attention, and application of DC NITESGON during encoding generating a more significant effect offline during memory consolidation that brings about more robust long-term memory processes.

Below, the discussion will begin with an overview of the basic and functional effects of NITESGON, particularly with how it purportedly influences the LC system to enhance memory performance. The discussion will then identify when 40 Hz AC NITESGON induces immediate neuromodulatory effects and describe the vital relationship between the LC-NA system and attention to improving memory processes. Subsequently, a more in-depth discussion of when DC NITESGON's offline effects occur and highlight the LC-NA system's role in initial memory consolidation and its relevance in improving memory processes will ensue. Here, outlined are important concepts such as the BT/STC hypotheses, followed by their relevance to dopaminergic signaling. Lastly, potential implications for AC and DC NITESGON's use as a preventive or ameliorative approach to AD and highlights of the current research's methodological strengths, weaknesses, and future direction for LC-based NITESGON studies will be discussed.

5.2 Basic and Functional Effects of NITESGON

5.2.1 How: Validation of the Peripheral Effect of NITESGON on the LC

Non-invasive neuromodulation methods have shown promise as a non-pharmacological approach to modulate the brain; however, a comprehensive understanding of its neurophysiological mechanism remains largely unknown (Liu et al., 2018). Today, the predominant consensus of tES primarily presumes that neuromodulatory effects are generated by directly modifying neurons (Reed & Cohen

Kadosh, 2018); however, researchers have recently begun to consider an alternative explanation, such that tES most likely affects neural circuits indirectly (i.e., via peripheral nerves) (Asamoah et al., 2019; Liu et al., 2018; Voroslakos et al., 2018). Per this, prior laboratory research incorporating various methodologies established NITESGON's ability to upregulate LC-NA activity via stimulation of the occipital nerve alone (Vanneste et al., 2020). Furthermore, the present thesis has validated the findings of the previous work and the utilization of peripheral nerve stimulation via NITESGON to upregulate the LC-NA pathway, evidenced by biomarker changes in sAA concentration levels and neural marker synchronization of gamma activity in the medial temporal lobe.

The postulated pathway in which NITESGON peripherally evokes LC-activation and subsequently distributes NA throughout the brain originates using two scalp electrodes to release a weak current that activates the ON transcutaneously. Distinguishing itself from other peripheral nerves, such as the vagus or trigeminal nerves, the ON contains numerous branches innervating the posterior scalp and as far anterior as the vertex of the scalp, thus lending itself as an ideal location to facilitate non-invasive stimulation (Weiner & Alo, 2018). Following ON activation, afferent fibers of the ON innervate the brainstem, projecting to the NTS (Couto et al., 2006). In response, the NTS can influence NA activity indirectly via connections linking the LC to the amygdala and hippocampus or directly via synapses with the LC whereby arousal is induced, and the distribution of NA activity is promoted (Couto et al., 2006). Hence, the utilization of NITESGON gives rise to an increase in NA release mediated by the LC.

In order to validate the effects of NITESGON on LC-NA activation, experiments throughout the present thesis heavily relied on the detection of biomarker changes, measurements of sAA concentration were measured immediately before and immediately after NITESGON application. The studies consistently demonstrated NITESGON's

ability to increase sAA concentration levels in both young and older adult populations, regardless of the nature of the type and frequency of current employed (i.e., AC or DC). These findings suggest that the neural mechanism underlying NITESGON modulation in older individuals corresponds to that which occurs in a younger population. In consideration of NITESGON becoming an application method capable of reliably upregulating LC-NA activity, the demonstration of generalizable application and results thus far are highly significant.

Further contextualizing the neuromodulatory effects of NITESGON's peripheral mechanism, these studies validated increased LC-NA activity as the neurophysiological mechanism responsible for NITESGON related memory improvement. Moreover, additional evidence of transcutaneous stimulation via NITESGON was observed in positive correlations between increased sAA concentration or increased gamma power in the medial temporal cortex and recall performance 7-days after initial learning, respectively, thus validating the enhancement of memory formation through the hippocampal LC-NA pathway.

Collectively, this mechanistic evaluation spotlights the indirect peripheral pathway in which non-invasive electrical stimulation reaches the LC and ignites the far-reaching noradrenergic neurotransmitter network. Accordingly, the demonstration of NITESGON's modulatory effects may assist in developing a protocol to induce LC-NA activity to influence diverse behavioral and physiological processes. In the following, a summary of the direct implications of using NITESGON targeting the LC and ways NITESGON has shown its ability to alter neural activity online during application (40 Hz AC) as well as induce long-lasting alterations of cortical excitability and activity offline during consolidation (DC) is provided.

5.2.2 When: 40 Hz AC NITESGON Online – Encoding

AC NITESGON was incorporated and exclusively used in Chapter 4 to explore the modulatory effects amongst different tES techniques. Behaviorally, 40 Hz AC NITESGON exhibited an immediate increase in the cumulative percentage of learned word pairs on Day 1; the first stimulation technique throughout this thesis to show such an effect. Moreover, improved memory recall was seen on Day 7, presumably due to LC-enhanced attentional mechanisms induced during learning. It is speculated that 40 Hz AC NITESGON targeting the LC is the mechanism behind the positive effects generated online during the encoding phase of memory; however, caution is due here given that this study did not investigate the underlying mechanism of action.

Cortical oscillations are fundamental processes underlying cognition and behavior (Buzsaki & Draguhn, 2004). For this reason, researchers have utilized the recognized practice of applying predetermined frequencies via tACS to enhance oscillatory synchronization (Tavakoli & Yun, 2017); however, lower-intensity frequencies have exhibited deficiency in entraining neural oscillations, suggesting greater intensities of stimulation are required for immediate and long-term effects (Lafon et al., 2017). Accordingly, no memory enhancement was observed in the 1 Hz AC NITESGON control group, whereas the 40 Hz AC NITESGON group displayed performance effects on Day 7, attesting to gamma frequencies' association with long-term memory processes (Fries, 2009; Jensen et al., 2007). However, this does not account for the immediate memory effect seen during learning, which may alternatively be explained by gamma oscillation's role in focused attention, a requisite for the processing of incoming stimuli and memorization (Fries, 2015; Tallon-Baudry et al., 2005; Vidal et al., 2021) as well as voluntary disengagement and reorienting of attention (Hopfinger et al., 2017).

The application of NITESGON to upregulate the LC at a frequency associated with gamma oscillations elicited a neuromodulatory influence on attentional processes. The observed effect is hypothesized to arise from a shift in the LC's neural firing pattern from an exploratory state (i.e., tonic firing) to a task-specific state (i.e., phasic firing) in response to AC NITESGON. Activation of the LC neuromodulatory system is responsible for responding to novel or salient stimuli and sensory signal processing; this can either be a result of bottom-up processes or top-down inputs from the prefrontal cortex (Avery & Krichmar, 2017; McBurney-Lin et al., 2019; Waterhouse & Navarra, 2019). In response, dense LC projections release NA into the prefrontal cortical areas, a region well supplied with α_2 adrenoceptors and highly involved in attention and behavioral arousal mechanisms (Arnsten & Li, 2005). The current hypothesis of the mechanism of AC NITESGON features the adaptive gain theory; however, there are other potential models of LC-NA function in arousal that may also provide other explanations, such as the network reset theory (Bouret & Sara, 2005) and the GANE (glutamate amplifies noradrenergic effects) model (Mather & Harley, 2016). In general, it seems possible that the application of AC NITESGON during the memory task may be boosting the LC-NA response and, therefore, assist in initiating brain state changes that facilitate the focused attention and cognitive flexibility needed for learning (Aston-Jones & Cohen, 2005b; Bouret & Sara, 2004; Vazey & Aston-Jones, 2012). Considering the highly influential role that the LC has on attentional mechanisms, it could be that the utilization of 40 Hz AC NITESGON induced an immediate effect by way of increased focused attention that led to more accurate task performance. In turn, suggesting 40 Hz AC NITESGON targeting the LC offers an alternative approach to boost memory.

In support of this phenomenon, two studies utilizing pupillometry response attest to the role phasic LC-NA activity plays in enhanced attentiveness and task performance

when in a selective (Hoffing & Seitz, 2015) or a sustained attentional state (Unsworth & Robison, 2016). Moreover, a recent rodent study indicated a causal link exists between activation of the LC-NA pathway and attention control via two distinct stimulation pathways, whereby stimulation of LC neuronal projections to the ventrolateral orbitofrontal cortex resulted in decreased distractibility; in addition, stimulation of LC neuronal projections to the dorsomedial prefrontal cortex subsequently resulted in increased goal-directed attention (Bari et al., 2020). Thus, pinpointing two distinct coeruleo-cortical pathways in which the LC may enhance attention and reduce impulsivity (Bari et al., 2020). Additionally, a recent study used a similar paradigm in which tACS (40 Hz) over the Pz electrode improved immediate memory recall and offline delayed recall of an episodic memory task in patients with MCI due to AD (Benussi et al., 2021). Upon consideration of the observed effects of 40 Hz AC NITESGON and previous reports of tACS indirectly affecting neural circuits (Asamoah et al., 2019), inquiries are postulated regarding if the prior study was a result of stimulation of the precuneus area or if it was potentially due to peripheral stimulation of the branching of the ON thus activating the LC. Given the relationship between the LC-NA system, attention, and the potential for the AC NITESGON neuromodulation of memory encoding, the evidence provided here suggests that future research in the pursuance of tACS application to upregulate gamma activity to improve cognitive performance should be continued. Moreover, the findings of AC NITESGON promoting attentional mechanisms may also contribute to therapeutic techniques aiming to regulate psychiatric disorders with symptoms of inattention and impulsivity, such as ADHD, or other neurocognitive disorders related to a decline in attention, such as frontotemporal dementia.

5.2.3 When: DC NITESGON Offline – Consolidation

The present thesis provided evidence identifying that non-invasive direct current stimulation of the LC generates positive effects on memory formation and storage – offline during the consolidation phase of memory. The initial indicator of this assertion was the observation of NITESGON having no immediate learning effects on Day 1, in addition to no immediate effect when NITESGON was applied on Day 7 during retrieval. However, findings produced consistent and reproducible long-term memory enhancement effects 7-days after initial learning with an effect that may last up to 28-days when NITESGON was applied during or immediately after the learning task in both healthy, young- and older-adult populations.

It is well established that memories are initially transient in nature shortly after encoding, however, have the potential to undergo initial memory consolidation, a continuous strengthening and stabilization process that involves LC-reliant structural and chemical changes in neural ensembles, within seconds (up to hours) of a learned experience (Dudai et al., 2015; Sara, 2009b; Squire et al., 2015). Moreover, the consolidation process can be greatly regulated by varying behavioral, hormonal, and neural influences during this labile period, thus altering the potential for LTM formation (Nader et al., 2000). Consequently, this has resulted in substantial investigations aiming to enhance and/or exploit the neural mechanisms involved in memory consolidation (Eschenko et al., 2017). The present thesis utilized the LC's role in memory processes to propose a technique capable of artificially upregulating the LC-NA system to prompt LC-induced modulatory effects generated by peripheral nerve stimulation originating via an extraneous source of novelty. To my knowledge, no previous investigations have explored such an effect, thus the current research adds to and will be of interest to the

growing body of non-invasive stimulation research aiming to enhance memory performance.

Evaluation of the current thesis' evidence has led to the speculation that the effects of DC NITESGON are a result of the LC playing a significant role within the neurobiological model of STC/BT. The STC hypothesis is a framework explaining how changes in synaptic plasticity are promoted and reinforced owing to two dissociable actions: (1) the creation of a local "synaptic tag" by way of a weak tetanization that in turn "capture" (2) plasticity related proteins synthesized by dopamine that arise from a strong tetanization immediately preceding, during, or succeeding the weak tetanization (Frey & Morris, 1997; Redondo & Morris, 2011), otherwise known as "synaptic cooperation" (Pinho et al., 2020). Accordingly, behavioral studies rendered an extension of this representation, known as the BT hypothesis, whereby a weak encoded event (i.e., a learning-behavioral tag) inducing STM may be converted to LTM when accompanied by a novel or strong event (i.e., capable of inducing the synthesized proteins) immediately prior to, during, or immediately after learning (Moncada et al., 2015; Moncada & Viola, 2007; Viola et al., 2014). Here, the methodological design of the present experiments enabled the fulfillment of prerequisites within the BT paradigm, in particular, the learning task (i.e., weak event) satisfied the setting of a behavioral tag and NITESGON operated as the essential strong stimulus delivered either simultaneously, during, or immediately after the learning task.

The framework proposed by the STC/BT hypotheses have spotlighted the LC as a brain region of interest. In the context of the BT model, the LC upholds a critical twofold role: (1) responding to novel or salient stimuli or experiences and (2) releasing the dopamine necessary for synthesizing proteins. Firstly, novel or salient events, in addition to stress, trigger LC-NA phasic activity which results in a heightened level of arousal

(Aston-Jones & Cohen, 2005a, 2005b), and in turn sparks reactions from the LC that influence LTM storage and enhance memory persistence in a comparable fashion to that of vivid "flashbulb memories" brought on by surprising or emotional experiences (Cahill & McGaugh, 1998; Lemon & Manahan-Vaughan, 2012; McGaugh, 2015; Vogel & Schwabe, 2016). Secondly, novelty-induced LC activation gives rise to dopaminergic signaling in the hippocampus, a requirement for the synthetization and availability of plasticity related proteins needed for synaptic potentiation and retention of novel memories (Moncada & Viola, 2007; Wagatsuma et al., 2018; Wang et al., 2010). Recently, discussions regarding the source of dopamine's origin have emerged and have shifted from dopamine originating in the VTA (Rossato et al., 2009) to proposals of LC tyrosine-hydroxylase neurons mediating post-encoding enhancement of memory in a manner consistent with corelease of NA and dopamine from dense LC axons in the hippocampus (Kempadoo et al., 2016; Takeuchi et al., 2016). Considering the twofold role of the LC in context of the BT model, it can be suggested that NITESGON initiated a novelty response from the LC causing a cascading effect to invoke initial memory consolidation.

Moreover, evidence of the dual-task assessments carried out within Chapter 3 further endorsed offline effects via NITESGON-induced LC-novelty, and additionally validated the essential disposition of a temporal-proximity associated with the weak training event that determines the occurrence of memory consolidation. Here, retroactive and proactive memory effects were observed – NITESGON retroactively credited the word associations learned prior to the object-locations as well as proactively credited the object-locations learned subsequently to the word associations – indicative of the BT model (Moncada et al., 2015; Viola et al., 2014).

Despite the sparse number of studies demonstrating the BT paradigm in human participants within the last decade, the present thesis in conjunction with prior studies have collectively strengthened the generalizability of ways to retroactively and proactively induce memory enhancement (Ballarini et al., 2013; Dunsmoor et al., 2015; Kalbe & Schwabe, 2021; Oyarzun et al., 2016; Patil et al., 2017; Ramirez Butavand et al., 2020). Variations in methodological designs, such as what weak training tasks and sources of novelty were used have successfully supported effects occurring within the consolidation of memory, thus suggesting that the effects of BT are potentially not specific to the task or the novel stimulus. Furthermore, the accumulation of evidence has exhibited BT in a wide range of age groups, as well as in a broad range of times elapsed between learning and memory performance assessments; all while utilizing a strong stimulus within the generally acknowledged +/- 1-hour "window of opportunity" surrounding the weak event (Ballarini et al., 2013; Dunsmoor et al., 2015; Kalbe & Schwabe, 2021; Oyarzun et al., 2016; Patil et al., 2017; Ramirez Butavand et al., 2020). However, separating the prevailing experiments from prior human BT research is the inaugural utilization of non-invasive brain modulation upregulating the LC-NA system as the novel stimulus. Consequently, this has potentially cast a new light on an offline effect whereby the administration of this artificial NITESGON-induced LC novelty reduces interference despite being utilized in a dual-task paradigm that employed two tasks highly similar in nature within the immediate succession of one another.

Memory formation and persistence are susceptible to interference occurring pre- and post-encoding (Zeithamova & Preston, 2017) and are heavily influenced by commonality amongst the learned and intervening stimuli (Varma et al., 2017); therefore, it was highly anticipated that effects of interference would be observed. Within the STC/BT literature, memory interference has been proposed to be the result of "synaptic competition," a

proposed “fight for proteins” that arises between tagged synapses when the amount available is insufficient to satisfy the requirements for all synapses within the specific time frame. Furthermore, competition amongst limited protein resources between two memory traces for their consolidation results in one memory converting to LTM at the expense of the other. Therefore, it is postulated that the LC released increased levels of dopamine in response to NITESGON-induced novelty, which in turn potentially enabled optimal availability of plasticity related proteins, thus eliminating, or significantly decreasing, synaptic competition and subsequent interference owing to learning tags set in the hippocampus not having to compete amongst one another to initiate the consolidation process. However, further investigations of NITESGON induced dopaminergic release is needed to confirm this postulation.

5.3 Implications

Evidence obtained throughout the present thesis has facilitated the endorsement of a proper proposal regarding implications concerning the observed NITESGON-initiated LC-induced effects of memory augmentation. Specifically, the exhibition of consistent results throughout the various experiments within the research has established a reproducible stimulation paradigm for neuromodulation of the LC-NA system via DC NITESGON and has also initiated the foundation of a potential stimulation model for AC NITESGON. Moreover, findings from this thesis will add to and be of interest to the growing body of non-invasive stimulation research aiming to upregulate the LC neuromodulatory system artificially or enhance cognitive functions considering that evidence within identifies when to stimulate and with what type of stimulation to induce meaningful effects. Overall, the findings allude to intriguing possibilities of how

NITESGON may be utilized in future clinical practices as a therapeutic strategy to prevent, delay, or alleviate the neuropathology or symptomatology of AD.

5.3.1 AC NITESGON

Forty Hz AC NITESGON improved online and offline associative memory performance through arousal-related LC activity. Here, 40 Hz AC NITESGON was employed due to gamma frequency's involvement in attention and LTM operations (Fries, 2009; Jensen et al., 2007) and acted as a novel stimulus to the LC, thus presumably initiating the transition into a phasic state. In response, the LC phasic firing pattern induced increased gamma power in the prefrontal cortex, resulting in the observed online memory improvements. These findings have important implications for developing our understanding of cognitive improvement; however, in light of recent research regarding gamma entrainment and novelty-induced activation of the LC, respectively, continued research utilizing AC NITESGON may lead to more significant implications that may hold substantial interest to the field of AD research.

Of note, due to technological advances in recent years, a mechanistic link between the increasing vulnerability of the LC and the progression of neurodegeneration in AD has been further acknowledged (Matchett et al., 2021), such that the LC has been identified as one of the earliest hosts of tau pathology (Braak & Del Tredici, 2011) and dysregulation of the LC-NA system as one of the neuromodulatory systems associated with memory impairment in AD (D. Weinshenker, 2008). Thus, the LC's significant role in memory and AD, as discussed in greater detail in the next chapter, has caused it to become a primary target for research.

Emerging animal models and a recent human study have brought attention to the potential protective properties of phasic LC-NA activity and utilizing sensory-evoked 40

Hz entrainment to restore the synchronization of gamma oscillations, respectively. One study induced LC phasic activity or excessive LC tonic activity via optogenetic activation to rodents seeded with human pretangle tau; rodents that were regularly exposed to phasic activity exhibited reduced memory impairment and displayed preserved LC axonal strength (Omoluabi et al., 2021). Furthermore, a second study utilized positron emission tomography imaging to demonstrate the association between increased LC neurochemical function and decreased AD-related pathology, as well as an association between increased LC neurochemical function and self-reported cognitive engagement and physical activity (Ciampa et al., 2022). Together, these associations posit that LC phasic activity induced by novelty-like experiences can uphold LC neuronal strength and may prevent LC impairment or susceptibility to AD (Ciampa et al., 2022). Moreover, additional rodent research brought attention to enhancing neuronal preservation and reducing AD pathology via sensory-evoked 40 Hz entrainment (Adaikkan & Tsai, 2020). Synchronized oscillations in the forebrain improved cognitive functions (Martorell et al., 2019); however, more strikingly, chronic gamma entrainment had a significant influence on neurons and non-neuronal cell types that contributed to the preservation of neuronal and synaptic density and exhibited neuroprotective effects against AD-associated neuropathology and neurodegeneration (Adaikkan et al., 2019; Martorell et al., 2019). Presented with these findings, it is not illogical to suggest the use of 40 Hz AC NITESGON to artificially shift the LC into a phasic state to promote NA's neuromodulatory effects to maintain cognitive function as well as neuroprotective effects to preserve axonal strength and influence non-neuronal cell types to reduce AD-related neuropathology, however, this implication will require future investigations.

5.3.2 DC NITESGON

DC NITESGON improved associative memory performance through novelty-induced LC-arousal that promoted the release of NA, and likely dopamine, required for synaptic memory consolidation, a process in which information captured during encoding is physiologically organized to become permanent LTM. Given this formidable effect, DC NITESGON may potentially improve memory formation by hampering pathological disruption of memory consolidation in a non-invasive and nonpharmacological manner. Recently, two emergent studies presented evidence indicating accelerated long-term forgetting (ALF), a phenomenon whereby newly acquired information is retained for up to 30-minutes but is then rapidly forgotten within hours to weeks, is a pre-symptomatic indicator of AD (Wearn et al., 2020; Weston et al., 2018). More specifically, ALF can predict cognitive decline in healthy older individuals (Wearn et al., 2020) and appears on average 7-years before estimated symptom onset (i.e., amnestic MCI) in a cohort of autosomal dominant AD families (Weston et al., 2018). Discriminating cognitively normal healthy individuals from individuals that exhibit ALF in the pre-symptomatic stage of AD may help lead to an earlier marker of AD-related cognitive decline (Wearn et al., 2020; Weston et al., 2018), thus giving rise to an earlier stage whereby preventative measures may be utilized.

ALF exhibits atypical forgetting despite normal encoding or early memory recognition, which is indicative of a breakdown occurring in the post-acquisition consolidation of long-term memories (Hoefeijzers et al., 2013). Contrary to the well-recognized diminishment of neuroplasticity and decline in episodic memory regarding AD, there is comparatively little knowledge of the contributions and/or impacts of long-term consolidation barring the understanding that the medial temporal lobe, especially

the hippocampus, plays a significant role (Braak & Del Tredici, 2015; Brier et al., 2016). ALF places a newfound emphasis on the consolidation process of memory in relation to AD, however, it should not go unmentioned that the neuromodulatory systems in charge of regulating memory are undoubtedly just as vital as the brain regions responsible for storing the memory (Cahill & McGaugh, 1996). Considering the substantial influence the LC-NA system has in hippocampal plasticity via synaptic connections supporting memory formation (Wagatsuma et al., 2018), the presumable diminished plasticity underpinning the detriment to long-term memory demonstrated in ALF, and the auspicious effects exhibited by DC NITESGON, NITESGON may prove to be a valuable therapeutic approach for alleviating the symptomology of AD. Through NITESGON induced LC-NA upregulation, NITESGON can be used as an early intervention technique to mitigate features of ALF and improve memory consolidation.

Altogether the findings presented in this thesis suggest that both AC and DC NITESGON may assist in mitigating AD-related memory impairment and neuropathology, albeit through seemingly different mechanisms.

5.4 Strengths, Limitations, and Future Directions

5.4.1 Methodological Strengths

Several strengths lie within the methodological designs described throughout the present research. One of the primary methodological strengths of the thesis was that all experiments were designed as randomized, double-blind, sham-controlled studies that included tES naïve human participants. Furthermore, there was no significant missing data of note for the studies included within this thesis. Additionally, the inclusion of human participants enabled the exhibition of NITESGON's ability to provoke an LC response in both younger and older adults, thereby excluding a potential age-

demographic limitation. Considering evidence has demonstrated that cognition may decline in relation to age (Hedden & Gabrieli, 2004; Park & Festini, 2017) and that there are myriad of individual neuroanatomical differences (e.g., scalp, skull thickness) (Voroslakos et al., 2018), including human participants demonstrated NITESGON's capability to overcome these naturally occurring phenomena. Moreover, prior investigations of BT have been predominately limited to animal research (Moncada et al., 2015), therefore the present studies establish that human memories may be formed through the BT model, which significantly contributes to the validity of the proposed hypothesis.

An additional strength of the methodological design was the position of the two (anode and cathode) scalp electrodes to target the ON during stimulation. A consistent and steadfast electrode placement has been emphasized as a critical consideration by technical guides to tES usage (Thair et al., 2017; Woods et al., 2016). Thus, the capability of NITESGON to incorporate an effective electrode placement without needing to be altered across a large population, with minimal to no adverse events, suggests that it is a practical, convenient stimulation technique that may be readily utilized to elicit favorable behavioral effects. Moreover, while acknowledging that there is still a great deal of investigations to be performed, NITESGON's uncomplicated and manageable electrode placement will help to facilitate NITESGON as a potential at-home treatment approach.

Additional methodological strengths were made evident in Chapters 2-4, whereby NITESGON featured as a critical component within the neurobiological model of the BT hypothesis. A key strength of these chapters was the use of NITESGON to artificially upregulate the LC-NA system to boost the transformation of STM to LTM. Furthermore, Chapter 3 incorporated dual-task paradigms, thus enabling the recognition of NITESGON's ability to generate retroactive and proactive memory effects and that

NITESGON effects are not confined to the stimulation period but instead operate within a window of opportunity. Incorporating the dual-task paradigms permitted the use of tasks of different domains, thus facilitating the substantiation that NITESGON effects were not task-specific. In addition, the dual-task paradigm permitted the use of similar domains, thus exhibiting NITESGON's presumptive effect of reducing memory interference. Utilizing NITESGON, unlike prior human-BT studies that employed educational lessons (Ballarini et al., 2013; Ramirez Butavand et al., 2020), aversive shock to the wrist or leg (Dunsmoor et al., 2015; Kalbe & Schwabe, 2021), or reward (Oyarzun et al., 2016; Patil et al., 2017) as their associated novel stimulus, respectively, enabled the demonstration of NITESGON's capability to improve memory for both tasks within the dual-task paradigms as well as the diminution of memory interference, two features that have not been observed in human-BT research previously.

The final strength encompassed within the methodological design was presented in Chapter 4 via a direct comparison of AC and DC NITESGON whereby the single divergent factor between the two protocols was whether alternating or direct current was employed during a word association memory task. Only a marginal number of studies have comparatively analyzed the behavioral modulatory effects obtained via tACS and tDCS, many of which have been restricted to working memory tasks, whereby either theta- or gamma-tACS was compared to anodal-tDCS and sham stimulations employed across various brain regions (even when incorporated within the same study) (Hoy et al., 2015; Jones et al., 2019; Lang et al., 2019; Rohner et al., 2018). This absence of comparative studies may be contributing to the gap in the current understanding of the underlying mechanism(s) of stimulation and behavioral outcomes. Therefore, the rare, direct comparison of AC and DC NITESGON effects with minimal stimulation paradigm

differences is a significant strength, particularly given that distinct behavioral differences were observed between the two techniques.

5.4.2 Methodological Limitations

When considering this thesis, it must be taken into account that some limitations exist within the research, such as small sample sizes, which warrant cautious interpretation of results and encourage replication studies with larger sample sizes.

Moreover, the current thesis explicitly focuses on behavioral data; thus, these studies were limited by the absence of neuroimaging techniques that may help establish the precise underlying mechanism of NITESGON. The mechanism of NITESGON is speculated due to the nature of the experimental design of the current studies unable to simultaneously measure brain activity during the task while also undergoing NITESGON. Therefore, a direct link between NITESGON-induced alterations in brain function and changes in associative memory performance remains to be determined. Further studies need to be conducted to determine whether NITESGON directly modulates cortical activity or other neuromodulatory pathways in addition to the LC-NA pathway, thereby contributing to the observed behavioral results. The non-invasive technique used in the present studies gives rise to the potential that these effects may be mediated partially by a transcranial mechanism along with the transcutaneous mechanism. Based on the electrode placement, NITESGON may also be activating the parietal cortex. The posterior parietal cortex has long been implicated in attention processes and episodic memory retrieval (Cabeza et al., 2008; Ciaramelli et al., 2008) and has recently been shown to improve face-word associative memory after tDCS (Vulic et al., 2021). These findings suggest examining the link between NITESGON and the posterior parietal cortex more closely.

Additionally, it must be recognized that the effects obtained could be explained by activating other neuromodulatory mechanisms. Previous animal research indicates that peripheral nerve stimulation, such as vagus nerve stimulation, also activates the dopaminergic (Perez et al., 2014), serotonergic (Hulsey et al., 2019), and cholinergic (Hulsey et al., 2016) pathways which release dopamine (Duszkiewicz et al., 2019) and acetylcholine (Maurer & Williams, 2017) both of which play an important role in inducing long-term plasticity changes related to memory consolidation. Further studies regarding the role of additional neuromodulators would be worthwhile.

A further limitation distributed across several facets is the omission of utilizing a proxy measurement for dopamine. The absence of a dopamine proxy measure resulted in the inability to make any inferences regarding the release of LC-dopamine. Furthermore, this prevented distinguishing the source of dopamine and thus if the VTA had a role in the improved memory performance effects induced by NITESGON. It could be argued that the VTA indirectly contributed to the formation of memories via other brain areas, given that recent animal research has suggested that VTA dopaminergic neurons project to the amygdala and bolster emotional memory in conjunction with the LC (Giustino et al., 2020; Tang et al., 2020), as well as contribute to synaptic consolidation independently and complementary to the LC (Moncada, 2017). Moreover, a recent opinion piece has suggested that the VTA-dopaminergic system may instead promote memory formation via sleep-dependent hippocampal reactivation or systems memory consolidation, whereby memories are reorganized and transferred from the hippocampus to the neocortex (Duszkiewicz et al., 2019); however, a consensus regarding the role of VTA-dopamine has yet to be achieved. VTA-dopamine's influence on synaptic and systems consolidation may have potentially influenced the observed effects of the thesis, however, the absence of a dopamine proxy measure limits the thesis to default to making inferences

about the source of dopamine based on evidence from previous studies and ongoing debates, thus highlighting future work is necessary to validate dopaminergic co-release from the LC.

Due to the previously acknowledged limitation of neuroimaging technique shortages, replication studies with additional EEG analyses and/or fMRI measurements will need to be conducted to provide confirmatory data regarding the findings of 40 Hz AC NITESGON presented in Chapter 4. Regarding the mechanism of 40 Hz AC NITESGON, further research is required to determine if or how AC NITESGON leads to activation of the LC-NA system to affect memory formation, as well as, given higher-order cognition's strong link to an extended range of communication between different brain regions, it is feasible that AC NITESGON is not only contingent on oscillatory frequency but also gamma oscillations being driven by a lower frequency in nested oscillations (Pagnotta et al., 2020); such replication studies will help establish tACS' interactions between brain regions. The results of AC NITESGON's potential role in improved attention mechanisms have considerable implications for AC NITESGON's potential as a future therapeutic technique, making these confirmatory studies an utmost priority.

Sleep is recognized as a critical element to memory consolidation (Rasch & Born, 2013), thus denoting a limitation of the thesis was the omittance of assessing potential effects of NITESGON on participants' quality and hours of sleep during the 7-days between learning and testing.

Lastly, despite participants being asked to refrain from practicing or searching for the word associations online, it was impractical to control the participants' actions outside of the studies, thus resulting in the chance that participants may have rehearsed the information between visits and ultimately influencing their performance.

5.4.3 Future Directions

Exceptional research to this point has led to a new appreciation for the LC-NA system. Expanding the knowledge of LC organization and modulatory effects in behavioral and cognitive processes will help lead to LC-based therapeutic approaches targeting neuropsychiatric and neurological diseases with more clinical effectiveness. The limitations mentioned above have highlighted potential future research that may be conducted to increase our understanding of LC neuromodulation and eliminate any previous oversites. In addition, a more natural progression of the current research should incorporate investigations regarding optimal parameters of AC and DC NITESGON administration and to extend investigations into other neurocognitive domains.

In order to discover the most advantageous framework for AC and DC NITESGON, future research investigating variables such as length of stimulation, number of sessions, and time between the stimulation and learning task is strongly recommended. Accordingly, longitudinal studies examining the longevity of NITESGON's effects are also needed. Such evaluations would determine if there is a need for supplementary memory maintenance stimulation sessions of NITESGON to induce longer-lasting memories. Furthermore, it is important to note that questions about optimal parameters remain for both AC and DC NITESGON usage and that parameters for superior cognitive improvement or exploiting potential neuroprotective properties may vary amongst the two techniques, thus indicating further exploration will be needed for both currents and various targeted effects.

Moreover, future research should explore if NITESGON-induced LC-arousal affects other neurocognitive domains, mainly executive functions, in addition to other types of memory. For instance, AC NITESGON's immediate effect raises queries regarding

whether it induces advantageous effects within cognitive domains that require more immediate action, such as working memory, decision-making, processing speed, and inhibition. These particular cognitive tasks rely heavily on attentional control and selection to execute goal-directed behaviors; two facets highlighting the dual involvement of LC-arousal in regulating behaviors and cognition. Furthermore, future research should examine the generalizability of DC NITESGON's effect in consolidation on other types of learning and memory tasks beyond associative memory and free recall. In addition, future research might explore how novelty-induced LC-arousal via NITESGON affects memories that undergo the process of reconsolidation, such as the rehearsal of learning a new language or relearning to play the guitar. DC NITESGON's potential to ensure restabilization or solidify robust memories may considerably impact implicit learning and semantic memory or motor learning and procedural memory.

Taken together, this portion of the thesis has addressed the first primary objective by highlighting the favorable end of the spectrum and substantiating a role for the LC-NA system in attentional mechanisms and hippocampal-based memory formation and, to a greater degree, put forth a NIBS protocol to artificially upregulate the LC-NA system to induce such effects. The current discussion has highlighted additional avenues of research to be pursued that may provide valuable evidence for optimal NITESGON parameters for the enhancement of learning and memory, in addition to future therapeutic approaches to impede AD-like behavioral deficits and neuropathological detriments.

To fully apprehend the significance and criticality that the LC-NA system possesses on brain function, the opposite end of the spectrum must be examined. Therefore, the subsequent chapter will highlight the second primary objective of this thesis and showcase the prominent role the degradation of the LC-NA mechanism holds in the emergence and progression of AD and the hypothesized rationale behind females'

predisposition. The stark contrast denoting a functional and nonfunctional LC-NA system enables the exhibition of the complete spectrum that is the LC-NA system.

6 Sex Differences in the LC: A Heuristic Approach that May Explain the Increased Risk of Alzheimer's Disease in Females

6.1 Importance of Subject

Today, there are a total of 55 million dementia patients across the globe, including 6.4 million Alzheimer's patients that reside in the United States alone ("2021 Alzheimer's disease facts and figures," 2021). Amongst the patients in the U.S., 3.9 million are female and 2.5 million are male, a matter of substantial interest regarding the present theory and AD research as a whole (Rajan et al., 2021). Considering the significant number of AD patients in the U.S., which is projected to double by 2050, and the two to one ratio it yields, this hypothesis has the potential to help in early identification and intervention of AD for 3.9 million females currently, and 7.6 million females by 2050 (Rajan et al., 2021). However, in order to do so, it is pivotal that we reassess the approach that is taken for treating this heterogenous disease, particularly given the fact that no effective treatment exists. A substantial amount of evidence acknowledges the role of the LC in AD pathogenesis and progression. Rapidly becoming one of the most emerging issues in AD research, sex differences and why females disproportionately develop AD has recently received greater attention. However, there are no existing investigations examining an association between sex differences and the LC in AD diagnosis, thus highlighting a major gap in the current understanding of risk and prevention of AD.

6.2 Background

6.2.1 Brief Overview on LC-NA Activity

Upon activation, NA is discharged from the LC and acts on both alpha (α)- and beta (ß)-adrenergic receptors present in neurons and glial cells, thus facilitating signal transduction and diverse functions within the central nervous system (CNS) (Ferrucci et

al., 2013), ranging from regulation of arousal and autonomic function to influencing episodic memory, attention, and working memory (Arnsten & Li, 2005; Berridge & Waterhouse, 2003; Cahill & McGaugh, 1996; McGaugh & Roozendaal, 2009; Robbins & Roberts, 2007; Sara, 2009b). NA also acts as a neuromodulator regulating synaptic connections and neuroplasticity, while also promoting neurogenesis and survival (Marien et al., 2004; Wagatsuma et al., 2018). NA additionally provides neuroprotection by suppressing neuroinflammation, and in under certain conditions, defending neurons from amyloid-induced toxicity, excitotoxicity, metabolic, and oxidative stress (Feinstein et al., 2002; Feinstein et al., 2016; Mather & Harley, 2016; Robertson et al., 2016; Zucca et al., 2017). LC neuron terminals also possess and release co-transmitters, such as neuropeptide Y and galanin, concurrently with NA (Holets et al., 1988), both of which have been proven to contribute to neuronal plasticity and neuroprotection (Angelucci et al., 2014; Borbely et al., 2013; Counts et al., 2010; Spencer et al., 2016).

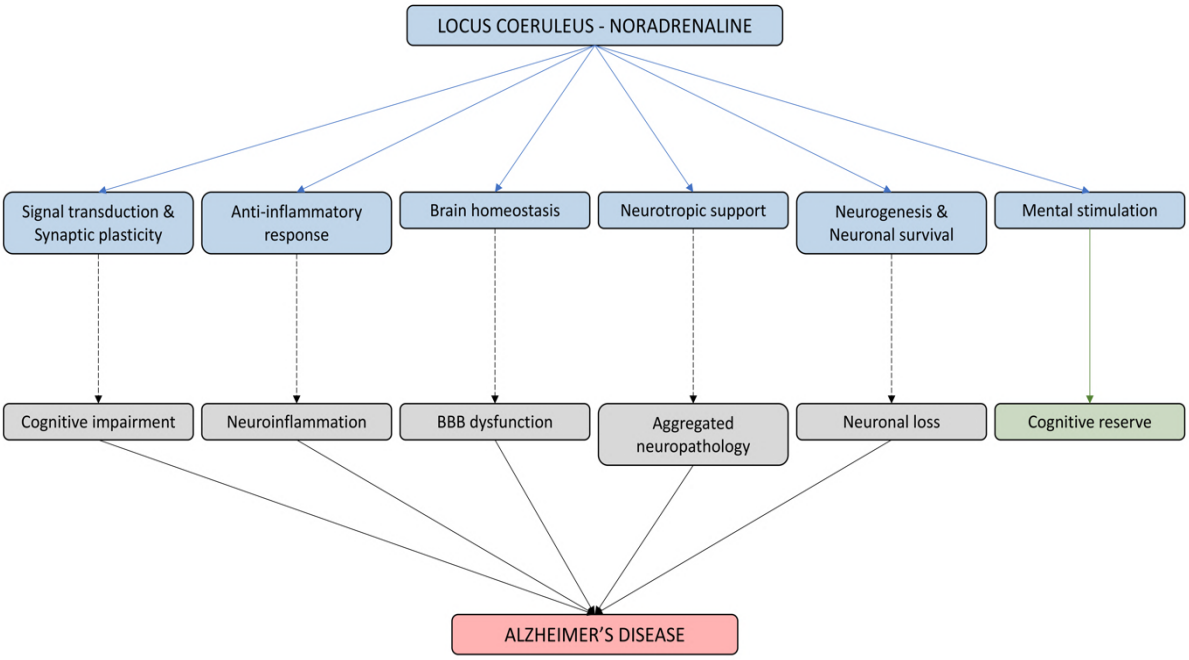
6.2.2 Evolution of Locus Coeruleus Involvement in AD

The LC has become the focus of numerous investigations due to its influential functions throughout the brain and its role as the earliest host of Alzheimer's pathology (Mravec et al., 2014; Satoh & Iijima, 2019). Renowned discoveries by Braak and colleagues have identified intraneuronal abnormal tau in the human LC prior to LC neuronal loss and subsequent LC neuronal death as tau burden increases (Braak & Del Tredici, 2015; Braak et al., 2011); this research has since been validated in a more recent rat model (Ghosh et al., 2019). Consequently, the presence and aggregation of extracellular amyloid beta protein (Aß) in the human LC has been shown to accompany neurofibrillary tangles (NFTs) in the late-stage processes of Braak and Braak (BB), particularly stages V and VI (Braak & Del Tredici, 2015; Braak et al., 2011). Moreover,

the afferent and efferent projections of human LC neurons are thin, vastly arborized, and poorly myelinated, causing them to be eminently fragile (Berridge & Waterhouse, 2003). This is particularly significant regarding the afferent nerve fibers that permeate through the gut to the vagus nerve and CNS microvasculature, this causes the LC-NA system to be particularly vulnerable to being exposed to harmful metabolites, pathogens, and viruses (Mabbott & MacPherson, 2006). These noxious microorganisms can then intensify tau hyperphosphorylation, each of which can be transmitted throughout the brain not only degrading the LC-NA pathway but also causing detrimental alterations of more extensive neuronal function. Surviving LC neurons containing abnormal pathologies spread trans-synaptically to other brain regions exacerbating synaptic dysfunction, resulting in cognitive decline and memory impairment, and neurodegeneration (Goedert, 2015; Grudzien et al., 2007; Mabbott & MacPherson, 2006; Mohamed et al., 2013). These pathological changes can also depress neurovascular coupling, and increase inflammation and blood brain barrier (BBB) permeability (Carter, 2017; Feinstein et al., 2016; Satoh & Iijima, 2019). The association between severe neuronal loss in the LC and AD is strongest in the rostral and medial-dorsal projecting neurons (Bondareff et al., 1982; Busch et al., 1997; German et al., 1992; Mann et al., 1985; Tomlinson et al., 1981), which has a major impact on disease outcome given that the loss of LC neurons reduces the availability of NA in the forebrain, hippocampus, and other target-regions; degree of LC cell loss has been shown to be highly correlated with global cognitive score, neuropathological accumulation, duration and severity of illness in elderly patients (Bondareff et al., 1982; Kelly et al., 2017; Zarow et al., 2003). Consequently, the disruption of the LC-NA pathway may very well be at the epicenter of the development of AD (Braak & Del Tredici, 2015; Mather & Harley, 2016; Mravec et al., 2014). Refer to figure 6.1 for the implications involving the LC-NA system in AD.

Figure 6.1

LC System: Physiological and Pathophysiological Implications



Note. The LC-NA system is involved in a host of physiological functions *(shown via blue line).* However, as the LC starts to falter, these mechanisms begin to weaken resulting in various AD symptomology *(shown via dashed line)*. In reverse, mental stimulation of the LC, provided by novelty and salience experiences, leads to improved cognitive reserve *(shown via green line).*

6.2.3 Evolution of Sex as a Risk Factor for AD

Studies examining patients have highlighted the disproportionate AD vulnerability of females over males, a ratio of approximately two to one (Mielke, 2018), has brought forth valuable discoveries of sex differences in areas such as onset and conversion (Altmann et al., 2014), rate of cognitive decline (Holland et al., 2013; Lin et al., 2015), and brain atrophy (Hua et al., 2010). Significant findings from these studies emphasizes the substantial consequence that disregarding sex may have for the vast range of current studies that do not sufficiently consider sex as a moderating variable in experimental,

clinical, and therapeutic studies, and in identifying potential gender-specific prevention and treatment approaches to AD. Ongoing research to explain higher female incidence AD rate is primarily interested in risk factor profiles and prevention of AD, specifically heeding the effects of the apolipoprotein ε4 allele (APOE ε4) variant and the diminution of ovarian hormones, however, current studies have yet to determine or justify why the ratio exists.

6.3 New Hypothesis

Upon reexamination of disseminated animal and human research alike, I hypothesize the escalated vulnerability of female AD is caused by hormonal changes effecting the LC-NA system that first results in a loss of estrogens and NA, and secondly, a consequential cascade of neuroinflammation, loss of BBB integrity, and neuropathological changes contributing to the onset and progression of AD.

6.3.1 For Consideration of Estrogens Research

Female and male sex hormones include progesterones, androgens, and estrogens, however, the levels in which these hormones are produced differ depending on their sex, whereby androgens are more relevant for males, estrogens are more critical for females. Although all aforementioned sex hormones have their role to play, estrogens are of primary concern regarding females, considerably more so when females begin approaching the age range of perimenopause, menopause, and postmenopause. In females' reproductive years, estrogens are predominantly produced by the ovaries and adrenal glands. Although estrogens are primarily known for their capabilities regarding female reproduction, these estrogens also circulate through the BBB in order to reach the brain and become a "master regulator" of biological functions as well as aid in

neuroprotective effects (Rettberg et al., 2014). The classification of estrogens contains three forms of the sex hormone: estrone (E1), estradiol (17β-estradiol or E2), and estriol (E3). Of these three estrogens, 17β-estradiol is the most prominent and highly researched form of estrogens. In addition to circulating estrogens produced by the females' reproductive organs, 17β-estradiol can also be produced endogenously in the brain, also known as brain-derived estrogen (Rettberg et al., 2014).

In the latter part of a female's lifetime, females will undergo stages of perimenopause, also known as menopausal transition, menopause, and postmenopause, respectively. Perimenopause typically begins at an average age of 47 and persist for approximately 5-8 years (Roberts & Hickey, 2016), and is denoted by females experiencing irregularity of duration and time between menstrual cycles, reproductive senescence, as well as, a gradual decline in ovarian hormones (Harlow et al., 2012; Wang et al., 2020). Menopause typically occurs among females who fall in the age range of 48 to 52 years of age on average (Inter, 2019), and is defined as the 12-month period of amenorrhea where estrogens (evaluated by 17β-estradiol levels per STRAW+10) begin to rapidly decline (Harlow et al., 2012). After 12 months of cessation of menses, the occurrence of the final menstrual period can be confirmed, as well as the beginning of postmenopause, a stage in which females cease to menstruate and estrogens (evaluated by 17β-estradiol levels per STRAW+10) continue to decline for approximately 1 year, after which the estrogens begin to stabilize at the lowest levels in a female's lifespan (Harlow et al., 2012; Wang et al., 2020); it is also of note both brain-derived and circulating 17β-estradiol mirror the same amount of significant decline when postmenopausal females were compared to females who were premenopausal (Rosario et al., 2011).

As previously mentioned, the classification of estrogens contains E1, 17β-estradiol or E2, and E3, however, the field of neuroscience commonly uses the umbrella term

"estrogen" as an all-inclusive term for estrogens. As a consequence of the utilization of this umbrella term, the type of estrogen being referred to in numerous studies consulted for this prospective piece was ambiguous and resulted in the assumption that studies were most likely discussing 17β-estradiol when using "estrogen." Therefore, it must be mentioned that although it is suspected that this report is highly representing the effects of 17β-estradiol, the potential contributions and effects of other hormones, such as, E1, E3, and progesterone, should not be neglected and must be considered when pondering females and AD. Furthermore, this limitation accentuates an area of research that neuroscience field must improve upon. Moving forward, it is critical for researchers to (1) be more specific and provide more thorough descriptions when discussing sex hormones including estrogens, as well as, (2) conveying whether they are referring to circulating or brain-derived estrogens in order to prevent obscurity of information, and (3) identify if females fall within reproduction, perimenopause, menopause, or postmenopause by using an outlined set of criterion, such as the STRAW+10 criteria (Harlow et al., 2012). This issue should be addressed promptly considering the upward trend in research regarding sex differences in AD. Finally, it must be noted that the specific form of estrogen will be documented in this chapter if it was identified, however, due to the forgone impediment, the majority of the evidence provided will use the term "estrogens."

6.3.2 Estrogens Influence on LC-NA Regulation

By way of various regulatory mechanisms, gonadal hormones, such as estrogens, have significant effects on human brain function, including cognition, bioenergetic systems, and homeostasis (Brinton et al., 2015; McEwen & Alves, 1999; Mielke et al., 2014; Rettberg et al., 2014). Estrogens' direct ability to modulate LC activity is promoted by

the presence of both nuclear estrogen receptors (ER) ER-α and ER-β and cell-membrane ER subtype G protein coupled estrogen receptor 1 (GPR30) found in wide distribution throughout the LC (Cui et al., 2013). Additionally, studies looking at various male and female rat species have depicted that estrogens plays a significant role in facilitating sex differences in LC-NA morphology, from embryonic development into adolescence (Bangasser et al., 2011; Pinos et al., 2001) *(for more details see subsection Selective Vulnerability and Multiple Clinical Phenotype)*. Likewise, studies using ovariectomized female rats have exhibited that estrogens have a considerable impact on LC function by acting as a presynaptic modulator of NA release by altering the level of enzymes synthesizing NA, such as tyrosine hydroxylase (TH) and dopamine ß-hydroxylase (DBH) (Lubbers et al., 2010; Vathy & Etgen, 1988). In addition, estrogens are a key regulator in the degradation of NA by reducing catechol-O-methyltransferase (COMT) levels, an essential enzyme needed for the breakdown of NA (Bangasser et al., 2016). Given the evidence regarding estrogens' effect on humans, and if the findings from the aforementioned rat studies are translatable to humans, this suggests the females could have an increased capacity for NA production and release.

Considering estrogens' strong influence on LC-NA regulation (Morrison et al., 2006; Rocca et al., 2011), and menopause causing a rapid loss of the ovarian sex-hormones in all females' midlife, I hypothesize that the loss of estrogens may explain the decline of the female LC-NA system, resulting in an increased risk of developing AD. This prospective piece highlights three key determinants of AD in which estrogens and the LC-NA make significant contributions that require further examination: namely, neuroinflammation, BBB, and APOE ε4.

6.3.2.1 Effects of LC and Estrogens Activity on Neuroinflammation.

Mounting evidence implicates glial-mediated inflammation as a major contributor to cognitive decline and neurodegenerative processes (Heneka et al., 2015). When reacting to neuroinflammation, microglia cluster around amyloid deposits, and can become chronically activated (Edison et al., 2008; McGeer & McGeer, 2002; Rogers et al., 2002). Overactive glia cells are unable to effectively clear Aß. Aß accumulation upregulates pro-inflammatory release and exacerbates the inflammatory cascade leading to synapse elimination, further amplifying neurodegeneration, and cognitive function declining (Hong et al., 2016; Presumey et al., 2017; York et al., 2018), along with increased neurotoxicity and neuroinflammation wherefore AD neurodegenerative processes are amplified (Eikelenboom et al., 2002; Fakhoury, 2018; Feinstein et al., 2002; Giorgi et al., 2019; Heneka & O'Banion, 2007). The above immunological cascade of events is gaining traction as having a fundamental role in the AD neurodegenerative processes (McGeer et al., 1987; Salminen et al., 2009; Shao et al., 1997).

Current evidence in animal studies favors the idea of an early fundamental link between neuroinflammation and LC degeneration that brings about AD pathogenesis (Giorgi et al., 2019). These studies and similar findings in humans have demonstrated that NA acts as an endogenous CNS immunomodulator by helping maintain homeostasis (Amor et al., 2010; Feinstein et al., 2002; O'Sullivan et al., 2009), influencing other neurons and glia to exert potent neuroprotective effects as an anti-inflammatory, and operating as a suppressor on pro-inflammatory gene induction (Chavarria & Cardenas, 2013; Feinstein et al., 2016; Giorgi et al., 2019; Lenz & McCarthy, 2015). As AD develops, as seen in BB stages V, there is a progressive neuronal degeneration and cell loss in the LC thereby decreasing NA availability (Braak et al., 2011). This decrease may carry negative implications given that *in vivo* amyloid precursor protein (APP) mice

models suggest deterioration of LC varicosities prevents the release of extra-synaptic NA to surrounding glial cells and consequently inhibits phagocytosis and interferes with the amyloid pathology generating cycle (Heneka et al., 2010), thus promoting chronic inflammation and the formation of Aβ in areas usually innervated by the LC (Heneka & O'Banion, 2007).

Estrogens, such as 17β-estradiol, exert influential modulatory effects on microglia and are shown to directly attenuate pro-inflammatory release from these cells, as well as, indirectly prevent the transcription of pro-inflammatory cytokines (Pfeilschifter et al., 2002). As females undergo menopause, the decline in 17β-estradiol synthesis and changes in the immune system significantly increase the levels of pro-inflammatory markers brought on by the loss of anti-inflammatory and pro-resolution activity, thus suggesting a pro-inflammatory phenotype in females (Pfeilschifter et al., 2002). Non-infectious diseases such as obesity, diabetes, and depressive illnesses are all associated with the same pro-inflammatory phenotype (Vina & Lloret, 2010). This is not coincidental, as those diseases can also be classified as female driven risk factors that increase the likelihood of developing a comorbid diagnosis of AD (Vina & Lloret, 2010).

Taken together, it can be inferred that postmenopausal females are more likely exposed to additional inflammation due to higher levels of Aß and immuno-stimulated glia. A noteworthy study detected the presence of Aß pathology in 1,031 postmortem human brains when using immunocytochemistry (4G8) and Campbell-Switzer staining (Braak et al., 2011). Of these cases, 4% exhibited Aß deposition in the fourth decade of life, however, the most prominent levels of Aß plaques were observed in the sixth decade and beyond. More indicative to the current proposal, a significant correlation between Aß deposition and age was found to progress more rapidly in females (Braak et al., 2011). Without estrogens, notably 17β-estradiol, and LC-NA neuroprotection, the ability to

downregulate the transcription of inflammatory genes in microglia and reduce amyloid neuronal toxicity is decreased (Miller & Duckles, 2008; Ross et al., 2015; Uchoa et al., 2016). Consequently, substantial depletion of NA increases the magnitude of neuroinflammation and gives rise to a relentless inflammatory mechanism that accelerates the neuropathology it was created to eradicate, leading to more rapid development of AD pathology in females (Akiyama et al., 2000; Heneka et al., 2010; Marien et al., 2004).

6.3.2.2 Effects of LC and Estrogens Activity on BBB.

Studies using various species of rats have shown the LC-NA to have a role in BBB maintenance, including the regulation of BBB permeability and neurovascular coupling (Chi et al., 1998; Harik & McGunigal, 1984; Kelly et al., 2019), and has also been shown to modulate morphological and functional properties of the BBB (Cohen et al., 1997; Kalinin et al., 2006; Kelly et al., 2019). More particularly, a morphometric analysis indicated intracortical astrocytes being one of the direct targets of LC-NA terminals thus implying that NA has a regulatory influence on astrocytic functions of BBB permeability and homeostasis (Ben-Menachem et al., 1982; Cohen et al., 1997; Seguela et al., 1990). Furthermore, NA, by means of an association with cyclic adenosine monophosphate (cAMP), regulates the formation and stability of tight junctions (TJs) (Dye et al., 2001; Mulder et al., 1990; Mutafova-Yambolieva et al., 2003; Rubin & Staddon, 1999). This can be seen in the way TJ disarrangement and gliosis is caused by noradrenergic fiber degeneration (Kalinin et al., 2006).

In addition, the emergence of neuroinflammation and neurodegeneration in the LC, due to diverse pathogens, are two physio-pathological aspects promoting BBB dysfunction (Carter, 2017; Carvey et al., 2009). As the BBB begins to lose functionality,

a decrease in barrier tightness is observed due to the degradation of TJs (Abbott et al., 2010). This maladaptive increase in permeability allows for the transport of cytokines and immune cells into the CNS and cerebrospinal fluid (Zlokovic, 2008). As a result, a secondary inflammatory response creates an environment suited for a pro-inflammatory take-over that compromises Aβ clearance, affects TJ integrity, causes endothelial cell (EC) damage, and insufficient nutrient supply; comparable to what is identified in AD (Baeten & Akassoglou, 2011; Kelleher & Soiza, 2013). In addition, defective neurovascular unit cells that prompt neurovascular impairment can result in neurodegeneration and the deterioration of cognitive abilities and advance the inception and progression of AD (Abbott et al., 2010; de la Torre, 2004; Farkas & Luiten, 2001; Kalaria, 2000; Kim et al., 2012; Montagne et al., 2017; Sagare et al., 2012; Viswanathan & Greenberg, 2011; Zlokovic, 2008, 2011).

Animal studies using endogenous estrogen have shown 17β-estradiol's ability to protect the vasculature of intercellular junctions via targeting and regulating claudin-5, which is specifically needed to enhance permeability (Burek et al., 2010), and annexin A1 protein (Hughes et al., 2013), an anti-inflammatory regulator of TJ formation, thus governing BBB integrity (Cristante et al., 2013; Solito et al., 1998). Moreover, 17β-estradiol has the capability to act as a homeostatic signal by limiting the disturbance of lymphocytes and enhancing TJ function and has a profound impact on the BBB, thus marked differences may occur as females age and the associated circulating 17β-estradiol declines (Bake & Sohrabji, 2004).

Another important target of both NA and estrogens is the cerebral microvasculature (Duckles & Krause, 2007; Kalaria & Harik, 1989; Kalaria et al., 1989). LC-NA deafferentation impairs forebrain cerebrovascular function during the preclinical and prodromal stages of AD (Kelly et al., 2017). NA's ability to regulate vascular related

functions, such as cerebral blood flow, can be seen in anatomical evidence of rat studies depicting LC ascending fibers delivering direct innervation to the microvascular endothelium (Cohen et al., 1997; Kalaria et al., 1989). Additional rat studies have observed estrogens' exposure guards against apoptosis of ECs, alters mitochondrial function, increases cerebral blood flow, as well as, induces anti-inflammatory actions on cerebral blood vessels, and in turn suppresses endothelial inflammation (Duckles & Krause, 2007; Sudoh et al., 2001). Due to the relatively high energy demands of these specialized ECs, cerebral vascular endothelium contains more mitochondria than endothelium in any other vascular beds, thus increasing energy production needed to maintain the BBB efficiency while also reducing oxidative stress that is associated with AD (Smith et al., 1996; Stirone et al., 2005). Collectively, the presented animal literature exhibits how the LC-NA and estrogens play a critical role in protecting and regulating the BBB, as well as, how the degradation of the BBB results in a significant decline in cognitive abilities and an increase in neurodegeneration. Taking this evidence into account advances the implication that a combined loss of estrogens and NA leads to females becoming more susceptible to consequences brought on by having a more permeable and less governed barrier. Nevertheless, considering the fact that present data is predominantly based on animal models, replicating the findings of these studies in humans will be advantageous to the current hypothesis.

6.3.2.3 APOE ε4 Interaction with LC and Estrogens.

To date, it is suggested that the strongest genetic risk factor for AD is the APOE ε4 allele (Riedel et al., 2016). Mice studies have linked APOE to BBB dysfunction via ε4 alleles' conduciveness for vascular atrophy resulting in increasing permeability and reducing cerebral blood flow (Alata et al., 2015; Bell et al., 2012). In addition, carriers of

the ε4 allele have been shown to have altered astrocyte bioenergetics that restrict astrocytes from allowing proper lipid and glucose transport, storage, and utilization (Fernandez et al., 2019). APOE ε4 carriers display a disease-associated microglia (DAM) profile, including increased pro-inflammatory cytokine production with impaired phagocytosis, deficient debris clearance, and impaired migration capability (Fernandez et al., 2019). These detrimental functional effects predispose carriers to neuropathological features associated with AD, such as impairments in cerebral glucose metabolism, neuronal signaling, neuroinflammation, and mitochondrial function (Brandon et al., 2018; Liu et al., 2013).

APOE ε4 status is also instrumental in the mediation of neuronal maintenance, growth, and synaptic repair. Synaptic disturbances in the LC-NA tract in APOE-deficient mice were more evident the farther away the axon nerve terminal is from the cell body (Chapman & Michaelson, 1998). These findings enhance the likelihood that the LC-NA projecting pathways composed of longer axons and branch formations could potentially be dependent on APOE for normal function. A supplementary analysis in patients that assessed the role of APOE ε4 on the compensatory mechanism neurons undergo when in distress resulted in LC neurons experiencing insufficient dendritic remodeling in addition to neuronal degeneration (Arendt et al., 1997). Taken together, this illustrates that LC neurons are likely compromised in APOE ε4 carriers and this could contribute to the breakdown of the LC-NA pathway more rapidly than in non-carriers, and potentially results in an earlier age at onset and a more expeditious development of the disease (Arendt et al., 1997; Chapman & Michaelson, 1998). In addition, a recent female-only rodent study indicated APOE ε4 inhibition of vesicular monoamine transporter 2 (VMAT2) in LC exacerbates Tau pathology in AD, signifying that the LC's initial role

in contributing to AD may conceivably be due to APOE ε4's pathological effect (Kang et al., 2021).

APOE ε4 also impacts females independent of AD diagnosis. Studies using non-cognitively impaired ε4 allele carriers reported an APOE ε4-by-sex interactions on human brain metabolism, structure, and functional connectivity (Damoiseaux et al., 2012; Heise et al., 2014; Sampedro et al., 2015). This indicates an unestablished mechanism of action between sex and APOE ε4 (Sampedro et al., 2015). Ultimately, APOE ε4 has a multifaceted effect on cell types through diverse pathways, consequently disrupting the homeostasis and leading to greater neurotoxicity (Fernandez et al., 2019). However, knowing that females' more complex dendritic structure leaves them susceptible to harsher synaptic disturbances, combined with APOE ε4 influencing dendritic remodeling, leads to speculation that the extent of synaptic degeneration of the LC-NA pathways in AD is more pronounced in female ε4 allele carriers. Furthermore, females have an increased risk in MCI and AD occurrence around menopausal age ranges (Inter, 2019), which, as outlined in this chapter, can potentially be explained by a loss of estrogens; this hypothesized interaction with APOE ε4 has yet to be investigated.

6.3.3 Future Experiments and Validation Studies

Hormone replacement therapy (HRT) studies are of great popularity given the link between estrogens depletion and the increased risk of AD. Although previous trials are not without debate and limitations (Cagnacci & Venier, 2019), their contributions regarding HRT and female AD are invaluable assets that have facilitated ideas for future research. The past two decades have provided evidence that has not strongly supported the hormonal loss hypothesis in relation to cognitive function and/or dementia risk (Gurvich et al., 2018). These studies have done well to incorporate various sex hormones

in multiple combinations through multiple application methods. The Women's Health Initiative (WHI) conducted a study on postmenopausal females 65 years and older (Shumaker et al., 1998). Upon examination of the effects of estrogens alone (conjugated equine estrogens (CEE)), estrogens plus progestin (CEE plus medroxyprogesterone (MPA)), Premarin, a CEE that contains estrone and 17β-estradiol, as well as MPA, the WHI concluded that not only are the use of these HRTs not recommended but that the risks (of cancer, cardiovascular, and dementia) outweighed the benefits (Rossouw et al., 2002; Shumaker et al., 2004). A similar study, the Cache County Study (CCS), included postmenopausal females of 65 years of age or older (oophorectomized and non-oophorectomized) who reported uses of either "unopposed" or "opposed" estrogens (Shao et al., 2012). The results of CCS indicated that postmenopausal females who began HRT 1-5 years from menopause exhibited a trend toward AD risk reduction, whereas participants who began HRT closer to or after menopause showed no association to lower AD risk (Shao et al., 2012). A third study, the Kronos Early Estrogen Prevention Study (KEEPS), examining postmenopausal, healthy, non-hysterectomized females found no cognitive benefits after participants initiated HRT for four years via oral CEE plus micronized progesterone or transdermal estradiol plus micronized progesterone (Gleason et al., 2015). Additionally, the Early Versus Late Intervention Trial with Estradiol (ELITE) study looked to identify if utilizing oral micronized 17β-estradiol with progesterone gel for five years would have any effect on cognitive abilities in females who were more than 10 years postmenopausal (Hodis et al., 2015). ELITE concluded with no affects being evident in verbal memory, executive functions, or global cognition (Hodis et al., 2015). Taken together, the major critique of the previous four studies includes participants being in the postmenopausal stage, thus prompting the notion that future experiments must consider hypotheses such as estrogens administered into a

healthy environment, “healthy cell bias” (Gurvich et al., 2018), and the efficacy of HRT in relation to the timing and onset of menopause, “window of opportunity” (Maki, 2013). There has been some evidence supporting the timing hypothesis of HRT in observational studies where females have self-reported menopause status, timing of HRT initiation, and if opposed or unopposed estrogens were utilized (Maki, 2013). Together, these observational studies provide support for the timing hypothesis given that each study’s results showed reduced risks of AD in groups that began HRT in earlier stages of their life (Henderson et al., 2005; Shao et al., 2012; Whitmer et al., 2011). These findings stand in stark contrast compared to the previous four studies mentioned, particularly a follow-up study to the CCS which identified females who began HRT 1-5 years before menopause had a 30% reduced risk of AD, and a 37% reduced risk when taking HRT for 10 or more years (Shao et al., 2012).

In order to validate the present hypothesis, it is essential for future studies to persist in identifying sex differences in the LC-NA system. Moreover, it is imperative that studies are translated from rodents to humans in order to draw inferences and confirm the effects in humans. Additionally, the examination of previous publications determined that a majority of animal research and experimental treatment trials are largely dominated by work carried out in males. In order for scientific research to excel, experimental and clinical design must undergo considerable modifications such as routinely stratifying data by sex and ensuring studies are statistically powered to detect interaction effects between sexes. Furthermore, a set list of guidelines must be established for reporting sex differences (Ferretti et al., 2018), otherwise inconsistencies and uncertainties will remain in the understanding of female AD.

Future experiments may embrace the goal of working towards establishing an interrelated timeline connecting the dysregulation of the LC-NA system, the decline of

sex hormones, and AD vulnerability together. This will allow for studies conducted to utilize a more relatable timeline that associates the females' stages of menopause with the advancement of neuropathology in the LC using the pre-established BB stages. The institution of this timeline will enhance the investigation of the hormonal loss hypothesis when testing how HRT affects the LC-NA system and enable studies to place consistent, corresponding timeframes for their findings regarding "window of opportunity" and "healthy cell bias." Taken together, these future investigations are instrumental in exploring methods to upregulate estrogens and LC-NA mechanisms in order to deter further neuropathological accumulation and neurodegeneration. It is vital to address this hypothesis promptly seeing that the increase in today's lifespan could indicate that on average females would spend up to one-third of their life without production of estrogens (Li & Singh, 2014).

6.4 Challenges Addressed by the Current Hypothesis

6.4.1 Aging

The increased longevity of females is one of the most argued justifications for the increased risk of AD and has recently come under more scrutiny as of late (Nebel et al., 2018). Evidence indicates aging alone is not a suitable determinant for LC degeneracy (Theofilas et al., 2017), however, the present theory insinuates that aging is notable given the association of age and onset of menopause in the majority of females. As mentioned previously, the current mean age of the onset of menopause is approximately 51 years of age, with a mean range of 48 to 52, and perimenopause typically begins at an average age of 47. This is significant due to these age ranges of the emergence of perimenopause and menopause, respectively, coinciding with BB stages Ia, Ib, I and II. At these ages, a vast majority of human autopsy brains were classified in BB stages Ia and Ib whereby

prominent AT8 immunoreactive pretanlge material was displayed, noted as the source of argyrophilic neurofibrillary lesions, thus facilitating neuronal vulnerability (Braak & Del Tredici, 2015). Additionally, approximately half were classified as BB stages I and II at which point Gallyas-positive neurofibrillary lesions were manifesting in the entorhinal regions, indicative of the potential initiation of neuronal deterioration that could occur in future BB stages (Braak & Del Tredici, 2015). A highly significant correlation between AD-related pathologic findings and age has been found in females, yet no current significant difference has been seen between females and males in early and mid BB stages. However, more significant variations are seen between males and females as individuals increase in age. Given the strong influence estrogens have on LC-NA regulation (Morrison et al., 2006; Rocca et al., 2011), and the fact that females suffer from greater implications than males the further away they get from perimenopause and menopause age, it must be considered if a decrease in estrogens may have a disproportionately negative effect on the LC-NA system of females, thus resulting in an increased risk of developing AD.

6.4.2 Selective Vulnerability and Multiple Clinical Phenotypes

Animal studies have depicted sexual dimorphism in LC morphology (Bangasser et al., 2016; Bangasser et al., 2011), with sex differences in LC size, make up, and neuronal number, depending on the strain of rat used. Furthermore, studies using Wistar rats show developing females to have a higher volume and neuronal count (Guillamon et al., 1988; Pinos et al., 2001), and this, together with sex differences in dendritic structure (Bangasser et al., 2011), may be due to the influence of estrogens during LC neurogenesis (Pinos et al., 2001; Rodriguez-Zafra et al., 1993); female dendritic trees have been shown to be longer and more complex, with greater numbers of branch points and higher-order

branching (Bangasser et al., 2011). If these findings could be reproduced in human studies, this may support the prospect that females receive higher levels of synaptic input and process additional information coming into the peri-LC, from regions such as the periaqueductal gray and NTS (Van Bockstaele et al., 2001). The NTS can influence NA activity both directly via synapses on neurons in the LC, and indirectly via connections linking the LC to the amygdala and hippocampus (Couto et al., 2006). Alternatively, the NTS is also involved in the regulation of gastrointestinal activity along with the vagus nerve, which, as mentioned previously, could result in exposing the LC to toxic agents (Mabbott & MacPherson, 2006).

Due to the LC mediating arousal and autonomic function, the potential multifaceted dendritic structure of the female LC and the increase in NA induced by estrogens allows for the potential of heightened emotional arousal response (Bangasser et al., 2011). A repercussion of the increased stimulation seen in the female LC could be the rationale for hyperarousal resulting in and contributing to the increased rates of disorders seen in females (Bangasser et al., 2016), such as post-traumatic stress disorder, depression, and sleep disturbance, all of which share clinical phenotypes with, and are comorbid risk factors of AD and coincidentally are more predominant in females (Toro et al., 2019). This connection between their modified LC-NA network and the presentations of behavioral manifestations in females emphasizes the need for further examination into the causes-and-effects of selective vulnerability in female AD.

An additional consideration regarding LC vulnerability stems from results of a recent mice study investigating the effects of 3, 4-dihydroxyphenylglycollaldehyde (DOPEGAL) on the LC indicated that monoamine oxidase A (MOA-A) metabolizes NA into DOPEGAL through oxidative stress, which in turn increased asparagine endopeptidase (AEP), and resulted in Tau N368 cleavage which is susceptible to

hyperphosphorylation, aggregation, and propagation, leading to noradrenergic cell death (Han et al., 2021). This study contributes significant information when considering the current hypothesis given that estrogens and progesterones are both regulators of oxidative stress (Irwin et al., 2008). As mentioned previously, females begin to experience perimenopause and menopause, respectively; approximately when BB stages Ia and Ib, I and II occur in humans, coinciding with the timing when pretangle and tangle material is observed, and neuronal vulnerability is facilitated. This is significant on account of the process of NA oxidizing to DOPEGAL and ultimately resulting in tau cleavage and noradrenergic cell death is conceivably protracted, and thus are not observable until later BB stages, leading to speculation that the loss of estrogens and progesterones in early BB stages due to perimenopause and menopause is potentially one of the explanations of NA metabolizing to DOPEGAL due to the lack of the female's ability to regulate oxidative stress.

Furthermore, findings from a recent study indicated similar results where increased levels of NA-metabolism were connected to greater cerebral spinal fluid (CSF) phosphorylated tau in both female and male memory clinic patients (Riphagen et al., 2021). Additionally, these findings were affiliated with a decrease in learning ability that was tracked over a 6-year timeframe; this observation is in line with the idea that changes in the LC prior to the accumulation of neuropathology takes time to intensify to a measurable degree which enables researchers to discern behavioral differences (Riphagen et al., 2021). Collectively, these studies indicate oxidative stress, which is regulated by estrogens and progesterones, metabolizing NA ultimately results in neuropathology. Upon consideration of these results and taking the current hypothesis into account, it is of substantial importance to advance this research by investigating the effects of sex differences, such as females undergoing the menopausal transition, may have on NA

oxidization and if this results in increased neuropathology load and neuronal death in the LC.

6.4.3 Modifying Risk Factors

Potential preventative measures that have vastly emerged in AD literature are those of brain reserve and cognitive reserve (Stern et al., 2019). Brain reserve pertains to the individual's anatomical brain structure, and suggests a more complex morphological framework that includes increased synaptic and neurotransmitter receptor densities that would potentially be able to withstand consequences of neuronal loss or tolerate more detriment. Alternatively, cognitive reserve spotlights individuals' functional resilience and suggests increased cognitive processes or more intricate neural networks that aid in creating a higher degree of built-in redundancy (Stern, 2009). Currently and collectively, both brain reserve and cognitive reserve highly favor males as opposed to females. For instance, brain reserve strongly suspects that males increased cerebral brain volume is superiorly capable of withstanding the accumulation of pathology to that of females (Giedd et al., 2012; Katzman et al., 1988). This is further supported by evidence indicating females had significantly greater odds of AD given the identical amount of pathology to males (Barnes et al., 2005), as well as female brain volumes declining faster in patients with MCI and AD (Skup et al., 2011). However, bearing in mind the previously discussed preeminence of the female rodent LC morphology and if this is translatable to female human LC morphology, what it means for female brain reserve remains to be answered.

Conversely, a link between the characteristics of cognitive reserve, such as education, occupational attainment, and lifetime experiences, and the upregulation of the LC-NA system in both animal models and *in vivo* human studies has been alluded to (Robertson,

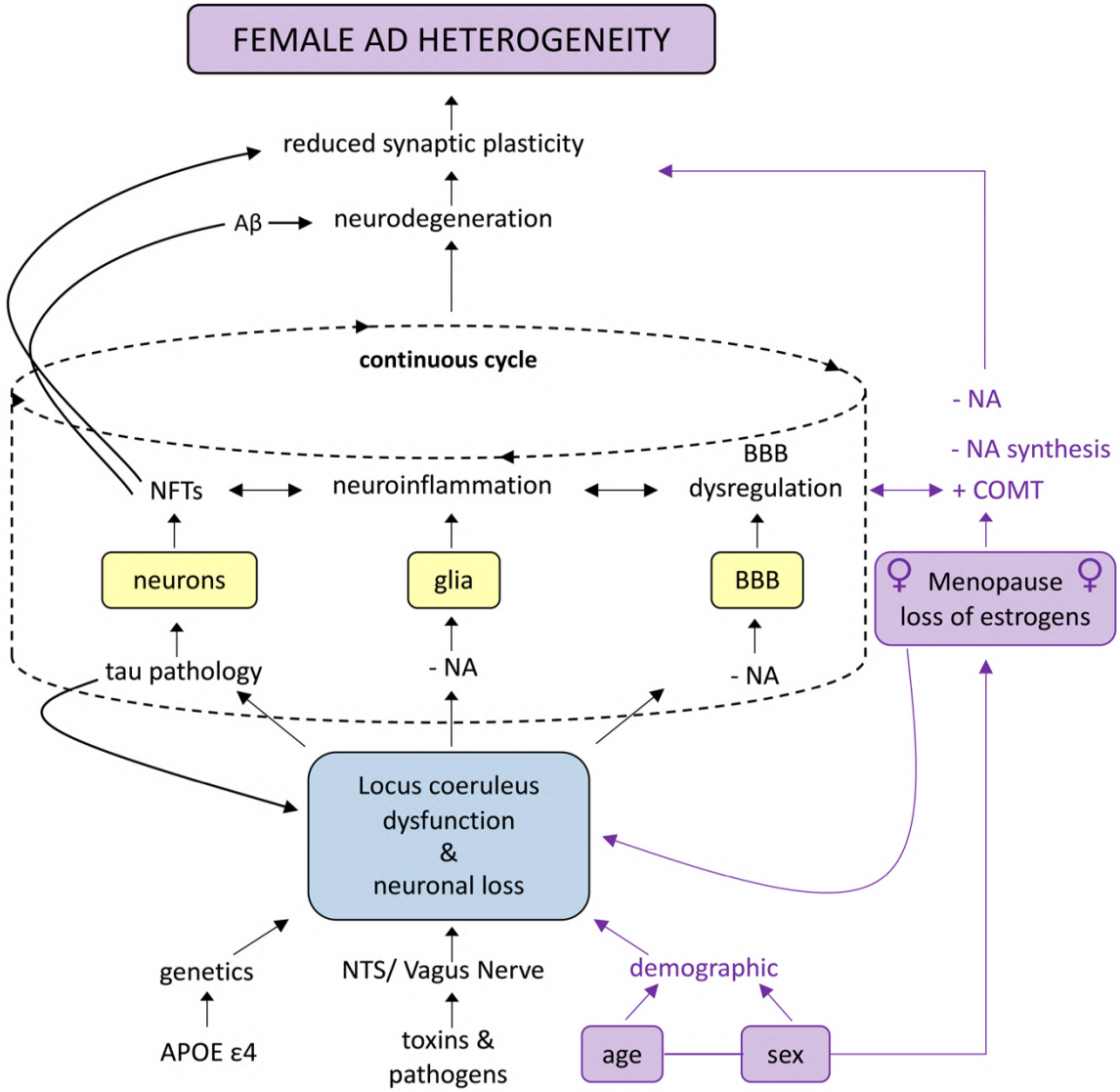
2013). An upregulated LC-NA system allows for an increase in the number of receptors on the target cell, permitting more effective signal transmissions needed for peak cognitive performance, while simultaneously releasing a protective mechanism against threats of inflammation and neuropathology (Feinstein et al., 2016; Mather & Harley, 2016; Robertson et al., 2016; Zucca et al., 2017). While the mechanism of cognitive reserve is still difficult to define, the model suggesting NA being the fundamental pillar that underpins a delay in AD pathology is something to consider. It is widely acknowledged that males/men surpass females/women in cognitive reserve on the basis of both sex and gender differences due to previous societal practices which resulted in less opportunity for cognitive enrichment for females/women. Moreover, a study comparing LC signal intensity and the established proxies of reserve (i.e., education level and occupational attainment) in a cognitively healthy population, showed older-adult females, on average, have ~20% less LC signal intensity than older-adult males (Clewett et al., 2016). Conversely, a more recent study observed no sex-differences in age-related LC intensity associations (Liu et al., 2020). These conflicting results may be due to different sample sizes and/or varying parameters of MRI, thus continuation of improving upon the methodological approach is required to validate such findings. Nonetheless, a potential sex difference in LC signal intensity raises the question of what this means in regards to an indisposed LC in menopausal females (i.e., during a marked time of the depletion of estrogens). Does this affect females/women's day-to-day cognitive capabilities or their ability to undertake cognitive reserve, and therefore losing out on the potential to compensate or modify the AD state? If so, a way to exploit females' ability to perpetuate cognitive reserve must be discovered, thereby upregulating the LC-NA system, earlier on in life, in order to mitigate the foreseeable AD pathology that comes as a result of the decline of estrogens and LC-NA dysregulation.

6.4.4 Pathogenic Predisposition and Sequence of Progression

Structural and metabolic characteristics of female APOE ε4 gene carriers contribute to a predisposition to pathophysiological dysfunction, resulting in neuropathological components of AD. The composition of phenotypic, LC morphological framework induced by estrogens, and demographic attributes establish the substructure that influences LC dysfunction and neuronal loss that accelerates AD pathogenesis. In the course of female AD, it is more likely that the combined neuroprotective functions of NA, the inflammatory response system, and the regulation of the BBB, with the additional support of estrogens, are able to hold off the disease until a critical threshold. It is conceivable that a drastic decrease in estrogens and NA production could result in the pathologic process of tau pathology and Aß so severe that it can be observed in BB stage V and VI through LC cell loss and impaired cognitive function. Moreover, an overall loss of tropic support occurs, thus leaving the female brain incapable of producing an anti-inflammatory response and governing BBB integrity, in turn conducing a toxic neuronal environment. Neuropathologies intensifying, BBB dysfunctioning, and neuroinflammation rising creates a continuous fed forward cycle that contributes to the progression of the disease, and causes clinical signs of AD such as a reduction of neuronal plasticity and neurodegeneration to become more apparent, and are currently unable to be halted or reversed Refer to figure 6.2 for this hypothesized route of female AD heterogeneity.

Figure 6.2

Hypothesized Route of Female AD Heterogeneity



Note. This chapter proposes that sex and age, as it relates to the onset of menopause, increases female vulnerability due to the rapid loss of estrogens and its effects on the LC-NA system, thus accelerating the load of neuropathology that give rise to neurodegeneration and reduced synaptic plasticity. This hypothesized route not only plays a critical role in AD development, but also disproportionately effects females. Redrawn from Mravec 2014

6.4.5 Mixed Pathology

The exploration into sex difference and their frequency patterns of mixed neuropathologies have only recently begun (Toro et al., 2019). However, according to a recent study involving autopsy-based investigations on human brains, females significantly exhibited signs of mixed AD and cerebrovascular pathology compared to males (Barnes et al., 2019). This finding raises intriguing questions regarding the nature and extent of the role in which the two critical components of the present hypothesis have given the current proposal's explanation of the vitality of the BBB being heavily dependent on favorable LC-NA signaling the production of estrogens. It is therefore likely that a further connection may exist between the downfall of both the female LC and estrogens and an increase in the comorbidity of mixed pathologies, thus warranting future studies.

6.4.6 Biomarkers

Currently, the amyloid/tau/neurodegeneration (ATN) classification framework has seen a significant rise in hopes that it will assist in the diagnostic procedure, development of preventative measures, and treatment strategies for AD. ATN classification for AD focuses on the presence of Aß pathology, tau pathology, and neurodegeneration. Prior research utilizing the ATN biomarkers, as well as, most LC investigations have yet to report any significant difference amongst males and females, however, sex differences have not been the predominant variable of consideration. In line with ongoing research, this proposal supports moving in the direction of focusing investigations on the LC as a new biomarker potentially capable of tracking prognosis and progression of AD, with a specific interest in sex differences. It is essential that further efforts utilize various methodologies, such as unbiased stereological analysis to assess LC cell population,

volume, and neuronal death (Theofilas et al., 2017). Additionally, capitalizing on enhancements new technologies provide to current techniques such as *in vivo* imaging (Betts et al., 2019) and PET imaging (Liu et al., 2021) will be a critical component in measuring LC integrity and functionality, allowing researchers to overcome previous obstacles such as its small size and location. Combining these various methodologies will further facilitate the ability to validate the LC as an early-stage biomarker for AD, and incite further exploration into sex differences.

6.4.7 Translation Potential (Diagnostic Implications)

Future research in pursuance of advancing early disease detection and improving upon investigational therapeutic approaches are of utmost priority. Neuropsychological testing upon exhibitions of cognitive deficits is one the primary courses of action to assess if AD has begun to manifest. However, the current assessment strategies not adjusting for sex differences indicates a significant shortcoming in the clinical diagnosis of AD, and may contribute to a myriad of misdiagnoses or late diagnoses in females.

Cognitive abilities have recently been recognized in both animal and human studies as being molded by gonadal steroids and the differences by which LC-NA mechanisms modulate the amygdala and hippocampus regions, and throughout the brain (Ycaza Herrera et al., 2019). These factors contribute to males and females having unique patterns of activation and performance on emotional autobiographical and episodic memory, spatial ability, and perceptual processing tasks (Ycaza Herrera et al., 2019). It has been proposed that the mechanism of action by which the LC assists males and females differently is in how they distinguish and redirect attention to salient stimuli and how they encode and retrieve specific information. Such discrepancies become behaviorally apparent as males outperform females on recall tasks for central

information, spatial rotation, and navigation tasks, while females excel on recall tasks for ancillary information, objection location, and verbal memory tasks (Li & Singh, 2014; Ycaza Herrera et al., 2019).

Acknowledging the role the LC and estrogens play in the sex-specific strengths and disadvantages consistently present in cognitive processes may have direct bearing on neuropsychological testing, so much so that it ought to be accommodated for in the formation of sex-adjusted normative data and sex-based cut-off scores used to differentiate normal aging and preclinical AD. Accordingly, this will increase the likelihood of preventing AD symptoms being masked during examination and further improve validity of AD diagnosis. Sex-specific neuropsychological assessments will have vast implications for the monitoring of AD progression, as well as, measuring the effectiveness of experimental treatments.

6.5 Linkage to Other Major Theories

Currently, there is no disease-modifying treatments available in spite of an enormous amount of research focused primarily on the role of Aβ plaques and NFT in neurodegeneration in patients (Chen et al., 2017; Gauthier et al., 2016; Montine et al., 2012). However, the underlying cause/causes of AD pathology remain at large, highlighting the critical urgency that must be devoted to researching new hypotheses such as the one presented in this chapter. The substantial amount of AD heterogeneity has led to the proposal of numerous theories with various focal points ranging from genetic predisposition, abnormal accumulation of pathogenic properties, dysregulation of the CNS, and environmental risk factors, implying a high probability of multiple pathways being involved in the clinical presentation and progression of AD. However, prior postulations have overlooked sex differences in AD heterogeneity. Therefore, I have put

forth a hypothesis that has pinpointed the LC-NA system as the hub of etiology in female AD, and furthermore distinguishes the decline in NA and the production of estrogens as the initiating drivers that spur the primary concentration of the aforementioned hypotheses. Although this theory is not fully supported by experimental research, dissecting and analyzing formerly disseminated publications provided the evidence needed for this defining contribution that will now require others in the field to embrace and utilize to stimulate ideas for future studies. Conducting experiments aimed at maintaining estrogens and LC-NA production may prove to provide the pertinent evidence needed in order to find suitable therapeutic interventions.

7 Conclusion

In conclusion, this thesis provides evidence that features the LC-NA system across a spectrum that entails the noradrenergic neuromodulatory system at its peak performance and its degeneration as a critical factor in AD progression. Across these two themes, I sought to demonstrate how and when NITESGON could be used to upregulate the LC system to improve cognition and highlight existing evidence that demonstrates how the disruption of the female LC-NA mechanism and the decline of estrogens leads to an increase in AD vulnerability.

Furthermore, the present thesis brought awareness to a peripheral pathway capable of activating the LC-NA system. AC and DC NITESGON elicited specific behavioral effects across three experiments, whereby AC NITESGON immediately generated activation of the LC that in turn affected encoding via the augmentation of attention, whereas DC NITESGON triggered a novelty-induced LC response that in turn initiated initial memory consolidation via the presumptive co-release of NA and dopamine. These observations highlighted NITESGON as a neuromodulation technique that has the potential to significantly influence brain function and behavior. Moreover, the prospective chapter hypothesizing the loss of estrogens and NA in females triggering a cascade of effects that include neuroinflammation, loss of BBB integrity, and neuropathological changes contributing to AD highlighted the need for in-depth investigations into the loss of estrogens and diminished production of the noradrenergic mechanism to combat AD sex differences.

Taken together, this thesis promotes further research to identify optimal and reliable stimulation parameters for NITESGON to induce LC-NA neuromodulatory effects as well as research aimed towards distinguishing the LC as a potential biomarker of AD that accounts for sex differences. Such studies will enable prevention trials to target the LC

(i.e., NITESGON) as a means to potentially mitigate AD-related memory impairment and neuropathology before it causes irrevocable neurodegeneration.

8 References

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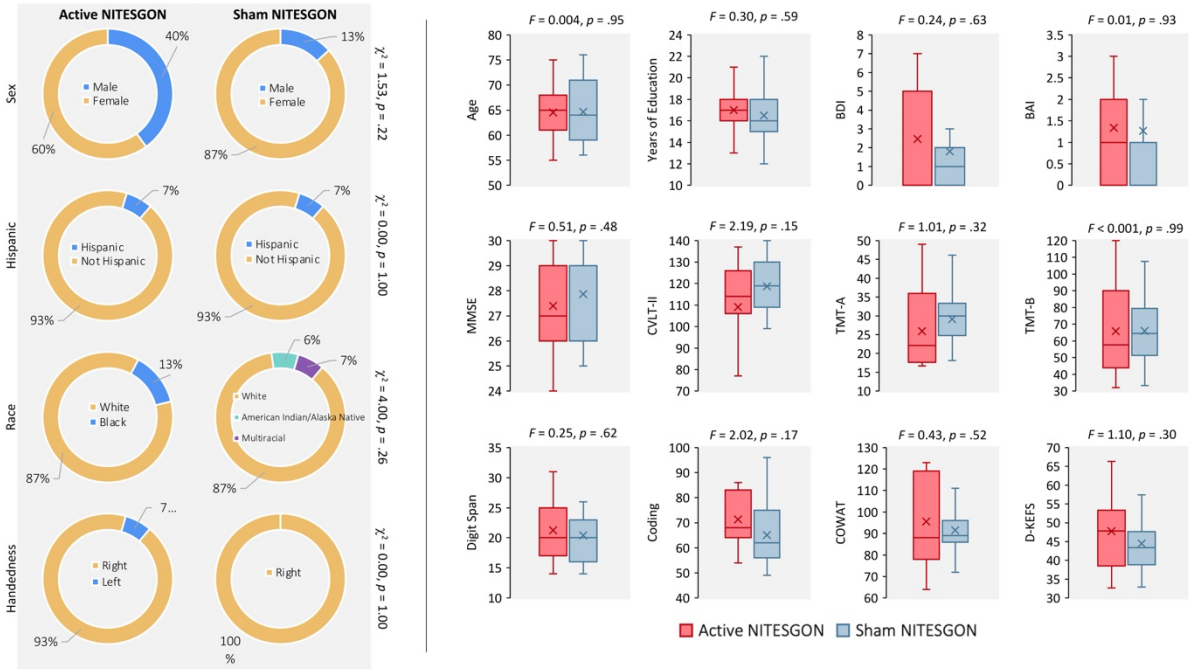
9 Appendix

9.1 Appendix I: Supplemental Figure for Chapter 2

Figure 1

Participant Overview



Note. No significant differences were observed for sex, Hispanic background, race, or handedness between the active and sham NITESGON groups. Furthermore, no significant differences were demonstrated for age, years of education, Beck Depression Inventory (BDI), Beck Anxiety Inventory (BAI), Mini-Mental State Examination (MMSE), cumulative California Verbal Learning Test (CVLT-II), Trial Making Test A (TMT-A) Trial Making Test B (TMT-B), Wechsler Intelligence Adult Scale (WAIS-IV) Digit Span and Coding, cumulative Controlled Oral Word Association Test (COWAT) or the average Delis-Kaplan Executive Function System (D-KEFS) between the active and sham NITESGON groups.