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Faculty of Health Science
Department of Psychology

Transcranial Direct Current Stimulation as a memory enhancer in healthy participants and patients with Alzheimer`s disease.

Martin Bystad

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Department of Psychology

Faculty of Health Sciences

University of Tromsø, Norway.

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List of research reports

Report I:

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Report II:

Bystad, M., Storø, B., Gundersen, N., Wiik, I.L., Nordvang, L., Grønli, O., Daae-Rasmussen, Aslaksen, P.M. (Submitted). **Can accelerated transcranial Direct Current Stimulation improve memory functions? An experimental, placebo-controlled study**.

Report III:

Aslaksen, P.M., Bystad, M., Ørbo, M.C. & Vangberg, T.R. (2018). **The relation of hippocampal subfield volumes to verbal episodic memory measured by California Verbal Learning Test II in healthy adults**. *Behavioral Brain Research*, *351*, 131-137.

Abstract

The aim of this thesis was to investigate the effects of transcranial direct current stimulation (tDCS) as a memory enhancer in Alzheimer's disease patients and healthy individuals. In addition, we wanted to study how verbal memory functions are related to hippocampus subfield volumes.

This thesis consists of three reports, in which two of the reports (I and II) aimed to study the effects of tDCS, and the other report (III) focused on verbal memory and subfields of the hippocampus. In all three reports, the California Verbal Learning Test II (CVLT-II) was used to assess verbal memory functions. The CVLT-II is normed for age and sex and is a widely used memory test, in both experimental and clinical settings.

In reports I and II, the effect of a stimulation method called "transcranial direct current stimulation (tDCS)" was investigated. This is a noninvasive method in which two or more electrodes are placed on the scalp. The electrode positioning depends on the area intended to be stimulated. A weak direct current is delivered through the scalp and aims to increase cortical excitability (i.e., aims to make the neurons more capable of responding to stimuli). The stimulation electrode (the anode) was placed over the temporal cortex, whereas the reference electrode (the cathode) was placed over the right frontal cortex.

In report I, we used a randomized controlled trial design in which 26 patients with Alzheimer's disease underwent six 30-minute sessions of tDCS stimulation during a two-week period. Half of them received active tDCS stimulation, while the other half received placebo tDCS. We found no significant differences between active and placebo tDCS, neither in the primary outcome nor in the secondary outcome measures.

In report II, 40 healthy participants underwent six tDCS sessions for two consecutive days. Half of the participants received active tDCS, and the other half received placebo tDCS. No

significant differences were found in verbal memory outcomes. However, in the young participants there was a significant difference between active and placebo tDCS in executive functions measured by the Trail Making Test, part B (TMT B).

In report II, we investigated the relation between verbal memory and hippocampal subfield volumes in 47 right-handed healthy adults. T1-weighted MRI results were obtained using a 1,5 Tesla scanner. The results showed a significant correlation between left hippocampal subfields volumes and verbal memory. However, no significant correlations were found between right hippocampal volumes and verbal memory.

The overall conclusions are as follows: 1) In patients with Alzheimer's disease, six 30-minute sessions of active tDCS over a period of two weeks did not offer any significant improvements in memory functions, compared to the placebo tDCS results. However, the generalizability is limited due to the small sample size. 2) In healthy participants, six 30-minute sessions of active tDCS for two consecutive days did not offer significantly better memory outcomes, compared to the placebo tDCS results. 3) In healthy adults, there was a significant correlation between verbal memory performance and left hippocampal subfield volume.

Sammendrag

Hensikten med denne avhandlingen var å undersøke om transcranial Direct Current Stimulation (tDCS) kan bedre verbale hukommelsesfunksjoner hos pasienter med Alzheimers sykdom og friske deltakere, samt å undersøke sammenhengen mellom verbal hukommelse og volum av hippocampus og sub-strukturer av hippocampus.

Avhandingen består at tre artikler, der to av artiklene (I og II) undersøkte effektene av tDCS, mens den siste artikkelen (III) studerte verbal hukommelse og hippocampus volum. I alle tre artiklene ble California Verbal Learning Test II (CVLT-II) brukt som mål på verbal hukommelsesfunksjon. CVLT-II er en nevropsykologisk test som er normert for både alder og kjønn og er mye brukt i forskning og klinikk.

I artikkel I og II ble effekten av tDCS undersøkt. Dette er en ikke-invasiv metode der to eller flere elektroder plasseres i hodebunnen, over det området man ønsker å stimulere. En svak likestrøm går gjennom hodeskallen og har til hensikt å påvirke kortikal eksitabilitet (dvs. at nevronene reagerer lettere på stimuli). Stimuleringselektroden (anoden) ble plassert over venstre temporal korteks, mens referanse elektroden (katoden) ble plassert over høyre frontal korteks.

I artikkel I brukte vi et randomisert kontrollert design der 26 pasienter med Alzheimers sykdom fikk seks behandlingssesjoner med tDCS i løpet av to uker. Varigheten på hver sesjon var 30 minutter. Halvparten av pasientene fikk aktiv tDCS, mens den andre halvparten fikk placebo tDCS. Vi fant ingen signifikant forskjell mellom aktiv og placebo tDCS, hverken på primære eller sekundære utfallsmål.

I artikkel II fikk 40 friske deltakere seks sesjoner med tDCS, fordelt på to påfølgende dager. Halvparten av dem fikk aktiv tDCS, men den andre halvparten fikk placebo tDCS. Det ble

ikke funnet noen signifikant forskjell i verbal hukommelsesfunksjon, men det var en signifikant forskjell mellom aktiv og placebo tDCS i eksekutiv funksjon hos de yngre deltakerne, målt med Trail Making Test B (TMT-B).

I artikkel II undersøkte vi sammenhengen mellom verbal hukommelsesfunksjon og hippocampus volum hos 47 høyrehendte voksne deltakere. En MR scanner med 1,5 tesla ble benyttet. Resultatene viste en signifikant korrelasjon mellom venstre hippocampus volum og verbal hukommelse, mens det derimot ikke var noen signifikant korrelasjon mellom høyre hippocampus volum og verbal hukommelse.

Konklusjonene fra de tre rapportene var følgende: 1) Hos pasienter med Alzheimers sykdom gir ikke seks 30 minutters seksjoner med aktiv tDCS i løpet av to uker noen signifikant forbedring i hukommelsesfunksjon, sammenliknet med placebo tDCS. Det var imidlertid få deltakere, noe om begrenser generaliserbarheten 2) Hos friske deltakere ga ikke seks sesjoner med 30 minutter aktiv tDCS over to påfølgende dager noen signifikant forbedring i hukommelsen, sammenliknet med placebo tDCS 3) Hos friske deltakerne var det en signifikant korrelasjon mellom verbal hukommelse og venstre hippocampus volum.

Transcranial direct current stimulation as a memory enhancer in healthy participants and patients with Alzheimer's disease

Introduction

The idea that electrical currents may affect our brain has persisted for two thousand years. Roman physician Scribonius Largus claimed that placing an electrical torpedo fish over the scalp could reduce headaches (Sarmiento, San-Juan, & Prasath, 2016). One of the first trials with electrical current treatment methods for melancholia was conducted in the middle of the 18th century (Sarmiento et al., 2016). However, during the 19th century, there was an increasing interest in investigating the possible electrical current treatment methods for mental disorders.

A method that gained ground during the last 20 years is transcranial direct current stimulation, abbreviated "tDCS". The application of tDCS is noninvasive, associated with few adverse effects, simple to use and inexpensive (Nitsche & Paulus, 2011). The current is a low direct current, usually as low as 1-2 mA, delivered through electrodes placed on the scalp (Nitsche & Paulus, 2011).

tDCS must never be confused with "electroconvulsive therapy" (ECT). The latter involves anesthesia, and the current used is far stronger and leads to seizures (Higgins & George, 2009). While ECT is primarily used in psychiatric hospitals as a treatment method for severe depression, tDCS has a broader area of application. The applications of tDCS range from the treatment of chronic pain (Fagerlund, Hansen, & Aslaksen, 2015) to enhancing cognitive functions in healthy individuals (Chi, Fregni, & Snyder, 2010). Patients can even administer tDCS treatment themselves with preprogrammed devices optimized for this purpose. This broad application, combined with few adverse effects (mainly redness, itching and tingling), may have led to an increased use of and interest in tDCS (Brunoni et al., 2011).

tDCS also differs from transcranial magnetic stimulation (TMS). TMS delivers a brief

electrical current through a coil that leads to a magnetic field. This magnetic field is delivered across the skull and alters neuronal activity (Priori, Hallett, & Rothwell, 2009). While tDCS relies on a weak direct current, TMS relies on a magnetic field (Priori et al., 2009). Both TMS and tDCS aim to change cortical excitability (i.e., make the neurons more capable of responding to stimuli) (Fregni & Pascual-Leone, 2007). tDCS is better suited for double blind studies than TMS. This suitability is as such because tDCS leads to minimal scalp sensations, while TMS may lead to strong scalp sensations, and it is challenging to induce such scalp sensations for placebo TMS (Priori et al., 2009).

Most tDCS studies use either "anodal" stimulation or "cathodal" stimulation. The difference between these two stimulation methods is the polarity. Anodal stimulation induces excitatory effects, while cathodal stimulation decreases excitatory effects (DaSilva, Volz, Bikson, & Fregni, 2011). Usually, the anode is referred to as the "stimulation electrode", whereas the cathode is referred to as the "reference electrode". The reports in this thesis used anodal stimulation, i.e., the anodal electrode was placed above the brain area to be stimulated.

It is estimated that approximately 50% of the current enters the cortex through the skull, in both humans and monkeys (Nitsche, Kuo, Paulus, & Antal, 2015). Undeniably, some of the current will not reach the cortex because of the skull, cerebrospinal fluid, blood, etc. One study (Underwood, 2016) claimed that only 10 % of the current reached the tissue. However, this result was obtained in a cadaver with dead brain tissue, making a comparison to living tissue difficult.

Electrode positioning can be important for the efficacy of tDCS stimulation. Both computational modeling studies and studies monitoring physiological changes from tDCS stimulation suggest that positioning can affect stimulation efficacy (Woods et al., 2016). In general, the stimulation electrode should be placed on the scalp above the cortical area to be stimulated (DaSilva et al., 2011; Woods et al., 2016). For instance, placing the anodal electrode

above the frontal lobe may be most appropriate for depression due to the assumption that depression is associated with hypoactivation of the frontal lobes (Palm, Hasan, Strube, & Padberg, 2016).

Most studies use a current strength of 1-2 mA (Bikson, Datta, & Elwassif, 2009). Some studies have investigated the effect of 4 mA (Chhatbar et al., 2017). However, for safety reasons, it is recommended to not exceed 2 mA (Iyer et al., 2005). Reports (I and II) in this thesis applied a current strength of 2 mA.

Neuroplasticity and the mechanisms of tDCS

The brain has a remarkable ability for adaptability and changing itself (Doidge, 2007). The prefix neuro refers to the "neuron" (the nerve cells in our brain), while the suffix plasticity means changeable, malleable and modifiable (Doidge, 2007). Learning and memory rely on neuroplasticity (Petrovic et al., 2017).

Neuroplasticity can be observed throughout the life span. For instance, Envig and colleagues (Engvig et al., 2010) investigated the effect of memory systems (mnemonics) in healthy elderly individuals. They found that specific memory systems/strategies may improve memory functions. Even more interestingly, they used magnetic resonance imaging and found that eight weeks of such memory training increased cortical thickness.

Such neuroplasticity was also found in a study by Maguire and colleagues (Maguire, Woollett, & Spiers, 2006). They investigated London taxi drivers by using neuroimaging. They revealed that these taxi drivers had greater posterior hippocampal volume compared to that of controls. It is reasonable to believe that such hippocampal volume was a result of their need to navigate and remember a huge number of routes. Furthermore, physical activity may enhance neuroplasticity (Hillman, Erickson, & Kramer, 2008). This enhancement was also demonstrated in a study in which elderly individuals began aerobic exercise (Erickson et al., 2011). Compared to the stretching group, the exercise group displayed both improved

memory and neuroplasticity. At the one-year follow-up, it was found that participants in the aerobic exercise group had increased hippocampal volume by two percent, whereas hippocampal volume declined in the stretching group.

The potential for neuroplasticity seems to decrease with aging (Barnes, 2003; Rossini, Ferilli, Rossini, & Ferreri, 2013). Such a decrease in neuroplasticity may explain why older individuals experience more memory deficits than younger individuals do (Barnes, 2003). Furthermore, Alzheimer's disease leads to inhibited neuroplasticity (Kumar et al., 2017). To improve memory functions in healthy individuals and patients with brain disorders, it is reasonable to assume that enhancing neuroplasticity could be useful.

Long-term potentiation (LTP) is crucial for neuroplasticity. LTP is a long-term increase in the excitability of neurons with respect to particular synaptic inputs caused by the repeated high frequency of that input (Carlson, 2013). LTP involves a long-term increase in synaptic strength (Bliss & Collingridge, 1993). This increase builds on the principle of "fire together, wire together" and was demonstrated experimentally by Lømo several decades ago (Carlson, 2013). A large number of studies have revealed that LTP involves an increase in the number of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the postsynaptic membrane (Carlson, 2013). Having more AMPA receptors leads to more glutamate being bound, thus causing a larger excitatory postsynaptic potential (Henley & Wilkinson, 2016). It is also assumed that LTP can be elicited by the activation of N-methyl-D-aspartate (NMDA) glutamate receptors (Lüscher & Malenka, 2012). We now know that LTP forms the basis for neuroplasticity and for learning and memory (Petrovic et al., 2017).

The opposite of LTP is long-term depression (LTD). LTD is a long-term decrease in the excitability of a neuron with respect to a particular synaptic input caused by terminal bouton stimulation, while the postsynaptic membrane is hyperpolarized or only slightly depolarized (Carlson, 2013). Thus, LTD involves a decrease in synaptic strength and a

reduction in AMPA receptors (Henley & Wilkinson, 2016).

In the field of neuroplasticity, brain-derived neurotrophic factor (BDNF) has gained ground (Tapia-Arancibia, Aliaga, Silhol, & Arancibia, 2008). BDNF is a neurotrophic factor and helps support the growth and survival of neurons (Cunha, Brambilla, & Thomas, 2010). BDNF promotes LTP (Cunha et al., 2010). Both aging and Alzheimer's disease are associated with lower levels of BDNF (Tapia-Arancibia et al., 2008).

The main mechanism of tDCS is to trigger neurons to stimulate or form new connections (Giordano et al., 2017). tDCS aims to alter the resting state potential of neurons and thereby induce neuroplasticity (Stagg & Nitsche, 2011). More specifically, tDCS facilitates neural function by modulating cortical excitability (Stagg & Nitsche, 2011). It is important to emphasize that tDCS does not directly cause neuronal firing but triggers conditions that make neuronal firing more likely (Reinhart, Cosman, Fukuda, & Woodman, 2017).

Studies using functional magnetic resonance imaging (fMRI), electroencephalography (EEG) and pharmacological approaches suggest that tDCS leads to neurophysiological changes in the cortex (Venkatakrishnan & Sandrini, 2012). For instance, Keeser applied EEG (Keeser, Padberg, et al., 2011) and fMRI (Keeser, Meindl, et al., 2011) and revealed that tDCS stimulation can increase excitability and strengthen connectivity within different resting state networks. Additionally, neuroimaging studies have found that during tDCS stimulation, regional cerebral blood flow increases by 17% (Zheng, Alsop, & Schlaug, 2011).

Madeiros and colleagues (Medeiros et al., 2012) suggested that tDCS alters the levels of neurotransmitters underneath the electrode. It has been found that a single tDCS session increases the levels of glutamate, which is the primary excitatory neurotransmitter (Hone-Blanchet, Edden, & Fecteau, 2016). Glutamate plays an important role in LTP (Granger, Shi, Lu, Cerpas, & Nicoll, 2013). Furthermore, tDCS can lower the levels of gamma-aminobutyric

acid (GABA), a neurotransmitter with inhibitory effects (Bachtiar, Near, Johansen-Berg, & Stagg, 2015; Stagg et al., 2009). In addition, citalopram (a selective serotonin reuptake inhibitor) can prolong the effects of tDCS stimulation of the motor cortex (Nitsche et al., 2009). Furthermore, tDCS also increases the concentrations of calcium ($CA^{2+)}$ and BDNF, which play important roles in neuroplasticity (Das, Holland, Frens, & Donchin, 2016).

The excitatory effects of tDCS stimulation persist after the stimulation ends (Nitsche & Paulus, 2001; Podda et al., 2016). Stimulation for nine minutes may elicit excitatory effects after 30 minutes, whereas 13-minute stimulations lead to alterations that last for up to 90 minutes (Thair, Holloway, Newport, & Smith, 2017). It could be reasonable to assume that longer stimulations will increase the duration of the after-effects compared to the duration induced by shorter stimulations. However, Monte-Silva and colleagues (Monte-Silva et al., 2013) revealed that tDCS sessions for longer than 26 minutes may lead to inhibitory effects rather than excitatory effects. This outcome may result from a calcium overflow that impairs neuroplasticity (Monte-Silva et al., 2013). In sum, the optimal duration of tDCS stimulation is still uncertain.

Despite the fact that tDCS has been studied extensively, relatively few studies have investigated the exact physiological mechanisms behind tDCS. Thus, the underlying mechanisms of tDCS are not fully understood (Bennabi et al., 2014; Mohammadi, 2016). Moreover, most mechanistic studies on the physiological effects of tDCS have focused on the motor cortex. This focus is highly relevant for stroke patients if the stroke is located in the motor complex but less relevant for other functions, e.g., memory functions (Medeiros et al., 2012).

tDCS is a cognitive enhancer in healthy participants

As a cognitive enhancer, tDCS has gained interest. Improving cognitive abilities has attracted attention. Psychologist Corneliu Giurgea even said, "*man is not going to wait*

passively for millions of years before evolution offers him a better brain." (Farah, 2015).

There is evidence that tDCS may improve cognitive functions in healthy people, i.e., verbal fluency, working memory, verbal episodic memory, visual memory, attention and language processing speed (Tremblay et al., 2014). For instance, Ross and colleagues found that tDCS could enhance name recall in both younger and elderly participants (Ross, McCoy, Coslett, Olson & Wolk, 2011). Likewise, another study found that tDCS could improve verbal memory in older and younger participants (Manenti, Brambilla, Petesi, Ferrari, & Cotelli, 2013).

A recent meta-analysis investigated 24 tDCS studies, with a total of 566 participants above 60 years of age. They concluded that tDCS may ameliorate episodic memory in both healthy and cognitively impaired older adults (Huo et al., 2019). Despite the evidence that tDCS can improve cognitive functions, it should be emphasized that the results are mixed (Tremblay et al., 2014). Over the past two decades, over 3000 articles have investigated the effect of tDCS on different brain functions. Nevertheless, the studies investigating the effect of tDCS on cognition rely on different tDCS protocols, and the results are inconsistent.

In addition, most studies rely on a single session of stimulation (Horvath, Forte, & Carter, 2015). Consequently, this approach may limit the physiological effects. Since the after-effects of a single tDCS session are relatively short lived (60-90 minutes), it is important to rely on multiple sessions (Nitsche et al., 2015). To enhance cognitive function in daily life, the effect must last longer than the experimental session. Horvath and colleagues (Horvath et al., 2015) conducted a review and concluded that single tDCS sessions had minimal cognitive effects in healthy participants. They also concluded that multiple sessions may generate better effects.

Extending the duration of the tDCS sessions (longer than 30 minutes) does not seem to produce better results (Nitsche et al., 2015; Woods et al., 2016). Hence, the use of multiple

stimulations and a small interval between each stimulation is recommended (Nitsche et al., 2015; Woods et al., 2016). Repeating the tDCS stimulation within a time window of 30 minutes may lead to more cumulative effects (Nitsche et al., 2015). Nitsche and colleagues (Nitsche et al., 2015) suggest "*simply prolonging stimulation duration seems not to be the optimal strategy. The alternative might be the repetition of stimulation sessions*" (p. 102). Based on this recommendation, in report II, we used a novel stimulation protocol with short intervals between each tDCS session. There is clearly a need for better standardization among tDCS protocols in healthy participants (Tremblay et al., 2014). In addition, the optimal tDCS protocol for healthy participants needs to be further investigated. We still do not know whether short intervals (as we investigated in report II) are better than long intervals between tDCS sessions.

Normal aging is associated with a steady decline in cognitive function, especially memory functions (Ward, Berry, & Shanks, 2013). As the older population continues to grow, methods to reduce age-associated cognitive decline have gained increasing interest (Hsu, Ku, Zanto, & Gazzaley, 2015). Thus, in report II in this thesis, tDCS was investigated as a memory enhancer in both young and elderly participants.

<u>tDCS is a cognitive enhancer in Alzheimer's disease</u>

Alzheimer's disease is a degenerative disease and the most common type of dementia, accounting for 60% of all cases of dementia (Blennow, de Leon, & Zetterberg, 2006). Due to increased life expectancy, it is estimated that the prevalence of Alzheimer's disease will double during the next 30 years (Alzheimer`s Association, 2019). The prevalence of Alzheimer's disease is less than one percent in people under 65 years of age, but for people over 85 years, the prevalence is between 24 and 33% (Blennow et al. 2006).

Alzheimer's disease leads to a progressive decline in cognitive domains. This decline manifests as a steady decline in memory functions, orientation capabilities, executive

functions, visuospatial abilities and verbal abilities (Alzheimer`s Association, 2019; Mayeux, 2010). Memory impairment is a core symptom of Alzheimer's disease. One meta-analysis found memory decline to be the most pronounced symptom of Alzheimer's disease (Bäckman, Jones, Berger, Laukka, & Small, 2005), especially in the "mild stage".

The progressive decline in Alzheimer's disease can broadly be defined by three stages: "mild Alzheimer's disease", "moderate Alzheimer's disease" and "severe Alzheimer's disease" (Henderson & Jorm, 2000). In the mild stage, or "early stage", individuals experience difficulties acquiring new information and memory loss for recent events (for instance, an inability to remember what happened yesterday). In the moderate stage, memory loss may be more serious, and new information is immediately lost, but previous knowledge can be retained (for instance, remembering children's names). In this stage, the declarative memory is profoundly affected, while the procedural memory is more intact. In the severe stage, the memory loss is monumental, and only fragments of the memory are left (for instance, remembering some events from childhood). In this stage, verbal function is usually very impaired. This impairment means that both the ability to understand and to produce words or sentences are severely affected. The life expectancy after diagnosis is estimated to be seven–ten years in many studies (Zanetti, Solerte, & Cantoni, 2009).

The exact cause and pathological mechanisms behind Alzheimer's disease are uncertain. A common hypothesis is that Alzheimer's disease leads to a massive loss of neurons as a consequence of excessive levels of plaques (beta-amyloid) and tangles (tau-proteins) in the brain (Alzheimer`s Association, 2019; Mayeux, 2010). In the early stages of Alzheimer's disease, these pathological changes are especially prominent in the medial temporal lobe, including the hippocampus and entorhinal cortex (Jack et al., 1997). Previous studies also suggest that Alzheimer's disease is associated with decreased acetylcholine and neuroplasticity (Blennow et al. 2006). Furthermore, Alzheimer's disease is associated with

neuroinflammation in the hippocampus (Valero et al., 2017). Hence, it seems likely that inflammatory processes are related to the pathology of Alzheimer's disease (Frozza, Lourenco, & De Felice, 2018).

Since Alzheimer's disease is highly complex, it remains extremely difficult to find a cure (Cummings, Morstorf, & Zhong, 2014). For instance, Cummings and colleagues (Cummings et al., 2014) reviewed clinical trials from 2002–2012 and found that the failure rate for drug development in Alzheimer's disease is 99,6 %. There are few treatment options for patients with Alzheimer's disease (Alzheimer's Association, 2017). Pharmacologic (medications) and nonpharmacologic (cognitive stimulation, physical exercise) therapeutic approaches cannot cure the disease or slow the patient's decline, but may provide a slight improvement in symptoms (Alzheimer's Association, 2017).

A groundbreaking cure for Alzheimer's disease may be decades ahead. Thus, in the near future, it will be important to investigate the effect of symptom-modifying treatments. This aim was also recommended in a report from the Alzheimer's Association in 2012, which specifically emphasized the importance of investigating symptom-modifying approaches (Alzheimers Association, 2012).

There are several suggestions regarding why tDCS may have beneficial effects in Alzheimer's disease (Hansen, 2012; Yu, Park & Sim, 2014). First, as previously noted, Alzheimer's disease is associated with impaired neuroplasticity (Koch et al., 2012). Thus, impaired neuroplasticity may be a potential target for intervention (Kumar et al., 2017; Rajji, 2019). Increased neuroplasticity through tDCS may lead to improved memory functions (Hill, Kolanowski, & Gill, 2011). Second, in Alzheimer's disease, there is generally reduced excitability within and atrophy of the temporal cortex (Tapia-Arancibia et al., 2008). tDCS stimulation aims to improve such reduced excitability. Third, tDCS may increase levels of acetylcholine (Seong Hun Yu, Seong Doo Park, & Ki Chel Sim, 2014), a neurotransmitter

important for learning and memory. Alzheimer's disease is linked to a reduction in acetylcholine, and increasing levels of acetylcholine may be beneficial in Alzheimer's disease (Naik et al., 2009). Cholinesterase inhibitors (e.g., rivastigmine) are widely used in Alzheimer's disease to enhance the levels of acetylcholine (Naik et al., 2009). Fourth, Alzheimer's disease is also associated with low levels of glutamate (Li & Tsien, 2009). tDCS may facilitate the glutamatergic process (Hone-Blanchet et al. 2016). Fifth, since Alzheimer's disease leads to a reduction in BDNF (Lee et al., 2005), tDCS may improve neuroplasticity by increasing BDNF (Fritsch et al., 2010).

If these mechanisms of tDCS can be beneficial for patients with Alzheimer's disease, it may slow the progression of the disease. However, it would be more realistic to expect that tDCS may be a symptom-modifying treatment. tDCS in Alzheimer's disease may serve as a symptom-modifying treatment by slowing cognitive decline and/or improving cognitive functions for a short period of time.

It is of utmost importance to test whether tDCS can be a symptom-modifying treatment in Alzheimer's disease. To date, nine published studies have investigated the efficacy of tDCS as a cognitive enhancer in patients with Alzheimer's disease. There is still limited evidence of tDCS as a symptom-modifying treatment in patients with Alzheimer's disease.

In the following studies, tDCS in Alzheimer's disease was investigated. Ferrucci and colleagues (Ferrucci et al., 2008) investigated the effect of three 15-minute sessions of tDCS stimulation for patients with Alzheimer's disease; temporoparietal areas were stimulated. They found that scores on a word recognition test significantly improved, by 17% for anodal stimulation compared to the results for placebo and cathodal tDCS stimulation.

Similarly, Boggio and colleagues (Boggio et al., 2009) delivered three 30-minute tDCS sessions. These sessions included tDCS stimulation of the temporal cortex, tDCS

stimulation of the frontal cortex and placebo stimulation in random order. The results revealed that stimulation of the temporal cortex led to significantly better scores on a visual recognition task.

Furthermore, Boggio and colleagues (Boggio et al., 2012) employed tDCS stimulation of the temporal cortex. Each session lasted 30 minutes and was delivered for five consecutive days. The results revealed that active tDCS stimulation improved visual recognition by nine percent compared to a two-and-a-half percent improvement for placebo tDCS. The improvement from the active tDCS stimulation persisted for a month after the last stimulation session.

Another study by Khedr and colleagues (Khedr et al., 2014) reported that ten sessions of 25 minutes of tDCS stimulation of the prefrontal cortex led to a significantly increased score on Mini Mental Status Examination (MMSE) compared to the scores achieved after placebo tDCS. The MMSE score improved by two points immediately after active tDCS stimulation. This improvement increased by two more points at the two-month follow-up. In comparison, placebo tDCS stimulation improved the MMSE scores by 0.4 points at the two-month follow-up.

Cotelli and colleagues (Cotelli et al., 2014) used frontal cortex stimulation with ten tDCS sessions in combination with computerized memory training. They investigated how this intervention could improve face-name associations. There was no significant difference between placebo and active tDCS stimulation in name-face associations.

Additionally, Suemoto and colleagues (Suemoto et al., 2014) applied tDCS over the frontal cortex for six sessions during a period of two weeks. The aim of the stimulation was to reduce apathy due to Alzheimer's disease. No significant differences were found between active and placebo tDCS.

More recently, Khedr and colleagues (Khedr et al., 2019) randomized patients into two

groups, a placebo group and an active group. Each patient underwent 10 sessions of tDCS stimulation for a total of 40 minutes. The stimulation sites were both the left and right temporoparietal cortices for 20 minutes on each side. They found that active tDCS led to significant improvements on cognitive test results (the MMSE, clock drawing test, and Montreal Cognitive Assessment), whereas no such results were found in the placebo group.

Furthermore, Im and colleagues (Im et al., 2019) randomized patients into either active or placebo tDCS groups that would undergo daily 30-minute stimulation sessions at home for six months. Compared to the placebo group, active tDCS led to significant changes on cognitive test results (MMSE, Boston Naming Test). However, no such effect was observed for delayed recall. The regional cerebral metabolic rate for glucose (rCMRglc) in the temporal/inferior gyrus was preserved in the active group but was reduced in the placebo group.

The results from some of these Alzheimer's studies are promising. However, there are central methodological limitations, and tDCS cannot be seen as an adjuvant intervention in Alzheimer's disease (Buss, Fried, & Pascual-Leone, 2019; Kim, 2016). First, an important limitation is that most tDCS studies focus on immediate effects (James Giordano et al., 2017; Hsu et al., 2015). The application of tDCS as a therapeutic for Alzheimer's disease seems unlikely without more evidence of its long-term effects. There is clearly a need to study the long-term effects of tDCS in Alzheimer's disease (Cruz Gonzalez et al., 2018).

In their study, Im and colleagues (Im et al., 2019) applied a long-term intervention (for six months). However, we do not know whether the effect of the six-month tDCS intervention persisted after the last stimulation session. In general, very few studies have investigated the long-term effects of tDCS, so the long-term effect is unknown (Cruz Gonzalez et al., 2018; Vestito, Rosellini, Mantero, & Bandini, 2014).

Second, most studies are small-scale clinical trials with fewer than 30 patients (Kim,

2016). Such small-powered studies limit generalizability. Thus, the results from the above studies must be interpreted with caution.

Third, previous studies have relied on less advanced cognitive outcome measures. Most studies relied on gross cognitive screening tools rather than neuropsychological tests with better accuracy for testing specific cognitive functions. Two previous reviews recommended that future studies on tDCS and Alzheimer's disease rely on more sophisticated cognitive outcome measures (Freitas, Mondragón-Llorca, & Pascual-Leone, 2011; Nardone et al., 2011).

In this thesis, report I sought to overcome some of these methodological shortcomings. We applied a randomized, placebo-controlled (RCT) design and applied more comprehensive cognitive outcome measures. This application was in accordance with the recommendations from previous reviews (Freitas et al., 2011; Nardone et al., 2011).

Memory functions

All reports (report I, report II, and report III) in this thesis involve memory functions. In report I and report II, memory functions were the primary outcome measures, whereas in report III cognitive and neurobiological aspects of memory were investigated.

Memory can be defined as the capacity of the brain to acquire and retain usable skills and new information (Baddeley, 1999). For both humans and animals, memory functions are core cognitive domains. Human memory can be divided into explicit and implicit memory (Schacter, 1992). Explicit memory relies on conscious effort, while implicit memory is more automatic/unconscious (Purves et al., 2008). Recalling information during an exam is an example of explicit memory, whereas riding a bike is an example of implicit memory.

Encoding, storing and recall are the core processes of explicit memory function. Encoding refers to processing the information so it can be stored, while storage is the retention of the information and recall is the process of retrieving the acquired information

(Gazzaniga & Heatherton, 2015). Hermann Ebbinghaus was the first psychologist who studied our ability to recall information (Gazzaniga & Heatherton, 2015). He created nonsense syllables and tried to remember a list of such syllables. He discovered, using himself as the only research participant, that the ability to recall the information occurred rapidly during the first hours and days, and later, there was a more steady, gradual decline (Pashler, Rohrer, Cepeda, & Carpenter, 2007). This theory is often termed "the forgetting curve" or "Ebbinghaus curve". In addition, he also found that overlearning and repetition decreased forgetting and could improve recall (Pashler et al., 2007).

Memory and learning are closely related concepts. However, there are some differences. Learning is the process of acquiring memory, while memory is a behavioral change caused by an experience (Gazzaniga & Heatherton, 2015). For instance, the ability to acquire new words is learning, whereas the ability to recall the words is memory (Okano, Hirano, & Balaban, 2000). tDCS aims to improve both learning and memory.

Explicit memory can be divided into episodic and semantic memory (Baddeley, 1999). Semantic memory relies on facts, knowledge and concepts, whereas episodic memory builds on events and experiences (Tulving, 2001). Contrary to semantic memory, episodic memory relies on the recollection of past experiences (Tulving, 1985). For instance, to know that a bike has two pedals is semantic memory and memories of riding a bike in the past are examples of episodic memory. The ability to recall a list of words is an example of episodic memory, more specifically verbal episodic memory.

Episodic memory can be divided into three parts: immediate recall, delayed recall and recognition (Delis, Kramer, Kaplan, & Ober, 2004). Immediate recall are memories we can recall without delay, for instance, repeating a history instantly. Delayed recall is our ability to remember knowledge, information or past experiences after either short (ten minutes) or long (months) intervals. Delayed recall requires a process referred to as consolidation. Squire and

colleagues (Squire, Genzel, Wixted, & Morris, 2015) define consolidation as follows: "*Consolidation refers to the process by which a temporary, labile memory is transformed into a more stable, long-lasting form*". Consolidation is our ability to transfer memory material from immediate memory to long-term memory (Carlson, 2013) and also describes a newly formed memory going through a transformation process in which the memory becomes stronger and more resilient (Alberini, 2005).

Weston and colleagues revealed that delayed recall after seven days is a predictor for developing Alzheimer's disease (Weston et al., 2018). Delayed recall is also found to be a stronger predictor for Alzheimer's disease than both structural imaging and cerebrospinal fluid biomarkers (Gomar, Bobes-Bascaran, Conejero-Goldberg, Davies, & Goldberg, 2011). To distinguish age-related memory decline from Alzheimer's disease, delayed recall (of a word-list) has a sensitivity and specificity of 89% (Weissberger et al., 2017). One study (Chandler et al., 2004) found that only three percent of healthy elderly adults had difficulties with delayed recall of three words. For patients with Alzheimer's disease, 87 % recalled no words or one word.

Unlike recall, recognition involves a cue. Recognition is the ability to recognize previous knowledge, for instance, to recognize family members and places. Both depression, cerebrovascular disease (vascular dementia) and Lewy body dementia are associated with delayed recall, whereas recognition is intact (Shankle et al., 2005).

A useful framework for understanding the relation between immediate recall and delayed recall is the Atkinson-Shiffrin model of memory (Atkinson & Shiffrin, 1968). In this model, short-term memory is responsible for short-term storage and is able to hold information for 20-30 seconds. Short-term memory has a rapid rate of forgetting. Immediate recall requires short-term memory. The information from short-term memory can be stored more persistently in long-term memory through consolidation. In the Atkinson-Shiffrin

model, short-term memory is similar to a bottleneck since the information must pass from short-term memory to be stored in long-term memory. Long-term memory has a large storing capacity, but quite the opposite is true for short-term memory (Higbee, 2001). Short-term memory can be compared to an in-basket on an office desk, whereas the long-term memory would be like the file cabinet in an office (Higbee, 2001).

The Atkinson-Shiffrin model is widely used, despite its simplicity. Studies of brain lesions provide evidence for this model (Gazzaniga & Heatherton, 2015; Squire, 2009). People with severe hippocampal lesions can have intact short-term memory and are able to recall information immediately, even if their consolidation process is severely affected (Squire, 2009). The Atkinson-Shiffrin model is criticized for being too simple and does not emphasize the importance of working memory (the active processing of information for current use) (Baddeley, 1994).

There may be a difference between verbal and visual memory when people recall information (Lezak, Howieson, Bigler, & Tranel, 2012). Verbal memory is our ability to remember verbal information, e.g., an instruction or a postal address. Visual memory refers to our ability to remember visual information/knowledge, e.g., a picture or an illustration. Thus, there are different neuropsychological tests for the assessment of either visual or verbal memory (Lezak et al., 2012). The present thesis focuses on verbal memory.

Memory functions can be assessed with standardized neuropsychological batteries (Lezak et al., 2012). A typical memory test for assessing verbal memory is a list-learning task in which the patients/participants are presented with a list of words. Then, they are instructed to recall the list immediately, after a delay and to recognize which words were presented (Delis et al., 2004). Assessing the ability to recall words from a list is one of the most common ways to investigate memory functions, both experimentally and clinically (Gavett et al., 2016).

Memory functions decline with increasing age. However, to state that all memory functions decline with aging is an oversimplification. Compared to episodic memory, semantic memory and implicit memory are much more resistant to aging (Schaie & Willis, 2010). In addition, there is large individual variability within the elderly population. For instance, it is found that subsamples of people aged 70 years and older outperformed people in middle age on memory tests (Habib, Nyberg, & Nilsson, 2007). Furthermore, the study design may also affect how age-related memory declines are detected. Cross-sectional designs seem to present earlier declines in age-related memory compared to that of longitudinal designs (Schaie & Willis, 2010).

Hippocampus

The hippocampus is located in the temporal lobe and is a key brain structure for consolidation (Ramirez et al., 2013). Patient H. M had both of his hippocampi removed after epilepsy surgery. Due to the surgical procedure, H. M lost his ability to consolidate new information (Scoville & Milner, 2000). His delayed recall was severely impaired. He could remember past events prior to the surgery but was unable to remember any new information after the surgery. His condition led neuroscientists to understand the importance of the hippocampus for the formation of new episodic and semantic memories.

The hippocampus tends to atrophy with aging. From the age of 60 years, the volume of the hippocampus has an annual reduction of one–two percent (Raz et al., 2005). A form of hippocampus atrophy is a part of normal cognitive aging and may be responsible for the reduction in episodic memory that most people experience in old age (Bartsch & Wulff, 2015). It is assumed that age-related memory decline is caused by a reduced ability to consolidate new information (Kukolja, Goreci, Onur, Riedl, & Fink, 2016).In Alzheimer's disease, the hippocampus is seriously affected, even in the early stage (Querfurth & LaFerla, 2010). One assumption is that plaque formation in Alzheimer's disease begins in the

hippocampus and then spreads throughout the brain (Khan et al., 2014). Early deterioration of the entorhinal cortex and hippocampus (Criscuolo et al., 2017) could explain why delayed recall is such a sensitive measure of Alzheimer's disease (Gomar et al., 2011).

Memory functions seem to correlate with the relative volume of the hippocampus. The volume of the hippocampus (when adjusted for intracranial volume and age) is associated with the ability to acquire and remember new words (list-learning) (Pohlack et al., 2014; Ystad et al., 2009). In general, verbal memory is more dependent on the left hippocampus than on the right hippocampus (Ezzati et al., 2016; Ystad et al., 2009). Furthermore, long-term stress and depression can lead to memory impairment as a consequence of hippocampal atrophy (Kim, Pellman, & Kim, 2015). Both long-term stress and depression are associated with the accumulation of cortisol. Such accumulation may be neurotoxic and can lead to atrophy of the hippocampus (Kim et al., 2015; Sapolsky, 1996). It has also been revealed that experimentally increased cortisol levels are associated with reduced delayed recall (Newcomer et al., 1999).

The hippocampus is composed of different segments or "subfields". The hippocampus can be divided into 13 different subfields (Iglesias et al., 2015). The differentiation of such subfields requires brain imaging with very high resolution (Iglesias et al., 2015). Among these subfields are four well-known subfields, called "cornu ammonis" (Andersen, Morris, Amaral, O'Keefe, & Bliss, 2007). These subfields range from CA1 to CA4 and seem to have specialized functions. In case-control studies using the California Verbal Learning Test II (CVLT-II), it was found that the volume of the CA1 correlated better with delayed recall, whereas CA2-3 and CA4 were more related to immediate recall (Mueller, Chao, Berman, & Weiner, 2011; Mueller et al., 2012). These studies also found that focal lesions in CA1 can aggravate autobiographical memory and mental time travel (Thorsten Bartsch, Döhring, Rohr, Jansen, & Deuschl, 2011). In report III, we aimed to investigate how verbal memory was

related to hippocampus volume and these subfields.

The medial temporal lobe consists of the hippocampus, entorhinal cortex, perirhinal cortex and parahippocampus (Carlson, 2013). It is difficult to separate these areas in detail with regard to distinct functions (Lipton & Eichenbaum, 2008). However, it seems that the hippocampus and parahippocampus contribute to consolidation, while the entorhinal and perirhinal cortex contributes more to recognition (Eichenbaum, Yonelinas, & Ranganath, 2007). One fMRI study found that the parahippocampus was significantly more activated when people viewed spatial information (e.g., rooms, landscapes) compared to faces or objects (Epstein & Kanwisher, 1998).

The medial temporal lobe is vital for declarative memory (Purves et al., 2008; Squire & Zola-Morgan, 1991). Medial temporal lobe injuries can lead to difficulties with memory function (Squire & Zola-Morgan, 1991). For the detection of dementia, medial temporal atrophy can be a sensitive measure (Burton et al., 2008). The sensitivity for distinguishing patients with Alzheimer's disease and healthy controls is 83-84% (Wei et al., 2019; Westman et al., 2011).

Bikson and colleagues found that tDCS stimulation could stimulate the hippocampus in rats (Bikson et al., 2004). One mechanism governing this response is that tDCS increases brain-derived neurotrophic factor (BDNF) in rats, which may affect the hippocampus (Yu, Wu, Chien, & Hsu, 2019). In humans, we do not know whether tDCS can affect the hippocampus. However, it is possible to stimulate the temporal lobe where the hippocampus is located. Therefore, the aim for reports I and II in this thesis was to stimulate the temporal lobe.

General research questions

The major research questions in this thesis were how tDCS can improve verbal memory functions and how performance on the California Verbal Learning Test II (CVLT-II) is

associated with hippocampus volume. Studies in this thesis aimed to supplement the existing literature.

The research questions in this thesis are as follows:

1) Can active anodal tDCS lead to significantly better verbal memory function compared to that observed after placebo tDCS in patients with Alzheimer's disease?
2) Can active anodal tDCS lead to significantly better verbal memory function compared to that after placebo tDCS in healthy elderly and heathy younger participants?
3) Are higher scores on CVLT-II associated with a larger volume of the subfields (CA1-CA4) of the hippocampus? We expect to find this association, in line with previous studies.

Methods

Overview of study design

	n (males)	Participants	Design	Memory assessment
Report I	25 (14)	Patients with Alzheimer's disease	Randomized placebo-controlled clinical trial (RCT)	California Verbal Learning Test-II (CVLT-II)
Report II	40 (11)	Healthy elderly and young participants	Experimental placebo-controlled study	California Verbal Learning Test-II (CVLT-II)
Report III	47 (16)	Healthy adults	Cross-sectional MRI study	California Verbal Learning Test-II (CVLT-II)

Participants

In report I, a total of 26 patients with Alzheimer's disease were enrolled in the study. One patient decided to withdraw due to a lack of motivation. We applied the revised

«NINCDS-ARDRA» criteria for Alzheimer's disease (McKhann et al., 2011). We followed section 4.2 in these criteria: "probable Alzheimer's disease with increased level of certainty." This determination of eligibility requires documentation of a progressive cognitive decline based on information from informants (relatives) and a cognitive and/or neuropsychological evaluation. There were 13 patients in the placebo group and 12 patients in the active group. Patients were not eligible if they had serious somatic disorders (cancer, chronic obstructive pulmonary disease, heart failure) or neuropsychiatric disorders (psychosis or severe depression) that could influence cognitive function.

In report II, a total of 40 participants were included in the study. There were two groups of participants: one group of young participants (age 20-30 years) and another group of participants in later adulthood (age 60-69 years). Participants were required to be healthy, i.e., could not suffer from any serious diseases (cancer, heart failure, stroke) or diseases/injuries in the central nervous system. In addition, participants were not eligible if they had any mental disorders (e.g., depression, anxiety, etc.). No participants decided to withdraw from the study.

In report III, a total of 47 right-handed participants (31 females, age 20-71 years) were included in the study and tested with the California Verbal Learning Test II (CVLT II) and two subtests of the Wechsler Abbreviated Scale of Intelligence (WASI). All participants were required to be healthy. They could not suffer from any serious somatic diseases or mental disorders. Since the study involved brain imaging with magnetic resonance imaging (MRI), pregnancy or body implants were exclusion criteria.

Recruitment methods/randomization

In report I, patients with Alzheimer's disease were recruited by advertisement in the local newspaper. In addition, a secretary at the Geriatric Department, University Hospital of North Norway sent an invitation letter to patients recently diagnosed with Alzheimer's

disease. In reports II and III, participants were recruited by both advertisements in the newspaper and at the university and by sending out an invitation letter to the Tromsø senior university (an organization where retired individuals meet and discuss science/politics).

In reports I and II, patients/participants were randomized to either active or placebo tDCS. Patients/participants were assigned to a list with codes provided by the tDCS manufacturer. Each patient/participant received his/her own unique code. The code decided whether the tDCS stimulator should deliver placebo or active stimulation. We used random.org (www.random.org) to randomize the order of the codes. It was not possible to identify the codes during the study. After the experiments in reports I and II were completed, the list was decoded. Neither the participant/patient nor the experimenter could identify if the stimulation was active or placebo, since they only had the code.

Memory assessment with the California Verbal Learning Test II (CVLT-II)

In all three reports (reports I, II and III), we used the California Verbal Learning Test-II (CVLT-II) to assess verbal memory functions. CVLT-II is a widely used memory test, normed by age and sex (Delis et al., 2004). CVLT-II assesses immediate recall, delayed recall and recognition (Delis et al., 2004). More specifically, CVLT-II measures verbal auditory episodic memory. Additionally, CVLT-II measures serial position effects (primacy and recency), cued recall, intrusions and interference (Delis et al., 2004).

In reports I and II, CVLT-II was our primary outcome measure, while the study conducted for report III investigated how CVLT-II correlated with hippocampal subfields. When using CVLT-II, the participant/patient was presented with a 16-word list. This presentation was performed five times. The patient/participant was instructed to recall the list immediately after each presentation. Recalling this list immediately assesses immediate memory. After a delay of 20 minutes, the patient/participant was asked to recall all the words from the word list. This task requires delayed recall. Then, the patient was presented with a

word-list containing 32 words and was instructed to say “yes”/“no” if the word was recognized (i.e., was presented on the 16 words list). Such “yes”/“no” responses requires recognition.

CVLT-II can be used both experimentally for healthy participants and to assess memory functions before and after a treatment, surgical procedure or disease (Delis et al., 2004). In general, test-retest practice effects can be prominent for memory tests (Benedict, 2005). However, using parallel versions of memory tests minimizes the test-retest practice effect (Benedict & Zgaljardic, 1998). Thus, CVLT-II consists of two parallel versions: “standard” and “alternate” versions. These two versions have different and independent word lists to reduce test-retest practice effects.

CVLT-II is widely used in both research and clinical practice to assess patients with Alzheimer’s disease (Delis et al., 2005). A patient with Alzheimer’s disease will typically find the CVLT-II delayed recall task very difficult (Rabin et al., 2009). The delayed recall task requires consolidation of verbal information, and in Alzheimer’s disease, such consolidation is impaired (Mayeux, 2010). Younger participants scored significantly better than healthy elderly on immediate and delayed recall tasks, whereas patients with Alzheimer’s disease scores significantly lower than healthy elderly on immediate recall, delayed recall and recognition tasks (Delis et al., 2004).

In a Norwegian study by Bosnes (Bosnes, 2007), a significant correlation was found between CVLT-II and Wechsler Memory Scale Revised (WMS-R) scores for delayed recall ($r = 0{,}58$, $p < 0{,}001$). Other studies have assessed patients using the CVLT-II, e.g., patients with depression (Hammar, Isaksen, Schmid, Årdal, & Strand, 2011), chronic pain (Landrø et al., 2013), bipolar disorders and schizophrenia (Simonsen et al., 2009). There are no Norwegian norm data for the CVLT-II (Siqveland, Sundseth, Dalsbø, Harboe, & Leiknes, 2014), and all norm data used in Norwegian studies are from the USA (Delis et al., 2004).

The age norms for the CVLT-II data are based on cohorts (i.e., cohorts were aged 60-69 years, 70-79 years, etc.) (Delis et al., 2004). The CVLT-II has good test-retest reliability. For immediate recall, the test-test reliability is 0,82, whereas for delayed recall, the test-retestreliability is 0,88 (Delis et al., 2004). For recognition, the test-retest reliability is 0,79 (Delis et al., 2004).

There is a short format of the CVLT-II, consisting of nine words. This short format is very suitable for patients with Alzheimer's disease and other forms of dementia (Delis et al., 2004). However, this format does not have any parallel versions and may increase the probability of test-retest practice effects.

Transcranial direct current stimulation (tDCS)

In reports I and II, we used a transcranial direct current stimulation (tDCS) device from NeuroConn, Ilmenau, Germany. In both studies (reports I and II), the stimulation duration for each session was 30 minutes, and the current intensity was 2 mA. This outcome was in line with previous recommendations (Brunoni et al., 2012; Monte-Silva et al., 2013; Thair et al., 2017). We used a pair of 35-cm^2 rubber electrodes covered with sponges to deliver the current. These electrodes were placed at the skull. The stimulation electrode ("the anode") was placed over the temporal cortex at the T3 position, according to the 10-20 system (a system used for electroencephalographic electrode positioning). This positioning was similar to that used by Boggio and colleagues (Boggio et al., 2012), and targeting the temporal lobe is recommended for memory improvement in Alzheimer's disease (Zhao et al., 2017). We aimed to enhance verbal memory function. The left temporal cortex plays a major role in verbal memory (Frisk & Milner, 1990; Johnson, Saykin, Flashman, McAllister, & Sparling, 2001), so we wanted to target this area. The reference electrode ("the cathode") was placed on the right frontal lobe (at the Fp2 position, according to the 10-20 system). The session

duration and electrode positioning were identical in both the placebo and active tDCS groups. Figure 1 and Figure 2 show the procedure of tDCS stimulation in reports I and II.

Figure 1: The tDCS procedure in report I.

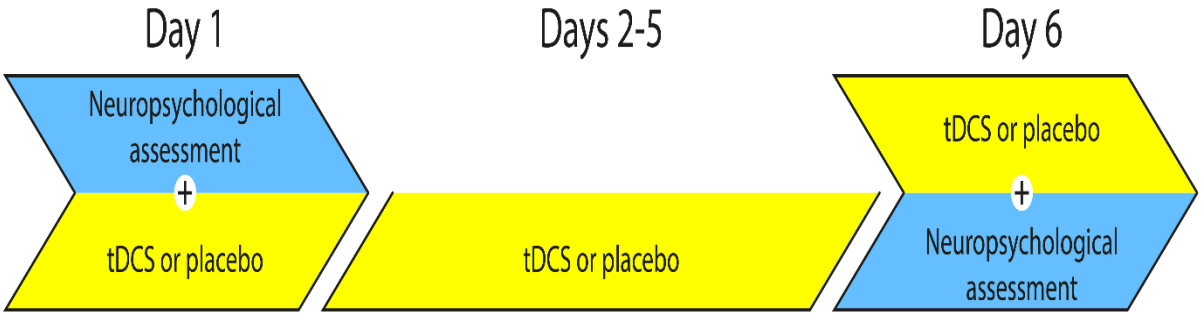
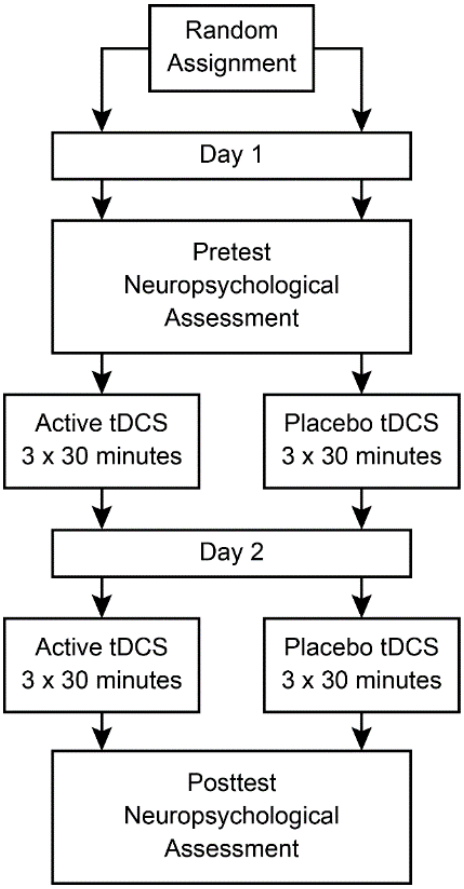


Figure 2: The tDCS procedure in report II.



tDCS and adverse effects

The small number of reported adverse effects may contribute to the increased interest in tDCS (Brunoni et al., 2011). In the literature, it is emphasized that tDCS is associated with very few and minor adverse effects. This outcome was also the conclusion from a systematic review conducted by Brunoni and colleagues (Brunoni et al., 2011). They identified the adverse effects of tDCS in 117 studies with human participants. Adverse effects were usually minor. In most cases, the adverse effects were itching, tingling, headache, a burning sensation and discomfort. However, one study reported that tDCS stimulation led to mania (Kalu, Sexton, Loo, & Ebmeier, 2012).

Despite the fact that tDCS is associated with very minor adverse effects, it is difficult to know exactly where the safety limit is in regard to current strength and duration. However, a previous safety review concluded that a duration below 40 minutes and a current strength of less than 4 mA did not produce any serious adverse effects or injuries (Bikson et al., 2016). These recommendations were based on a review of 33200 sessions and 1000 participants with repeated sessions.

A registration questionnaire is available that queries participants about adverse effects. This questionnaire was developed by Brunoni and was translated to Norwegian by Fagerlund (Fagerlund et al., 2015). When using this questionnaire, the experimenter is instructed to ask for adverse effects (adverse effects, i.e., itching, headache, nausea, and redness). In report II, we included this questionnaire. We found it especially important to be aware of possible adverse effects since we used a novel tDCS stimulation protocol (with short intervals between each session, also referred to as "accelerated tDCS"). In report I, we decided to reject the questionnaire about adverse effects. We found it difficult for patients with Alzheimer's

disease to report adverse effects based on a questionnaire since the questionnaire requires retrospective memory. Thus, we asked all patients and their caregivers (who accompanied them to the lab) to observe and report possible adverse effects.

In both report I and report II, no adverse effects were reported or observed. We cannot generalize these findings to other studies. However, we can assume that tDCS is associated with few adverse effects in healthy participants and patients with Alzheimer's disease. The tDCS protocols applied in report I and report II (six 30-minute sessions over a two-week period and three 30-minute sessions for two consecutive days, respectively) seem to be very well tolerated.

Ethical considerations

The procedures and methods in report I, report II and report III were approved by the Regional Committee for Research Ethics in Medicine and Health Sciences (2012/1890) and were conducted in accordance with the Declaration of Helsinki.

In report I, patients with Alzheimer's disease had to sign an informed consent form. To provide this consent, they had to understand the consequences of their participation. All patients and their caregivers received verbal and written information about the study. Prior to participation, we had a meeting with each patient and his/her caregiver during which we discussed different aspects of the informed consent and consequences of participation. During the meeting, we ensured that the patients understood the information. The potential benefit of participation (improved memory function) outweighed the risks (minor adverse effects). Additionally, we ensured that the patients relative (e.g., wife or son) understood the purpose of the study and that all information was stored and published anonymously.

Summary of reports I-III

Report I: Bystad, M., Grønli, O., Rasmussen, I.D. Gundersen, N., Nordvang, L., Wang-Iversen, H. & Aslaksen P.M. (2016). Transcranial direct current stimulation as a memory enhancer in patients with Alzheimer`s disease: A randomized placebo controlled trial. *Alzheimer`s Research & Therapy, 8*, 1-7.

The aim of this randomized placebo-controlled trial was to investigate tDCS as a memory enhancer in patients with Alzheimer's disease. We aimed to improve verbal memory functions since impaired verbal memory is a core symptom of Alzheimer's disease (Mayeux, 2010). Hence, the stimulation electrode (anodal) was placed above the left temporal lobe. As a background for further research, we relied on the results of Boggio and colleagues (Boggio et al., 2012), who found that tDCS stimulation of the temporal lobe improved recognition memory for a month after the last stimulation session.

We used a double-blinded, randomized placebo-controlled trial (RCT) to compare the effects of active tDCS with that of placebo tDCS. Patients with Alzheimer's disease were randomized into two groups: an active group and a placebo group. Both groups underwent the same procedure, except that the placebo group did not receive active current during the stimulation. Patients in the active group received 2 mA stimulation, lasting for 30 minutes. Six stimulation sessions were delivered over a period of two weeks.

The primary outcome measure was verbal memory, assessed with the California Verbal Learning Test II (CVLT-II). This is a neuropsychological test, normed by age and sex. To reduce test-retest practice effects, we used two parallel versions of the CVLT-II (standard and alternative versions). These versions have different lists of words to remember. Secondary outcome measures included the Mini Mental Status Examination (MMSE), the clock-drawing test and Trail Making Test A and B.

We used the nonparametric Mann-Whitney U test to investigate differences between

active and placebo tDCS. A nonparametric Mann-Whitney U test was used due to the violation of normally distributed data. This analysis failed to reveal any significant differences between active and placebo tDCS on both primary and secondary outcomes.

For the primary outcome measure (verbal memory function), we did not find any significant differences between active tDCS and placebo tDCS for either CVLT-II immediate recall, delayed recall or recognition. For secondary outcome measures (Trail Making Test A, the Mini Mental Status Examination and the clock-drawing test), no significant differences between active and placebo tDCS results were found. None of the patients had any adverse effects.

Report II: Bystad, M., Storø, B., Gundersen, N., Wiik, I.L., Nordvang, L., Grønli, O., Daae-Rasmussen, Aslaksen, P.M. Can accelerated transcranial Direct Current Stimulation improve memory functions? An experimental, placebo-controlled study. *Submitted to Heliyon*).

The aim of this experimental study was to assess the effect of tDCS on memory functions in healthy participants. We relied on previous recommendations (Nitsche et al., 2015) where short intervals between each tDCS session could improve the effects. We wanted to investigate how tDCS with short intervals could affect memory functions. Such short intervals involve giving tDCS for 30 minutes and then repeating the tDCS session within a 30-minute timeframe. This type of protocol is novel and is referred to as "accelerated tDCS".

We used a double-blind placebo-controlled design. Half of the participants received active tDCS, while the rest received placebo tDCS. This study was randomized. Neither the participant nor the research assistant knew if the tDCS device delivered placebo or active stimulation.

Each participant received a total of six tDCS sessions. These six sessions were conducted for two consecutive days. Three 30-minute sessions of tDCS were conducted each day. The interval between each session was less than 30 minutes. The current intensity was 2

mA. The stimulation electrode (anodal) was placed above the left temporal lobe.

The primary outcome measure was verbal memory. This measure was assessed with the California Verbal Learning Test II (CVLT-II) before the first session and after the sixth session. We used two parallel versions of the CVLT-II (standard and alternative) to limit the test-retest practice effects. Secondary outcome measures were digit span from the Wechsler Memory Scale (WMS) and Trail Making Test A and B. We also used the vocabulary and matrix reasoning aspects from Wechsler's Abbreviated Scale of Intelligence (WASI) initially to control for general intellectual abilities.

We conducted independent t-tests to investigate the differences in the mean score change between placebo and active tDCS groups. MANOVA was conducted to investigate group differences between placebo and active tDCS groups adjusted for age. We did not find a significant difference between placebo and active tDCS groups for verbal memory functions, neither for the young nor for the elderly participants. For all the participants (N = 40), our analysis showed no significant differences in CVLT-II score changes for the active and the placebo tDCS groups (baseline – post 2). No significant differences were found between the active and placebo tDCS groups for CVLT-II immediate recall, CVLT-II delayed recall, or CVLT-II recognition scores. For TMT B, active tDCS led to significantly better scores than placebo tDCS. For WMS digit span scores, there was no significant difference between the active and placebo groups.

For the group of elderly participants (N = 20), we found no differences in the CVLT-II scores between the active and placebo tDCS groups. We found no significant differences between active and placebo tDCS groups for CVLT-II immediate recall, CVLT-II delayed recall and CVLT-II recognition scores. There was no significant difference between the active and placebo groups for TMT A and TMT B and digit span scores. In the group of younger participants (N = 20), we found no difference in CVLT-II scores between active and placebo

tDCS groups. No significant differences were found between the active and placebo tDCS for CVLT-II immediate recall, CVLT-II delayed recall and CVLT-II recognition. There were no significant differences between the active and placebo groups for TMT-A and digit span scores. For the young participants, the active tDCS group performed significantly better than the placebo tDCS group on TMT-B.

Report III: Aslaksen, P.M., Bystad, M.K., Ørbo, M.C. & Vangberg, T.R. The relation of hippocampal subfield volumes to verbal episodic memory measured by California Verbal Learning Test II in healthy adults. *Behavioral Brain Research*, *351*, 131-137.

The aim of the study was to investigate the association between separate hippocampal subfields and verbal memory performance in healthy participants. The hippocampus can be divided into 13 segments. In our study, we aimed to investigate four subfields: CA1-CA4. These four subfields may have specialized functions, and the volume of the hippocampal subfields seems to be positively correlated with memory function. However, few studies have investigated the relationship between hippocampal subfields and cognitive functions. Verbal memory seems to be associated with the left hippocampus rather than the right hippocampus (Ezzati et al., 2016).

A total of 47 healthy adults participated in the study. Of these participants, there were 31 females, and the age range was 20-71 years. The mean education level was 13,78 years (SD = 2,02). All participants were right handed.

To assess general cognitive functions, we applied two subtests of Wechsler's Abbreviated Scale of Intelligence (WASI) (Pearson, 1999). These two subtests were matrix reasoning and vocabulary. Matrix reasoning is a nonverbal subtest that assesses visuospatial problem solving, while the vocabulary subtest requires the participant to explain words with increasing levels of difficulty. Scores on these two subtests are converted to standardized

scores using age norms. To assess verbal memory, we applied a Norwegian version of the California Verbal Learning Test II (CVLT-II).

Participants were scanned with a 1,5 T Phillips Intera MR scanner using an 8-channel head coil. Within a month after the cognitive testing, MRI scanning was performed for all participants. Hippocampal subfield segments were analyzed using FreeSurfer 6.0. (https://surfer.nmr.mgh.harvard.edu/).

The data were normally distributed. Thus, independent samples t-tests were used to investigate group differences in unadjusted volumes, whereas paired samples t-tests were applied to investigate differences between the right and left formations. Correlations between CVLT-II subtests and hippocampal volumes were evaluated with Pearson correlations. To reduce the probability of type I errors and to adjust p-values for multiple testing, p-values were adjusted with the false discovery rate (FDR) procedure. There were no significant correlations between the CVLT-II delayed recall scores and the right hippocampal subfields. However, significant correlations were found between CVLT-II immediate recall scores and volumes of the left CA 1-4 subfields. For the left CA1 subfield, the correlation for immediate recall was r = 0,30, while the correlation for delayed recall was r = 0,43. For the left CA2-3 subfields, the correlation for immediate recall was r = 0,43, while the correlation for delayed recall was r = 0,41. For the left CA4 subfield, the correlation for immediate recall was r = 0,42, while the correlation for delayed recall was r = 0,47.

Our results support the assumption that verbal memory is related to the left hippocampus volume. It also suggests that the left hippocampus volume reflects the CVLT-II score. This relation strengthens the utility of the CVLT-II as a measure of verbal memory.

Discussion

The aim of the experimental tDCS studies (reports I and II) was to investigate the efficacy of tDCS as a memory enhancer in both healthy participants and patients with Alzheimer's disease. In addition, we wanted to investigate the relationship between verbal memory performance and volume of the subfields of the hippocampus.

In both reports I and II, we expected to find that active tDCS led to significantly improved memory compared to that assessed after placebo tDCS. In report III, we expected to reveal a significant correlation between hippocampal subfield volumes and verbal memory performance.

Both report I and report II failed to reveal a significant difference between active and placebo tDCS groups for memory improvement. In both studies, we used the CVLT-II to assess verbal memory functions. As report III shows, when corrected for age and sex, there was a significant positive correlation between CVLT-II scores and the volume of the left subfields (CA1-CA4) of the hippocampus. This correlation is in line with previous studies (Pohlack et al., 2014) and confirms our hypothesis.

The results from report I and report II did not agree with our hypothesis. Thus, our results are not in line with results from some of the previous Alzheimer's disease studies (Boggio et al., 2012; Boggio et al., 2009; Ferrucci et al., 2008; Khedr et al., 2014) or healthy participants (Manenti et al., 2013; Ross et al., 2011). However, it should be noted that tDCS seems to have mixed results in both Alzheimer's disease (Kim, 2016) and healthy participants (Horvath et al., 2015; Tremblay et al., 2014). This outcome makes it difficult to draw precise conclusions about the efficacy of tDCS.

The lack of significant differences between placebo and active tDCS groups for memory enhancement in our studies (report I and report II) can likely be attributed to several

different causes. The tDCS protocols in our studies may not be as efficient as we expected. In report I, we delivered six tDCS sessions over two weeks. It is possible that having only six stimulation sessions over a period of two weeks may be insufficient for increasing excitability. In addition, Alzheimer's disease is often associated with cerebrovascular lesions in the cortex (Attems & Jellinger, 2014). Such lesions may affect the distribution of the current to the tissue and current direction, reducing neuroplasticity and regional blood flow (Datta, Baker, Bikson, & Fridriksson, 2011; Hong et al., 2017; Pavlova, Semenov, & Guekht, 2019). Furthermore, in Alzheimer's disease, a reduction in neuroplasticity may be especially pronounced in the temporal cortex (Tapia-Arancibia et al., 2008) and impair the effect of tDCS. It is reasonable to assume that these factors can inhibit the effect of tDCS stimulation.

Since CVLT-II scores seem to reflect left hippocampus size (as revealed in report III), it could be assumed that the CVLT-II scores in report I and report II would have improved if tDCS stimulation had affected the hippocampus. Thus, our nonsignificant results in report I and report II could indicate that the current failed to reach the hippocampus. Without any neurophysiological measures, such an explanation should be interpreted with caution.

A dose-response relationship is suggested between the number of tDCS sessions and efficacy (Brunoni et al., 2012). Two case studies have found that a high number of tDCS sessions led to improved memory functions in Alzheimer's disease (Bystad, Rasmussen, Abeler, & Aslaksen, 2016; Bystad, Rasmussen, Grønli, & Aslaksen, 2017). However, the optimal tDCS protocol for Alzheimer's disease has not yet been determined.

In report II, we applied a stimulation protocol with short intervals between each tDCS session ("Accelerated tDCS"). To our knowledge, our study is the first to investigate accelerated tDCS as a memory enhancer. The lack of a significant difference between active and placebo tDCS groups could be due to our novel tDCS protocol. Such short intervals between sessions may be less effective than we assumed. A previous meta-analysis

(Dedoncker, Brunoni, Baeken, & Vanderhasselt, 2016) of 188 tDCS trials investigated how the interval between sessions could influence the effects of tDCS on cognitive outcome measures. Intervals between sessions ranged from less than 1 hour to up to 2 weeks. It was found that the interval between sessions had no influence on cognitive outcome measures, neither in healthy participants nor neuropsychiatric patients. The optimal time period between sessions and number of sessions remain to be determined in future studies (Cappon, Jahanshahi, & Bisiacchi, 2016).

However, in report II, we found a significant difference between active and placebo tDCS groups for executive functions in the younger participants. A test-retest practice effect is a possible explanation since TMT B is prone to test-retest practice effects. For instance, one study found that TMT B scores improved by nearly 10 seconds after two weeks (first retest session) and by 20 seconds after three months (fourth session) (Bartels, Wegrzyn, Wiedl, Ackermann, & Ehrenreich, 2010).

Another possible explanation is that cortical areas other than the temporal cortex may have been affected (e.g., frontal cortex) in our study, since tDCS may lead to widespread alterations of functional connectivity (Keeser, Meindl, et al., 2011). It has been suggested that tDCS may enhance alerting attention (Coffman, Trumbo, & Clark, 2012). For instance, it has been demonstrated that tDCS stimulates vigilance for up to six hours (McIntire, McKinley, Goodyear, & Nelson, 2014), which could lead to better TMT B scores.

General limitations with tDCS

A limitation with tDCS is that there is large individual variability in tDCS responses. To illustrate the large variability, Tremblay and colleagues (Tremblay, Beaulé, Lepage, & Théoret, 2013) can serve as an example. They reported that one participant experienced a 251% increase in motor evoked potentials, whereas another participant had a 41% decrease. It

can be difficult to identify potential reasons for such variability. However, it is reasonable to assume that individual factors can account for at least some of this variability. Differences in anatomy (e.g., skull size/thickness) and neurophysiology are important individual differences (Woods et al., 2016). Such differences may affect the distribution of current flow to the cortex. Hence, a "one size fits all" approach is probably not useful for tDCS.

A further limitation is that electrode placement is still an area of uncertainty (Zhao et al., 2017). We aimed to stimulate the temporal cortex. We decided to stimulate this area due to promising results found in the study by Boggio and colleagues (Boggio et al., 2012). For memory enhancement, it was found that the left temporal cortex was a better target for tDCS stimulation than the frontal cortex (Zhao et al., 2017). However, for conventional tDCS, the precision (spatial resolution) is considered to be low because the target of the stimulation usually relies on a cortical area (Datta et al., 2009). Conventional tDCS is associated with diffuse electrical fields, which are affected by individual brain anatomy and head shape (Mikkonen, Laakso, Tanaka, & Hirata, 2020).

Another limitation with tDCS is that interference may affect the tDCS results. It has been suggested that cognitive or motor activity during tDCS stimulation can enhance or inhibit the effect of tDCS (Horvath, Carter, & Forte, 2014). For instance, in their meta-analysis, Hsu and colleagues (Hsu et al., 2015) suggested that tDCS treatment in elderly individuals and patients with Alzheimer's disease may be more effective if a cognitive task is given during tDCS stimulation. However, one study revealed that imaginary tDCS stimulation reduced the effect of anodal tDCS stimulation, whereas it increased the effect of cathodal stimulation. The imaginary task was to visualize a motor movement (Antal, Terney, Poreisz, & Paulus, 2007). Further, another study applied the motor evoked potential (MEP) measure. This application indicates that motor evoked potentials are recorded from muscles due to tDCS stimulation of the motor cortex. They found that cognitive tasks (asking the participant

about language, mathematics and history) during tDCS stimulation could interfere with the effects (Miyaguchi et al., 2013). They found that such cognitive input reduced the effect of both anodal and cathodal tDCS stimulation. Thus, interference may be a limitation with tDCS that is difficult to control in experimental studies.

Walsh previously summarized the major limitations with tDCS; he emphasized that a lack of standardization regarding electrode placement, uncertainty about ecological validity (how results from tDCS studies may manifest outside the research lab) and mixed results are some of the major shortcomings of tDCS (Walsh, 2013).

Limitations with our studies

All our studies (reports I, II and III) have some central methodological limitations that need to be addressed. For report I, a central limitation is our small sample size. There is clearly a need to conduct larger clinical trials to assess the effect of tDCS in Alzheimer's disease. A small sample size is also a limitation of report II and report III. A large number of tDCS studies rely on small sample sizes. For instance, in a meta-analysis by Hsu and colleagues, only two out of 12 studies had a sample size slightly above 30 participants (Hsu et al., 2015). A small sample size may increase the risk for a "type II error". This error can occur when a false null hypothesis is retained, i.e., a "false negative". There may also be a risk for a "type I error", where the false null hypothesis is rejected, i.e., a "false positive".

Recruitment difficulties were the main reason for our small sample sizes in report I and report II. Recruiting healthy participants was difficult due to skepticism about the current stimulation, i.e., the word "current" can have negative associations. Some participants even confused tDCS with electroconvulsive therapy (ECT). We lack data about this confusion. However, such considerations should be noted. Failure to recruit the planned number of patients within the expected timeframe is a common problem in Alzheimer's trials (Grill & Karlawish, 2010). Low motivation, comorbidity, high age and the lack of a caregiver who can

accompany patients to the research lab are typical barriers for studies involving patients with Alzheimer's disease (Grill & Karlawish, 2010).

Another limitation is that it can be very difficult to compare our results with results from previous tDCS studies since we relied on more advanced memory assessments. While the use of a more sophisticated memory assessment is a strength, it also makes comparisons with previous studies more difficult. For report I and report II, we do not know if our results would have been different if we had relied on the same type of tests that previous studies relied on (for instance, the "visual recognition task" or that used by Boggio and colleagues (Boggio et al., 2012)).

A further methodological limitation with reports I and II is that we relied solely on neuropsychological/cognitive outcomes. We did not includeneurobiological /neurophysiological outcome measures (e.g., neuroimaging). Consequently, it is uncertain whether our stimulation protocol in reports I and II caused any neurophysiological changes.

Another limitation is that we lack information about the educational level of the participants in report I and report II, so we could not control for this variable. Educational level seems to influence episodic memory in both healthy individuals (Angel, Fay, Bouazzaoui, Baudouin, & Isingrini, 2010; Ronnlund, Nyberg, Backman, & Nilsson, 2005) and among patients with Alzheimer's disease (Scarmeas, Albert, Manly, & Stern, 2006).

In report III, we relied on a relatively small sample size, and the participant's age span was large. These shortcomings limit the generalizability of the results. In addition, we used an MRI scanner with a 1,5 Tesla magnetic-field strength. Compared to the 1,5 Tesla magnetic field strength, the 3 Tesla magnetic field strength has a higher resolution and is more sensitive for the detection of subfields (Winterburn et al., 2013).

Limitations with memory assessment

In all three reports, we relied on memory assessment with the CVLT-II. There are some

limitations with this memory assessment that should be taken into account. We used the Norwegian version of CVLT-II. However, this Norwegian version is based on American norms. While there are similarities with Norwegian and American culture, we do not know how representative these norms are in terms of education and health.

Another limitation with the CVLT-II and memory assessment in general is ecological validity (Dubreuil, Adam, Bier, & Gagnon, 2007). It is difficult to know exactly how scores from the CVTL-II in an experimental setting may manifest in real life situations. In other words, it is unknown to what degree CVLT-II scores can be related to everyday memory, for instance, remembering errands, appointments, new names or details from conversations. The ecological validity may be further investigated (Dubreuil et al., 2007). Furthermore, there may be a gap between self-reported memory and delayed recall performance (Sohel, Tuokko, Griffith, & Raina, 2016). One explanation for this gap could be that memory assessment partly fails to detect everyday memory and implicit memory (Dubreuil et al., 2007).

A third limitation is that memory assessment can be demanding. Learning a long list of words can be overwhelming for patients with memory impairments (e.g., those with Alzheimer's disease). Standard forms of the CVLT-II consist of 16 words, which may be excessive. For patients with memory impairments, a short form of CVLT-II exists with only nine words (Delis et al., 2004). However, this short form of CVLT-II does not have a parallel version and may be prone to test-retest practice effects.

Strengths with our studies

Our studies have several methodological strengths that should be emphasized. A strength for report I and report II is that we applied a double-blind placebo-controlled design. Therefore, both the experimenter and the participant were unaware of which condition the patient/participant was allocated to (placebo or active tDCS).

Another methodological strength is that we used standardized neuropsychological

testing (CVLT-II) to assess verbal memory functions. As previously noted in this thesis, CVLT-II is normed for both sex and age, and it is a widely used neuropsychological tool (Delis et al., 2005; Delis et al., 2004). The advantage of relying on a standardized memory test is that the results are more likely to have high internal validity and test-retest reliability compared to a nonstandardized test.

Thus, we relied on a more sophisticated memory assessment than that used in previous studies. Two previous reviews suggested that further tDCS studies in Alzheimer's disease should rely on more sophisticated cognitive outcome measures (Freitas et al., 2011; Nardone et al., 2011). The application of CVLT-II was in line with that recommendation. Neuropsychological testing is the most reliable method for assessing cognitive functions in both Alzheimer's disease and healthy individuals (Lezak et al., 2012).

For report III, a strength is that we combined verbal memory assessment (CVLT-II) with brain imaging (MRI). MRI is an advanced method that provides the opportunity to explore neural aspects of verbal memory functions. In report III, we both have cognitive (memory assessment) and neurobiological correlates.

Implications

Our studies may have implications for further research and clinical applications. For report I, we demonstrated the need for further larger-powered studies, and we also found that our protocol (with six tDCS sessions for two weeks) may not be the optimal protocol. In addition, our results also indicate that tDCS as a therapeutic in Alzheimer's disease still needs more evidence to be considered as an evidence-based intervention.

For report II, we applied a novel tDCS protocol, with short intervals between sessions ("accelerated tDCS"). We failed to demonstrate that this protocol was efficient for enhancing memory. Our results contradict previous recommendations (Nitsche et al., 2015). However, no adverse effects were observed/reported, which may indicate that tDCS is generally a well-

tolerated method.

In report III, we demonstrated the association between episodic verbal memory functions assessed with CVLT-II and the left hippocampus subfield. This finding may support CVLT-II as a valuable and reliable tool for assessing verbal memory functions.

Further research

There is clearly a need to conduct larger-scale studies to investigate tDCS as a memory enhancer. Overcoming recruitment barriers will make it possible to conduct larger studies. Further studies will likely benefit from an increasing number of trial sites. A multicenter approach may be ideal for obtaining a larger number of healthy participants or patients with Alzheimer's disease (Grill & Karlawish, 2010).

Further studies should also apply psychophysiological methods, such as MRI or (electroencephalography) EEG, to investigate how tDCS may affect cortical activity and neuroplasticity. MRI and/or EEG can be used in combination with memory assessments as outcome measures. As Medeiros and colleagues suggest (Medeiros et al., 2012), investigating neurobiological effects may be important to optimize tDCS protocols.

In addition, further studies should also compare the effects of tDCS with active control groups. Currently, most tDCS studies compare the effect of tDCS with placebo tDCS. It could be important to determine how tDCS results compare to those of common interventions. For instance, comparing active tDCS with anticholinergic drugs in Alzheimer's disease may be one approach. Another possible approach is to compare active tDCS with memory strategies (e.g., mnemonics) in healthy participants. For the utility of tDCS, it can be important to determine whether tDCS is more or less effective than "treatment as usual" or memory strategies. A prime example of a study where tDCS was compared to "treatment as usual" is a study by Brunoni and colleagues (Brunoni et al., 2017). In a randomized controlled trial, they compared the effectiveness of tDCS to that of escitalopram (a common antidepressive

medication) in patients with depression.

As previously noted, individual differences may affect the current distribution of current flow to the cortex (Woods et al., 2016). Further studies should use individual calibration to overcome the limitations of individual differences. This approach is possible through the use of a computer simulation and functional magnetic resonance imaging (Datta et al., 2011). Such an approach makes visualization of the spatial distribution possible. Furthermore, magnetic resonance imaging can also be helpful for finding the optimal electrode placement (Jog et al., 2016). In addition, it makes it possible to determine whether a specific tDCS dose and/or duration could alter activation in the targeted area (Bikson, Rahman, & Datta, 2012). Such information could permit individual calibration of the tDCS protocol.

Furthermore, high-definition tDCS (HD-tDCS) should be explored in future studies. This approach employs a more focal stimulation by using multiple electrodes (Hampstead, Sathian, Bikson, & Stringer, 2017). Typically, the anode is placed in the center, while the cathodes are placed around the anode, forming a "ring". The most common montage is the 4 x 1 ring montage with a center active electrode surrounded by four return electrodes that is used to focus transcranial current within a cortical area of interest circumscribed by the ring (Edwards et al., 2013). It was found that such stimulation could be more focal and lead to better excitatory effects than the tDCS placement used in this thesis (Kuo et al., 2013). HD-tDCS is in its infancy but should be investigated in future studies.

We should not overlook the fact that Alzheimer's disease is very complex, and the neuropathological mechanisms behind the disease are not fully understood (Querfurth & LaFerla, 2010). Less than one percent of all clinical trials have revealed significant results (Cummings et al., 2014). Accordingly, our results may reflect the fact that Alzheimer's disease is highly complex and that treatment effects are difficult to obtain (Honig et al., 2018).

Overall conclusions

The main findings in the present thesis merge into three conclusions. These three conclusions can be summarized as follows:

1) Six sessions of active tDCS for 30 minutes delivered over the temporal cortex for two weeks did not lead to significantly improved verbal memory functions in Alzheimer's disease patients compared to the results obtained by placebo tDCS. However, a small sample size makes it difficult to draw precise conclusions and limits the generalizability of the results. No adverse effects were found in this study.

2) Six sessions of active tDCS for 30 minutes over two consecutive days ("accelerated tDCS") delivered over the temporal cortex did not lead to significantly better verbal memory functions than placebo accelerated tDCS. No adverse effects were found in this study, and tDCS seems to be very well tolerated.

3) Using MRI, it was found that verbal memory functions, assessed with the CVLT-II, were significantly associated with the volume of the left hippocampus subfields in healthy adults. Thus, CVLT-II scores may reflect the volume of the left hippocampus.

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Paper I

Bystad, M., Grønli, O., Rasmussen, I.D., Gundersen, N., Nordvang, L., Wang-Iversen, H. & Aslaksen, P.M. (2016). Transcranial direct current stimulation as a memory enhancer in patients with Alzheimer`s disease: A randomized placebo controlled trial. *Alzheimer`s Research & Therapy, 8*, 13.

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Alzheimer's Research & Therapy

RESEARCH Open Access

Transcranial direct current stimulation as a memory enhancer in patients with Alzheimer's disease: a randomized, placebo-controlled trial

Martin Bystad[1,3*], Ole Grønli[3], Ingrid Daae Rasmussen[1,3], Nina Gundersen[1], Lene Nordvang[1], Henrik Wang-Iversen[1] and Per M. Aslaksen[1,2]

Abstract

Background: The purpose of this study was to assess the efficacy of transcranial direct current stimulation (tDCS) on verbal memory function in patients with Alzheimer's disease.

Methods: We conducted a randomized, placebo-controlled clinical trial in which tDCS was applied in six 30-minute sessions for 10 days. tDCS was delivered to the left temporal cortex with 2-mA intensity. A total of 25 patients with Alzheimer's disease were enrolled in the study. All of the patients were diagnosed according to National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association criteria. Twelve patients received active stimulation, and thirteen patients received placebo stimulation. The primary outcome measure was the change in two parallel versions of the California Verbal Learning Test–Second Edition, a standardized neuropsychological memory test normalized by age and gender. The secondary outcome measures were the Mini Mental State Examination, clock-drawing test, and Trail Making Test A and B.

Results: Changes in the California Verbal Learning Test–Second Edition scores were not significantly different between the active and placebo stimulation groups for immediate recall ($p = 0.270$), delayed recall ($p = 0.052$), or recognition ($p = 0.089$). There were nonsignificant differences in score changes on the Mini Mental State Examination ($p = 0.799$), clock-drawing test ($p = 0.378$), and Trail Making Test A ($p = 0.288$) and B ($p = 0.093$). Adverse effects were not observed.

Conclusions: Compared with placebo stimulation, active tDCS stimulation in this clinical trial did not significantly improve verbal memory function in Alzheimer's disease. This study differs from previous studies in terms of the stimulation protocol, trial design, and application of standardized neuropsychological memory assessment.

Trial registration: ClinicalTrials.gov identifier NCT02518412. Registered on 10 August 2015.

Keywords: Alzheimer's disease, Randomized controlled trial, Transcranial direct current stimulation, Memory, Neuropsychology, Neuromodulation

* Correspondence: martin.k.bystad@uit.no
[1]Department of Psychology, Research Group for Cognitive Neuroscience, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway
[3]Department of Geropsychiatry, University Hospital of North Norway, Tromsø, Norway
Full list of author information is available at the end of the article

Background

Neuroimaging studies have suggested that Alzheimer's disease is associated with pathological and structural changes in the brain, especially in the temporal cortex [1]. Several studies have demonstrated that stimulation of the temporal cortex with transcranial direct current stimulation (tDCS) can enhance name recall in healthy elderly persons [2] and improve recognition memory in patients with Alzheimer's disease [3–5]. tDCS is non-invasive and works by inducing a low direct current in the cortical area of interest [6]. Small electrodes are placed on the scalp above the brain area that is targeted by tDCS. This stimulation facilitates cortical excitability and thereby neuroplasticity [6].

The results of previous studies are promising [3–5]. However, there is still insufficient evidence that supports tDCS as an intervention for Alzheimer's disease. Randomized, placebo-controlled trials are warranted to assess the efficacy of temporal cortex tDCS in patients with Alzheimer's disease. Trials should include more comprehensive outcome measures to explore the effect of tDCS on memory function. The aim of the present study was to investigate the effect of tDCS on verbal memory functions in patients diagnosed with Alzheimer's disease.

Methods

Study design and participants

A randomized, placebo-controlled trial with a parallel group design was performed. Two groups were included in the intervention: an active tDCS group and a placebo tDCS group. The allocation ratio was 1:1.

Patients diagnosed with Alzheimer's disease were invited to participate in the study via a letter from the Department of Geriatric Medicine at the University Hospital of North Norway, and healthy participants were recruited through a newspaper advertisement. The eligibility criteria were living at home and fulfillment of the research criteria for the likelihood of having Alzheimer's disease according to the revised criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association criteria [7]. We followed section 4.2 in these criteria: "Probable Alzheimer's disease with increased level of certainty." This determination of eligibility for the study requires evidence of a progressive cognitive decline based on information from informants (relatives) and a cognitive and/or neuropsychological evaluation [7].

We excluded patients who scored <18 on the Mini Mental State Examination (MMSE) [8]. Other exclusion criteria included serious somatic disorders (cancer, chronic obstructive pulmonary disease, and heart failure) or neuropsychiatric disorders (e.g., severe depression and psychosis) that might reduce cognitive abilities. The patients with comorbid cerebral conditions, such as cerebrovascular injuries and/or stroke, brain tumor, or Parkinson's disease, were not eligible to participate in the study. Patients using cholinesterase inhibitors had to have been using them for at least 3 months before enrolling in the study. A total of 25 patients with Alzheimer's disease were included in the study.

A total of 22 healthy elderly volunteers, aged 59–83 years, served as controls for the neuropsychological test performance at baseline. None of them had cognitive impairment or other serious diseases. These healthy volunteers were recruited through an advertisement. The control group did not receive any tDCS stimulation. They completed the Hospital Anxiety and Depression Scale [9], a questionnaire used to screen for depression and anxiety.

The neuropsychological test battery used for healthy volunteers and patients with Alzheimer's disease was identical. The study was executed in a research laboratory at the University of Tromsø Institute of Psychology. The study was ethically approved by the regional committee for medical and health research ethics (2012/1890) and was registered in the ClinicalTrials.gov database with the identifier NCT02518412. All of the patients and healthy control subjects signed a written informed consent form in line with the Declaration of Helsinki before participating in the study. Each patient received a gift card worth 600 NOK (67 EUR, 75 USD) for their participation. Figure 1 contains a flow diagram of the trial.

Outcome measures

The primary outcome measure was verbal memory function. We used a validated and standardized Norwegian version of the California Verbal Learning Test–Second Edition (CVLT-II) to assess three aspects of verbal memory function: immediate recall, delayed recall, and recognition [10]. CVLT-II is normed by age and gender and is widely used to assess patients with Alzheimer's disease [10]. To reduce test-retest effects, the CVLT-II consists of two parallel versions: the CVLT-II standard and alternate forms, which contain two different and independent word lists. We used the standard form at baseline and the alternative form in the posttest.

The secondary outcome measures included the MMSE, clock-drawing test, and Trail Making Test parts A and B (TMT A and B). The MMSE is a screening tool used for assessing cognitive impairment (e.g., orientation, recall, arithmetic, language, and ability to follow simple instructions) [8]. The clock-drawing test is another screening tool used for detecting cognitive impairment and is also used to assess visuoconstructive ability [11]. The TMT consists of part A and part B. TMT A measures sustained attention, whereas TMT B assesses executive function [12].

To control for general cognitive abilities, we used the Wechsler Abbreviated Scale of Intelligence with the matrix reasoning and vocabulary subtests [13]. To screen for depressive symptoms, we used the Cornell Scale for

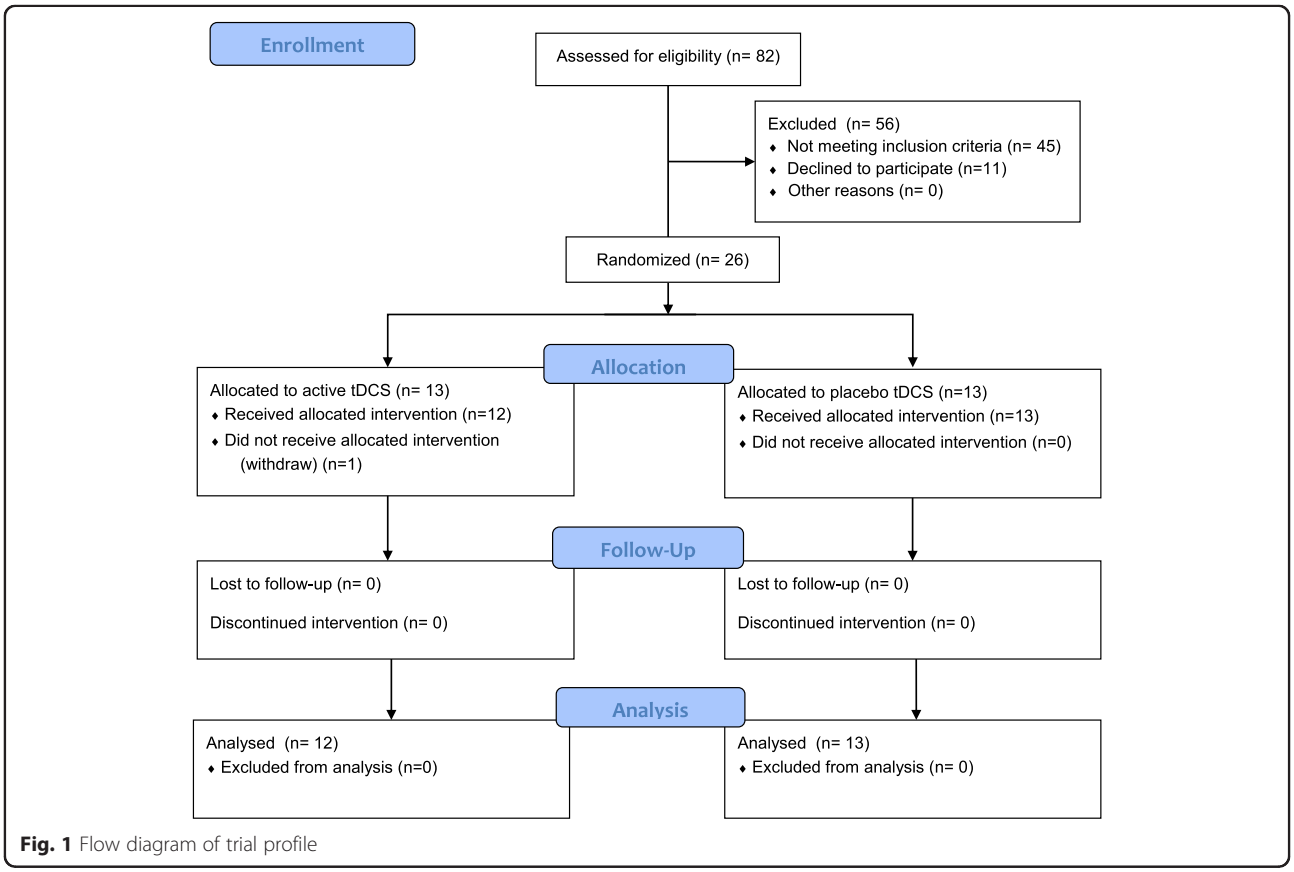

Fig. 1 Flow diagram of trial profile

Depression in Dementia [14], which is a questionnaire completed by an informant (i.e., a relative). A score above 13 indicates depression, which was an exclusion criterion in the present study. We documented progressive decline using the Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE) [15], which was also completed by an informant. To assess for potential confusion during neuropsychological testing, the Confusion Assessment Method [16] was applied by a research assistant. This questionnaire is based on the observation of core symptoms of confusion (e.g., inattention, disorganized thinking, and altered level of consciousness).

Intervention

The intervention was treatment with tDCS using a direct current stimulator (neuroConn, Ilmenau, Germany), which is battery-driven and delivers a direct current. The current intensity was 2 mA, and the stimulation duration was 30 minutes. A pair of 35-cm^2 rubber electrodes transferred the direct current. These electrodes were inserted into sponge pads soaked with 10 ml of sterile water. To stimulate the left temporal lobe, the anode (positive electrode) was placed at the T3 position in the 10–20 system for electroencephalographic electrode positioning. The cathode (negative electrode) was placed on the right frontal lobe at the Fp2 position. For the placebo tDCS, the electrode placement and session duration were identical to those for active tDCS. However, in the placebo tDCS, the current was delivered for 30 seconds at the beginning of the stimulation, then the current was turned off automatically.

Randomization and blinding

The patients were assigned to a list with five-digit codes provided by the manufacturer of the tDCS stimulator. Each patient had his or her own code. The codes instructed the stimulator to deliver either placebo or active stimulation. The order of the codes was randomized using the Random.org website (https://www.random.org/). To ensure double-blinding, the list of code assignments was not disclosed during the entire tDCS intervention. The list was decoded when the study was completed to identify the patients in the active and placebo groups. The tDCS stimulator did not display information that could be used to identify the placebo or active stimulation.

Procedure

After their inclusion in the study, the patients and their relatives visited the research laboratory and received

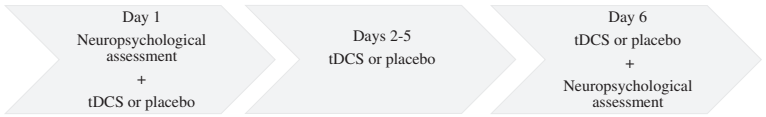

Fig. 2 Overview of the procedure

information regarding the project. During this meeting, the patient completed an informed consent form. Subsequently, the patient underwent neuropsychological testing (baseline). The neuropsychological assessment lasted for approximately 60 minutes, including several short breaks. After the neuropsychological assessment was completed, the first tDCS stimulation commenced. Each patient underwent six sessions of tDCS or placebo tDCS stimulation for 10 days. Each tDCS stimulation session lasted 30 minutes. An experienced research assistant administered the tDCS stimulation. When the last tDCS stimulation was completed, the patient performed the neuropsychological post-testing and received a gift certificate. Figure 2 gives an overview of the procedure.

Power and statistical analyses

In previous studies in which tDCS was used to stimulate memory functions in patients with Alzheimer's disease, researchers reported significant results ($p < 0.05$) with a total of ≤15 patients [3–5] in a within-group design. Thus, we aimed to include a larger sample than those described in previous studies [3–5] to ensure accurate analysis of the effects of the intervention.

We used IBM SPSS version 22 software (IBM, Armonk, NY, USA) to perform the statistical analysis. Because of a violation of the assumption of a normal distribution, a nonparametric Mann-Whitney *U* test was conducted to compare the placebo tDCS and active tDCS groups at baseline. A nonparametric Kruskal-Wallis test was used to assess the baseline characteristics for all three groups (placebo tDCS, active tDCS, and healthy control subjects at baseline).

For the primary analyses, the data had a normal distribution. However, because of a small sample size and a large variance, we decided to use a nonparametric Mann-Whitney *U* test for the analysis. With the Mann-Whitney

Table 1 Baseline characteristics

	Active tDCS ($n = 12$)	Placebo tDCS ($n = 13$)	*p* Value	Controls ($n = 22$)	*p* Value
Age, years	70.0 (8.0)*70.5 (21.0)*	75.0 (8.7)*75.0 (30.0)*	0.12	68.8 (6.8)*69.0 (24.0)*	0.062
Males	7 (58 %)	7 (53 %)	0.85	4 (18 %)	
DM	12 (100 %)	12 (92 %)	0.76		
CVLT-II IR	25 (7.9)*22.0 (25.0)*	23 (6.8)*23.0 (22.0)*	1.00	52.7 (10.0)*54.0 (33.0)*	0.01[a]
CVLT-II DR	−2.7 (0.5)*−2.5 (2.0)*	−2.3 (0.8)*−2.5 (2.5)*	0.4	−0.4 (0.9)*−0.5 (3.5)*	0.01[a]
CVLT-II RG	0.6 (0.9)*0.7 (3.0)*	1.0 (0.5)*1.1 (1.8)*	0.24	1.5 (1.0)*2.4 (3.3)*	0.01[a]
TMT A	91.0 (45.0)*81.0 (138.0)*	143.0 (65.0)*131.0 (191.0)*	0.059	48.5 (18.6)*46.5 (87.0)*	0.01[a]
TMT B	*266.0 (123.0)215.0 (266.0)*	347.0 (225.0)*259.0 (693.0)*	0.67	93.0 (34.8)*90.5 (149.0)*	0 · 01[a]
Clock	3.33 (1.4)*3.5 (5.0)*	1.5 (1.6)*1.0 (4.0)*	0.024[a]	4.86 (0.86)*5.0 (2.0)*	0.01[a]
MMSE	20.0 (2.8)*21.0 (8.0)*	21.2 (3.9)*23.0 (13.0)*	0.71	29.5 (1.09)*30.0 (5.0)*	0.01[a]
WASI Ma	43.0 (9.2)*44.5 (27.0)*	42.5 (6.9)*42.0 (26.0)*	0.81	58.05 (9.0)*61.5 (34.0)*	0.01[a]
WASI Vo	41.7 (9.3)*39.0 (31.0)*	41.6 (14.3)*44.0 (48.0)*	0.76	57.0 (9.9)*57.0 (40.0)*	0.01[a]
Cornell Scale for Depression in Dementia	5.7 (4.3)*6.0 (12.0)*	4.8 (3.4)*5.0 (12.0)*	0.65		
CAM	0.0	0.0	1.0		
IQCODE	3.9 (0.3)*4.1 (1.2)*	4.1 (0.3)*4.2 (1.1)*	1 · 0		

DM dementia medications, *CVLT-II IR* California Verbal Learning Test–Second Edition Immediate Recall, *CVLT-II DR* California Verbal Learning Test–Second Edition Delayed Recall, *CVLT-II RG* California Verbal Learning Test–Second Edition Recognition, *WASI* Wechsler Abbreviated Scale of Intelligence, *IQCODE* Informant Questionnaire of Cognitive Decline in the Elderly, *CAM* Confusion Assessment Method, *MMSE* Mini Mental State Examination, *TMT* Trail Making Test, *tDCS* transcranial direct current stimulation

Data are the mean (SD) or n (%). Median and range are displayed in italic type. The first *p* value column shows the differences between the placebo and active groups at baseline. The second *p* value column displays the differences between the active, placebo, and control groups at baseline. For CVLT-II, delayed recall is displayed as age- and gender-adjusted *z*-scores (normalized mean 0, SD 1). For immediate recall the score is displayed as a T-score (normalized mean 50, SD 10), and for recognition the score is an adjusted d' score (relationship between total hits and false-positive results). For TMT A and B, results are displayed in seconds. Maximum score on the MMSE is 30. Scores <24 indicate cognitive impairment [8]. Scores on the WASI are displayed as T-scores (normalized mean 50, SD 10). The cutoff score on the IQCODE for Alzheimer's disease is >3.5 [15]. For the Cornell Scale for Depression in Dementia, a cutoff >12 indicates depression [14]. CAM ranges from 0 to 4, where 0 indicates no symptoms of confusion. The clock-drawing test scores range from 0 to 5, where 5 indicates no errors.

[a]$p < 0.05$ denotes statistically significant values

Table 2 Outcome measures

	Active tDCS ($n = 12$)	Placebo tDCS ($n = 13$)	Difference	p Value
Primary outcomes				
CVLT-II immediate recall	5.0 (25.0)	0.0 (31.0)	5.0	0.270
CVLT-II delayed recall	0.0 (1.5)	0.0 (2.5)	0.0	0.052
CVLT-II recognition	0.3 (4.0)	−0.08 (1.6)	0.47	0.089
Secondary outcomes				
MMSE	1.0 (9.0)	1.0 (10.0)	0.0	0.799
Clock-drawing test	0.0 (4.0)	0.0 (5.0)	0.0	0.378
TMT A	3.5 (262.0)	−7.0 (219.0)	10.5	0.288
TMT B	22.0 (204.0)	−96.0 (443.0)	118.0	0.093

CVLT-II California Verbal Learning Test–Second Edition, *MMSE* Mini Mental State Examination, *TMT* Trail Making Test, *tDCS* transcranial direct current stimulation
Data are the median (range) values. The median values are the estimated change from baseline to posttesting. The positive values indicate positive changes. For the CVLT-II immediate recall, the median value is displayed as a T-score. For the CVLT-II delayed recall, the median value is displayed as a scaled z-score. For CVLT recognition, the median value is an adjusted d' score. The differences between the placebo and active tDCS were calculated using a nonparametric Mann-Whitney U test

U test, we examined the change from baseline to posttest. The raw scores for the neuropsychological tests (CVLT-II and WASI) were scaled according to standardized norm tables [13, 17]. The significance level was set at $p < 0.05$.

Results

A total of 82 patients diagnosed with Alzheimer's disease were assessed for eligibility. Of these patients, 45 were excluded because of comorbid and serious somatic diseases, MMSE score <17, and psychiatric diseases. A total of 11 patients declined to participate in the study. One patient decided to withdraw from the study. Twenty-five patients were enrolled in the study and completed the intervention between June 2013 and June 2015. Table 1 shows the patients' baseline characteristics.

In our analysis, we found significant differences between healthy control subjects and patients with Alzheimer's disease at baseline. Except for the clock-drawing test, there were no significant differences in the baseline characteristics between the placebo and active groups (Table 1).

For the primary outcome measures, scores between the active and the placebo group did not differ significantly on the CVLT-II immediate recall (95 % confidence interval [CI] −9.00 to 2.00; U = 99.00, z-score = 1.14, p = 0.270, $r = 0.22$), CVLT-II delayed recall (95 % CI −1.0 to 0.0; U = 113.50, z-score = 2.132, $p = 0.052$, $r = 0.42$), or CVLT-II recognition (95 % CI −1.25 to 0.18; U = 96.00, z-score = 1.38, $p = 0.089$, $r = 0.27$). The scores on the secondary outcome measures (MMSE, clock-drawing test, and TMT A and B) did not differ significantly between the active and placebo tDCS groups (Table 2). Table 3 display the number of patients showing improvement on primary outcome measures.

Table 3 Frequency table

	Active tDCS ($n = 12$)	Placebo tDCS ($n = 13$)
CVLT-II immediate recall	9	6
CVLT-II delayed recall	4	1
CVLT-II recognition	7	4

CVLT-II California Verbal Learning Test–Second Edition, *tDCS* transcranial direct current stimulation
The data represent the number of patients showing improvement on primary outcome measures. Improvement was displayed as positive changes from baseline to posttest

Safety and tolerability

Both patients and their relatives were told to report likely adverse effects (e.g., headache, itching, skin irritation). However, no adverse effects were reported, which indicates that the tDCS intervention was both safe and well-tolerated.

Discussion

The aim of the present randomized, placebo-controlled study was to assess the effect of tDCS stimulation on verbal memory function in patients with Alzheimer's disease. We were unable to reveal significant differences between the placebo and active tDCS groups in both primary and secondary efficacy outcomes. We found a tendency for improved delayed recall in the active tDCS group, albeit not significant.

Boggio and colleagues stimulated [4] the temporal cortex in patients with Alzheimer's disease using a 30-minute tDCS stimulation for 5 consecutive days. This stimulation increased visual recognition memory scores by 8.9 %, and the improvement persisted for 1 month after the last simulation session.

Our results are not in agreement with the results of previous studies [3–5], which can be attributed to several likely explanations. First, we used a fixed stimulation protocol for all patients. Several recent studies suggested that anatomical differences (e.g., skull thickness) can

affect current distributions to the cortex [18]. Future tDCS studies will likely take advantage of computational models to ensure individual calibration of the stimulation procedure.

Second, the patients in our study may have been less receptive to tDCS because of the severity of their disease. tDCS stimulation seems to be less effective in the advance stages of Alzheimer's disease [19, 20]. According to our baseline measures of memory function, a majority of our patients had severe memory impairment (see CVLT-II characteristics in Table 1). Alzheimer's disease is associated with reduced neuroplasticity (i.e., a considerable reduction in long-term potentiation) [21]. This condition is especially pronounced in the temporal cortex [22] and may inhibit the effect of temporal cortex stimulation when memory impairment is severe.

Third, our study differs from previous studies [3–5] by its limited sample size and in terms of the stimulation procedure, study design, and outcome measures. According to Elder and Taylor [23], different stimulation paradigms should be investigated in Alzheimer's disease. The optimal stimulation procedure for Alzheimer's is still uncertain. Thus, the present study is in line with these recommendations and applied a new stimulation paradigm. Clinical application of tDCS is still in its infancy [24]. It is important to find the most effective tDCS paradigm for patients with Alzheimer's disease.

A major difference between the present study and previous studies [3–5] is our application of standardized memory assessment. This accords with recommendations derived from previous reviews [19, 20]. Neuropsychological testing is considered to be the most reliable method for assessing cognitive function in Alzheimer's disease [25]. Furthermore, in the present study, we applied a randomized, placebo-controlled design. To the best of our knowledge, this is the first randomized, placebo-controlled study of tDCS stimulation of the temporal cortex in Alzheimer's disease. Additionally, none of our patients experienced any adverse effects due to the intervention, which indicates that tDCS is safe and well-tolerated.

We recommend future studies with outcome measures that include neuropsychiatric symptoms, neuropsychological assessment, and activities of daily living. The Neuropsychiatric Inventory [26] and the Amsterdam Instrumental Activities of Daily Living Questionnaire [27] are recommended in that regard.

Large-scale randomized controlled studies are warranted. Recruitment is a main barrier. Recruitment presents a challenge for clinical studies of tDCS [18] and trials in Alzheimer's disease [28]. One way to facilitate the recruitment process is to increase the number of trial sites [28]. In addition, increasing the repetition rate (e.g., stimulation twice per day) could be more feasible and might require fewer separate days of visits to the research laboratory. Such stimulation may even prolong the aftereffects of stimulation [29, 30]. Fewer visits can be beneficial for recruitment [28].

Conclusions

This randomized, placebo-controlled study failed to reveal any significant results. There was a nonsignificant improvement in delayed recall for the active tDCS condition. This trial showed high tolerability of tDCS. In future research, investigators should use both neuropsychological and neurophysiological outcome measures, study patients in early stages of Alzheimer's disease, and overcome recruitment barriers to increase power.

Abbreviations

CAM: Confusion Assessment Method; CI: confidence interval; CVLT-II: California Verbal Learning Test–Second Edition; DM: dementia medications; IQCODE: Informant Questionnaire on Cognitive Decline in the Elderly; MMSE: Mini Mental State Examination; tDCS: transcranial direct current stimulation; TMT: Trail Making Test; WASI: Wechsler Abbreviated Scale of Intelligence.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB planned the study, recruited patients, collected the data, wrote the first draft of the manuscript, revised and reviewed the final draft, and analyzed the data. OG planned the study, recruited patients, and drafted and revised the manuscript. IDR collected the data, recruited patients, and drafted and revised the manuscript. NG, LN, and HWI collected the data and were involved in writing the first draft of the manuscript and revising the final manuscript. PMA planned the study, wrote the first draft of the manuscript and revised it, analyzed the data, revised the final manuscript, and served as a supervisor for MB. All authors read and approved the final manuscript.

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Author details

[1]Department of Psychology, Research Group for Cognitive Neuroscience, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway. [2]Department of Child and Adolescent Psychiatry, University Hospital of North Norway, Tromsø, Norway. [3]Department of Geropsychiatry, University Hospital of North Norway, Tromsø, Norway.

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Paper II

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Can accelerated transcranial Direct Current Stimulation improve memory functions? An experimental, placebo-controlled study.

Martin Bystad14; Benedicte Storø2, Nina Gundersen2, Ida Larsen Wiik2, Lene Nordvang2, Ole Grønli, M.D, PhD4; Ingrid Daae Rasmussen14, Per M. Aslaksen, PhD13.

Author affiliations:

1Department of Psychology, Research Group for Cognitive Neuroscience, Faculty of Health Sciences, University of Tromsø, Norway.

2Department of Psychology, Faculty of Health Sciences, University of Tromsø, Norway

3Department of Child and Adolescent Psychiatry, University Hospital of North Norway, Norway.

4Department of Geropsychiatry, University Hospital of North Norway, Norway

Martin Bystad. martin.k.bystad@uit.no

Benedicte Storø. benedicte.storoe@gmail.com

Nina Gundersen. nina872@gmail.com

Lene Nordvang. lno034@post.uit.no

Ida Larsen Wiik. iwi012@post.uit.no

Ole Grønli. ole.k.gronli@unn.no

Ingrid Daae Rasmussen. ingrid.d.rasmussen@uit.no

Per M. Aslaksen. per.aslaksen@uit.no

Corresponding author:

Martin Bystad. Department of Psychology, Research Group for Cognitive Neuroscience Faculty of Health Sciences, University of Tromsø. Postbox 6050, N-9037, Norway. +47 77 62 08 09, e-mail address: martin.k.bystad@uit.no

Potential conflicts of interest:

The authors declare that they have no potential conflicts of interest to disclose.

Abstract

The aim of this study was to investigate whether transcranial Direct Current Stimulation (tDCS) could improve verbal memory functions in healthy elderly and younger participants. We hypothesized that active tDCS led to significantly improved memory function, compared to placebo tDCS. Forty healthy participants (20 elderly and 20 younger participants) were included in the study. We applied a novel stimulation protocol, where six sessions of anodal tDCS were administrated during two consecutive days. Each tDCS session lasted 30 minutes. The current intensity was 2mA and the stimulation area was the left temporal lobe at T3 in the 10-20 EEG system. Immediate recall, delayed recall and recognition memory were assessed with California Verbal Learning Test II (CVLT-II) and executive functions were assessed with the Trail Making Test (TMT) before the first tDCS session and after the last tDCS session. Half of the participants received placebo tDCS, whereas the other half received active tDCS. We did not reveal any significant differences between active and placebo tDCS in memory functions. However, there was a significant difference between active and placebo tDCS in executive function measured by the Trail Making Test (TMT). This experimental study failed to reveal significant differences between active and placebo accelerated tDCS for verbal memory functions. However, accelerated tDCS was found to be well-tolerated in this study.

Keywords: Memory, transcranial direct current stimulation, neuropsychology, neuromodulation, cognitive enhancer.

Introduction

A method that may improve memory functions in healthy individuals is called transcranial direct current stimulation (tDCS) (Manenti, Brambilla, Petesi, Ferrari, & Cotelli, 2013). This is a non-invasive stimulation method aimed to enhance plasticity and learning (Prehn & Flöel, 2015). tDCS treatment is performed by placing two or more electrodes on the scalp (one stimulation electrode and one reference electrode). The position of the stimulation electrode depends on the cortical area targeted for stimulation. Then, a weak current (2 mA or less) is delivered through the stimulation electrode. tDCS is simple to administer and it is associated with few adverse effects (Brunoni et al., 2012).

tDCS works by modulation of cortical excitability and neuroplasticity (Nitsche & Paulus, 2001). Thus, tDCS aims to increase neuroplasticity through the process of long-term potentiation (LTP) (Monte-Silva et al., 2013). This involves an increase in synaptic strength and is crucial for neuroplasticity and memory (Lynch, 2004). tDCS does not directly cause neuronal firing, but trigger conditions that makes neuronal firing more likely (Reinhart, Cosman, Fukuda, & Woodman, 2017).

Previous studies suggest that tDCS may enhance memory functions (Kristin Prehn & Flöel, 2015). For instance, Sandrini and colleagues (Sandrini et al., 2014) found that a 15 minute active tDCS session could significantly improve recall of a wordlist after 30 days. Furthermore, another study found that tDCS could improve verbal memory functions in both old and young participants (Manenti et al., 2013). Prehn and colleagues found that a combination of selective serotonin reuptake inhibitor (SSRI) and tDCS could give significant better immediate memory in both younger and older participants (Prehn et al., 2017). However, no such effects were found for delayed recall.

Verbal memory functions decline with age (Cargin, Maruff, Collie, Shafiq-Antonacci, & Masters, 2007). Thus, it could be assumed that aging can affect the efficacy of tDCS, when tDCS is used as a memory enhancer. For instance, Ross and colleagues found that tDCS stimulation of the temporal lobe could improve name recall for faces in both younger and older participants (Ross, McCoy, Coslett, Olson, & Wolk, 2011). However, older participants improved more compared to younger participants. One assumption is that that aging weakens cortical connections and that tDCS may enhance neuronal firing in a higher degree than for younger participants (Gutchess, 2014). tDCS may work better for elderly, since younger individuals have a nearly optimal level of neuroplasticity and thus smaller potential for improvement. However, a recent study (Leach, McCurdy, Trumbo, Matzen, & Leshikar,

2018) found that younger participants improved more than older participants and that older participants may be less receptive to tDCS. It is uncertain whether older participants benefits more from tDCS than younger participants. Hence, there is a need to investigate if the effect of tDCS differs between elderly and younger individuals.

It is also found that tDCS can improve memory functions in patients with Alzheimer`s disease. Boggio and colleagues (2012) found that 30 minute sessions of active tDCS for five consecutive days could lead to a nearly 10 % improvement in recognition memory. This improvement was prolonged for one month and was significantly higher in patients who underwent tDCS than in those who received placebo tDCS, which only led to a 2.6 % improvement.

On the other hand, Bystad and colleagues (2016a) found no significant differences in memory improvement between active and placebo tDCS in patients with Alzheimer`s disease. In Alzheimer`s disease, studies using tDCS have shown inconsistent results (Kim, 2016). In healthy individuals, tDCS is also associated with mixed results (Tremblay et al., 2014).

Before tDCS can be validated as a therapeutic tool, it is important to investigate different stimulation protocols in healthy individuals, in order to find the optimal stimulation protocol. In addition, since cognitive functions are relevant for our function in daily life it can be useful to investigate if tDCS leads to cognitive improvement.

The optimal number of tDCS sessions and the interval between sessions remain uncertain (Woods et al., 2016). For both experimental and clinical application of tDCS, the lack of standardized protocols possesses a problem when conducting new studies or comparing results between studies (Cappon, Jahanshahi, & Bisiacchi, 2016).

It is assumed that a high repetition rate, with short intervals between each tDCS sessions can probably be more efficient than increasing the duration of the stimulation (Nitsche, Kuo, Paulus, & Antal, 2015; Woods et al., 2016). Such high repetition rate may lead to longer lasting effects, since the neurophysiological after-effects of tDCS is relatively short lived. For instance, a recent study suggested that 13 minutes of tDCS stimulations of 2mA leads to 90 minutes after-effect (Thair, Holloway, Newport, & Smith, 2017).

To prolong the effect of tDCS, it has been proposed to use short intervals (< 30 minutes) between sessions (Woods et al., 2016). Such short intervals between each session can be referred to as “accelerated tDCS” (Bystad, Rasmussen, Abeler, & Aslaksen, 2016). A previous case study found that such application of tDCS could improve memory functions in patients with early stage Alzheimer`s disease (Bystad et al., 2016b). However, to date, this protocol has limited evidence.

Based on previous studies (Manenti et al., 2013; Sandrini et al., 2014), we aimed to investigate the effect of accelerated tDCS on memory functions and executive functions in both healthy elderly and healthy younger participants. We applied an accelerated tDCS protocol, with short (30 minutes) intervals between each session. We hypothesized that active tDCS would lead to a significantly improved verbal memory function (immediate recall, delayed recall and recognition), compared to placebo tDCS.

Materials and methods

Participants

A total of 40 individuals participated in the study. There were 20 elderly (59-69 years, mean age = 63 years, 16 females) and 20 young (19-30 years, mean age = 22 years, 13 females) participants. The eligibility criteria were absence of any serious somatic or psychiatric conditions or injuries to the central nervous system that could impact cognitive functions. Such conditions included cancer, cerebrovascular diseases, chronic obstructive pulmonary disease, heart failure, depression / anxiety and psychosis. All participants completed the Hospital Anxiety and Depression Scale (HADS) (Mykletun, Stordal, & Dahl, 2001), a questionnaire used to screen for depression and anxiety. Patients with scores above 15 on the HADS were excluded because depression may affect cognitive functions (Lam, Kennedy, McIntyre, & Khullar, 2014).

Participants were recruited by advertisement. All participants were informed that the experiment aimed to investigate if tDCS could improve memory functions. The study was executed in a research laboratory at the University of Tromsø, Department of Psychology. All participants signed a written informed consent prior to participation. They were compensated with a gift-card, worth 500 NOK (approximately 59 USD) after the participation. The study was approved by the Regional Ethical Committee for Research Ethics in Medicine and Health Sciences (2012/1890).

Outcome measures

In the present study, the primary outcome measure was verbal memory functions, assessed with the California Verbal Learning Test–Second Edition (CVLT-II) (Delis, Kramer, Kaplan, & Ober, 2004). The CVLT-II is a standardized neuropsychological test, normalized by age and gender. The CVLT-II assess immediate recall, delayed recall and recognition. The CVLT-II is widely used (Delis et al., 2004) and based on list-recall, where the participant is instructed to recall a list with 16 words. The CVLT-II has good test-retest reliability (Delis et

al., 2004). To reduce test-retest practice effects, we used the standard version at baseline, and the alternative form after the last tDCS session. The standard and alternative forms have different word-lists.

The secondary outcome measures included the Trail Making Test A and B (TMT A and TMT B) (Tombaugh, 2004) and the Digit Span test from the Wechsler Memory Scale (WMS) (Wechsler, 1998). TMT A measures sustained attention, speed and motor function, whereas TMT B also assesses executive functions. WMS Digit Span measures attention / working memory. The participant is instructed to repeat a cumulative sequence of numbers forward and backward.

To control for general cognitive abilities, the Matrix Reasoning and Vocabulary tests from the Wechsler Abbreviated Scale of Intelligence (WASI) (Pearson, 1999) were conducted at baseline. To screen for cognitive impairment among the elderly participants we used the Mini Mental Status Evaluation (MMSE-NR) (Folstein, Folstein, & McHugh, 1975).

To assess possible adverse effects, we used a questionnaire from Brunoni and colleagues that was translated into Norwegian (Brunoni et al., 2011). This questionnaire asks specifically about adverse effects from the tDCS procedure, specifically regarding itching, tingling, headache and discomfort (Brunoni et al., 2011).

Transcranial Direct Current Stimulation (tDCS)

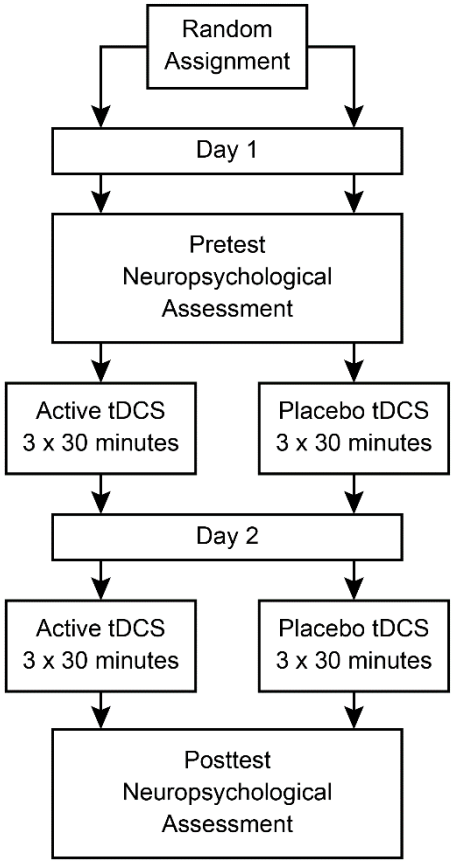
The stimulation was delivered using a direct current stimulator (neuroConn, Ilmenau, Germany). The stimulation duration was 30 minutes and the current intensity was 2mA. The current was transferred to the skull through a pair of 35-cm2 rubber electrodes. The anode (stimulation electrode) was placed at the T3 position in the 10-20 system (a system used for electroencephalographic electrode positioning). The cathode (reference electrode) was placed at the Fp2 position, i.e on the right frontal lobe. For both the placebo and the active tDCS, the electrode placement and session duration were similar. In the placebo tDCS, a current was delivered only for the first 30 seconds. After these 30 seconds, the stimulator turned the current off automatically.

All participants were assigned their own five-digit code. This code determined if the tDCS device should give the placebo or active stimulation. Neither the experimenter nor the participant knew if the tDCS stimulator delivered the active or placebo stimulation. Thus, the study was double blind. The order of the codes was randomized using the Random.org website (https://www.random.org/).

Procedure

Participants met individually for two consecutive days in a research laboratory at the university. First, each participant received information about the study. Then, the participant underwent the neuropsychological assessment. The duration of this assessment was approximately 60 minutes. When the assessment was completed, the first tDCS session began. Three sessions were given on both the first day and the second day. Each tDCS session lasted for 30 minutes. The break between the sessions was about 30 minutes. After the final tDCS session, the participant underwent neuropsychological assessment. See figure 1 for an overview of the procedure.

Figure 1:



Statistical and power analysis

All data were analyzed in SPSS Version 22. We calculated the change scores between baseline and post stimulation neuropsychological assessment scores to investigate the effect of the tDCS stimulation. We conducted independent t-tests to investigate the differences in

the mean change of scores between placebo and active tDCS. A MANOVA was conducted to investigate group differences between placebo and active tDCS adjusted for age. Data were normally distributed, shown by Shapiro Wilk test.

A previous study (Sandrini et al., 2014) with healthy participants found that active tDCS led to significant improvement in verbal memory functions, compared to placebo tDCS. In that study, tDCS was delivered only once, with a 15-minute duration. Based on mean scores from Sandrini et al., 2014, we used a power estimation calculator (clincalc.com) and estimated that our study had 80 % power in order to achieve a significant effect with a least 32 participants (16 placebo and 16 active tDCS). Thus, we wanted to include a total of 40 participants. The alpha-level was 0.05.

Results

Table 1 displays the number of participants who improved on different outcome scores from baseline to post-test. The analysis showed no significant differences in CVLT-II scores between the active and the placebo tDCS (Table 2). For CVLT-II immediate recall F = 0.067, df = (1.0), p = 0.79, CVLT-II delayed recall (F = 0.24, df = (1.0), p = 0,62) and CVLT-II recognition (F = 0.092, df = (1.0), p = 0.76), no significant differences were found between the active and the placebo tDCS. However, we found that the active group scored significantly better on change scores than the placebo group on TMT-B, F = 4.54, df = (1.0), p = 0.040.

Table 1. Frequency table

	Active tDCS (N = 20)	Placebo tDCS (N =20)
CVLT-II immediate recall	9	6
CVLT-II delayed recall	4	4
CVLT-II recognition	5	3
TMT-A	17	13
TMT-B	18	18
Digit Span	11	17

The data represent the number of patients who showed improvements (>) on the outcome measures. Improvement was considered as improved scores from baseline to the post-test.

Table 2. Changes in cognitive scores for all participants (N = 40)

	Group		Age Group		Age Group * Group	
Outcome	p	F	p	F	p	F
CVLT immediate	0.797	0.67	0.70	0.14	0.57	0.32

CVLT delayed	0.622	0.24	0.092	3.00	0.46	0.544
CVLT recogntion	0.20	0.092	0.535	0.39	0.61	0.25
TMT-A	0.91	3.02	0.59	3.81	0.93	0.007
TMT-B	0.040	4.54	0.66	0.18	0.97	0.33
Digit Span	0.365	0.84	0.78	0.77	0.81	0.055

Note: "Group" is active or placebo, "Age Group" is younger or elderly and "Age Group * Group" is the interaction between the group and the age group.

For the group of elderly participants, we found no differences in the CVLT-II scores between active and placebo tDCS (see Table 3). For CVLT-II immediate recall ($t(15.69) = -0.90$, $p = 0.37$), CVLT-II delayed recall ($t(14.83) = 0.18$ $p = 0.85$) and CVLT-II recognition ($t(11.19) = 0.43$, $p = 0.67$), no significant difference were found between active and placebo tDCS. For TMT A ($t(17.94) = 2.02$, $p = 0.058$) and TMT B ($t(15.92) = 0.64$, $p = 0.52$), and Digit Span ($t(12.45) = -0.98$, $p = 0.91$), there was no significant difference between active and placebo group.

Table 3. Change in scores for elderly participants (N = 20).

	Group	Mean	Std. Deviation	*P value*	*Hedges g*
CVLT immediate	Placebo	-4.90	10.98	0.37	0.38
	Active	-1.10	7.34		
CVLT delayed	Placebo	-0.35	0.97	0.85	0.07
	Active	-0.43	1.01		
CVLT recognition	Placebo	-0.25	0.58	0.67	0.18
	Active	-0.44	1.23		
TMT-A	Placebo	-2.10	7.50	0.058	0.86
	Active	-9.10	7.93		
TMT-B	Placebo	-13.80	28.05	0.52	0.28
	Active	-21.25	20.66		
Digit Span	Placebo	10,20	1,47	0,91	0.04
	Active	10,10	2,51		

Note: The mean values are the estimated change from baseline to post-testing (post testing minus baseline). For the CVLT-II immediate recall score, the mean value is displayed as a T-score. For the CVLT-II delayed recall and recognition scores, the mean value are displayed as Z-scores. An independent t-test was applied to calculate the differences between the placebo and active tDCS groups. For the CVLT scores and Digit Span scores, a positive values indicates a positive change. For TMT A and B, negative values indicate improvements. Significant values ($p = < 0.05$) are marked with *.

For the group of younger participants, we found no difference in CVLT-II scores between active and placebo tDCS (see Table 4). For CVLT-II immediate recall ($t(18.00) = 0.22$, $p = 0.82$), CVLT-II delayed recall ($t(17.69) = -0.82$, $p = 0.42$) and CVLT-II recognition ($t(17.82) = -0.58$, $p = 0.56$), no significant difference were found between active and placebo tDCS. For TMT-A ($t(17.99) = 1.08$, $p = 0.29$ and Digit Span ($t(16.90) = -0.48$, $p = 0.63$) there were no significant differences between the active and placebo groups. However, on TMT-B ($t(11.47) = 3.26$, $p = 0.007$), the active group scored significantly better than the placebo group.

Table 4. Changes in scores for younger participants (N = 20)

Outcome	Group	Mean	Std. Deviation	*P* value	*Hedges g*
CVLT immediate	Placebo	-4.30	9.80	0.82	0.09
	Active	-5.30	9.83		
CVLT delayed	Placebo	-1.25	1.29	0.42	0.35
	Active	-0.80	1.13		
CVLT recognition	Placebo	-0.60	0.90	0.56	0.26
	Active	-0.35	1.00		
TMT-A	Placebo	-8.00	9.92	0.29	0.46
	Active	-12.80	9.79		
TMT-B	Placebo	-10.20	6.90	0.007*	1.39
	Active	-30.50	18.43		
Digit Span	Placebo	0.50	2.06	0.63	0.20
	Active	0.90	1.59		

Adverse-effects

No adverse-effects were reported, neither in young participants or elderly participants, based on a questionnaire (Brunoni et al., 2011) for adverse-effects in tDCS procedures.

Discussion

The aim of the present study was to investigate whether accelerated tDCS could improve verbal memory functions in healthy young and healthy elderly participants. We also investigated whether tDCS could affect executive functions in both young and elderly participants. In addition, we wanted to study if age was a significant factor of tDCS efficacy.

We did not reveal significant differences between placebo and active tDCS in verbal memory functions. This was not in accord with results from two previous studies (Manenti et

al., 2013; Sandrini et al., 2014). Furthermore, we did not find any significant differences in verbal memory between placebo and active tDCS, whilst adjusting for age.

However, we found a significant difference between placebo and active tDCS for executive functions, as measured with TMT-B. This significant difference was only found among the younger participants. It should be noted that none of our participants reported any adverse-effects, despite the short intervals between each the tDCS sessions. Accelerated tDCS seems to be both safe and well-tolerated in our study.

The reason for our non-significant effect of tDCS on verbal memory functions may be attributed to several different causes. First, we applied a novel stimulation protocol (i.e., accelerated tDCS, with short intervals between each session). To our knowledge, no studies have investigated such an intensive protocol. Accelerated tDCS is based on recommendations from Nitsche and colleagues (2015), rather than evidence. This protocol may not be as efficient as we expected.

Second, it also uncertain if tDCS actually leads to cognitive (Horvath, Forte, & Carter, 2015b) and neurophysiological (Horvath, Forte, & Carter, 2015a) changes in healthy individuals. Horvath and colleagues argue that tDCS has some major shortcomings (e.g., electric current influences, inter-subject variability) (Horvath, Carter, & Forte, 2014). For instance, Trembley (Tremblay et al., 2014) reported that one participant experienced a 251 % increase in motor evoked potentials, whereas another participant experienced a 41 % decrease. Anatomic differences (e.g skull thickness) and neurophysiology are individual factors that may affect the distribution of current flow to the cortex (Horvath et al., 2014). The effect of tDCS on cognitive function in healthy participants is associated with conflicting results (Tremblay et al., 2014). Hsu and colleagues (2015) argues that tDCS may work best in pathological states and benefit those who need it most, since there may be a ceiling effect in healthy participants and tDCS may serve to strength weaken pathological neural circuits. Consequently, we cannot disregard a lack of effect from our tDCS stimulation.

We revealed a significant effect on executive functions in the young participants. A possible explanation is the limitation of the TMT-B test, which was used to measure executive functions. TMT-B seems to have substantial test-retest practice effects, especially over a short interval (Bartels, Wegrzyn, Wiedl, Ackermann, & Ehrenreich, 2010). For instance, a study (Bartels et al., 2010) found that retest with TMT-B after three weeks could improve the score with nearly 10 seconds. Since memory test usually have parallel versions, TMT-A and TMT-B are more susceptible to test-retest practice effects. According to our results (Table 1), most participants improved on the TMT-B test, regardless of placebo or active tDCS. We cannot

completely rule out a test-retest practice effect.

Further, tDCS has low specificity (Csifcsak, Boayue, Puonti, Thielscher, & Mittner, 2018). Even if our aim was to stimulate temporal cortex, other cortical areas may also have been affected (e.g frontal cortex), since tDCS may lead to a widespread alterations of functional connectivity (Keeser et al., 2011). It is suggested that tDCS may enhance alerting attention (Coffman, Trumbo, & Clark, 2012). This could lead to better scores on TMT-B.

Limitations

The present study has several limitations that needs to be addressed. One limitation is that we relied solely on cognitive functions for our outcome measures. Consequently, we do not know if the tDCS stimulation induced any neurophysiological changes. There may be a chance that our tDCS protocol affected neuroplasticity and neural activity. However, this remains unknown in our study.

Further, a second limitation is our "one size fits all" approach. It is reasonable to assume that anatomical differences (e.g., skull thickness) can affect the efficacy of the tDCS stimulation, i.e how the current is distributed to the cortex. We did not apply a computational model to calibrate the tDCS stimulation for each participant. Our lack of individual calibration is a limitation, since individual differences can be an important factor (Sarkar, Dowker, & Cohen Kadosh, 2014).

A third limitation is that we did not combine tDCS with any cognitive stimulation. We only applied tDCS. This could affect the efficacy of our tDCS protocol, since the effect of tDCS may improve when tDCS and cognitive stimulation are used simultaneously (Hsu et al., 2015).

Future research

Further research should take advantage of both neuropsychological assessment and psychophysiological measures (e.g., event-related potentials or neuroimaging). A combination of such outcome measures will provide insight into the cognitive and neurophysiological effect of tDCS. There is clearly a need to investigate the potential effect of tDCS on neurobiological changes in healthy individuals. For future research, it can also be useful to calibrate the tDCS procedure for each participant. A computation model can be applied in order to determine out how individual differences will affect the current distribution. In

addition, it could be of potential interest to study the effect when tDCS and cognitive stimulation are delivered simultaneously.

Conclusions

This experimental study did not reveal a significant difference between active and placebo accelerated tDCS for verbal memory functions. However, we found that the tDCS stimulation led to a significant improvement in executive function in younger participants, assessed with TMT-B. Our accelerated tDCS protocol, with short intervals between each session, was well tolerated with no side effects of the stimulation. Future research should combine neuropsychological and neurophysiological outcome measures.

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List of abbreviations

tDCS = transcranial Direct Current Stimulation
CVLT-II = California Verbal Learning Test II
TMT = Trail Making Test
MMSE = Mini Mental Status Examination
HADS = Hospital Anxiety and Depression Scale
WASI= Wechsler Abbreviated Scale of Intelligence.
WMS = Wechsler Memory Scale

Contributors

M.B. planned the study, recruited patients, collected the data, wrote the first draft, revised and reviewed the final draft and analyzed the data. He is the corresponding author. O.G drafted and revised the manuscript. I.D.R. drafted and revised the manuscript. N.G, B.S, I.L.W, and L.N collected the data. P.M.A. planned the study, wrote the first draft, revised the manuscript, analyzed the data and revised the final manuscript. He served as a supervisor for M.B. All authors approved the final manuscript.

Potential conflicts of interest

The authors declare that they have no potential conflicts of interest to disclose.

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Paper III

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Research report

The relation of hippocampal subfield volumes to verbal episodic memory measured by the California Verbal Learning Test II in healthy adults

Per M. Aslaksen[a,b,*], Martin K. Bystad[a], Marte C. Ørbo[c], Torgil R. Vangberg[d]

[a] Department of Psychology, The Arctic University of Tromsø UiT, Tromsø, Norway
[b] Department of Child and Adolescent Psychiatry, The Regional Unit for Eating Disorders, The University Hospital of North Norway, Tromsø, Norway
[c] Department of Cardiothoracic and Vascular Surgery, Heart and Lung Clinic, The University Hospital of North Norway, Tromsø, Norway
[d] Department of Radiology, University Hospital of North Norway, Tromsø, Norway

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ABSTRACT

Total hippocampal volume has previously been shown to correlate with performance on tests for verbal episodic memory. However, there are sparse evidence on how hippocampal subfield volumes are related to verbal episodic memory in healthy adults. The present study investigated the association between volumes of separate hippocampal subfields and verbal episodic memory performance in healthy volunteers. Forty-seven participants (31 females) between 20–71 years age underwent testing with the California Verbal Learning Test II (CVLT II), and the Wechsler Abbreviated Scale of Intelligence (WASI) to obtain an estimate of cognitive functioning. T1-weighted MR images were obtained after cognitive testing, and volumetric estimates adjusted for age and estimated total intracranial volume were calculated in the FreeSurfer 6.0 software suite for cerebral -and hippocampal structures. The sample performed within the statistical normal range on both CVLT II and WASI. Significant correlations adjusted for multiple testing were found between CVLT II subtests of total learning, free immediate recall and free delayed recall and volumes of the left Cornu Ammonis (CA) 1–4 subfields. There were no significant correlations between right hippocampal subfields and CVLT II performance, and no significant correlation between WASI results and hippocampal subfields. The present results suggest that better verbal episodic memory measured by the CVLT II is associated with relative larger volumes of specific left CA hippocampal subfields in healthy adults. Due to the small sample size and large age-span of the participants, the present findings are preliminary and should be confirmed in larger samples.

1. Introduction

Episodic memory, which is the ability to remember experiences that occurred at a particular place and time, has been related to hippocampal functions in several studies [1–3]. The hippocampus is usually subdivided into the Cornu Ammonis (CA) CA1, CA2-3, CA4/dentate gyrus, the presubiculum and the subiculum, which are the larger substructures of the hippocampus. Previous studies have suggested that these subfields have separate and specialized functions with regard to memory processes, and the subfield division are therefore not merely an anatomical classification [4,5].

Development of advanced magnet resonance imaging (MRI) techniques during the last decades have provided the opportunity to study both anatomical features and cerebral activation with high precision. Volumetric MRI studies that combines structural assessment of the brain with concomitant cognitive measures provides the opportunity to study the inter-individual variability in brain structures that can be statistically related to certain categories of cognition [6]. The structural approach is useful when assessing whether structural individual differences in cerebral areas are associated with normal or impaired cognition [7].

The volume of the hippocampal subfields is assumed to be positively correlated with episodic memory functioning [8,9], even if some studies have reported a negative association between hippocampal volume and memory processes [10,11]. However, most previous volumetric studies of verbal memory and hippocampal size have used data for the hippocampus without separating the subfields and the number of studies investigating the relation between hippocampal subfields and cognitive functions are sparse. Furthermore, the results in the existing studies are not entirely consistent, and differences in findings may arise from differences in characteristics of the samples, method for estimating hippocampal subfields and selection of cognitive tests.

A recent study using subfield segmentation of MRI data from healthy elderly showed that verbal memory performance measured by

* Corresponding author at: Department of Psychology, The Arctic University of Norway UiT, 9037, Tromsø, Norway.
E-mail address: per.aslaksen@uit.no (P.M. Aslaksen).

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the Repeatable Battery for Assessment of Neuropsychological status (RBANS) shows that larger volumes of the CA1 and the subiculum were correlated with better verbal memory retrieval [12]. Another study on healthy younger adults using high-field MRI with manual hippocampal segmentation revealed that verbal memory performance [8] measured by the Wechsler Memory Scale III [13] was correlated with larger volumes of the CA1, CA2-3 and CA4. Using a measure for autobiographical episodic memory [14] in healthy young adults, Palombo et al. [15], found that volumes of the left CA2/3 and the bilateral subiculum were associated with higher number of details generated from an autobiographical interview. In case-control studies using the California Verbal Learning Test (CVLT-II) [16] to assess verbal memory, larger volumes of CA2-3 and CA4 were associated with better immediate verbal recall, whereas CA1 volume correlated with better delayed verbal recall [17,18].

Early lesion studies indicated that verbal auditory memory is more dependent on the left hippocampus compared to the right [19], which have been supported in some more recent human volumetric studies [15,20,21]. Furthermore, several studies in healthy adults have found structural asymmetry with larger volumes of the right hippocampus compared to the left [22–24]. This asymmetry has also been shown to be associated with preserved memory functions in elderly persons, where memory impaired subjects had no significant volume asymmetry between the left and the right hippocampus [25]. The underlying mechanism for the structural asymmetry is unknown, but it may be related to the functional specialization of the temporal lobes, where the left side normally is more associated with verbal memory whereas the right is associated with non-verbal memory functions [22,26]. Thus, a lateralization effect of verbal episodic memory on hippocampal subfield volumes can be anticipated.

In the present study, we used the CVLT-II to assess verbal memory functions in healthy adults of both sexes in a wide age-span to test whether episodic verbal memory shows a lateralization effect in the hippocampus. The CVLT II is extensively used in both clinical and scientific settings [27], but there is limited data on the hippocampal anatomical correlates of CVLT II performance in healthy volunteers. Thus, data for structural correlates of the CVLT II is important for both clinical and scientific purposes. Based on findings in previous studies using similar methodology, we expected that different outcome measures of the CVLT-II had specific correlates of the hippocampal subfield volumes. Specifically, we hypothesized that the left CA2-3 and CA4 volumes should be associated with the learning score and immediate recall memory score, whereas the left CA1 and the subiculum should be associated with delayed recall performance.

2. Methods

2.1. Participants

Forty-seven, right-handed volunteers (31 females) in the age range 22–71 (Mean = 38.36, SD = 20.16) years were recruited on the campus of the University of Tromsø, Norway. The mean educational level of the sample was 13.78 (SD = 2.02) years (median = 14 years). All participants signed an informed consent stating that they were healthy and had no present or history of severe disease or injuries. The study was approved by the Regional Committee for Research Ethics in Medicine and Health Sciences (project 2012/1588) and was conducted in accordance with the Declaration of Helsinki. The participants received a gift card worth 300 Norwegian Kroner (approx. 37 EUR/47 USD) as compensation for their participation. Exclusion criteria were previous concussions, traumatic brain injury, or other injuries or diseases involving the central nervous system, including psychiatric conditions. Patients on prescribed medications were excluded, with the exception of oral contraceptives in women. Medical conditions, pregnancy, or body implants not compatible with participants' safety in the MR-scanner were also exclusion criteria.

2.2. Neuropsychological tests

Verbal episodic memory was measured by the Norwegian version of the California Verbal Learning Test II (CVLT-II), standard version [16]. The CVLT-II measures verbal auditory learning, recall- and recognition memory, and was administrated and scored according to the standardized instructions. The learning trial gives a total score from five separate recalls of a 16-item word list (List A) that is read aloud to the examinee who is to repeat the words from the list in random order after each reading. Thereafter, a second list (List B) is introduced as a distractor before the examinee is asked to recall as many items as possible from List A. The correct items remembered from A after the distractor list, comprises the Immediate recall trial. Then, the examiner asks the participant to categorize the word list into four categories (i.e., furniture, vegetables, clothes, and animals) to obtain a measure of Immediate cued-recall abilities. Twenty minutes after the total learning trial, the free and cued recall of List A are repeated, and comprise the delayed recall trial and the delayed cued recall trial, respectively. At last a recognition trial and a forced recognition trial is performed.

The results from the cued recall trials were not included in the present data analyses due to high correlations with the free recall data ($r > 0.70$) and the small sample size restricting the number of comparisons to be performed.

The raw scores from the CVLT-II total learning trial, immediate and delayed recall trials and the recognition trials were converted to standard scores using published normative data that corrects for both age and sex. Validity studies of the Norwegian version of the CVLT-II have shown good fit between the Norwegian translation and American norms [28].

Visual-spatial abilities and crystallized intelligence were assessed by two subtests (Matrix Reasoning and Vocabulary) from the Norwegian version of the Wechsler Abbreviated Scale of Intelligence (WASI) [29]. The Vocabulary subtest demands that the examinee gives overt explanations of words with an increasing level of difficulty. The Norwegian version of the WASI has shown acceptable fit to American normative data [30]. The Matrix reasoning is a non-verbal subtest measuring visual-perceptual- and problem solving ability. WASI scores are converted to standardized scores by normative data, correcting for age, but not sex.

2.3. MRI acquisition

Subjects were scanned in a 1.5 T Phillips Intera MR scanner using an 8-channel head coil. The T1-weighted structural scans were 3D turbo field echo scan with TR = 1.825 ms, TI = 855 ms, TE = 4.0 ms, flip angle = 8°, and voxel resolution = $0.94 \times 0.94 \times 1.25$ mm^3. The MRI scanning was performed within a month after cognitive testing for all participants.

2.4. Volumetric MRI analysis

Hippocampal subfields were calculated by an automated segmentation process [31] implemented in Freesurfer 6.0 (https://surfer.nmr.mgh.harvard.edu/). FreeSurfer automatically labels each voxel of the T1 MR-images to one of 40 predefined structures by using probabilistic brain atlases [32–34]. In the present study, the volumetric data for the hippocampal subfields, estimated total intracranial volume and total left and right hippocampus volumes were used. See Fig. 1 for an example of segmentation based on Freesurfer 6.0 from the present study. The volumes used in the correlation analyses were adjusted for age, sex and estimated total intracranial volume (eTIV). The adjustments were performed by linear regressions where brain volumes were dependent variables, and age, sex and eTIV were entered as predictors. The standardized residuals from the regressions were then saved and used for the analyses. The adjusted volumes (residuals) for the left and the right Cornu Ammonis (CA) CA1, CA2/3, CA4/Dentate Gyrus (DG),

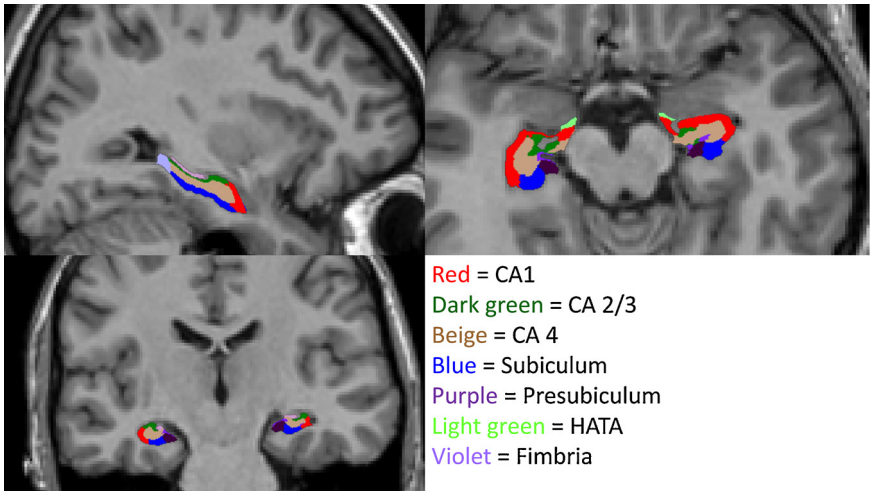

Fig. 1. Example of segmentation of hippocampal subfields based on T1-weighted MRI. Voxel resolution = 0.94 × 0.94 × 1.25 mm^3.

presubiculum, subiculum and the total volumes of the right and the left hippocampus were correlated with performance on cognitive tests.

2.5. Statistical analyses

The distribution of the calculated residuals was not significantly different from a normal distribution shown by the Shapiro-Wilk test, and this was further confirmed by inspection of Q—Q plots of the residuals. Thus, parametric statistical testing was performed. Independent samples *t*-test were used to test group-differences in unadjusted volumes. Paired samples *t*-tests were used to compare differences between the left and the right hippocampal formations. Correlations between hippocampal volumes and CVLT II subtests were performed with Pearson correlations, and in order to adjust p-values for multiple testing and reducing the probability of type I errors, p-values were adjusted with the False Discovery Rate (FDR) procedure with q = 0.05 [35,36]. After FDR adjustments performed with a script for SPSS (http://www-01.ibm.com/support/docview.wss?uid=swg21476447) the level of significance was $p < .0044$ for the correlational analyses between cognitive performance and hippocampal subfields. Elsewhere, p-values < .05 were considered significant. To test whether correlations were significantly different based on their z-score distribution, the Fisher r-to-z transformation test was employed.

3. Results

3.1. Cognitive data

Descriptive data for CVLT II, WASI, and volumetric measures are presented in Table 1. The sample means of the cognitive tests were within one standard deviation from the normative means, however, the results on the CVLT II showed that one abnormally low score (below 2 SD from the normative mean) from separate participants occurred on all CVLT subtests. All participants performed within the statistical normal range (T-score > 40) measured by the WASI tests, and the frequency of abnormal scores was not deviant from other studies using neuropsychological methodology [37]. There were no significant sex differences in CVLT II adjusted scores (all t's < 1.82), but females performed better compared to males based on the unadjusted raw scores on the recognition subtest ($t(45) = 2.26$, $p = .03$). No other comparison between males and females performance on the CVLT II reached significance.

Table 1
Descriptive statistics. SD = Standard deviation. WASI = Wechsler Abbreviated Scale of Intelligence, CVLT = California Verbal Learning Test II. eTIV = Estimated total intracranial volume. WASI and CVLT II Total learning scores are shown in T-scores (normative mean = 50, SD = 10), other CVLT II scores are shown in Z-scores (normative mean = 0, SD = 1). N = 47, 31 females.

Variable	Mean	Median	SD	Minimum	Maximum
Age at scanning	38.4	34	20.2	20	71
WASI Matrix reasoning	56.9	57	7.1	37	69
WASI Vocabulary	57.5	58	7.9	40	70
CVLT Total learning	53.4	53	10.3	24	69
CVLT Immediate recall	.47	.50	.99	−2	2
CVLT Delayed recall	.42	.50	.97	−2	1.5
CVLT Recognition	−.01	0	.63	−2	1
eTIV, cm^3	1572	1544	134	1370	1932
Left hippocampus, mm^3	3428	3410	285	2875	4202
Right hippocampus, mm^3	3511	3483	298	2937	4226
Whole hippocampus, mm^3	6939	6966	575	5812	8428
CA1, mm^3	1270	1265	119	1041	1558
CA2/3, mm^3	426	424	45	335	552
CA4/DG, mm^3	520	526	47	438	644
Subiculum, mm^3	861	844	88	711	1093
Presubiculum, mm^3	612	597	67	473	781

3.2. Unadjusted volumetric data

Males had larger right hippocampus ($t(45) = 2.15$, $p = .037$) and larger eTIV ($t(45) = 5.07$, $p < .001$), but there was no sex difference in left hippocampal volume ($t(45) = 1.17$, $p = .25$). There were significant differences in volumes between the left and the right hemisphere on the hippocampal subfield measures shown by paired samples t-tests (all t's (46) < 5.01, all p's < .001) with exception of the comparison left versus right subiculum ($t(46) = 0.45$, $p = .65$). The right subfields were larger ($p < .05$) compared to the left subfields, with the exception of the left presubiculum being larger than the right ($t(46) = 5.03$, $p < .001$). All t-tests on the left versus right comparisons of volumetric data are presented in Table 2. Univariate Pearson correlations between the left hippocampus, the right hippocampus and total hippocampal size and age and eTIV revealed that age had no significant association with any of the volumetric data, but there was a non-significant tendency to negative correlations between age and volumes. eTIV was significantly associated with left hippocampal volume ($r = .49$, $p < .01$), right hippocampal volume ($r = .59$, $p < .001$) and total hippocampal volume ($r = .55$, $p < .001$).

Table 2
Comparison of left and right hippocampal subfield volumes with paired samples t-tests.

	Left Mean	Right Mean	t	p
Hippocampus - whole mm^3	3428	3511	−5.51	< .001
CA1 mm^3	621	648	−5.44	< .001
CA2/3 mm^3	201	224	−8.20	< .001
CA4/DG mm^3	252	268	−6.77	< .001
Subiculum mm^3	431	429	.45	.65
Presubiculum mm^3	315	297	5.01	< .001

Table 3
Pearson correlations between CVLT-II subtests and volumes of hippocampal subfields and hippocampus. Volumes are standardized residuals from linear regressions in order to adjust for total intracranial volume, sex, and age. L = Left, R = Right. * = p < .05 unadjusted, bold ** = p < .0044 FDR adjusted. CA = Cornu Ammonis, CVLT = California Verbal Learning Test II. DG = Dentate gyrus. N = 47.

	CVLT II Total Learning	CVLT II Immediate Recall	CVLT II Delayed Recall	CVLT II Recognition
Hippocampal subfields				
L hippocampus - whole	.27	.33*	.32*	.08
L CA1	.30*	.30*	**.43****	.17
L CA2/3	.34*	**.43****	**.41****	.27
L CA4/DG	**.40****	**.42****	**.47****	.15
L Subiculum	.05	.19	.12	.01
L Presubiculum	−.19	−.11	−.07	−.23
R hippocampus - whole	.18	.27	.23	.02
R CA1	.24	.27	.28	.17
R CA2/3	.14	.29	.22	−.07
R CA4/DG	.22	.32*	.26	−.03
R Subiculum	.01	.10	.09	−.05
R Presubiculum	−.24	−.14	−.08	−.19
Merged (L + R) subfields				
Whole hippocampus	.26	.31*	.26	−.03
CA1	.28	.30	**.40****	.20
CA2/3	.25	.39*	.33*	.10
CA4/DG	.33*	.38*	.39*	.15
Subiculum	.09	.14	.21	.11
Presubiculum	−.15	−.16	−.11	.03

3.3. Correlation analyses on data adjusted for age, sex and eTIV

The correlation analyses showed that there were no significant associations between the right hippocampal subfields residuals and the CVLT II subtests. There were several significant correlations ($p < .05$) between the CVLT II subtests and left hippocampal subfields residuals, however when applying the FDR adjustments ($p < .0044$), only the CA1, CA2/3 and the CA4 had significant associations with verbal memory performance (Table 3 and Fig. 2). There were no significant correlations between performance on the WASI and hippocampal measures when adjusting p-values with the FDR procedure. The Fisher r-to-z transformation tests showed that none of the significant correlations between subfields in the left and CVLT II performance was significantly different from the same correlations in the right subfields when using the z-score as criterion. The z-scores of the difference ranged from $z = 1.34$, $p = .09$ to $z = 0.99$, $p = .16$ (one-tailed). The correlations between the merged (left + right) subfields and CVLT II performance showed that larger volume of the CA1 was significantly associated with better delayed recall performance. However, the whole hippocampus, the CA2/3 and the CA4/DG had correlations with all CVLT II measures with the exception of the recognition score, but the significance of these correlations did not pass the FDR criterion (see Table 3).

4. Discussion

In accordance with our hypothesis, the present results showed significant associations between larger volumes of the left hippocampal subfields (CA1, CA2/3, CA4/DG) corrected for age, sex and eTIV with verbal learning and memory performance. Specifically, volume of the CA1 correlated with delayed recall, whereas volume of the CA2/3 was related to immediate and delayed recall, and volume of the CA4/DG were significantly associated with learning, immediate and delayed recall. Volumes of the right hippocampal subfields were not significantly related to verbal learning or recall performance. This lateralization effect in hippocampal subfields is not previously shown for verbal memory tests that include list learning, which is the type of test most commonly used for measuring verbal episodic memory [27]. Two previous studies [20,21] found a similar effect of the whole left hippocampus without separating the subfileds. Moreover, a recent study by Palombo et al. [15], found that performance on the Autobiographical Interview [38] correlated significantly with the left CA2/3 but not the right CA2/3.

The analyses of structural volumes showed that the right CA1, CA2/3 and the CA4/DG were larger compared the same structures in the left hemisphere. The presubiculum was the only subfield in the left hippocampus being larger than the subfields in the right hemisphere. This is in line with several previous studies [22–24] on healthy volunteers showing a structural asymmetry of hippocampal volumes. In patients with Alzheimer's disease, episodic memory deficits are more associated with volumes of the left subfields compared to the right subfields [39], and the structural asymmetry of the hippocampal volumes is absent. It has been suggested that the left compared to the right hippocampus is more affected by atrophy caused by vascular and neurodegenerative processes in Alzheimer's disease [40]. Thus, the episodic verbal memory deficits in Alzheimer's disease could to some extent be caused by increased left hippocampus vulnerability compared to the right hippocampus [41]. Taken together, several studies in both healthy volunteers and patients suggest that the left hippocampus is more involved in verbal episodic memory in healthy volunteers compared to the right hippocampus.

Previous studies have suggested that CA2/3 and CA4/DG are structures more related to encoding and learning than recall, whereas the CA1 is an output structure mainly related to retrieval functions [42–44]. The results from the present study support these suggestions, except that volumes of the CA2/3 and the CA4/DG were related to performance in the recall stage of verbal memory, and not solely associated with the learning phase. This is in line with a 7 T fMRI study showing that the CA2/3 and CA4/DG are activated both during learning and recall, however more in the learning phase [45]. In comparison to Zammit et al [12], we did not find any correlation between volume of the left or right subiculum and verbal memory. Zammit et al [12] used only data for the merged left and the right subfields in their analyses in contrast to the present study.

The partially divergent findings on the relation between hippocampal morphometric data and verbal memory performance may be due to several methodological differences between studies. Different verbal memory tests have been employed across volumetric studies on hippocampal subfields. The Free and Cued Selective Reminding Test [12], The Autobiographical Interview [15] and the Wechsler Memory Scale (WMS-IV) [8] have been used in healthy samples, whereas the WMS-III [46], the CVLT [17,46] and the Brief Assessment of Cognition in Schizophrenia [47] have been used in studies with mixed samples of healthy controls and patients. Even if tests for episodic verbal memory are highly correlated, the concordance is not perfect between tests [48,49]. Hence, different tests designed for measuring episodic memory might measure different aspects of the construct and may produce variability in hippocampal correlates. In addition, Zammit et al [12]

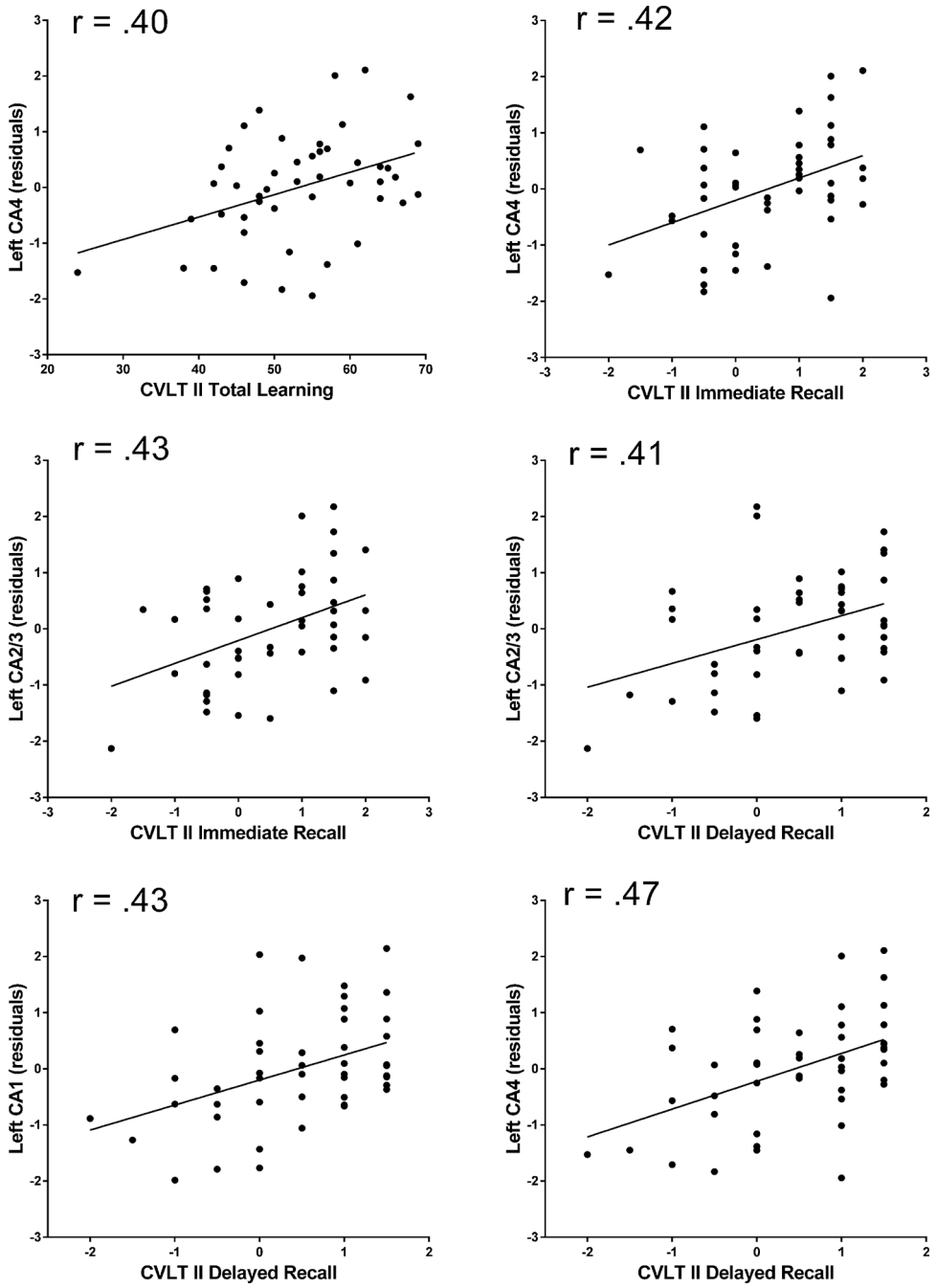

Fig. 2. Scatterplots for correlations between CVLT II subtests and hippocampal volumes adjusted for age, sex and estimated intracranial volume (eTIV). r = Pearson correlation coefficient.

employed FreeSurfer for automatic volumetric segmentation in healthy volunteers, whereas Travis et al [8] and Palombo et al [15] used a manual segmentation procedure. Different methods for estimating volume may produce small but measurable differences in estimates of cerebral structures [50]. Additionally, the age span in studies on healthy volunteers differ across studies, from participants below 35 years [8,15] to elderly with a mean age at approx. 79 years [12]. The present study recruited volunteers in the age span 20 to 71 years, but there were no significant linear association between unadjusted hippocampal volumes and age. Previous studies have suggested that the linear effect of age is small, but still significant with negative correlations between age and volume in healthy cognitively preserved elderly [51], even if the rate of age-releated athrophy is suggested to be low ($\leq$ 0.2% per year) [52]. On the other hand, age effects and brain maturation do often show complex and non-linear patterns and other statistical models than the linear approach might be better suited for this purpose [53]. Furthermore, even if there were no significant association between age and unadjusted hippocampal subfield volumes in the present study, previous studies have found that structural changes of the hippocampus during development may contribute to age-related differences in episodic memory [54]. In healthy elderly, a positive relationship between preserved memory functions and hippocampus volume is generally supported, even if some studies found no such association or a negative association, for an overview see Kaup et al. [9].

Females perform generally better on tests related to episodic memory compared to males, and the CVLT II norms are adjusted for sex [16]. Data from the present study did not show any sex difference on adjusted CVLT II scores, but females performed better on the

recognition subtest based on the unadjusted raw scores. The lack of sex differences in the CVLT II raw score data may be attributed to the small sample size and the educational level of the participants. Furthermore, there are sex differences in hippocampal subfield volumes where females have larger volumes than males adjusted for intracranial volume, but males and females display similar decrease of hippocampal volumes with age [52]. In the present study, we adjusted for both the effects of sex and age in the correlation analyses between CVLT performance and volumetric data, and the results cannot inform about the impact of sex and age on episodic memory.

The main limitations of the present study are the small sample size and the large variability in age of the included participants. The Fisher r-to-z transformation tests showed that the distributions based on the correlations in the left and the right subfields did not differ significantly, even if the FDR-adjusted p-value from the correlations were significant. Furthermore, when using the FDR correction for controlling familywise error rates, there is a risk of type-II errors when rejecting correlations with p-values close to the FDR criterion. Hence, the generalizability of the findings is questionable and the results should be regarded as preliminary findings that need conformation in larger samples. Nonetheless, the significant results were in line with findings from studies with related methodology [8,15,17,18]. Several associations between hippocampal subfields volumes and verbal memory were close to significance, and a larger sample may have provided clearer findings. The Freesurfer segmentation process implemented in earlier versions (5.3 and earlier) of the software has received criticism for providing inaccurate estimates that conflicts with structural findings in anatomical studies [55]. In this study, we used Freesurfer 6.0 where the accuracy and correspondence with anatomical studies has been improved [31], and this version of Freesurfer has shown good test-retest reproductibility estimates for hippocampal segmentation in studies with large samples [56]. However, results based on 1 mm segmentation of internal subfields such as the CA4 should be interpreted with caution and further validation of the software with higher resolution should be performed to confirm the results. Thus, improvements in software and increased field-strength of MR-images might produce more accurate findings in future larger studies.

5. Conclusion

In summary, the present study showed that verbal learning, immediate- and delayed recall measured by the CVLT II had significant relations with separate subfields of the left hippocampus in healthy adults in the ages between 22–71 years. Furthermore, there were no significant associations between the right hippocampal subfields and verbal memory performance suggesting that auditory verbal memory is associated with volumetric lateralization effects in the hippocampus. The present results should be replicated in larger samples, and should be interpreted with caution due to the small sample size and large variability in age of the included participants.

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