UNIVERSITÀ
CAMPUS BIO-MEDICO DI ROMA

CORSO DI DOTTORATO DI RICERCA IN SCIENZE E INGEGNERIA PER L'UOMO E PER L'AMBIENTE / SCIENCE AND ENGINEERING FOR HUMANS AND THE ENVIRONMENT

Curriculum di *Bioscienze*

XXXVII CICLO

Prefrontal transcranial Direct Current Stimulation (tDCS) restores dopaminergic function and mitigates Alzheimer's Disease-like deficits in Tg2576 mice

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ANNO ACCADEMICO 2023/2024

INTRODUCTION

EXPERIMENTAL WORK

INTRODUCTION

1. ALZHEIMER'S DISEASE

Alzheimer's Disease (AD), representing one of the most profound challenges in contemporary neuroscience and medicine, is characterized by complex biological mechanisms and severe impacts on affected individuals. Beyond its classification as a neurological disorder, AD progressively impairs memory, cognition and personality, resulting in the gradual loss of an individual's sense of self and autonomy.

The first clinical recognition of AD dates back to 1906 when a pioneering German psychiatrist, Alois Alzheimer, presented the case of Auguste Deter, a 51-year-old woman exhibiting early signs of what would later be recognized as AD. He described clinical symptoms such as memory loss, personality changes and sleep disturbances, defining the condition as *"a peculiar and severe disease process of the cerebral cortex"*. Upon her death, Alzheimer analysed Deter's *post-mortem* brain with the help of advanced staining techniques developed by Franz Nissl, revealing hallmark pathological features: extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs), now recognized as the defining characteristics of AD (1–3). Alois Alzheimer's pioneering observations not only transformed our understanding of dementia but also ignited an enduring quest to decipher the complex mechanisms underlying this devastating condition. As scientific exploration advances, the aspiration to develop effective therapies that mitigate or prevent AD represents a critical frontier in medicine, embodying both the promise of innovation and the hope for a better future for patients and their families.

1.1 Epidemiology and Risk Factors

More than a century after its discovery, AD remains the most common form of dementia, accounting for approximately 60-70% of all cases (4). Currently, over 55 million individuals worldwide are living with dementia, and this number is projected to rise to 152 million by 2050. In 2019, the global economic burden of dementia was estimated at $1.3 trillion, with nearly half attributed to care provided by caregivers, underscoring the growing public health and economic impact of this condition (4–6).

While the exact mechanisms behind the progressive decline in brain function and cognition remain uncertain, the diverse range of symptoms points to the involvement of both genetic and environmental influences (7). As a result, AD is now recognized as a complex, multifactorial disorder with a highly individualized clinical presentation. Regardless, age remains the strongest risk factor for AD, with a prevalence of 38% among those aged 85 and older, and approximately 4% among individuals under 65 (6). Lifestyle and cardiovascular risk factors play a critical role in the pathogenesis of AD.

Conditions such as hypertension, diabetes, hypercholesterolemia, hyperlipidaemia and obesity, as well as habits like smoking and excessive alcohol consumption, are consistently associated with an elevated risk of developing AD (8,9). Conversely, protective factors have been identified, including regular physical activity, adherence to a Mediterranean-style diet rich in unsaturated fats and antioxidants and the maintenance of an active social and intellectual lifestyle (5,10,11). These behaviours are thought to mitigate risk by reducing inflammation, promoting vascular health and enhancing the cognitive reserve (CR). CR is a pivotal concept encompassing *neural reserve* (the structural and functional capacity of neural networks) and *neural compensation* (the ability to adapt cognitive strategies to counteract brain damage). Factors such as higher educational attainment, professional achievement and engagement in intellectually and socially stimulating activities contribute to building CR. Individuals with greater CR are thought to tolerate brain pathology longer before manifesting clinical symptoms, effectively delaying the onset of cognitive decline (12). Recent studies have also highlighted additional risk factors, including traumatic brain injuries and hormonal changes, particularly in women during menopause, when estrogen levels decline. Estrogens are hypothesized to play a protective role against neurodegeneration, potentially explaining the higher prevalence of AD among women compared to men (13).

While modifiable risk factors significantly influence the onset and progression of AD, genetic factors also play a crucial role in its development. AD, in fact, can be categorized into familial and sporadic AD based on the age of onset and the presence of specific genetic mutations. Familial AD is a form of Early-Onset AD (EOAD) that typically manifests in individuals under the age of 65, accounting for approximately 4-6% of all cases (10). This form of the disease is caused by autosomal dominant mutations in several key genes, most notably presenilin 1 (PSEN1), presenilin 2 (PSEN2) and Amyloid Precursor Protein (APP). These mutations result in an overproduction of amyloid beta (Aβ) peptides, which are prone to aggregation and are implicated in the pathological hallmark of AD, the formation of amyloid plaques (10,14). On the other hand, Late-Onset AD (LOAD), which normally affects individuals over the age of 65, is primarily associated with the presence of the ε4 allele of the apolipoprotein E (APOE) gene (15,16). APOE is a three-allele polymorphism (ε2, ε3 and ε4), with the ε4 variant being the strongest genetic risk factor for AD. The presence of this allele increases disease risk approximately threefold in heterozygotes and fifteenfold in homozygotes (17). The APOE gene encodes a protein that plays a central role in the transport of lipids and cholesterol within the brain, critical for maintaining neuronal health and synaptic function (18,19). Despite advances in understanding genetic contributions, the precise mechanisms underlying sporadic AD remain unclear, emphasizing the need for further research into the complex interactions between environmental influences and inherited predispositions. This complexity underscores the need for an

integrative approach to both research and clinical management, which should encompass not only the genetic and molecular underpinnings but also modifiable risk factors. Indeed, it is essential to develop personalized preventive strategies and early therapeutic interventions, tailored to an individual's specific risk profile, in order to delay or mitigate the onset and progression of the disease.

1.2 Symptomatology, Progression and Diagnosis of Alzheimer's Disease

AD is characterized by a long preclinical phase, with pathological changes occurring progressively over several years before the appearance of clinically evident signs (20–22). AD patients often exhibit significant changes in personality, neuropsychiatric symptoms (NPS; such as aggression, depression, apathy and psychosis), aphasia, alterations in circadian rhythms, loss of coordination, movement difficulties, epilepsy and seizures. Over time, cognitive symptoms emerge, including loss of orientation, impaired judgment and decision-making and memory deficits (10,23–25).

Although symptom variability exists, the disease course is marked by a progressive decline in cognitive, non-cognitive and motor functions, leading to a gradual loss of independence for patients. This progression is referred to as the "*Alzheimer's Disease continuum*" (**Fig. 1**; 6), which includes three stages:

- Preclinical AD (cognitively unimpaired or subjective cognitive decline);
- Mild Cognitive Impairment (MCI);
- Dementia, further classified into mild, moderate and severe stages, based on symptom severity.

The duration of each phase varies greatly and is influenced by conditions such as age, sex, genetics and risk factors (26).

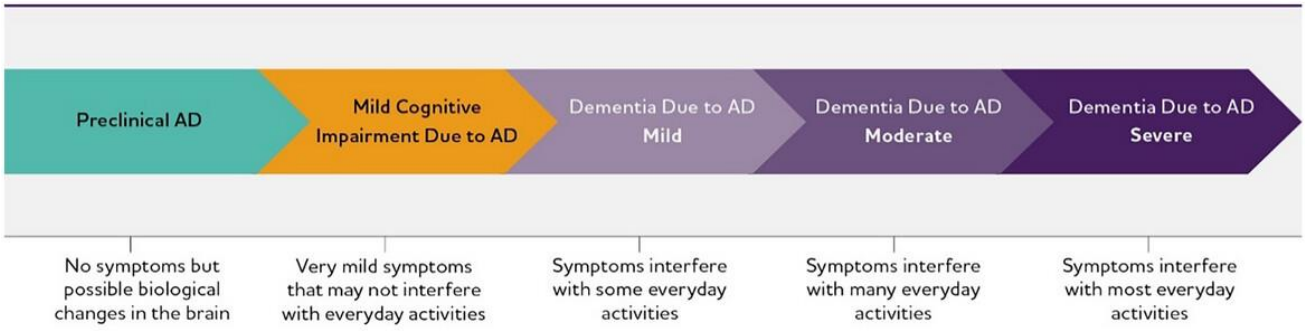

Figure 1 Alzheimer's Disease (AD) *continuum*. The image shows the progression of AD through five stages using equally sized arrows; however, this visual representation does not reflect the actual clinical and temporal variability of each phase, which can vary significantly. The preclinical phase can span years or even decades, as biological changes in the brain begin long before noticeable symptoms appear. Conversely, the moderate and severe dementia stages often progress more rapidly, with a pronounced decline in cognitive function and daily activities. Furthermore, the graphic simplifies the complexity of AD, which does not progress linearly. Symptoms can overlap between stages, and the disease's progression is influenced by individual factors such as genetics, age and overall health (6).

The preclinical phase of AD marks the onset of neurodegenerative processes in the absence of symptoms. During this stage, functional connections between various brain regions begin to deteriorate, which is why AD is also referred to as a "*Disconnection Syndrome*" (27). Compensatory mechanisms are activated to mitigate, at least initially, the neurodegeneration, thus delaying symptom onset.

In the MCI stage, first described by the neurologist Ronald Petersen (28), neuronal impairment and progressive brain atrophy emerge. Initially confined to cortical regions, these damages gradually extend to subcortical areas, such as the hippocampus. These changes occur when the brain is no longer able to compensate for the progression of the disease (6). During this phase, significant memory impairment and daily functional decline are not yet observed, although NPS—such as mood changes, depressive symptoms, apathy, increased anxiety and disruptions to the sleep-wake cycle—may appear. These can evolve into disorientation, confusion, aggression, agitation and hallucinations (29–31). The MCI stage is particularly crucial for early intervention, as individuals with MCI who exhibit NPS have a notably higher risk of progressing to AD. The appearance of these symptoms acts as a key prognostic indicator, suggesting that they should be considered as critical factors in predicting the trajectory of the disease. Therefore, the importance of recognizing and addressing these symptoms cannot be overstated, as they provide valuable insight into the future course of AD and may aid in preventing or delaying its onset (32).

In the dementia stage (mild and moderate), when neuronal atrophy extends beyond cortical and subcortical areas to include PFC and parietal cortex (PC), memory functions deteriorate and recognition of loved ones becomes increasingly difficult. Behavioural alterations become more prominent. In the later severe stage, extensive neuronal damage affects the entire brain, and patients are typically bedridden and no longer independent. Common complications include difficulties in bladder and bowel control, swallowing and the development of infections, such as aspiration pneumonia (6).

The diagnosis of AD currently involves a multidisciplinary team of specialists who use family history, comprehensive neuropsychiatric and cognitive testing and medical imaging techniques such as Magnetic Resonance Imaging (MRI) or functional MRI (fMRI), to rule out tumours, thyroid dysfunction, vitamin deficiencies or infections. Positron Emission Tomography (PET) is used to assess levels of Aβ and glucose metabolism alterations, while lumbar puncture allows the measurement of Aβ and tau protein levels in cerebrospinal fluid (CSF) (6). Despite these diagnostic methods, the diagnosis is often made at an advanced stage, when neuronal damage is already

extensive. For this reason, early diagnosis is crucial to prevent rapid cognitive decline and slow disease progression (33).

Over the years, diagnostic tools for AD have been refined significantly, culminating in the adoption of biomarker-based approaches that now allow for earlier and more accurate identification of the disease. The latest diagnostic criteria emphasize the use of Core 1 and Core 2 biomarkers, which provide a robust biological framework for detecting and staging AD pathology with unprecedented precision (34). In particular, Core 1 and 2 biomarkers differ in the timing of their pathological alterations and their specific clinical applications:

- Core 1 biomarkers, including amyloid PET imaging, CSF ratios such as Aβ42/40 and p-tau181/Aβ42, as well as advanced plasma-based assays of high precision (a prominent example being p-tau217), serve as early indicators of the disease. These biomarkers typically exhibit pathological changes prior to the onset of overt clinical symptoms, reflecting the hallmark neuropathology of AD (i.e. neuritic plaques and NFTs). They are indispensable for confirming an AD diagnosis in both symptomatic and asymptomatic individuals.
- In contrast, Core 2 biomarkers, which encompass imaging techniques like tau PET and specific soluble tau fragments (e.g., MTBR-tau243), become pathological at later stages of the disease. These biomarkers are closely linked to the emergence and progression of clinical symptoms, finding their primary utility in assessing the biological severity of the disease and its staging. However, unlike Core 1 biomarkers, Core 2 biomarkers are not generally employed as standalone diagnostic tools.

The integration of these two biomarkers sets anchors the diagnosis in a robust biological framework, representing an innovative and rigorous approach to enhancing diagnostic accuracy and reliability across the disease *continuum*. Indeed, this diagnostic paradigm reflects a sophisticated understanding of the molecular and clinical mechanisms underlying the pathology, marking a significant advancement in the management of AD.

1.3 Pathogenesis – Inside the Alzheimer's brain

As mentioned in the paragraph above, AD is microscopically characterized by two hallmark features: extracellular plaques and NFTs in the intracellular environment, predominantly located in medial temporal structures and the cerebral cortex and associated with extensive neuronal degeneration. In recent decades, research has sought to elucidate the pathogenic mechanisms underlying these lesions. The most studied mechanisms include the aggregation of Aβ peptides into

plaques and the hyperphosphorylation of tau protein, which is responsible for the formation of NFTs. Additional pathological processes involve neurovascular dysfunction, neuroinflammation, oxidative stress, mitochondrial dysfunction, neuronal death and the loss of synapses (10,35–37), all of which are believed to play a role in progressively exacerbating both cognitive and non-cognitive impairments (**Fig. 2**).

1.3.1 Neurobiological Alterations

Molecular analyses of neuronal plaques, following their morphological characterization, have identified Aβ as the principal peptide involved in plaque formation (38–40). Aβ consists of 40–42 amino acid residues and was initially believed to be exclusively produced under pathological conditions. However, nearly two decades ago, this view was fundamentally revised by the discovery that soluble Aβ is generated during normal cellular metabolism (41). Two major isoforms, Aβ40 and Aβ42, are present at low physiological levels throughout life. This observation suggests that Aβ has physiological roles that differ quantitatively and qualitatively from its detrimental effects when elevated in pathological states (42).

This finding has directed significant scientific attention toward the enzymes involved in APP processing, specifically α-, β- and γ-secretases, which are responsible for Aβ production. Among α-secretases, members of the ADAM (a disintegrin and metalloprotease) family—ADAM9, ADAM10 and ADAM17—have been identified as key enzymes (43). The β-secretase activity primarily resides in the membrane-bound aspartic protease BACE1 (β-site APP-cleaving enzyme 1) (44,45); γ-secretase is a multiprotein complex comprising PSEN1 and PSEN2, nicastrin, PEN-2 and APH-1. While presenilin forms the catalytic core of the complex, all components are essential for its activity. Loss or dysfunction of any single component abolishes the enzymatic function (46–49). APP is a transmembrane protein that undergoes extensive post-translational modifications during its transport from the endoplasmic reticulum to the plasma membrane. These modifications include proteolytic cleavages that release protein fragments into extracellular spaces or intracellular vesicles.

APP can be cleaved along two distinct pathways (50):

- The Non-Amyloidogenic Pathway: α-secretase cleaves APP at residue 83 of the C-terminus, producing a large extracellular fragment (sAPPα) and a membrane-anchored C83 fragment. The C83 fragment is further processed by γ-secretase into a small extracellular fragment (p3) and an intracellular domain (AICD), which is subsequently degraded.
- The Amyloidogenic Pathway: β-secretase cleaves APP at residue 99 of the C-terminus, releasing an extracellular N-terminal fragment (sAPPβ) and a membrane-anchored C99

fragment. This C99 fragment is then cleaved by γ-secretase between residues 38 and 43, producing extracellular Aβ peptides of 40 or 42 amino acids (Aβ40 and Aβ42) and an intracellular domain that undergoes cytoplasmic degradation. Notably, Aβ40 accounts for the majority of secreted peptides, while Aβ42 comprises approximately 10%.

Alongside Aβ, hyperphosphorylated tau protein has been identified as the primary constituent of intracellular NFTs. NFTs are composed of bundles of paired helical filaments of the microtubule-associated protein tau (51,52), a cytoskeleton-associated protein critical for microtubule polymerization and stabilization (53–55). Tau binds microtubules via specific domains, ensuring their assembly and stability. The phosphorylation of tau is tightly regulated by kinases (e.g., GSK-3β, CDK5) and phosphatases (e.g., PP-1, PP-2A) (56). Hyperphosphorylation disrupts this balance, leading to tau detachment and microtubule disassembly. This causes impaired axonal transport and neuronal dysfunction. However, similar to Aβ, it remains unclear whether tau hyperphosphorylation and tangle formation are causative or secondary to the progression of AD.

1.3.2 Neurodegenerative Processes and Neuronal Loss: exploring the Amyloid Cascade Hypothesis

The correlation between the presence of abnormal protein deposits in the brain—believed to have neurotoxic potential—and the clinical manifestations of AD has established Aβ plaques and NFTs as the disease-defining pathological features. These findings laid the groundwork for the "Amyloid Cascade Hypothesis (ACH)", a pivotal framework proposed in the early 1990s that has shaped the understanding of AD. The hypothesis posits that the accumulation of Aβ peptides in the brain plays a central role in initiating a pathological sequence of events, culminating in progressive cognitive decline (25; **Fig. 2**). The cascade begins with the overproduction or impaired clearance of Aβ, leading to the formation of toxic aggregates. These aggregates disrupt synaptic function, trigger neuroinflammation and promote tau hyperphosphorylation and NFT formation. In fact, Aβ peptides are not inert deposits but actively interact with several key neuronal receptor systems, like the α7 nicotinic acetylcholine receptor (α7-nAChR), ionotropic NMDA (N-methyl-D-aspartate) receptors, metabotropic glutamate receptors (mGluR5) and β-adrenergic receptors (β-AR). These interactions initiate intracellular signalling cascades that propagate neurotoxic effects, including dysregulation of calcium homeostasis, oxidative stress and the activation of apoptotic pathways (57–62). Such dysregulation undermines neuronal survival, leading to synaptic dysfunction and eventual neuronal death (59,63,64), core features of AD.

Beyond the direct neurotoxic effects, Aβ oligomers and fibrils are recognized by microglial pattern-recognition receptors (e.g., TREM2, CD36). This interaction triggers microglial activation

and an acute inflammatory response aimed at clearing Aβ aggregates. While initially protective, sustained microglial activation results in chronic neuroinflammation. This chronic inflammatory state involves prolonged secretion of pro-inflammatory cytokines such as IL-1β, TNF-α and chemokines, as well as astrocyte reactivity, further amplifying neuronal damage and contributing to a deleterious feedback loop between neuroinflammation and neurodegeneration (65,66).

Aβ also perturbs synaptic networks by altering neuronal electrical activity and fostering network desynchronization even before the overt formation of plaques. Such aberrant activity can stimulate the excessive synaptic release of Aβ, perpetuating its accumulation. This bidirectional interplay suggests a vicious cycle wherein Aβ aggregation and network hyperexcitability mutually reinforce each other. The presence of Aβ plaques in regions exhibiting aberrant connectivity underscores their potential role in driving pathological neuronal hyperactivity and subsequent synaptic remodelling (67,68).

Acknowledging the centrality of Aβ in AD pathology, the U.S. Food and Drug Administration (FDA) has recently approved anti-Aβ monoclonal antibodies as therapeutic drugs. These therapies, aiming at the reduction of brain Aβ, have demonstrated moderate efficacy in slowing cognitive decline in patients with early-stage AD. However, the treatment is associated with dose-dependent side effects, including amyloid-related imaging abnormalities (ARIA), oedema and other systemic complications, underscoring the need for further evaluation in extended clinical trials (69,70).

Importantly, recent paradigms suggest that while Aβ accumulation is a critical factor in AD, it cannot fully account for the disease's complexity. Rising evidence highlights the role of tau pathology, neuroinflammation, synaptic dysfunction, metabolic disturbances and vascular contributions, necessitating a broader framework to contextualize Aβ's role within a multifactorial disease mechanism. This reconceptualization supports a system-level approach, integrating Aβ-related pathways with other biological processes underlying cognitive and functional decline that defines the clinical course of AD (25).

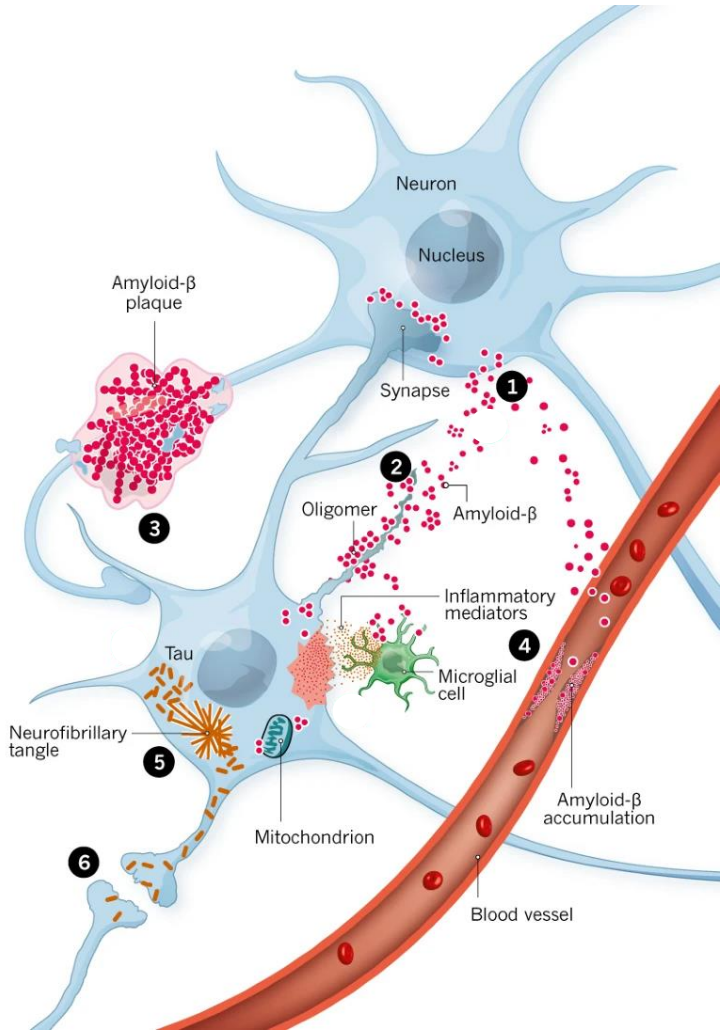

Figure 2 The neuropathology of AD. The image provides a visual representation of the key cellular processes associated with AD:

1. The Amyloidogenic Pathway is the initial step in the pathological cascade of AD, involving the cleavage of APP in the neuronal membrane and resulting in the release of Aβ peptides.
2. Once in the extracellular space, Aβ forms oligomers, which are thought to disrupt synaptic function, leading to synaptic loss and impairing communication between neurons.
3. These oligomers aggregate into plaques that further accumulate in extracellular spaces, interfering with the proper functioning of neurons and contributing to AD progression.
4. Amyloid-β deposits also appear in the blood vessels, where they activate microglial cells. These immune cells release inflammatory mediators, contributing to neuroinflammation and worsening neuronal damage.
5. Misfolded tau proteins accumulate inside neurons as NFTs, which disrupt mitochondrial transport contributing to neurodegeneration.
6. Finally, tau can spread from neuron to neuron through synapses, causing further misfolding and damage.

The image captures key events at the cellular level but does not fully illustrate their systemic consequences. Although the pathological processes are depicted within a single neuron, AD involves extensive brain networks, eventually leading to widespread degeneration and functional decline across the brain (adapted from **(71)**.

2. The Dopaminergic System and Its Role in Alzheimer's Disease

The dopaminergic system, historically associated with movement regulation and reward processing, has garnered increasing attention for its involvement in cognitive and emotional functions. Emerging evidence suggests that dysfunction within the dopaminergic system may contribute to the pathophysiology of AD. Thus, understanding the anatomy and functioning of the dopaminergic circuit provides a critical foundation for exploring its role in AD progression.

2.1 The Dopaminergic System

The dopaminergic system is a complex network of neurons that use dopamine (DA) as their primary neurotransmitter. This system is crucial for regulating motor activity, reward mechanisms and higher-order cognitive functions. Dopaminergic neurons originate predominantly in two midbrain nuclei: the Substantia Nigra pars compacta (SNpc) and the Ventral Tegmental Area (VTA). These nuclei give rise to distinct dopaminergic pathways that project to various regions of the brain, forming specialized circuits with unique functional roles.

2.1.1 Anatomy of Dopaminergic Circuits

The mesencephalic dopaminergic neurons, first mapped by Dahlström and Fuxe in 1964 (72), represent the primary source of DA, an endogenous monoamine neurotransmitter in the Central Nervous System (CNS) belonging to the catecholamine family, which also includes norepinephrine (NE) and epinephrine. DA is synthesized through a two-step process, beginning with the hydroxylation of the amino acid L-tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH), leading to the production of L-3,4-dihydroxyphenylalanine (L-DOPA), which is subsequently decarboxylated into DA by aromatic amino acid decarboxylase (73).

In the midbrain, DA-releasing neurons are anatomically and functionally heterogeneous; they are located in different subfields and are grouped into three nuclei (74):

- A8 (retrorubral field), which projects to the striatum and various areas of the limbic system. Neurons in the retrorubral field account for only 4-5% of the total DA neurons in the brain, and their function remains largely unclear;
- A9 (SNpc), which primarily projects to the caudate nucleus and putamen (also known as the dorsal striatum), mediating the control of voluntary movements (75). It is part of the **nigrostriatal system** and functionally integrated with the basal ganglia; its degeneration has been extensively studied in the context of Parkinson's Disease (PD) (76);

- A10 (VTA), which is part of the **mesocorticolimbic system** (**Fig. 3A**), with diffuse projections to the Nucleus Accumbens (NAcc), hippocampus, septum, amygdala and olfactory tubercle *(mesolimbic system)*, as well as to the cingulate cortex (CC), entorhinal cortex (EC) and prefrontal cortex (PFC) *(mesocortical system)* (77–79). These two pathways, largely overlapping (hence the term *mesocorticolimbic system*), are involved in reward and motivation, memory and cognition, mood regulation, affect, emotional response and social behaviours (74,80–82).

The Locus Coeruleus (LC) also produces DA due to the presence of noradrenergic TH-positive (TH^+) neurons, which co-release both DA and NE (74,83–85). This dual neurotransmitter release plays a pivotal role in a variety of essential cognitive and non-cognitive functions, including memory consolidation, emotional processing, attention and regulation of the sleep-wake cycle. The dopaminergic projections from the LC innervate extensive regions of the brain, such as the cortex, hippocampus, brainstem and spinal cord, thus contributing significantly to the integration of neural processes that underpin behaviour and neural plasticity (84–86).

Both of the aforementioned pathways (nigrostriatal and mesocorticolimbic system) demonstrate extensive connectivity with other neural circuits, including those involved in memory, attention and executive function. This anatomical and functional integration underscores the potential impact of dopaminergic dysfunction on multiple domains.

2.1.2 The VTA mesocorticolimbic dopamine system

The VTA is an essential component of the midbrain, recognized for its significant role in multiple neural processes due to its extensive connections with various brain regions. Located adjacent to important structures like the mammillary bodies, posterior hypothalamus and the oculomotor nerve fibers, the VTA also connects to other areas of the brain, notably through the forebrain medial bundle (MFB), which links it to the brainstem nuclei (**Fig. 3A**).

The VTA can be subdivided into medial and lateral portions, with distinct nuclei: the parabrachial pigmented nucleus (PBP) and the paranigral nucleus (PN) in the lateral part and the interfascicular nucleus (IF) in the medial part (87). These regions contribute to the overall functions of the VTA, with the PBP, PN and IF comprising the majority of the VTA's volume.

Functionally, the VTA is highly heterogeneous, with approximately 50-80% of its neurons being dopaminergic, while the remainder includes GABAergic (30%) and glutamatergic (5%) neurons (88). This diversity contributes to the complexity of the VTA's role in behaviour and its neurochemical

composition, making it challenging to define the specific contributions of each given subpopulation clearly. Thus, the VTA is viewed more as a functional area rather than a distinct nucleus (89–91). The VTA's neuronal diversity also extends to its electrophysiological properties and synaptic inputs, with some dopaminergic neurons co-releasing glutamate or GABA, adding complexity to the region (92,93). Due to its intricate structure and function, the VTA continues to be a focal point for research, furthering our understanding of its critical role in the brain's dopaminergic system and its involvement in behaviour, cognition and neurodegenerative conditions.

As mentioned above, the VTA plays a vital role in regulating a wide range of behaviours through the mesocorticolimbic dopaminergic pathway. These include motivation, reward, pleasure, attention, emotional processing, mood regulation, cognitive, executive and memory functions (94–100). The mesolimbic pathway, which connects the VTA with the limbic areas, is also often referred to as the *"reward system",* since it is crucial in processes such as motivation, addictive behaviours and aversion. Particularly, the DA signalling in the NAcc appears to play a crucial role in regulating emotional processes, including motivation, reinforcement of rewarding experiences and the regulation of mood (101). Dysregulation of DA signalling in this area has been linked to disorders such as food and drug addiction, when the brain's reward system is overactive or altered, leading to compulsive behaviours (96).

Technological advances, such as virus-based tracing, electrophysiology and optogenetics (102–106), have allowed researchers to better understand the broader role of DA in behaviour, going beyond addiction and reward to include integrative aspects of cognition. For example, VTA projections to the PFC are essential for cognitive functions like decision-making, attention, goal-directed behaviours and executive functions (97,100,107–110). Additionally, the dopaminergic projections from the LC and VTA to the hippocampus are involved in the regulation of mnemonic functions and learning—including novel object and environment recognition—spatial memory, parvalbumin-positive interneuron (PV-IN) excitability and also in stress response (84,85,111–116).

Thus, alterations in DA signalling in the mesocorticolimbic pathway can contribute to a wide range of cognitive and behavioural symptoms, such as memory impairments, mood disturbances, sleep issues and brain hyperexcitability (117–119). Given its role in emotional processing and mood regulation, the mesocorticolimbic system is also particularly implicated in disorders like anxiety, schizophrenia and depression (120–122). This highlights the complex and integrative role of the VTA and its dopaminergic projections in various brain functions and disorders.

DA and its receptors play also a pivotal modulatory role as an immune regulator by influencing diverse inflammatory pathways and immune cell dynamics. Emerging evidence underscores DA's capacity to inhibit neuroinflammatory responses, particularly through its interaction with activated microglia, while conversely, proinflammatory molecules can attenuate dopaminergic transmission, thereby establishing a bidirectional interplay between neuroinflammation and dopaminergic signalling (123). This relationship is especially pertinent in neurodegenerative diseases such as PD and AD, where neuroinflammatory processes contribute to the progressive degeneration of dopaminergic neurons (124). Central to DA's anti-inflammatory action is the inhibition of the Nucleotide-binding oligomerization domain-Like Receptor Pyrin domain-containing 3 (NLRP3) inflammasome, a key mediator of innate immune responses. DA receptors, categorized into D1-like and D2-like families, differentially modulate inflammatory processes. DA exerts this regulatory effect primarily *via* the D1-like receptor, which activates downstream cyclic AMP (cAMP) signalling pathways, leading to NLRP3 ubiquitination and subsequent proteasomal degradation (124–126). This mechanism effectively suppresses NLRP3 inflammasome-dependent inflammation, as demonstrated in both neuroinflammatory and systemic inflammatory contexts. In addition, DA also modulates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, further attenuating inflammatory signalling (127). On the other hand, D2-like receptors regulate the renin-angiotensin system in glial cells, inhibiting angiotensinogen production in astrocytes and modulating microglial angiotensin receptor activity. This modulation reduces the activity of the pro-inflammatory AT1/NADPH-oxidase/superoxide axis, thereby mitigating oxidative stress and inflammation (128). In this context, the inherent anti-inflammatory and antioxidant properties of DA have made it a promising candidate for therapeutic intervention in inflammation-associated diseases (129).

These findings highlight the potential of DA and its receptors as critical molecular targets for the treatment of neurodegenerative conditions and advocate for continued research integrating dopaminergic and neuroinflammatory imaging techniques to further elucidate this complex interplay.

2.2 Alterations of the Dopaminergic System in Alzheimer's Disease

For decades, the role of DA systems in AD has captivated scientific inquiry (130–133), driven by both post-mortem brain studies and groundbreaking animal model research (130,132,134). These investigations have yielded valuable insights into the complex interplay between DA signalling and the pathophysiology of AD, shedding light on potential therapeutic avenues.

As the brain ages, a natural decline in DA release from terminals is observed, accompanied by a reduced expression of DA receptors, particularly D2 receptors, and a decrease in DA transporter (DAT) expression within key brain regions such as the striatum, hippocampus and frontal cortex

(135–137). This decline in dopaminergic function is not surprising, given the crucial role of DA in hippocampal-dependent memory and neural plasticity. Indeed, numerous studies have linked AD to disruptions in mesocorticolimbic DA signalling, affecting both patients and experimental AD models (111,112,114,138,139). Post-mortem examinations of AD brains reveal a marked reduction in DA receptors, DAT and TH in the PFC, hippocampus and, notably, the NAcc, while the nigrostriatal pathway and dorsal striatum remain relatively unaffected (**Fig. 3B**; 131,140–143). This phenomenon has led to significant interest in the possibility that early alterations in the mesocorticolimbic dopaminergic system could play a role in the progression of AD. Consequently, research has increasingly focused on identifying early-stage DA dysfunctions as potential biomarkers or therapeutic targets in AD, sparking a wealth of preclinical and clinical studies to deepen this hypothesis.

2.2.1 Preclinical Evidence of Dopaminergic Dysfunction in Alzheimer's Disease

Preclinical research has been instrumental in uncovering the role of the dopaminergic system in AD, providing a detailed mechanistic framework to understand its involvement in the disease's onset and progression. Studies using validated transgenic animal models, have highlighted early and specific vulnerabilities within the dopaminergic system, particularly affecting the VTA.

Research from our laboratory showed in Tg2576 mice, a widely used AD model that overexpresses the human APP with the Swedish mutation (K670M/M671L), dopaminergic deficits emerging well before overt amyloid plaque formation. This mouse model is particularly suited for studying the early stages of AD, due to the slow progression of the pathology. As early as 3 months of age, these mice exhibit a reduction in VTA DA neuron numbers, with degeneration worsening progressively with age (144–146). This loss of dopaminergic inputs correlates with reduced DA outflow and diminished TH expression in critical projection regions such as the NAcc and hippocampus (145–148). Such disruptions manifest as reduced excitability of subicular pyramidal neurons, downstream glutamatergic transmission deficits, impaired synaptic plasticity—most notably in long-term potentiation (LTP) at CA3-CA1 synapses—as well as a reduction in dendritic spine density and significant cognitive, memory and reward deficits, reflecting early AD pathophysiology (144,145,147,149–153). Notably, these alterations cannot be attributed to amyloid plaque deposition, as plaques are not observed in this model until approximately 11 months of age (149). An intriguing aspect of this model is the preservation of synaptic plasticity at ventral CA3–CA1 synapses, which contrasts with the significant impairments observed in the dorsal region. This dorsal-ventral distinction may reflect differences in dopaminergic input, as the ventral hippocampus shows relatively higher dopaminergic tone even in the context of VTA degeneration (154). Moreover, the

loss of dopaminergic modulation leads to hippocampal hyperexcitability. This occurs because reduced DA availability in hippocampus impacts on PV-INs, diminishing their firing activity, and on perineuronal networks that protect these INs, which instead regress, disrupting functional connectivity and destabilizing hippocampal network dynamics in Tg2576 mice (116).

A defining feature of AD-related dopaminergic dysfunction is the selective vulnerability of VTA neurons compared to other dopaminergic populations—such as those in the SNpc or the LC— which remain intact at least until 7 months of age (145,155,156). Research into the increased vulnerability of VTA dopaminergic neurons in AD is ongoing, but certain features make these neurons more susceptible (157–159). Their long, often unmyelinated axons and pacemaker activity demand high energy and efficient mitochondria (160). Disruptions in mitochondrial function and autophagy can lead to the accumulation of damaged organelles, excess calcium release and reactive oxygen species (ROS), exacerbating neuronal damage. Our lab demonstrated that VTA dopaminergic neurons in Tg2576 mice show impaired autophagic flux, accumulation of damaged mitochondria and cytoplasmic Ca^{2+} dysregulation, overall increasing neuronal vulnerability (146,148). In parallel to these deficits, VTA neurons in Tg2576 mice also exhibit electrophysiological abnormalities (146–148). These impairments culminate in apoptosis mediated by apoptosis-inducing factor (AIF), highlighting a cell-autonomous mechanism underlying their degeneration (146).

Similarly, 3xTg-AD mice, which model both Aβ and tau pathology, demonstrate cortical DA depletion due to the loss of TH^+ terminals. This depletion contributes to pronounced cognitive impairments and deficits in cortical plasticity, suggesting that the disease interferes with dopaminergic neuromodulatory processes (161). Intriguingly, pharmacological interventions aimed at restoring dopaminergic balance, such as the administration of DA receptor agonists like apomorphine or reuptake inhibitors like Nomifensine, have shown promise in rescuing these deficits. These treatments highlight DA's essential role in maintaining cortical and hippocampal integrity (162,163).

Interestingly, preclinical models have also revealed compensatory adaptations within the dopaminergic system. For instance, 3xTg-AD mice show an upregulation of D2/3 autoreceptors in response to reduced DA release, suggesting an attempt to mitigate neurotransmitter deficits (164). Similarly, Tg2576 mice show increased expression of the D1 receptor in the hippocampus (145). Such compensatory changes may represent potential therapeutic windows for interventions aimed at enhancing dopaminergic tone.

Indeed, therapies targeting the dopaminergic system have shown considerable promise in preclinical settings. Sub-chronic *in-vivo* treatment of Tg2576 mice with L-DOPA (the natural DA

precursor) or selegiline (an inhibitor of DA catabolism) have been effective in improving hippocampal-dependent memory, synaptic plasticity and reward behaviours (116,145,147). Moreover, treatments that prevent the loss of TH^+ VTA neurons have successfully preserved DA release in the dorsal hippocampus (146), further underscoring the importance of VTA integrity in AD-related cognitive decline.

The dysfunction of the midbrain dopaminergic system extends beyond localized impairments, contributing to a breakdown of broader brain networks. EEG studies in 5xFAD mice reveal altered cortical activity patterns closely associated with midbrain DA neuron degeneration and reduced DAT levels in projection areas (165). Similarly, in Tg2576 mice, hippocampal gamma rhythms—critical brain oscillations in the coordination of neuronal communication, synaptic plasticity, cognitive processing and memory encoding—are significantly impaired because of the degeneration of midbrain dopaminergic inputs and the resulting hippocampal hyperexcitability. Importantly, pharmacological treatments targeting dopaminergic pathways—such as L-DOPA or sumanirole administration— can reestablish the excitation/inhibition balance and, consequently, restore gamma oscillations to near-normal levels (116). These findings further underscore the critical role of the midbrain dopaminergic input in sustaining the rhythmic activity of the hippocampus and, thus, the integrity of larger networks, the disruption of which may, in turn, contribute to the progression of cognitive deficits and NPS in AD by accelerating its progression.

The wealth of data from preclinical studies highlights the central role of dopaminergic system dysfunction in AD. The early and selective degeneration of VTA DA neurons, coupled with the downstream effects on synaptic plasticity and network integrity, underscores the importance of this neurotransmitter system in maintaining cognitive health. As research progresses, targeting dopaminergic dysfunction presents a promising avenue for therapeutic intervention, with the potential to delay or mitigate the cognitive decline associated with AD.

2.2.2 Clinical Evidence of VTA Dysfunction in Alzheimer's Disease

Preclinical evidence suggesting that defects in the VTA play a pivotal role in the progression of AD were corroborated also in clinical studies, which highlighted the early involvement of the VTA in human AD. Structural and functional imaging studies have been instrumental in shedding light on the VTA's role in AD. Resting-state functional MRI (rs-fMRI) and structural MRI studies have consistently shown that patients with amnestic-MCI (aMCI) display a reduced VTA volume, which is associated with a diminished functional connectivity between the VTA and several brain regions critical to memory and cognition, such as the hippocampus, parietal cortex and thalamus (166,167). Notably, this disconnection pattern becomes more pronounced as the disease progresses. In this

regard, longitudinal studies have shown, through PET imaging, that early dopaminergic dysfunction, including alterations in VTA connectivity, is associated with an increased risk of developing AD (168). For instance, aMCI patients who later converted to AD showed significantly reduced connectivity between the VTA and critical regions of the default-mode network (DMN)—a network of brain regions active during rest and involved in self-referential thought and memory processes—such as the posterior cingulate and precentral cortex, supporting the notion that VTA dysfunction is tied to early behavioural and emotional disturbances in AD progression, thus its connectivity loss can serve as a biomarker for predicting the progression from aMCI to AD (168–170).

Subsequent research revealed that the mesocorticolimbic dopaminergic system, which connects the VTA to regions implicated in emotion and motivation shows significant atrophy and metabolic deficits in both MCI and AD patients. These deficits are associated with memory performance and the expression of NPS, as shown by studies using structural MRI and ^{18}F-FDG-PET. For instance, Iaccarino et al. (171) found evidence of widespread loss of grey matter and reduced glucose metabolism in VTA targets within the medial temporal lobe and emotional regulation areas. In contrast, the nigrostriatal pathway was relatively spared, showing fewer alterations. This suggests a selective vulnerability of the mesocorticolimbic system in AD, consistent with the previous preclinical findings (171,172). Additionally, neurochemical alterations in the dopaminergic system—specifically DAT density—have been found to correlate with disease severity. Using Single-Photon Emission Computed Tomography (SPECT) with the radiolabelled tracer [123I]FP-CIT, a study by Sala et al. (173) demonstrated significant reductions in DAT binding in the ventral striatum and hippocampus in both aMCI and AD patients compared to healthy controls. The study also highlighted a loss of connectivity between the mesocorticolimbic network and the cingulate gyrus, particularly in the advanced stages of AD. This suggests that early dopaminergic dysfunction, specifically within the mesocorticolimbic pathway, may be a sensitive marker of disease progression and NPS.

Another important aspect to consider is the potential for early pharmacological intervention targeting dopaminergic dysfunction in the early stages of AD, particularly during the MCI phase. The possibility of using dopaminergic treatments during the MCI stage is highlighted as a promising strategy to delay the progression to AD, improve quality of life and mitigate DA-related symptoms. Although some dopaminergic drugs have demonstrated positive effects in mild-AD patients (133,137,174–177), their capacity to prevent or delay the conversion from MCI to AD has not been rigorously tested. Earlier trials of dopaminergic drugs have predominantly targeted cognitive deficits rather than addressing NPS. In fact, there is no FDA-approved medication specifically for NPS in AD. Current treatment often involves the use of anxiolytics, atypical antipsychotics and

antidepressants or mood stabilizers. These drugs, though prescribed off-label, have questionable efficacy and often produce serious side effects, making them suboptimal for AD management (178–182). Thus, there arises an urgent need for dedicated clinical trials that evaluate treatments specifically for NPS in MCI and AD stages. Promising research is emerging, particularly focusing on catecholamine reuptake inhibitors such as Methylphenidate, a DAT inhibitor, which has shown potential in improving apathy and other NPS in early-stage AD patients. Studies have indicated that Methylphenidate can improve cognitive performance, reduce apathy and alleviate depressive symptoms in mild AD, suggesting that dopaminergic modulation could offer a viable therapeutic approach for treating some of the neuropsychiatric manifestations of the disease (183–186).

In summary, the VTA and its dopaminergic circuits are crucially involved in the pathophysiology of AD. Evidence from imaging studies, neurochemical assessments and longitudinal analysis clearly indicates that dopaminergic dysfunction is an early and significant event in AD progression. The loss of VTA connectivity to key brain regions, especially the DMN, is a hallmark of cognitive decline and the emergence of NPS, highlighting the potential of targeting the VTA (187,188).

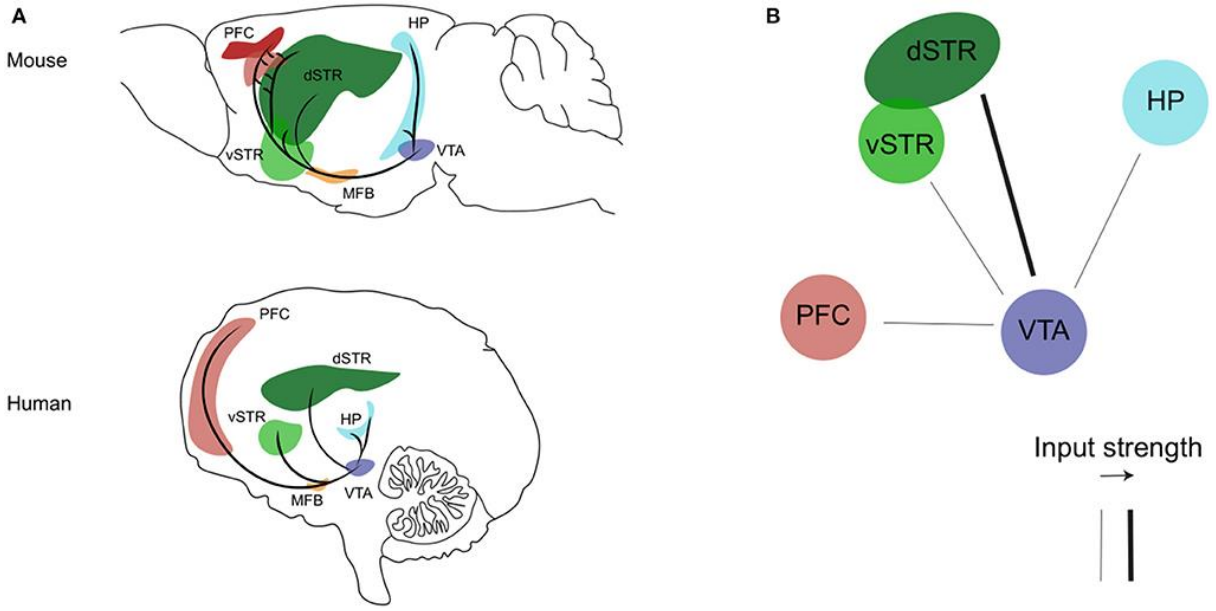

Figure 3 VTA Dopaminergic circuits in murine and human brains.

A) Schematic comparison of the mouse (upper) and human (bottom) brain, highlighting the major dopaminergic circuits. Projections originate from the VTA (purple) and extend to the hippocampus (HP, light blue), PFC (red), dorsal striatum (dSTR, dark green) and ventral striatum (vSTR, light green) *via* the MFB (orange). **B)** Simplified representation of functional connections between brain regions. illustrating the progressive weakening of VTA dopaminergic inputs (thin lines) to downstream regions in AD. The connectivity strength is represented by line thickness, highlighting significant reductions in dopaminergic influence particularly to the HP and vSTR, regions involved in memory and emotional regulation. This alteration mirrors early dysfunctions of the mesocorticolimbic system, potentially preceding overt neuronal loss in target areas. Since the MCI stage, these disruptions may contribute to memory deficits, NPS and broader functional disconnections, emphasizing their potential role as biomarkers for early AD diagnosis (189).

3. Transcranial direct current stimulation (tDCS): a new frontier of neurotherapy

Transcranial Direct Current Stimulation (tDCS) has emerged as a groundbreaking approach in the field of neurotherapy, offering a versatile and non-invasive method to modulate brain activity. Actually, this technique is part of a broader category of non-invasive brain stimulation (NIBS) methods, which have emerged as transformative tools in neuroscience and clinical research, providing the ability to modulate brain activity without surgical intervention.

3.1 The Rationale Behind transcranial Electrical Stimulation (tES)

The concept of harnessing electricity for curative purposes dates back to ancient times, with the earliest recorded experiments involving electricity for therapeutic purposes occurring in the Greco-Roman era. More specifically, torpedo fish were applied to patients' skin to deliver natural electric shocks, easing pain and other ailments such as headaches and gout (190,191). This ingenious use of bioelectricity foreshadowed today's sophisticated NIBS techniques, which now leverage targeted currents to unlock new possibilities in brain modulation and therapy.

Each NIBS technique is defined by its specific type of current and mechanism of action, broadly categorized into Transcranial Magnetic Stimulation (TMS) and Transcranial Electrical Stimulation (tES), each offering distinct methods to modulate brain activity. TMS uses rapidly alternating magnetic fields to induce localized electrical currents in the brain, modulating neuronal activity in an activity-dependent manner (192,193). This allows TMS to target specific cortical areas with high spatial precision, making it a powerful tool for studying localized brain functions and neuroplasticity. tES, on the other hand, applies low-intensity electrical currents directly to the scalp to influence neuronal excitability (194,195). It encompasses three main modalities: tDCS, transcranial Alternating Current Stimulation (tACS) and transcranial Random Noise Stimulation (tRNS): tDCS delivers a constant, direct current, modulating the resting membrane potential of neurons to either enhance (anodal stimulation) or inhibit (cathodal stimulation) neuronal excitability in a diffuse, non-focal manner (196–198); tACS employs sinusoidal currents at specific frequencies to entrain neuronal oscillations, making it particularly effective for modulating rhythmic brain activity associated with specific cognitive and behavioural functions (199–201); finally, tRNS uses random, high-frequency electrical noise to enhance neural plasticity through stochastic resonance, promoting increased sensitivity and connectivity across neuronal networks (202,203).

Understanding and optimizing tES is crucial for both preclinical and clinical research. In preclinical studies, tES provides a powerful tool for unravelling the mechanisms of brain plasticity

and neural network dynamics, especially in models of neurological diseases such as AD and PD. Clinically, tES holds promise for developing non-invasive, non-pharmacological, cost-effective therapies for a wide range of neurological and psychiatric conditions. Advancing research in this area will not only improve our understanding of brain function but also refine therapeutic interventions to maximize efficacy and safety.

3.1.1 Side Effects and Safety Profile

tES is widely recognized as a safe and well-tolerated neuromodulatory technique. Its safety profile is supported by extensive preclinical and clinical evidence, demonstrating minimal adverse effects. Among these, the most frequently reported are mild and transient and include itching, tingling or a mild burning sensation at the electrode sites, which are generally attributed to skin impedance and localized heating during stimulation, Additionally, some individuals may experience temporary redness at the electrode contact points due to prolonged application of electrodes, headaches, and, in rare cases, transient fatigue (204–206). These effects are typically self-limiting and resolve without medical intervention, underscoring the reliability of tES as a non-invasive approach to brain modulation (204,207,208). Beyond these minor side effects, rigorous evaluations in various populations—including healthy adults, children and individuals with neurological or psychiatric conditions—have consistently affirmed the absence of major adverse events. Such findings provide a foundation for the safe implementation of tES across diverse populations.

Today there are carefully delineated safety parameters to mitigate potential risks. Recommended current intensities are restricted to 1–2 mA, with session durations not exceeding 20–30 minutes (207). These limits ensure a low risk of adverse outcomes such as tissue damage or prolonged neural overstimulation. Preclinical studies in animal models have further validated this profile, indicating no histopathological alterations even with repeated or high-intensity stimulation protocols (209).

While adverse events associated with tES are exceedingly rare, individual-specific considerations—i.e., age, baseline neurophysiological state and underlying medical conditions—are critical for its safe application (210). For instance, patients with implanted electronic devices, such as pacemakers or deep brain stimulators, require careful consideration to prevent electromagnetic interference. Additionally, although rare, individuals with epilepsy or conditions associated to heightened cortical excitability may exhibit a marginally increased risk of seizure induction. Furthermore, polarity-dependent effects of tES may inadvertently influence non-targeted brain regions; for example, enhancing excitability in one cortical area might suppress activity in adjacent regions, potentially disrupting network-level dynamics (211).

The long-term implications of repeated tES sessions remain a critical area of ongoing investigation. While repeated stimulation is essential for achieving sustained therapeutic benefits, concerns persist regarding the cumulative impact on neural homeostasis and potential maladaptive plasticity in non-targeted regions. Emerging studies incorporating advanced neuroimaging, computational models and biomarkers aim to elucidate these effects, refining stimulation protocols to maximize efficacy and safety. By integrating these insights, researchers aim to address any residual concerns about neural homeostasis and maladaptive plasticity while maximizing the therapeutic potential of this versatile neuromodulatory tool. Evidence so far suggest that the technique is well-tolerated even with prolonged application. Indeed, long-term studies on healthy individuals have reported no significant risks, and the incidence of adverse events in active tES groups was often equivalent to or even lower than that of sham groups (212,213).

3.2 Focus on transcranial Direct Current Stimulation (tDCS).

Despite the groundbreaking discovery of electrical neuromodulation dating back to ancient times, which gained renewed attention with the advent of the electric battery in the 18th century, brain stimulation techniques then entered a period of experimental obscurity. This was primarily due to inconsistencies in experimental protocols, insufficient standardization and the lack of advanced tools for precise control and measurement, which hindered scientific progress. However, the field experienced a revival in the early 2000s, catalysed by technological advancements in NIBS. This resurgence was driven primarily by two landmark studies, Priori et al. (196) and Nitsche & Paulus (197), which were the first to demonstrate that tDCS effectively modulates cortical excitability, as measured trough Motor-Evoked Potentials (MEPs, i.e., electrical responses recorded from muscles following TMS of the motor cortex). These studies provided robust evidence that anodal tDCS enhanced cortical excitability, while cathodal tDCS suppressed it, with these effects lasting for a significant period post-stimulation.

These pioneering findings marked a pivotal moment, reigniting interest in tDCS both in preclinical and clinical research. Following these early breakthroughs, a substantial increase in studies further elucidated the mechanism of action of tDCS and its potential therapeutic applications across a range of neurological and psychiatric conditions. This surge of research contributed to the development of standardized protocols, which in turn facilitated the creation of evidence-based clinical guidelines. Notably, the guidelines proposed by Lefaucheur et al. (214) have become a cornerstone in the clinical use of tDCS, helping to refine its therapeutic efficacy and safety in treating conditions such as depression, stroke rehabilitation and chronic pain. The methodological advancements, combined with

a growing body of evidence, have firmly established tDCS as a leading contender in the rapidly expanding frontier of neurotherapeutics.

3.2.1 Mechanisms Underlying tDCS: How Does It Work?

tDCS operates by delivering a constant, low-intensity direct current (typically within the range of 0.5 to 4 mA) through electrodes (designated as anode and cathode) strategically positioned on the scalp (209). Unlike other NIBS techniques, such as TMS, tDCS induces subthreshold modifications in the resting membrane potential of neurons. These changes alter ionic conductance—particularly involving sodium and calcium channels—and receptor dynamics, particularly NMDA receptor activity, ultimately influencing spontaneous neuronal firing rates without directly eliciting action potentials (194,197,215). This distinct mechanism not only defines the unique functionality of tDCS but also minimizes task interference during simultaneous stimulation and behavioural assessments, thereby enhancing its applicability in experimental and clinical research contexts.

Thus, during tDCS, being a form of subthreshold brain stimulation, current penetrates the skull to interact with neuronal tissue, altering the resting membrane potential and thereby shifting neuronal excitability, increasing or decreasing it. Preclinical studies have demonstrated that the effects mediated by tDCS are contingent on the polarity of the applied current. Anodal stimulation depolarizes the membrane, resulting in enhanced spontaneous neuronal firing, while cathodal stimulation hyperpolarizes the membrane, leading to reduced firing rates (197,216,217; **Fig. 4**).

This phenomenon could be partly explained by a major Magnetic Resonance Spectroscopy (MRS) study that provided valuable insights into how tDCS involves electrophysiological modulation of neurotransmitters, such as glutamate and GABA, at the site of stimulation (**Fig. 4**): while anodal tDCS reduced local gamma-aminobutyric acid (GABA) concentrations, thus enhancing excitatory synaptic activity, cathodal tDCS appeared to reduce glutamatergic neuronal activity, which is closely correlated with a reduction in GABA levels. This interplay likely arises from the biochemical coupling between glutamate and GABA neurotransmitter systems, as their synthesis and metabolism are interconnected (218).

However, while this may be perceived as a straightforward dichotomy— anodal-excitation and cathodal-inhibition (AeCi)—this simplification fails to encapsulate the multifaceted nature of tDCS mechanisms. The outcomes of tDCS are highly influenced by various factors, such as the orientation of neurons with respect to the applied electric field, the intricate architecture of neural networks, as well as the baseline neural activity of the targeted brain regions (219). Neurons with high intrinsic activity often exhibit responses distinct from those in less active states, which underscores the

context-dependent effects of tDCS. For example, motor studies frequently observe the polarity-dependent effects of AeCi. However, this pattern is less consistent in cognitive domains, as cognitive tasks tend to involve expansive, often bilateral brain networks. A meta-analysis by Jacobson et al. highlighted that the AeCi effects are not reliably replicated in cognitive tasks, indicating that the complexity of these tasks likely interacts with tDCS mechanisms in unique ways (220). In fact, in some cases, cathodal stimulation has been found to enhance performance by mitigating neuronal competition (221). This phenomenon is particularly evident in tasks demanding sophisticated cognitive operations, such as language processing, where network-level effects may override simple polarity-based outcomes. Thus, the application of tDCS, especially in cognitive contexts, must consider these multifactorial influences to optimize its efficacy and interpret its effects accurately. This approach highlights the importance of tailoring stimulation protocols to specific experimental or therapeutic goals, rather than relying solely on generalized assumptions about polarity-based responses.

Regardless of the polarity of the current applied, at the cellular level, tDCS interacts with ongoing synaptic activity by modulating the efficacy of synaptic inputs, resulting in immediate effects (online effects). On the other hand, however, continuous stimulation over several minutes can induce changes in neuronal excitability that persist for up to an hour (222). These longer-lasting effects (offline effects) are mediated by both synaptic and non-synaptic mechanisms, involving processes related to neuroplasticity, particularly long-term potentiation (LTP) and long-term depression (LTD) (215,223).

tDCS influences presynaptic calcium channels, such as P/Q and N-type channels, which are essential for synaptic vesicle release and facilitation (224). This interaction with calcium channels alters axonal terminal dynamics and neurotransmitter release. Importantly, the sustained aftereffects of tDCS are associated with NMDA receptor activity and calcium influx, which trigger downstream signalling cascades and lead to prolonged alterations in synaptic efficacy (215,225,226; **Fig. 4**). Indeed, the NMDA receptor-dependent mechanisms play a pivotal role in tDCS-induced LTP. For example, when NMDA receptor antagonists are applied, the long-lasting effects of tDCS are suppressed, confirming the receptor's critical involvement in synaptic potentiation (215,225,227). Anodal tDCS, in particular, enhances NMDA receptor-mediated synaptic potentiation, which is further amplified by repetitive synaptic activation. This process is closely linked to brain-derived neurotrophic factor (BDNF) signalling (223,227; **Fig. 4**). BDNF enhancement under tDCS involves epigenetic modifications such as CREB phosphorylation and histone acetylation, both of which upregulate BDNF expression (228). Additionally, the activation of TrkB receptors, which are critical for motor learning and broader neuroplastic changes, mediates these effects (223). Collectively, these

findings emphasize the multifaceted and potent impact of tDCS on synaptic and neural network plasticity.

The effects of tDCS on synaptic plasticity are also mediated by its influence on GABA and glutamate neurotransmission, which thus contribute to both immediate and long-term effects. For example, in older adults, anodal tDCS has been shown to markedly reduce GABA levels, which correlates with enhanced local plasticity and improved functional connectivity (229). Similarly, when anodal tDCS is applied to the motor cortex, it results in a persistent decrease in GABA concentration that extends well beyond the stimulation period (230,231). The reduction in GABA levels following tDCS has been linked to changes in the activity of enzymes responsible for GABA synthesis (232).

tDCS has shown potential also in modulating neuroinflammation by influencing key inflammatory processes within the CNS (**Fig. 4**). It can alter the activity of microglia and astrocytes, the primary immune cells in the CNS, reducing the release of pro-inflammatory cytokines such as IL-1β and IL-6, while increasing anti-inflammatory cytokines like IL-4, IL-1α and IL-10 (233–236)(Lopes et al., 2019; Santos et al., 2020; Guo et al., 2020). Experimental animal models have further provided evidence of tDCS-induced neuroinflammatory modulation, showing a decrease in markers such as TNF-α and IL-1β alongside improved behavioural outcomes (237–239). In addition, human trials have revealed similar trends, with tDCS reducing plasma levels of pro-inflammatory cytokines, such as IL-6 and IL-8, and correlating with symptom improvement in conditions associated with neuroinflammation (240,241). The mechanisms underlying these effects may involve tDCS-driven modulation of brain immune cells and the regulation of signalling pathways that control cytokine release, ultimately reducing neuroinflammatory states and enhancing neuronal function.

Finally, on a network scale, tDCS enhances functional and structural connectivity between brain regions (**Fig. 4**). Research has demonstrated that repeated sessions of tDCS can facilitate neuroplastic changes by modulating the expression of activity-dependent genes and altering dendritic spine morphology (242–244). These processes contribute to the reorganization of cortical networks and offer therapeutic promise, particularly for conditions like AD, where disrupted connectivity and synaptic loss are central pathological features.

3.2.2 How tDCS Enhances Dopamine Neurotransmission

While the widely discussed mechanisms of tDCS focus on its effects on cortical excitability and plasticity, a less explored yet equally intriguing dimension is its influence on dopaminergic systems. This mechanism is gaining increasing attention in the scientific community, given its profound implications for modulating reward, motivation and cognitive processes. Far from being confined to

local cortical effects, tDCS demonstrates a remarkable ability to interact with and modulate deep brain circuits, including those rich in dopaminergic activity (**Fig. 4**).

Preclinical studies have provided foundational insights into how tDCS engages dopaminergic systems. A study on rats directly showed that cathodal tDCS, but not the anodal one, when applied to the cortex, produced an increase in extracellular DA levels in the striatum. Particularly, this effect became significant up to 120 minutes post-stimulation and persisted throughout the observation period (245). Although cathodal tDCS is typically thought to inhibit cortical excitability, this study contradicted prior assumptions about its suppressive effects on neuronal activity. It was hypothesized that cathodal tDCS might induce changes in cortical plasticity and facilitate dopaminergic activity in a manner distinct from the anodal effect: specifically, while anodal tDCS may directly induce activations of the frontal cortex and NAcc (which receives strong projections from the frontal cortex), cathodal tDCS may trigger long-term plastic changes in the brain, indirectly influencing dopaminergic systems.

Adding to this body of research, Peanlikhit et al. demonstrated that a single session of tDCS applied to the frontal cortex in mice elicited increased expression of c-Fos, a marker of neuronal activity, in several brain areas including the ventromedial prefrontal cortex (vmPFC) and associated subcortical circuits, such as the VTA, dorsal hippocampus, NAcc shell, paraventricular thalamus and hypothalamus, underscoring the capacity of tDCS to modulate the entire central network of motivation, reward and memory (246). Importantly, the activation of the VTA is particularly relevant, because, as described in the previous chapter, this area is known to be impaired in AD. This finding is extremely groundbreaking as it represents the first direct evidence of tDCS-induced activation of this specific circuit in mice, highlighting its potential to restore VTA function in pathological states, i.e., conditions in which this system is compromised.

Following these preclinical studies that have outlined the impact of tDCS on dopaminergic systems, research on humans has further validated these findings, offering a clearer understanding of how non-invasive neuromodulation affects brain networks. One pioneering study by Polanía et al. (247) was the first to provide direct evidence in humans, using resting-state functional magnetic resonance imaging (fMRI) to examine the effects of tDCS on brain connectivity. This study showed that anodal tDCS over the motor cortex, combined with cathodal stimulation over the contralateral frontopolar cortex, enhanced the functional connectivity of cortico-striatal and thalamo-cortical circuits. These effects included increased coupling between motor-related areas and subcortical regions, while reducing the functional coupling between the striatum and the DMN. This work was groundbreaking because it confirmed that tDCS could induce modulations beyond the primary

stimulation site but also induces widespread changes in distant, non-stimulated areas, which was previously unproven in human studies.

On one hand, the aforementioned study was crucial as it supported the idea that tDCS influences not only local brain activity but also modulates large-scale brain networks. On the other hand, the findings are difficult to interpret, as a direct link between the observed brain activations and any behavioural changes induced by the stimulation could not be established (247). However, this issue was addressed by subsequent studies. For instance, anodal tDCS was applied to the ventromedial prefrontal cortex (VMPFC) while participants performed a facial attractiveness rating task—an established behavioural paradigm associated with the dopaminergic system and reward processing. This study revealed that tDCS enhanced functional connectivity between the VMPFC and the ventral midbrain. More importantly, these neurophysiological changes were linked to a significant increase in attractiveness ratings, thereby establishing a direct connection between tDCS-induced neural changes and measurable behavioural outcomes (248). Weber et al. (249) examined the effects of tDCS on brain activity and connectivity during the Balloon Analog Risk Task, a well-established measure of risk-taking. Their fMRI results replicated the hemodynamic signatures of previous studies, showing a significant effect of bifrontal tDCS on both task-related brain activity and resting-state connectivity. Notably, tDCS increased the brain's response to losses in the right DLPFC and anterior cingulate cortex and reduced resting cerebral blood flow in the orbitofrontal cortex and caudate nucleus, with associated changes in global brain connectivity (249). Although no behavioural changes were observed, an effect that the authors thought could be attributed to reduced variability in the fMRI version of the task, the results provided insights into how prefrontal stimulation influences brain regions involved in decision-making. While Weber's study did not directly address the VTA, the findings is particularly relevant when considering the role of this dopaminergic area in processing reward and risk signals (250). Finally, another work examined the effects of prefrontal tDCS on dopaminergic activity using three indirect measures—facial attractiveness ratings, visuospatial bias and eye-blink rate (EBR). Although no significant overall effects were observed, the results revealed that participants with higher baseline DA showed greater changes in EBR following active stimulation, suggesting that individual differences, particularly in baseline dopaminergic functioning, may influence the effectiveness of tDCS interventions (251).

Research providing the first direct evidence of increased DA levels following tDCS has primarily focused on examining dopaminergic responses in the striatum. A particularly significant study in this area is that of Fonteneau et al. (2018). Using positron emission tomography (PET) with the radiotracer [11C]-raclopride, this study demonstrated that a single session of tDCS applied to the DLPFC resulted

in enhanced dopaminergic release, particularly in the right dorsal striatum and left putamen (252). This finding offers the first evidence of tDCS's direct effects on the dopaminergic system. Following this, subsequent studies aimed to confirm these findings and the underlying mechanisms. Notably, Fukai et al. (253) extended and deepened the results of Fonteneau by showing that DLPFC stimulation not only potentiates DA release in the ventral striatum but also correlates this increase with improvements in cognitive performance, particularly in attention. This study is pivotal as it provides direct evidence of a link between elevated DA levels and cognitive enhancement, demonstrating that dopaminergic modulation via tDCS can yield cognitive benefits. A couple of years later, the same research group explored the mechanisms of dopaminergic modulation by introducing the role of the GABAergic system. In particular, in a double-blind, crossover study, healthy subjects were subjected to tDCS with an anode placed on the left DLPFC and a cathode on the right DLPFC. The results showed that tDCS increased DA release in the right striatum and GABA concentrations in the left striatum. Notably, changes in cortical GABA were positively correlated with changes in striatal DA, indicating a complex modulation of the DA-GABA system by tDCS (254). The study suggests that tDCS can modulate these systems at a molecular level in the basal ganglia-cortical circuit, with potential therapeutic implications for complex neuropsychiatric disorders.

Finally, Meyer et al. (255) compared the effects of prefrontal tDCS with the pharmacological administration of L-DOPA, yielding striking results. fALFF (fractional Amplitude of Low-Frequency Fluctuations) analysis revealed that tDCS induced changes similar to those observed with pharmacological treatment, suggesting that prefrontal stimulation could be equally, if not more, effective in enhancing the dopaminergic system activity. This study is particularly significant as it opens new therapeutic avenues, indicating that tDCS may offer a viable alternative or complement to pharmacological treatments in neuropsychiatric conditions characterized by dopaminergic dysfunction.

Indeed, while the numerous aforementioned studies posit that tDCS may offer a promising method for non-invasive modulation of deep brain structures—like the VTA—in healthy animal models and human subjects, it also showed promise as a therapeutic tool in pathological conditions, such as PD—a disorder characterized by degeneration of dopaminergic neurons in the SNpc— and Attention Deficit Hyperactivity Disorder (ADHD) (256,257). For instance, in a study involving MPTP-treated mice, an established animal model of PD, tDCS resulted in increased DA levels in the striatum. More importantly, the study reported that tDCS significantly increased the content of TH (256). This augmentation in the MPTP model is indicative of a reduction in the loss of TH^+ neurons. This implies that tDCS might not only boost DA production but also help preserve the function of dopaminergic

neurons in PD, potentially offering a neuroprotective effect. In ADHD, a disorder characterized by alterations in dopaminergic transmission and reduced fronto-basal ganglia activation, tDCS applied to the frontal cortex of Spontaneously Hypertensive Rats (SHR)—the most commonly studied animal model of ADHD—increased DA levels in both the hippocampus and striatum, which are critical regions for cognitive and memory functions, but not in the PFC or brainstem (257).

The ability of tDCS to modulate DA systems has far-reaching implications. By leveraging the extensive connections between the prefrontal cortex and subcortical regions, prefrontal tDCS provides a means to influence deep brain areas traditionally inaccessible with other non-invasive techniques. Of course, challenges still remain in optimizing tDCS protocols for targeted outcomes. Moreover, understanding individual variability in response to tDCS is essential. Factors such as baseline neurochemical states, electrode placement and stimulation parameters influence outcomes. Personalized stimulation protocols, informed by biomarkers and real-time monitoring, could enhance both the efficacy and safety of tDCS.

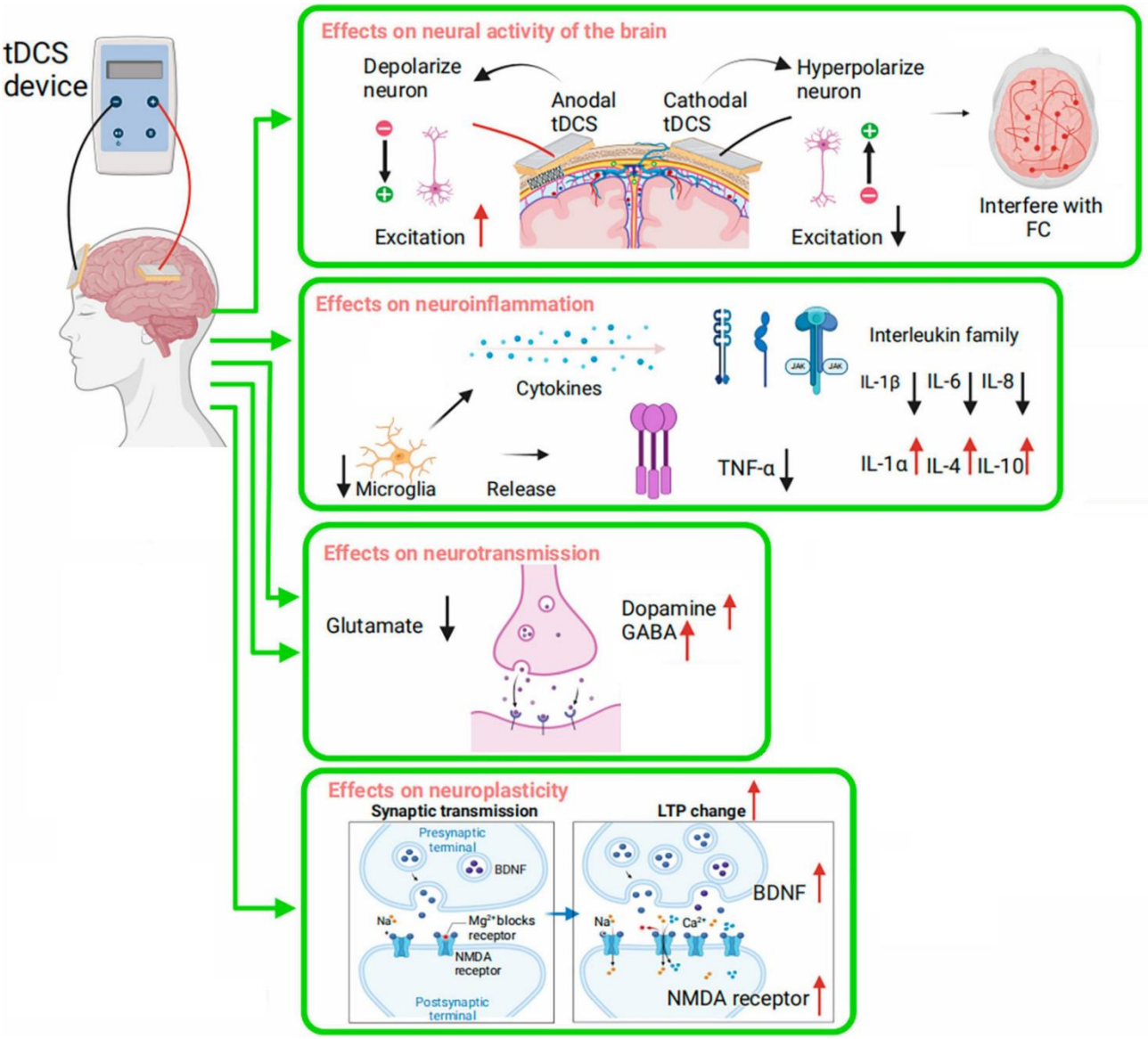

Figure 4. Mechanisms of action of tDCS on brain function.

The figure illustrates the multifaceted mechanisms of action of tDCS on brain function. tDCS, which involves the application of weak electrical currents through electrodes placed on the scalp, modulates neuronal activity by either depolarizing or hyperpolarizing neurons. Anodal tDCS enhances neural excitation, while cathodal tDCS diminishes it, impacting functional connectivity (FC) across brain networks. Beyond its effects on neural excitability, tDCS influences several critical processes in the brain. It plays a significant role in reducing neuroinflammation by attenuating microglial activation and modulating cytokine release, leading to a decrease in pro-inflammatory markers such as TNF-α and IL-1β, while promoting anti-inflammatory cytokines like IL-4 and IL-10. In addition, tDCS modulates neurotransmission, notably reducing glutamate levels while increasing key neurotransmitters such as DA and GABA, thereby regulating the excitation/inhibition balance. Furthermore, tDCS enhances neuroplasticity by promoting synaptic transmission and LTP through the upregulation of BDNF and the modulation of NMDA receptor activity. These changes strengthen synaptic connections and contribute to improved plasticity within neural networks. Collectively, these mechanisms underline the therapeutic potential of tDCS in modulating brain activity and treating a range of neurological and psychiatric disorders. Adapted from (258).

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EXPERIMENTAL WORK

4. WORKING HYPOTHESIS

During my doctoral studies, I focused on investigating the interplay between dopaminergic deficits and the neurophysiological dysfunctions characteristic of AD from multiple perspectives. Central to my research was the hypothesis that targeting the dopaminergic system early on during disease progression could serve as a promising therapeutic approach. Indeed, in recent years, there has been a great advance in our understanding of AD pathology, which has been channelled, in part, into the study of dopaminergic dysfunction as a critical early component in the disease progression. This impairment is now considered a key contributing factor to both cognitive impairments (e.g., memory and executive dysfunction) and NPS (e.g., apathy and reduced motivation) —important hallmarks of AD (137,187,189,259–261).

Structural and functional analyses have revealed significant neurodegeneration in mesocorticolimbic dopaminergic circuits, particularly within the VTA, even during the prodromal stages of AD (166–168). These deficits are mirrored in the Tg2576 mouse model of the disease, where progressive and selective loss of VTA dopaminergic neurons compromises the functionality of this region, leading to decreased DA availability in target areas, including the hippocampus and ventral striatum, while also negatively impacting a variety of cognitive, behavioural and neurophysiological functions (116,145,147). Notably, in these mice, VTA neurodegeneration precedes amyloid plaque deposition, manifesting as early as 3 months of age, a stage comparable to the subclinical phase of AD in humans, with effects that progressively worsen as the disease advances, further exacerbating its progression.

Therefore, this degeneration can explain both memory and reward deficits observed in Tg2576 mice (145,149,151,262–264) but can also justify the beneficial results of pharmacological interventions, such as L-DOPA or selegiline, aimed at restoring DA levels and enhancing the dopaminergic tone in VTA projections areas. These therapeutic strategies have demonstrated the ability to ameliorate synaptic plasticity deficits, cognitive decline and behavioural impairments in both preclinical models (145,156,162,163,265–270) and AD patients (133,137,174,175). These findings reinforce the pivotal role of DA in the disease's pathogenesis and the therapeutic potential of targeting the dopaminergic system.

In this context, tDCS emerges as a promising and cutting-edge method for the treatment of AD. Indeed, this technique has shown efficacy in modulating cortical excitability, promoting synaptic plasticity and improving cognitive functions in the AD *scenario*, as evidenced by both preclinical studies in animal models and clinical trials in human patients (271–278). Furthermore, emerging evidence indicate that tDCS, through stimulation of the prefrontal cortex, may also indirectly affect

deep-brain structures, including the VTA, suggesting a whole mesocorticolimbic circuit activation. Specifically, in both non-AD rodent models and humans, tDCS has been shown to have the potential to modulate dopaminergic neurotransmission, promote DA release and improve motivation-related behaviours (246–249,251–257,279). However, its capacity to counteract or mitigate dopaminergic deficits within the specific context of AD (i.e., in the presence of long-standing degeneration of the VTA) remains largely unexplored.

This gap formed the foundation of my research question: *Can repeated sessions of prefrontal tDCS induce selective activation of the VTA in Tg2576 mice, enhance dopaminergic output and, as a result, restore hippocampus and ventral striatum functionality (two important VTA projection areas closely linked to AD)?*

To address this, I conducted a two-week stimulation protocol of anodal prefrontal tDCS in Tg2576 mice at two key ages, 7 and 12 months, to evaluate distinct pathological contexts within the progression of AD symptoms. In fact, 7 months represent a pre-plaque, early-phase pathology (MCI-like stage), while 12 months reflect a more advanced disease state, with significant cognitive deficits and hippocampal Aβ plaque accumulation. By targeting these time points, the study aimed to explore the effects of tDCS during both mild and more advanced stages of AD pathology.

My personal contribution to this work spanned multiple critical aspects of the experimental design and execution. I was directly responsible for planning and performing all surgical procedures necessary for the experiments, including those required for electrode implantation and placement of the microdialysis probe. Furthermore, I conducted the tDCS stimulation sessions with rigorous methodological consistency to ensure reproducibility. Beyond this, I carried out electrophysiological recordings to assess synaptic plasticity in the hippocampal CA3-CA1 circuit and performed behavioural testing to evaluate the cognitive and neuropsychiatric outcomes of the intervention. These tasks required precision, technical expertise and a deep understanding of the disease model. Thus, my role encompassed the majority of experimental procedures, allowing me to develop a comprehensive perspective on the functional and physiological implications of prefrontal tDCS in this AD model.

Specifically, the objectives of this study were to investigate whether prefrontal tDCS could:

- Modulate dopaminergic signalling and markers of dopaminergic activity;
- Enhance synaptic plasticity in the hippocampal CA3-CA1 circuit;
- Improve cognitive and non-cognitive functions, including recognition memory, locomotion and depressive-like behaviours;
- Influence pathological biomarkers, such as Aβ deposition and neuroinflammation.

Overall, with this thesis work I sought to explore the limitations and potential of prefrontal tDCS in a context of advanced neurodegeneration, with the goal of providing new insights into the possible therapeutic impact of this method in counteracting AD pathology. This work contributes to the growing body of research supporting the translational potential of tDCS, not only as a therapeutic tool for AD or as a means of non-invasive stimulation of deep brain areas like the VTA, but also as a widely accessible intervention capable of improving quality of life for patients across diverse care settings.

5. MATERIALS AND METHODS

5.1 Animals

Heterozygous male Tg2576 mice (149) (APPSWE - Model #1349 TACONIC) and WT littermates were used at 7- and 12-months of age, as described in the text. Animals were housed with *ad libitum* food and water, with a 12-hour light/dark cycle. All experimental procedures complied with the ARRIVE guidelines and were carried out in accordance with the ethical guidelines of the European Council Directive (2010/63/EU). Experimental approval was obtained from the Italian Health Ministry (protocol #501-2019PR).

5.2 Surgical Implantation of Epicranial Cannula and tDCS Stimulation Protocol

Implantation of a prefrontal epicranial cannula was performed 2 days before the start of the stimulation sessions. Mice were anesthetized with a combination of Rompun (20 mg/mL, 0.5 mL/kg; Bayer) and Zoletil (100 mg/mL, 0.5 mL/kg; Virbac; intraperitoneally i.p.) before the surgical procedure. The animals were then placed in a stereotaxic apparatus and a small incision was made in the skin of the head. After skull exposure and cleaning, a tubular cannula (2.5 mm diameter) was implanted on the midline under stereotaxic control (David Kopf Instruments) over the PFC (AP +3.2 from bregma). To secure the cannula to the skull at the desired position, we first used instant glue to fix it firmly, followed by a thin layer of non-toxic glass ionomer cement (GlasIonomer Cement CX-Plus Kit; Shofu Inc.). To ensure an uninterrupted current flow during stimulation, the inside of the tubular cannula was left free of cement; additionally, to prevent debris/sawdust accumulation the canula was blocked with soft cotton between experimental sessions. At the end of the procedure, the animals were placed on a heated pad to maintain stable body temperature until fully awakened. Upon complete awakening, the animals were housed individually to prevent inadvertent implant removal by conspecifics.

After a post-surgical recovery period of 2 days, mice were assigned to one of four experimental groups (WT Sham, WT tDCS, Tg Sham and Tg tDCS). The animals then underwent 10 sessions of tDCS or Sham stimulation (5 days per week, with a 2-day break between sessions; **Fig. 5A**). To avoid potential confounding effects related to anaesthesia, animals were awake throughout the stimulation procedure. Animals were fitted with a custom-made Velcro jacket, securely fastened with a metal clip. The jacket contained a sponge pocket filled with conductive gel (ECI Electro-Cap Electro-Gel, Neuroevolution - Sistemas Médicos, Lda). The same gel was also applied to the inner surface of the prefrontal cannula implanted during surgery to facilitate stable electrode placement and signal

conductance. Animals were then restrained in Plexiglas holders to maintain consistent positioning during the stimulation session.

Stimulation was administered using a device (DC-Stimulator Plus, NeuroConn, Germany) with the anodal (active) electrode positioned in the prefrontal cannula and the cathodal (reference) electrode in the gel-filled pocket on the jacket. Both tDCS and Sham-treated mice underwent identical handling protocols, with the tDCS group receiving a continuous DC of 25 µA for 20 minutes per session, while Sham animals were connected to the stimulator without current flow. To avoid painful current transitions, controlled DC ramp-up and ramp-down phases were implemented by setting fade-in and fade-out to 20 sec values. After each stimulation session, animals were promptly returned to their home cages, where they remained for an additional 1–2 days, depending on the subsequent experimental protocol.

For the analysis of c-Fos expression, a modified stimulation protocol was employed to assess immediate early gene activation post-stimulation. In contrast to the repeated stimulation used for other experimental endpoints, this protocol involved a single prefrontal tDCS session administered under isoflurane anaesthesia. Anaesthesia was induced with 2–3% isoflurane, maintained at 0.8–1.5%, with an oxygen flow of 1.6–1.8 L/min. Animals were sacrificed one hour after the stimulation session.

5.3 In-vivo Microdialysis and Ultra-Performance Liquid Chromatography (UPLC)

To evaluate hippocampal DA and NE levels, mice were prepared for microdialysis through unilateral probe implantation, conducted 1-2 days before sampling. Mice were anesthetized with Zoletil and Rompun, then secured in a stereotaxic apparatus to vertically implant a concentric microdialysis probe (AN69 fibers, Hospal Dasco) targeted at the hippocampus (AP +3.0, ML ±3.0 mm from bregma) with a total length of 5 mm (of which 3 mm consisted of the dialysis membrane; **Fig. 5C**). After probe placement the surgical area was sutured and animals were returned to individual housing. To ensure stability and protection of probe connections, the inlet and outlet tubing of the probe were connected to two PE-20 segments (1–1.5 cm each) *via* flexible swivels.

Membrane recovery efficiency was validated *in vitro* prior to implantation to warrant consistent sampling. On the day of the experiment, 2 days after recovery from tDCS or Sham stimulation, the animal was placed in a circular cage filled with bedding, and the probe was connected to the microdialysis system, allowing the animal to move freely. Specifically, the small PE-20 tubing segments initially attached to the microdialysis probe's inlet and outlet were removed and replaced with longer PE-20 tubing. These extended segments were then connected to the CMA/100

microinfusion pump (Carnegie Medicine) *via* an ultra-low torque multichannel swivel (MCS5 model, Instech Laboratories), providing continuous perfusion of the probe during the experiment. A steady infusion of artificial cerebrospinal fluid (aCSF; in mM: NaCl 140, KCl 4, $CaCl_2$ 1.2, $MgCl_2$ 1) was maintained through the probe at a rate of 2.1 µL/min. After initiating perfusion, animals were allowed to acclimate for 1 hour to establish a stable baseline. Three consecutive baseline samples were then collected at 20-minute intervals over a 60-minute period. Baseline collection was followed by a 20-minute perfusion with KCl-enriched aCSF solution (100 mM KCl) to induce depolarization and neurotransmitter release, after which a single dialysate sample was collected. The perfusion was then switched back to aCSF, and three additional samples were collected over the next hour.

To verify probe placement, brains were postfixed in 4% paraformaldehyde, sectioned coronally (100 µm), stained with methylene blue and then examined under a microscope. Data from animals with improperly allocated probes were excluded from subsequent analyses.

Dialysate samples (20 µL) were analysed via an Ultra-Performance Liquid Chromatography system (UPLC; ACQUITY, Waters Corporation) equipped with an amperometric detector (Decade II, Antec Leyden). The UPLC system consisted of an electrochemical flow-cell (VT-03, Antec Leyden) with a 0.7 mm glassy carbon working electrode, maintained at a potential of 400 mV, and an *in-situ* Ag/AgCl reference electrode. The flow cell was positioned downstream of a BEH C18 column (2.1 × 50 mm, 1.7 µm particle size; Waters Corporation) kept at 37°C with a mobile phase flow rate of 0.07 ml/min. The mobile phase composition was (in mM): 50 orto-phosphoric acid 85%, 8 KCl, 0.1 EDTA, 2.5 1-octanesulfonic acid sodium salt, 14% methanol, adjusted to pH 6.03 with NaOH. DA and NE peak heights were identified by comparison with internal standards.

5.4 Acute Brain Slicing and Electrophysiological Recordings

Acute brain slices for electrophysiological recordings were obtained following the protocol outlined in Ting et al. (280). Briefly, solutions for brain slicing were prepared fresh (stored at +4°C for up to 5 days) and saturated with a 95% O_2 and 5% CO_2 mixture before use. Animals were anaesthetized with halothane and then transcardiacally perfused with cold (0-2°C) oxygenated solution containing (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 Glucose, 2 Thiourea, 5 Na-Ascorbate, 3 Na-Pyruvate, 0.5 $CaCl_2$ and 10 $MgSO_4$ (~295mOsm; pH 7.3-7.4 with 5 M HCl). Afterwards, the animals were decapitated, the brain was rapidly removed and parasagittal brain slices containing the dorsal hippocampus (300 µm thickness) were cut with a vibratome (VT1200S, Leica) in the same chilled-bubbled perfusion solution. After slicing, slices were transferred to a holding chamber containing the NMDG-based solution at 32-34°C and left to recover for 35 min, during which NaCl, prepared fresh on the experimental day, was added gradually to the

solution at 5-min intervals (see 241). Subsequently, slices were transferred at room temperature to a long-term holding chamber containing (in mM): NaCl 92, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 30, HEPES 20, Glucose 25, Thiourea 2, Na-Ascorbate 5, Na-Pyruvate 3, $CaCl_2$ 2 and $MgSO_4$ 2 (~ 300-310mOsm; pH 7.3-7.4 with NaOH 10 N) for at least 1 hour before experiments commenced.

5.4.1 Multielectrode Array Recordings

A single acute slice containing the dorsal hippocampus was transferred onto an 8x8 array of planar multi-electrodes, each 50x50 µm in size with an interpolar distance of 150 µm (MED-P5155, Alpha MED Sciences). Slice position and contact with electrodes was secured by a nylon mesh glued to a flattened piece of platinum wire. The brain slice was continuously perfused with oxygenated aCSF containing in mM: NaCl 124, KCl 3, NaH_2PO_4 1.25, 26 $NaHCO_3$, 10 Glucose, 1 $MgSO_4$, 2 $CaCl_2$ and 0.5 mM L-glutamine (10 mL/min, 32-34 °C). The brain slice was visualized at 2.5x magnification of an upright microscope (Leica DM-LFS, Leica Microsystems), while raw data were recorded with the MED64 System (Alpha MED Sciences), digitized at 20 kHz with a 6071E Data Acquisition Card (National Instruments), and low-cut filtered at 1 Hz. Amplitude, duration and frequency of stimulation were controlled by Mobius software (Alpha MED Sciences).

In order to record *f*EPSPs, Schaffer collateral stimulation was performed (200 µs duration), after choosing one stimulation electrode in the Stratum Radiatum and another recording electrode in the pyramidal cell layer in CA1 area, maintaining a constant distance between electrodes of 300 µm. After at least 20-30 minutes of baseline stable responses (single half-maximal stimulus, every 30 s), LTP was induced by applying two conditioning trains of 100 Hz stimuli for 1 sec administered at an interval of 20 s, followed by test stimulation for at least 1 h. The magnitude of LTP was assessed by calculating the mean peak of *f*EPSP during the final 5 minutes following the high-frequency trains, normalized to the mean baseline peak. All recordings obtained were analysed using Mobius (Alpha MED Sciences) and Origin6.0 (Microcal Software Inc.) software.

5.5 Behavioural Testing

All behavioural assessments were conducted two days following the completion of the tDCS sessions. Prior to each session, subjects were acclimated in an adjacent room where ambient lighting (15 lux) and noise levels (35 dB) were matched to those of the experimental environment. These environmental conditions were kept constant throughout all phases of testing. To minimize olfactory cues from influencing the mice's exploration patterns, all experimental apparatus, including objects used for object recognition, were thoroughly cleaned with 5% ethanol between sessions and experimental subjects.

5.5.1 Open Field (OF) and Novel Object Recognition (NOR) Tests

The experimental protocol commenced with the Open Field (OF) test, conducted over two consecutive days to assess locomotor activity in a circular arena (60 cm in diameter, 40 cm in height), made of plexiglass with a transparent base and opaque grey walls. On Day 1, each mouse was positioned at a fixed starting point near the arena's edge and allowed to freely explore for 10 minutes. The same procedure was repeated on Day 2. Data on locomotor activity were provided only by the test conducted on D1, but the dual-day exploration served as pre-habituation for the subsequent NOR test, ensuring that the animals were adequately familiarized with the environment. but also provided

On Day 3, the NOR test was performed in the same OF arena. The training phase began with the placement of two identical wooden spheres within the arena, positioned equidistant from the arena center. Mice were allowed to explore for 10 minutes. After a 24-hour interval (Day 4), each mouse was reintroduced to the arena for a 10-minute testing phase during which one of the wooden spheres was replaced with a metal column of similar size. The spatial configuration of the objects remained identical to that of the training phase, with the novel object positioned on alternating sides to control for potential side bias.

In both the OF and NOR test, exploratory behaviour was recorded using VirtualDub software (version 1.10.4), which tracked the mouse's movements and interactions with the objects. Behaviour analysis for the OF test was performed using ImageJ software (version 1.53q; National Institute of Health, USA), allowing for the tracking of the total distance travelled (in cm) by each animal in the arena, recorded on the experimental D1. For the NOR test, data were analysed using the free, open-source software BORIS (version 8.21.10; University of Torino, Italy), which provided the total exploration time (in sec) spent on each object. Exploration was defined as any interaction with the object, including touching it with the nose or forepaws, climbing on it, or sniffing within a 2 cm radius.

5.5.2 Tail Suspension Test (TST)

A separate cohort of animals, distinct from those used in other behavioural assays, was employed for the Tail Suspension Test (TST). The apparatus employed consisted of a rectangular wooden base (20 x 15 cm) with three perpendicular vertical wooden walls (55 cm high). A rectangular metal bar, securely affixed to the top of the apparatus, was utilized for suspending the mice by their tails during the experiment. Prior to testing, each subject's tail was carefully inserted into a black plexiglass cylinder (4 cm length, 1.6 cm outer diameter, 1.3 cm inner diameter, 1.5 g weigh) to prevent tail-climbing behaviour, which could confound test results. Subsequently, a strip of paper tape (15 cm length, 1.5 cm width) was applied to the tail approximately 3 mm from the distal end, ensuring a

secure attachment to the metal bar at the top of the apparatus. During the test, each mouse was suspended approximately 20 cm above the base of the apparatus for a total of 6 minutes. The experimental session was continuously recorded with VirtualDub and data were analysed with BORIS to assess the duration time (in sec) of both immobility, classified as passive behaviour and defined as a complete lack of movement except for minor adjustments to maintain balance, and active behaviour, encompassing all movements aimed at escape or repositioning (such as limb movements and attempts to reach the bar). At the end of the session, the paper tape and cylinder were carefully removed and each subject was gently returned to its home cage. Each subject underwent testing individually to avoid potential influence from other animals.

5.6 Immunofluorescence

Following anaesthesia (Rompun/Zoletil), mice were perfused transcardially with phosphate buffer (PB; 0.1 M, pH 7.4) and 4% paraformaldehyde in PB. After being postfixed in 4% paraformaldehyde for at least 4 h, brains were dehydrated and cryoprotected in 30% sucrose in PB at 4°C until sinking. Thirty µm-thick coronal sections were cut with a cryostat and slices were collected in PB-Sodium Azide 0.02% until processed for experiments.

Free floating slices were incubated with primary antibodies in PB containing 0.3% Triton X-100 overnight at 4°C.

For Aβ/Iba1 immunostaining, sections were pretreated with M.O.M.® (Mouse on Mouse) Blocking Reagent (1:1000; Vector laboratories, #MKB-2213-1, 2 h, RT) diluted in permeabilization solution (PB with 0.3% Triton X-100), and then incubated with primary antibodies overnight at 4°C in permeabilization solution.

Sections were subsequently washed in PB and incubated with secondary antibodies in the permeabilization solution (2 h, RT) followed by washes in PB and incubation with DAPI (1:1000, Serva). After mounted, slices were examined using a Nikon Eclipse Ti2 confocal microscope. The labelling specificity was confirmed by omission of primary antibodies and use of normal serum instead (negative controls).

For all analyses images were acquired with 10x or 20x-objectives by performing Z-stacks, then processed by maximum-intensity projection.

For the quantitative analysis, images were processed simultaneously and analysed with Fiji-ImageJ (http://imagej.nih.gov/ij/): after 8-bit conversion and background subtraction, the signal was quantified by measuring the relative fluorescence intensity. The F/A ratio defines mean fluorescence intensity (F) over a defined surface area (A). All samples were captured with identical Z-stack thickness and laser settings.

Relative intracellular Aβ expression levels were quantified by setting 10 randomly-distributed squared frames (70x70 pixel).

The mean number of Aβ plaques in the hippocampus was quantified manually from 10x-objective images.

The mean number of c-Fos$^+$/TH$^+$ neurons over the defined area captured by the camera was quantified by using NIS-Elements software (Nikon® Instruments Inc.).

Images were collected and quantification was done at least on 3-4 slices per mouse. Data were then averaged per mouse for figures and statistical analysis.

Primary antibodies: c-Fos (1:1000, Abcam #ab190289; RRID: AB_2737414), hAPP695 (6E10; 1:500, BioLegend #803001; RRID: AB_2564653), IBA1 (1:600; Wako #019-19741; RRID:AB_839504), TH (1:1000; Millipore #MAB318; RRID:AB_2201528).

Secondary Antibodies (ThermoFisher): Alexa Fluor-555 donkey anti-rabbit (1:200; #A31572; RRID:AB_162543), Alexa Fluor-488 donkey anti-mouse (1:200; #R37114; RRID:AB_2556542), Alexa Fluor-647 donkey anti-mouse (1:200; Catalog # A31571; RRID: AB_162542).

5.7 Stereology

Immunofluorescence sections were used to estimate microglia cell numbers in the dorsal hippocampus, outlined using the 5x-objective; Iba1$^+$ cells were marked with a 40x-objective (x, y, z dimension of probe was 100×100×25 µm). The total cell number was estimated according to the formula (Equation 1):

$$N = SQ \times (1/ssf) \times (1/asf) \times (1/tsf) \quad \textbf{(1)}$$

where SQ represents the neuron number counted in all optically sampled fields of the ROI, ssf is the section sampling fraction, asf is the area sampling fraction and tsf is the thickness sampling fraction.

5.8 Morphological Analysis

For quantitative 3D-analysis of the entire cell microglia were imaged with a Zeiss Microscope (Axio Imager KMAT) with a motorized stage and a camera controlled by the Neurolucida software (7.5v; MBF Bioscience) for 3D quantitative analysis of cell morphology, including soma and perimeter (154). We examined only non-overlapping cells with clear soma and branching. Sholl analysis included counting the number of dendritic intersections, nodes and endings and the lengths of processes at fixed distances from the soma in 10 µm-distanced concentric circles away from the soma. Analysis was done with a 100x-oil objective. Nine representative cells/animal were randomly analysed, and data were averaged for each mouse.

5.9 Sample size, randomization, blinding

The number of experimental units per group and experiment was determined with power analysis (G*Power software, version 3.1.9.7) using a power of 0.8 and errors of 0.05; standard deviations of were obtained from our previous publications (145).

To decide how mice from the same litter would be "destined" to different groups, we randomized animals using with a random-number table.

All researchers were blinded to the animal group and un-blinding occurred only after analysis.

Details for the experimental units for each experiment are described in figure legends.

5.10 Statistical Analysis

Statistical analyses were conducted using Prism software (version 8.01; GraphPad). Data distribution was assessed for normality utilizing the Shapiro-Wilk, Kolmogorov-Smirnov or D'Agostino & Pearson tests.

Comparisons between two experimental groups (e.g., Tg Sham vs. Tg tDCS) were performed using 2-tailed parametric tests (unpaired *t*-test or Welch's *t*-test) for data meeting the assumptions of Gaussian distribution, and non-parametric Mann-Whitney tests for data that did not meet normality criteria. For datasets involving paired measurements, such as the training and testing phases of the NORT, paired *t*-tests were used for normally distributed data, or Wilcoxon matched-pairs signed rank tests otherwise.

Analyses involving comparisons among more than two groups (e.g., WT Sham, WT tDCS, Tg Sham, Tg tDCS) were performed using 2-way Repeated-Measures ANOVA, followed by Tukey's or Sidak's multiple comparison post hoc test. If no significant interaction was observed between the independent variables, statistical comparisons were conducted using *t*-tests.

Microdialysis data (analysed with genotype, treatment and dialysis time point—baseline, KCl, post-KCl—as independent factors) were evaluated using 3-way Repeated-Measures ANOVA with Tukey's multiple comparison post hoc tests for further pairwise comparisons.
Statistical significance was defined as $p \leq 0.05$. In box-and-whisker plots, the central line represents the median, the box edges indicate the upper and lower quartiles, whiskers show minimum and maximum values and points represent individual experiments. All other data are presented as mean ± S.E.M.

For additional details on statistical analyses and sample sizes, refer to figure legends.

6. RESULTS

6.1 Prefrontal tDCS activates dopamine neurons in the VTA and enhances DA release in Tg2576 mice

We showed that the Tg2576 model of AD is affected by selective degeneration of DA neurons in the VTA, that is associated with many of the neuronal dysfunction and synaptic deficits resulting in behavioural impairments detectable in pre-plaque stage (at 7 months of age) (116,145,147). Here, we first aimed to test the efficacy of prefrontal tDCS in activating the remaining dopaminergic neurons in the VTA of Tg2576 mice, despite the ongoing neurodegenerative process. Accordingly, two groups of Tg2576 mice were chosen for prefrontal tDCS (thereafter Tg tDCS) or Sham stimulation (Tg Sham) under isoflurane anaesthesia. The tDCS group received a continuous direct current of 25 μA for 20 minutes, while Sham animals were connected to the stimulator without current flow (**Fig. 5A**). One hour after the delivery of the single tDCS session we conducted immunofluorescence analyses for c-Fos expression—an immediate-early gene commonly used as a marker of neuronal activation. Analysis of c-Fos revealed a significant increase in the number of c-Fos$^+$/TH$^+$ neurons in the VTA of Tg tDCS mice compared to Tg Sham mice, indicating that prefrontal tDCS robustly activates DA neurons (**Fig. 5B,** upper panel). We extended our analysis to the noradrenergic LC, another brainstem region primarily affected in AD (283). The LC showed no modifications in c-Fos$^+$/TH$^+$ neuron numbers following tDCS treatment in Tg2576 mice, indicating that LC is not activated by the stimulation (**Fig. 5B**, bottom panel).

We next examined whether repeated, 2-week sessions of prefrontal tDCS could modulate hippocampal dopaminergic signalling in freely moving Tg2576 and age-matched littermate controls. After two weeks of prefrontal tDCS or Sham treatment (**Fig. 5A**), using *in vivo* microdialysis we measured hippocampal DA levels evoked by extracellular KCl (**Fig. 5C**). No changes were observed in WT mice that underwent tDCS (WT tDCS; **Fig. 5D,** left panel). In contrast, Tg tDCS mice exhibited significantly higher levels of hippocampal DA outflow compared to Tg Sham (**Fig. 5D,** right panel). Of note, hippocampal noradrenaline (NE) levels were unaffected across all experimental mice (**Fig. 5E**), in line with the c-Fos data showing the lack of LC activation by tDCS.
Collectively, our results prove that prefrontal tDCS elicits an increase in evoked hippocampal DA outflow in Tg2576 mice through targeted activation of VTA DA neurons, suggesting that this stimulation can effectively stimulate dopaminergic pathways known to be impaired in AD.

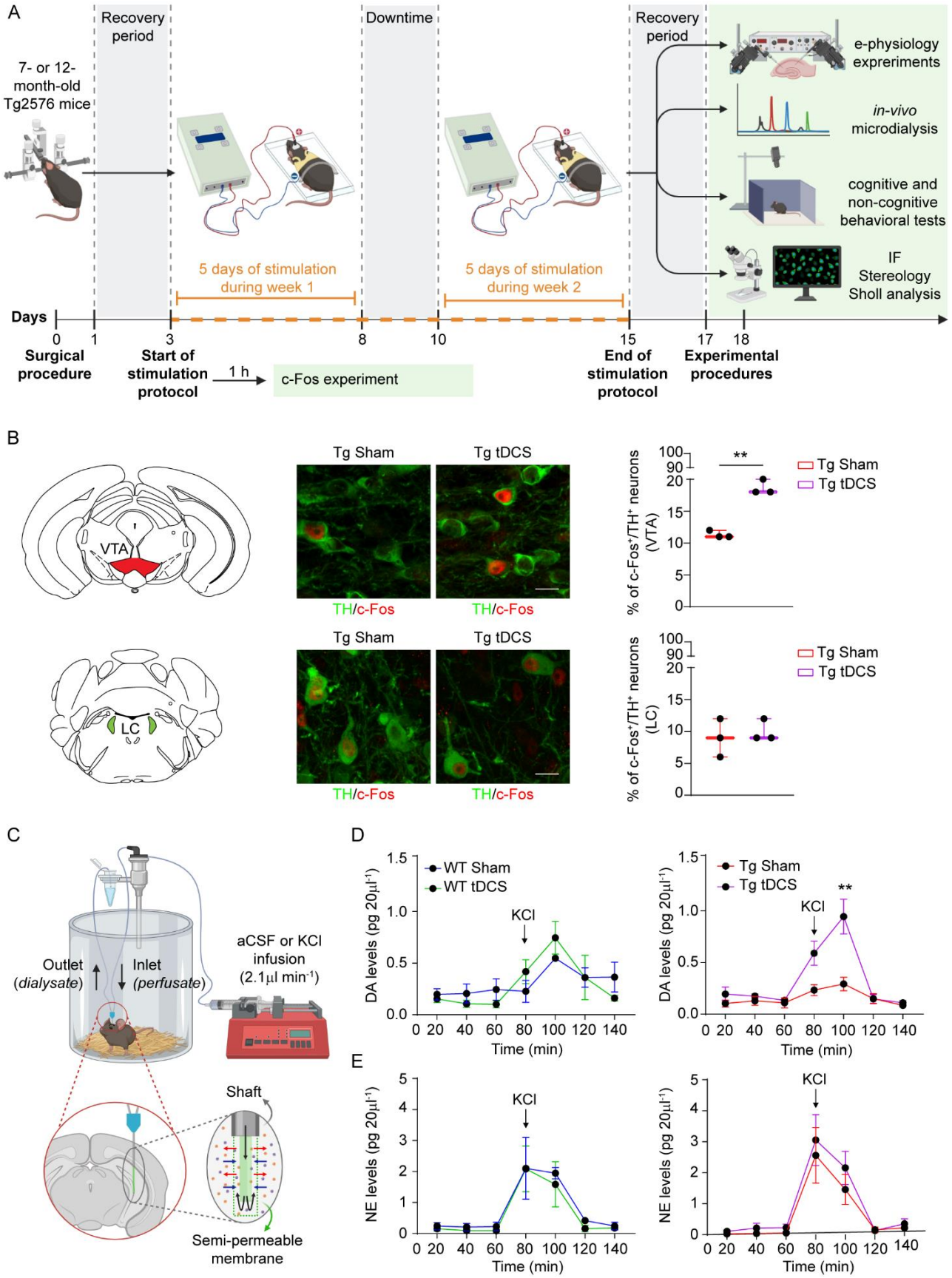

Figure 4. Prefrontal tDCS enhances KCl-induced dopaminergic outflow in the hippocampus *via* activation of dopaminergic neurons in the VTA in 7-month-old Tg2576 mice.

A) Schematic representation of the experimental timeline: Tg2576 and WT mice (7 or 12 months old) were implanted with prefrontal cannulae, followed by a 2-day recovery period. Mice then received either daily tDCS (20 minutes per day,

5 days per week) or Sham stimulation for two consecutive weeks (note that, for c-Fos analysis only, a single session of tDCS or Sham stimulation was administered under isoflurane anaesthesia, and animals were sacrificed 1 hour post-stimulation). After 2 more days of recovery, animals underwent subsequent behavioural or neurophysiological experiments. **B)** Representative coronal section and confocal images of the VTA (*upper panel*) and LC (*bottom panel*) showing c-Fos (red) labelled nuclei of TH^+ neurons (green) of 7-month-old Tg Sham and Tg tDCS mice (scale bar: 100 µm). Plots on the right show the number of $c\text{-}Fos^+/TH^+$ neurons, respectively within the VTA or the LC (Tg Sham: n=3; Tg tDCS: n=3; *VTA*: **p=0.0047 with 2-tailed unpaired *t*-test). **C)** Schematic representation of *in-vivo* microdialysis setup for measuring neurotransmitter release. The apparatus consists of an inlet for the infusion of aCSF or KCl at a constant flow rate (2.1 µL/min) and an outlet to collect dialysates for subsequent analysis. The first inset shows the anatomical placement of the unilateral hippocampal microdialysis probe in a coronal section of the mouse brain (probe and dialyzing membrane length: 5 and 3 mm, respectively; coordinates: AP+3.0, ML±3.0 mm from bregma), enabling extracellular fluid sampling for real-time neurotransmitter analysis. The second inset shows the semi-permeable membrane at the tip of the microdialysis probe, allowing the diffusion of molecules from the extracellular fluid of the brain into the perfusate. **D-E)** *In-vivo* microdialysis plots showing basal and KCl-induced hippocampal DA **(D)** and NE **(E)** levels in 7-month-old WT Sham (n=4), WT tDCS (n=4), Tg Sham (n=4) and Tg tDCS (n=6) mice measured at different time points (mean ± S.E.M.; **D**, 3-way RM-ANOVA: time × genotype × treatment, $F_{4,56}=0.7572$, p=0.5575; *time*, $F_{4,56}=16.60$, p<0.0001; *genotype*, $F_{1,14}=1.608$, p=0.2254; *treatment*, $F_{1,14}=4.785$, p=0.0462; Tg Sham Post KCl 1–Tg tDCS Post KCl 1 **p=0.0014, WT tDCS Basal Mean–WT tDCS Post KCl 1 p=0.0059, Tg tDCS Basal Mean–Tg tDCS Post KCl 1 p<0.0001 all with Tukey's post hoc test. **E**, 3-way RM-ANOVA: time × genotype × treatment, $F_{4,48}=0.1822$, p=0.9465; *time*, $F_{4,48}=26.24$, p<0.0001; *genotype*, $F_{1,12}=0.1302$, p=0.7245; *treatment*, $F_{1,12}=0.0500$, p=0.8268; Tg Sham Basal Mean–Tg Sham KCl p=0.0118, Tg tDCS Basal Mean–Tg tDCS KCl p=0.0003 both with Tukey's post hoc test).

6.2 Restoration of Synaptic Plasticity in the CA3-CA1 Synapses of Tg2576 Mice Following Prefrontal tDCS

The dopaminergic signalling from the VTA is critical for hippocampal-dependent memory formation and long-term potentiation (LTP), increasing synaptic strength in response to activity patterns or motivational demands (113,284). Indeed, we and others have previously shown that many hippocampal deficits in neuronal function and behaviour typical of Tg2576 mice can be restored by brief DA-based pharmacological treatments (116,145,147). Given the observed increase in hippocampal DA levels in Tg2576 mice following prefrontal tDCS, we next wondered whether this could be translated into functional improvements in LTP in the CA3-CA1 hippocampal pathway.

In line with previous observations (145,147), 7-month-old Tg Sham mice exhibited significant impairments in CA3-CA1 LTP compared to WT Sham mice. Importantly, prefrontal tDCS promoted a full restoration of LTP in Tg tDCS mice, comparable to the levels of WT sham mice (**Fig. 6**). The rescue of synaptic plasticity defects in Tg2576 mice suggests that prefrontal tDCS may counteract AD-related impairments in hippocampal function.

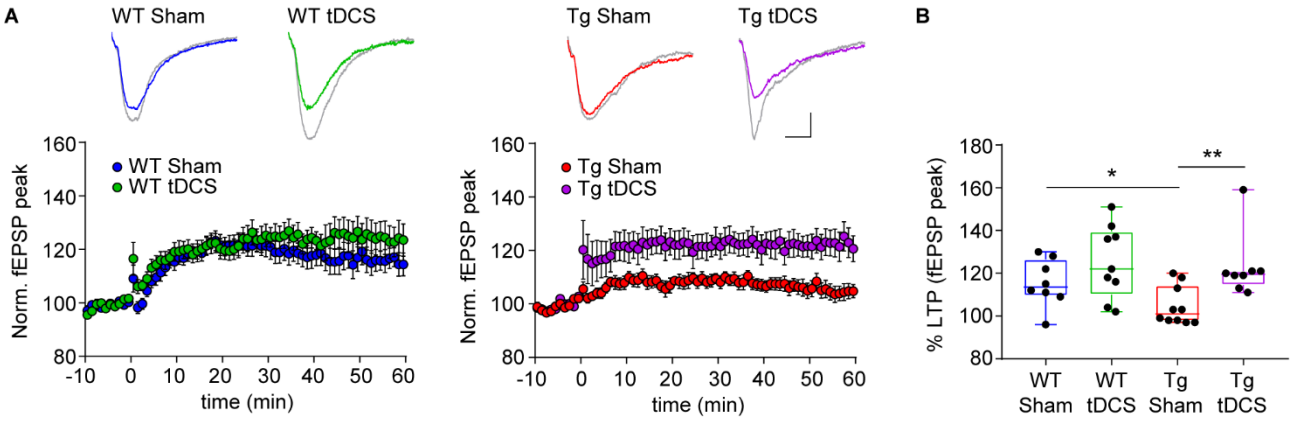

Figure 5. Prefrontal tDCS rescue synaptic plasticity in 7-month-old Tg2576 mice.
On the left are time-course plots showing LTP at CA3-CA1 synapses in acute hippocampal slices from 7-month-old Tg2576 and WT mice following tDCS or Sham treatment. Each plot presents the normalized average *f*EPSP peak amplitude (mean ± S.E.M) recorded in the CA1 region, with an initial 10-min baseline period, after which a HFS was applied to the Schaffer collateral pathway in CA3. Above are representative traces (scale bar: 0.1 mV; 0.5 s) illustrating *f*EPSPs at half-maximum stimulation recorded during baseline (*coloured trace*) and at 1-hour post-conditioning stimulus (*grey trace*). On the right is the box-and-whisker plot shows the percentage of potentiation measured 60 minutes post-HFS (WT Sham: n=8 slices/3 mice; WT tDCS: n=9 slices/4 mice; Tg Sham: n=10 slices/6 mice; Tg tDCS: n=8 slices/4 mice; WT Sham–Tg Sham *p=0.0368 with 2-tailed unpaired *t*-test; Tg Sham–Tg tDCS **p=0.0028 with Mann-Whitney test).

6.3 Prefrontal tDCS Restores Cognitive and Non-Cognitive Deficits in Tg2576 Mice

DA release in the hippocampus promotes novel-object recognition and spatial memory, reward-associated memory and memory consolidation (84,85,111–115). Thus, driven by the enhanced DA levels in the hippocampus, and the rescue of hippocampal synaptic plasticity following prefrontal tDCS, we next sought to determine whether the tDCS-mediated neuromodulation of the dopaminergic drive could translate into cognitive and/or non-cognitive improvements in Tg2576 mouse behaviour. We first focused on object recognition memory, a core domain of declarative memory often compromised in AD, and critically reliant on the integrity of the hippocampal network. Indeed, Tg2576 mice have repeatedly demonstrated significant defects in object recognition memory (285,286) that can be prevented by reducing the VTA DA neuron degenerative process (146). During the training phase of the Novel Object Recognition Test (NORT), no significant differences were observed in exploration times for the two identical objects across all groups (**Fig 7**), confirming an unbiased baseline for object preference.

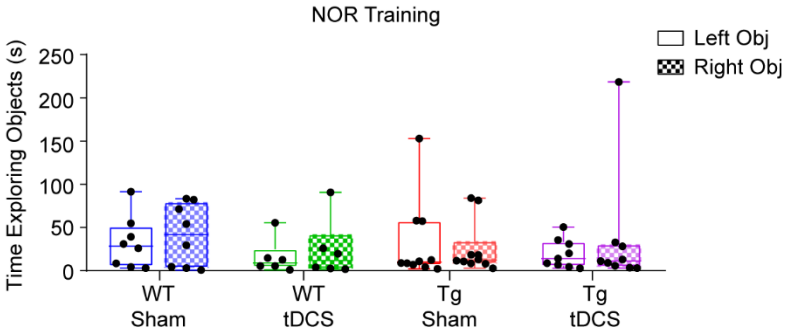

Figure 6. NORT training phase.

The graph shows the total time spent exploring two identical objects during the training phase of the NORT by 7-month-old WT and Tg2576 mice receiving tDCS or Sham stimulation (WT Sham: n=8; WT tDCS: n=6; Tg Sham: n=10; Tg tDCS: n=9).

During the testing phase 24 h after training, both WT Sham and WT tDCS groups showed a robust preference for the novel object, whereas Tg Sham mice did not display differential exploration between the two objects, confirming an impairment in recognition memory. In contrast, Tg tDCS mice behaved similarly to WT animals, showing significantly higher exploration of the novel object compared to the familiar one, indicating restored recognition memory comparable to WT controls (**Fig. 8A**).

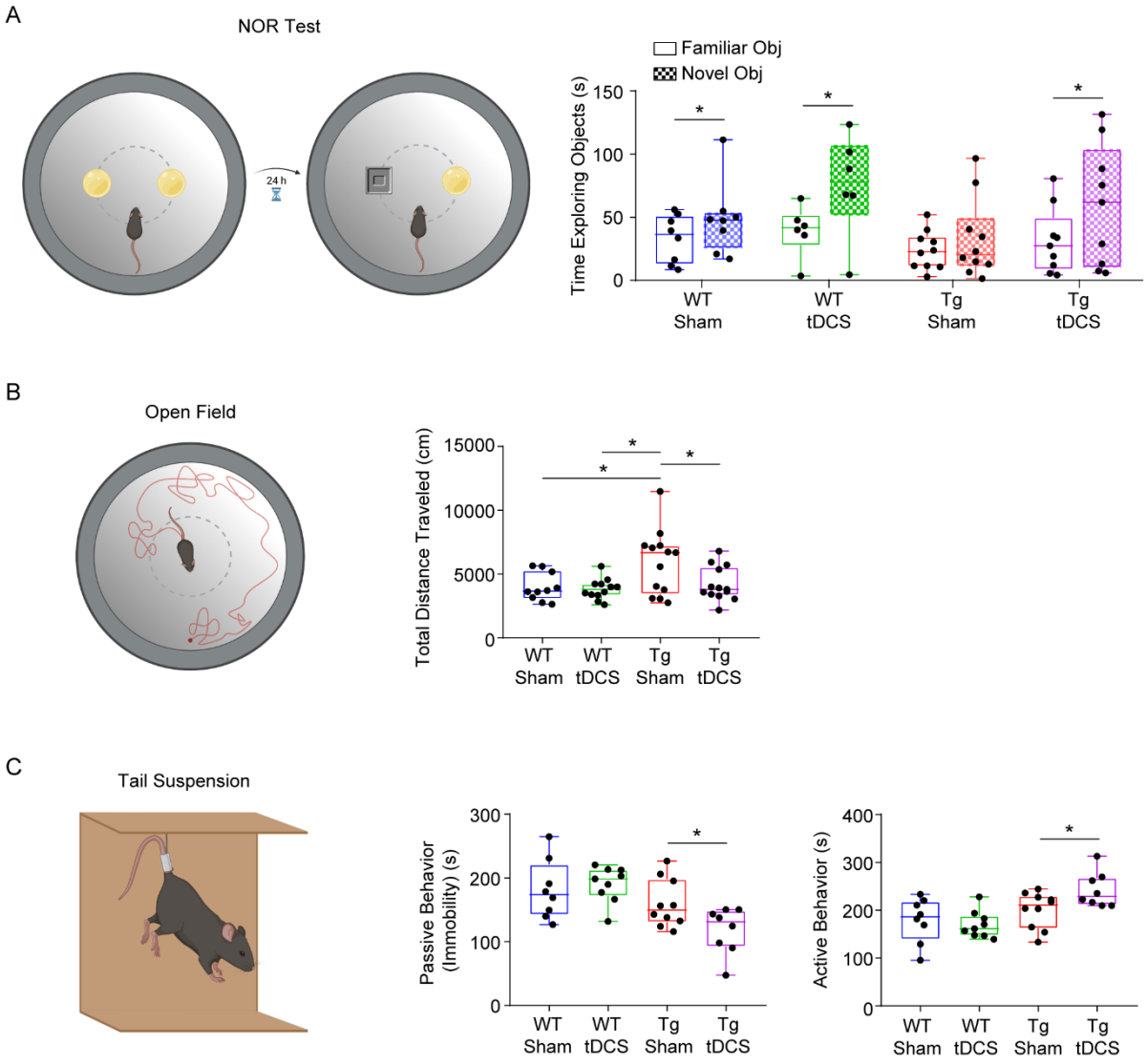

Figure 7. Prefrontal tDCS restores cognitive and non-cognitive functions in 7-month-old Tg2576 mice.
A) The graph shows the total time spent exploring objects (novel and/or familiar) during NORT, conducted 24 h after the training phase, by 7-month-old WT and Tg2576 mice receiving tDCS or Sham stimulation (WT Sham: n=8; WT tDCS: n=6; Tg Sham: n=10; Tg tDCS: n=9; WT Sham *p=0.0391 with Wilcoxon matched-pairs test; WT tDCS *p=0.0285, Tg tDCS *p=0.0425 both with Paired *t*-test). **B)** The plot represents the total locomotor activity, quantified as the distance traveled during the exploration of a novel environment in the OF test, by 7-month-old WT and Tg2576 mice following Sham or tDCS treatment (WT Sham: n=10; WT tDCS: n=12; Tg Sham: n=13; Tg tDCS: n=13; WT Sham–Tg Sham *p=0.0246; WT tDCS–Tg Sham *p=0.0129; Tg Sham–Tg tDCS *p=0.0444, all with 2-tailed Welch's *t*-test). **C)** The two box-and-whisker plots show antidepressant-like responses in the TST, quantified by the time spent in passive (immobility, *right*) and active (*left*) behaviours, reflecting coping strategies in 7-month-old WT Sham (n=8), WT tDCS (n=9), Tg Sham (n=10) and Tg tDCS (n=8) mice (Tg Sham–Tg tDCS *p=0.0303, for both active and passive behaviours, with 2-tailed unpaired *t*-test). [Figure created using BioRender.com]

Based on the known role of DA in driving motivation and reward *via* the VTA-NAcc pathway, and the fact that the VTA takes part in locomotion in novel environments (287), we tested mice through the OF test and TST to evaluate locomotor activity and depressive-like states, dysregulated in AD models (288,289). In the OF test, Tg Sham mice demonstrated increased locomotor activity,

covering a significantly greater distance compared to WT controls (**Fig. 8B**). However, tDCS treatment normalized the locomotor behaviour in Tg tDCS mice, reducing the total distance covered to WT levels. In the TST, Tg Sham mice unexpectedly showed no differences when compared with WT controls, indicating an apparent lack of depressive-like behaviours. This result may be attributable to the hyperlocomotion observed in this model, which could mask potential depressive-like immobility in this test. Instead, tDCS-treated Tg mice exhibited a strong reduction in immobility time and an increase in active behaviours compared to Tg Sham mice (**Fig. 8C**). Considering the reduced locomotor activity observed in Tg tDCS mice in the OF test, the increased active behaviours in the TST likely reflect a genuine improvement in motivation rather than an exacerbation of hyperactivity. To reinforce this hypothesis, we examined the levels of DAT in the NAcc core and shell regions, as a measure of dopaminergic fibers. As expected, DAT levels were strongly reduced in Tg Sham mice compared to WT animals in both NAcc regions (**Fig. 9**), mirroring the previously observed reduction in DA levels in naïve Tg2576 mice (145,290). Importantly, the prefrontal tDCS caused an increase in DAT levels in both regions of NAcc of Tg tDCS mice, in line with an enhancement of the dopaminergic drive to the NAcc.

Together, these data indicate that the neurochemical and functional changes induced by prefrontal tDCS contribute directly to the observed restoration of cognitive and non-cognitive symptoms of this AD model.

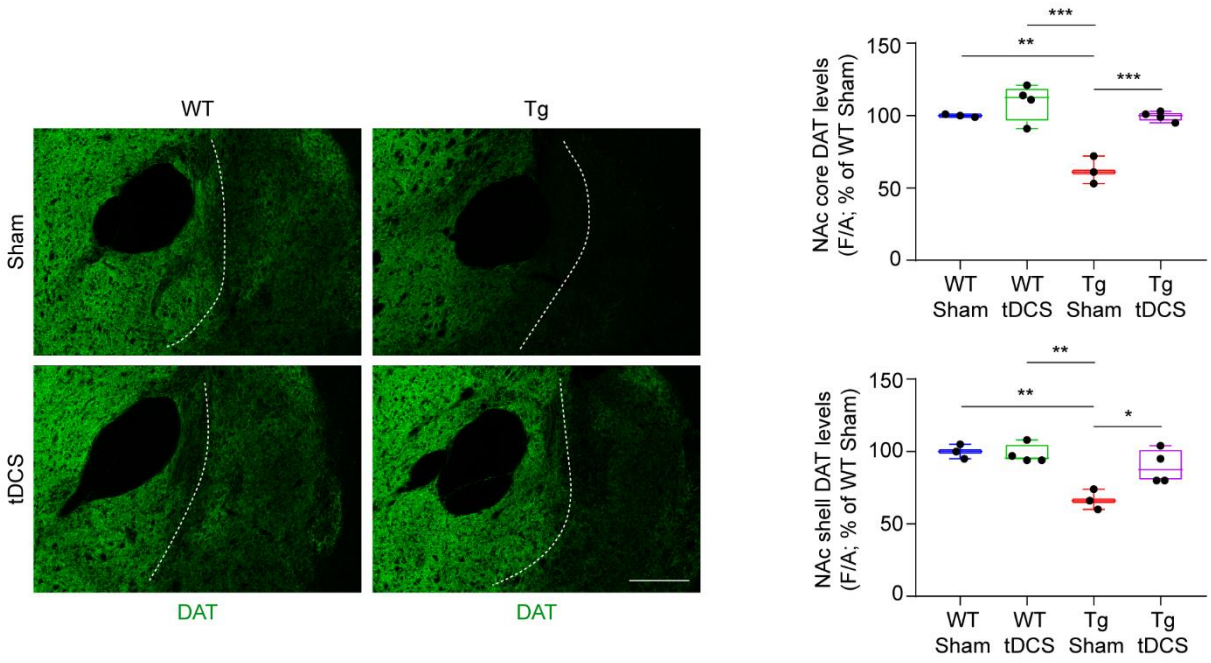

Figure 8. Prefrontal tDCS restores DAT levels in the NAcc of 7-month-old Tg2576 mice.

Representative confocal images of NAcc brain sections (scale bar: 200 μm) stained for DAT (green) of 7-month-old WT and Tg mice subjected to tDCS or Sham treatment. Dashed lines delineate the analysed region of the NAcc core (*on the left of the line*) and shell (*on the right of the line*). The adjacent plots display the DAT levels in the NAcc core (*upper*

panel) and NAcc shell (*bottom panel*), quantified through fluorescence intensity analysis (expressed as % of WT Sham levels), of 7-month-old WT Sham (n=3), WT tDCS (n=4), Tg Sham (n=3) and Tg tDCS (n=4) mice (*NAcc core:* 2-way RM-ANOVA: interaction, $F_{1,10}$=9.557, p=0.0114; *treatment*, $F_{1,10}$=26.17, p=0.0005; *genotype*, $F_{1,10}$=27.30, p=0.0004; WT Sham–Tg Sham **p=0.0012; WT tDCS–Tg Sham ***p=0.0001; Tg Sham–Tg tDCS ***p=0.0008, all with Tukey's post hoc test; *NAcc shell*: 2-way RM-ANOVA: interaction, $F_{1,10}$=7.528, p=0.0207; *treatment*, $F_{1,10}$=5.556, p=0.0402; *genotype*, $F_{1,10}$=21.36, p=0.0009; WT Sham–Tg Sham **p=0.0030; WT tDCS–Tg Sham **p=0.0028; Tg Sham–Tg tDCS *p=0.0209, all with Tukey's post hoc test).

6.4 Prefrontal tDCS ameliorates neuroinflammation and amyloid-β levels in Tg2576 mice

Dopaminergic receptors are expressed in different glia cells and their activation by DA has been shown to inhibit glial cell reactivity and release of pro-inflammatory cytokines (291–294). Given this inverse relationship between DA levels and neuroinflammation, we next investigated whether prefrontal tDCS, by activating DA neurons in the VTA, could improve neuroinflammatory responses in Tg2576 mice at pre-plaque stage and at an age marked by Aβ plaque accumulation.

At pre-plaque stage (7 months of age), Tg Sham mice showed an increased number of hippocampal $Iba1^+$ microglial cells compared to WT mice, with markedly altered morphology characterised by increased somatic area and higher complexity of dendritic branching, including more intersections, nodes, endings and increased dendritic length, suggesting morphologically reactive microglia. The prefrontal tDCS protocol determined a mild, yet insignificant, decrease in the number of $Iba1^+$ cells in Tg tDCS mice (**Fig. 10A,B**), while it significantly reduced microglial somatic area and changes in morphology toward a less activated state (**Fig. 10C,D**). At 12 months of age, an advanced disease stage characterised by Aβ plaque deposition in the hippocampus and cortex of Tg2576 mice, tDCS was equally effective in ameliorating hippocampal neuroinflammation. As shown in **Fig. 10E,F**, stereological cell counting revealed a marked reduction of $Iba1^+$ cell numbers in Tg tDCS mice compared to all other experimental groups. Additionally, tDCS application in Tg mice was effective in reducing both microglial somatic area and in reducing microglia branching (**Fig. 10G,H**), indicating a suppression of microglial activation even at this advanced disease stage (295–297).

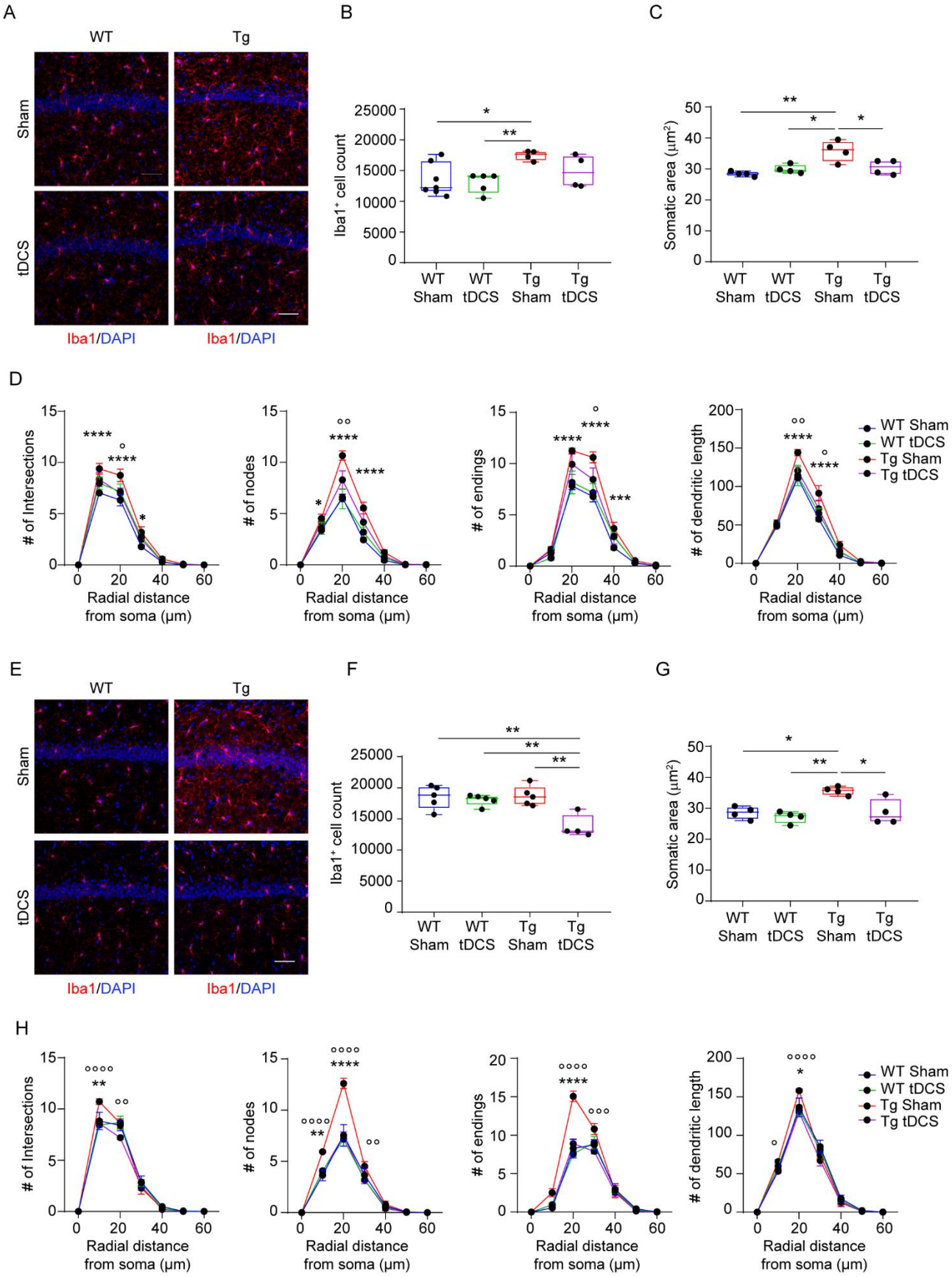

Figure 9. Prefrontal tDCS mitigates hippocampal neuroinflammatory activation and modulates microglial morphology in 7- and 12-month-old Tg2576 mice.

A) Representative confocal images (scale bar: 50 µm) of Iba1$^+$ cells (red) in the hippocampus of 7-month-old WT and Tg2576 mice receiving tDCS or Sham stimulation. Hippocampal neurons are counterstained with DAPI (blue). **B)** Plot

showing the stereological cell count of Iba1$^+$ cells in each experimental group (WT Sham: n=7; WT tDCS: n=5; Tg Sham: n=4; Tg tDCS: n=4; WT Sham–Tg Sham *p=0.0184; WT tDCS–Tg Sham **p=0.0017, all with 2-tailed unpaired *t*-test). **C)** Graph illustrating somatic area of hippocampal microglia in 7-month-old WT and Tg2576 (Sham vs tDCS) mice (n=4 mice per group; 2-way RM-ANOVA: interaction, $F_{1,12}$=9.344, p=0.0100; *treatment*, $F_{1,12}$=2.947, p=0.1117; *genotype*, $F_{1,12}$=12.86, p=0.0037; WT Sham–Tg Sham **p=0.0025, WT tDCS–Tg Sham *p=0.0128, Tg Sham–Tg tDCS *p=0.0246, all with Tukey's post hoc test). **D)** Plots representing morphological analysis of microglial complexity, including the number of intersections, nodes, endings and length of processes at different radial distance from soma in 7-month-old WT and Tg2576 following Sham or tDCS treatment (significant changes are marked by asterisks for WT Sham vs. Tg Sham and by dots for Tg Sham vs. Tg tDCS; n=4 mice per group; 2-way RM-ANOVA: Intersection (genotype): interaction, $F_{6,36}$=9.002, p<0.0001; *distance*, $F_{6,36}$=396.2, p<0.0001; *genotype*, $F_{1,6}$=12.18, p=0.0130; WT Sham–Tg Sham at 10 and 20 µm ****p<0.0001, and at 30 µm *p=0.0152; Intersection (treatment): interaction, $F_{6,36}$ =2.129, p=0.0737; *distance*, $F_{6,36}$=295.0, p<0.0001; *treatment*, $F_{1,6}$=2.752, p=0.1482; Tg Sham–Tg tDCS at 20 µm °p=0.0140; Nodes (genotype): interaction, $F_{6,36}$=27.65, p<0.0001; *distance*, $F_{6,36}$=433.9, p<0.0001; *genotype*, $F_{1,6}$=35.53, p=0.0010; WT Sham–Tg Sham at 10 µm *p=0.0189, at 20 and 30 µm ****p<0.0001; Nodes (treatment): interaction, $F_{6,36}$=2.420, p=0.0455; *distance*, $F_{6,36}$=153.9, p<0.0001; *treatment*, $F_{1,6}$=2.698, p=0.1516; Tg Sham–Tg tDCS at 20 µm °°p=0.0065; Endings (genotype): interaction, $F_{6,36}$=20.62, p<0.0001; *distance*, $F_{6,36}$=509.7, p<0.0001; *genotype*, $F_{1,6}$=29.38, p=0.0016; WT Sham–Tg Sham at 20 and 30 µm ****p<0.0001, and at 40 µm ***p=0.0004; Endings (treatment): interaction, $F_{6,36}$=1.909, p=0.1060; *distance*, $F_{6,36}$=235.2, p<0.0001; *treatment*, $F_{1,6}$=2.009, p=0.2061; Tg Sham–Tg tDCS at 30 µm °p=0.0326; Dendritic length (genotype): interaction, $F_{6,36}$=14.33, p<0.0001; *distance*, $F_{6,36}$=599.5, p<0.0001; *genotype*, $F_{1,6}$=13.18, p=0.0110; WT Sham–Tg Sham at 20 and 30 µm ****p<0.0001; Dendritic length (treatment): interaction, $F_{6,36}$=3.057, p=0.0160; *distance*, $F_{6,36}$=314.0, p<0.0001; *treatment*, $F_{1,6}$=3.523, p=0.1096; Tg Sham–Tg tDCS at 20 µm °°p=0.0085 and at 30 µm °p=0.0349, all with Sidak's multiple comparisons test). **E–H)** Analogous assessments in 12-month-old WT and Tg2576 mice following Sham or tDCS treatment, showing (**E**) representative confocal images (scale bar: 50 µm), (**F**) Iba1$^+$ cell count, (**G**) somatic area and (**H**) Sholl analysis of hippocampal microglial cells (**F**, WT Sham: n=5; WT tDCS: n=5; Tg Sham: n=5; Tg tDCS: n=4; 2-way RM-ANOVA: interaction, $F_{1,15}$=9.072, p=0.0088; *treatment*, $F_{1,15}$=13.51, p=0.0023; *genotype*, $F_{1,15}$=7.581, p=0.0148; WT Sham–Tg tDCS **p=0.0025, Tg Sham–Tg tDCS **p=0.0018, WT tDCS–Tg tDCS **p=0.0061; **G**, n=4 mice per group; 2-way RM-ANOVA: interaction, $F_{1,12}$=4.654, p=0.0520; *treatment*, $F_{1,12}$=10.45, p=0.0072; *genotype*, $F_{1,12}$=11.10, p=0.0060; WT Sham–Tg Sham *p=0.0102, WT tDCS–Tg Sham **p=0.0028, Tg Sham–Tg tDCS *p=0.0115; **H**, significant changes are marked by asterisks for WT Sham vs. Tg Sham and by dots for Tg Sham vs. Tg tDCS; n=4 mice per group; 2-way RM-ANOVA: Intersection (genotype): interaction, $F_{6,36}$=2.414, p=0.0460; *distance*, $F_{6,36}$=281.1, p<0.0001; *genotype*, $F_{1,6}$=0.5911, p=0.4712; WT Sham–Tg Sham at 10 µm **p=0.0065; Intersection (treatment): interaction, $F_{6,36}$=5.130, p=0.0007; *distance*, $F_{6,36}$=436.1, p<0.0001; *treatment*, $F_{1,6}$=23.63, p=0.0028; Tg Sham–Tg tDCS at 10 µm °°°°p<0.0001 and at 20 µm °°p=0.0051; Nodes (genotype): interaction, $F_{6,36}$=15.29, p<0.0001; *distance*, $F_{6,36}$=244.2, p<0.0001; *genotype*, $F_{1,6}$=9.721, p=0.0206; WT Sham–Tg Sham at 10 µm **p=0.0026 and at 20 µm ****p<0.0001; Nodes (treatment): interaction, $F_{6,36}$=26.63, p<0.0001; *distance*, $F_{6,36}$=446.2, p<0.0001; *treatment*, $F_{1,6}$=43.48, p=0.0006; Tg Sham–Tg tDCS at 10 and 20 µm °°°°p<0.0001 and at 30 µm °°p=0.0084; Endings (genotype): interaction, $F_{6,36}$=12.22, p<0.0001; *distance*, $F_{6,36}$=182.5, p<0.0001; *genotype*, $F_{1,6}$=11.72, p=0.0141; WT Sham–Tg Sham at 20 µm ****p<0.0001; Endings (treatment): interaction, $F_{6,36}$=13.59, p<0.0001; *distance*, $F_{6,36}$=247.0, p<0.0001; *treatment*, $F_{1,6}$=43.93, p=0.0006; Tg Sham–Tg tDCS at 20 µm °°°°p<0.0001 and at 30 µm °°°p=0.0003; Dendritic length (genotype): interaction, $F_{6,36}$=3.616, p=0.0066; *distance*, $F_{6,36}$=397.1, p<0.0001; *genotype*, $F_{1,6}$=0.4088, p=0.5462; WT Sham–Tg Sham at 20 µm *p=0.0123; Dendritic length (treatment): interaction, $F_{6,36}$=6.156, p=0.0002; *distance*, $F_{6,36}$=682.4, p<0.0001; *genotype*, $F_{1,6}$=12.90, p=0.0115; Tg Sham–Tg tDCS at 10 µm °p=0.0426 and at 20 µm °°°°p<0.0001, all with Sidak's multiple comparisons test).

Finally, we next investigated the impact of prefrontal tDCS on Aβ load on the basis of recent works reporting that DA or dopaminergic drugs can promote the degradation of Aβ and the disaggregation of amyloid fibrils (295–297). Analysis of intracellular Aβ levels at 7 months of age showed no differences in Tg tDCS mice compared to Tg Sham mice (**Fig 11A**). Yet, Tg tDCS mice at 12 months showed a significant reduction in the number of hippocampal Aβ plaques compared to Tg Sham mice, although plaque area remained unchanged across groups (**Fig 11B**).

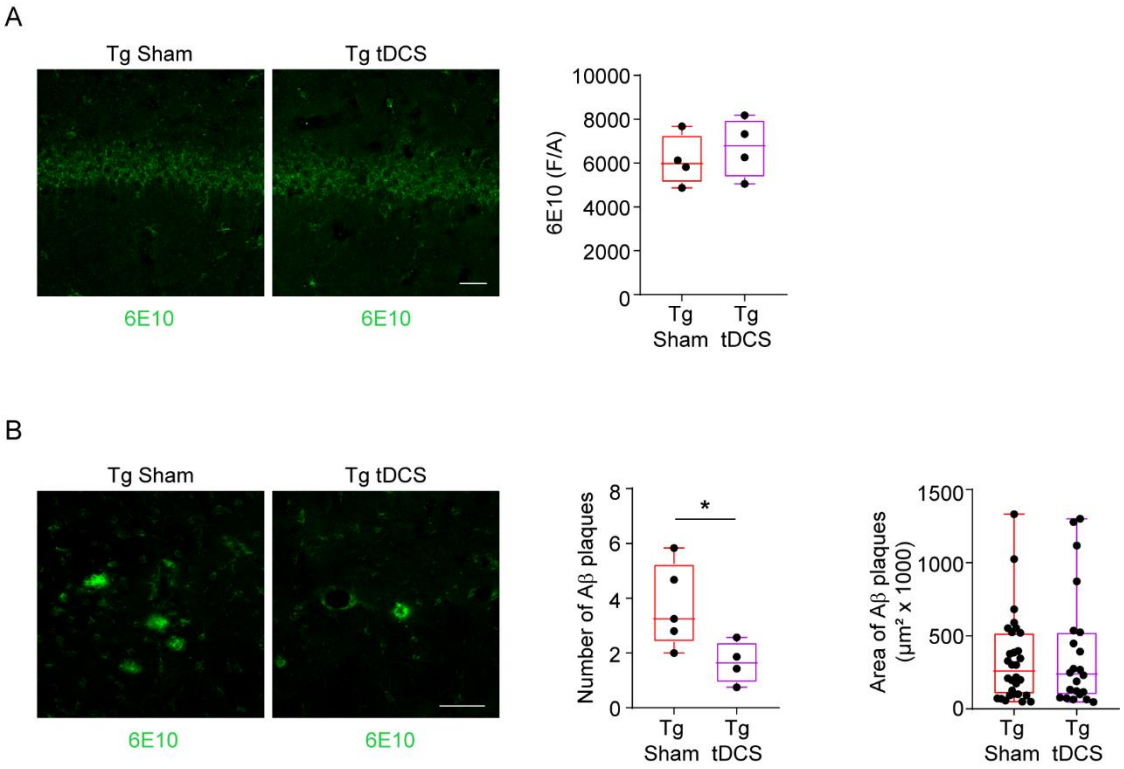

Figure 10. Prefrontal tDCS reduces aβ plaque burden in 12-month-old Tg2576 mice without affecting intracellular aβ at 7 months

A) Representative confocal images showing immunostaining of intracellular Aβ levels (6E10, green) in the hippocampal CA1 region of 7-month-old Tg Sham and Tg tDCS mice (scale bar: 50 μm). The adjacent graphs display intracellular Aβ quantification, measured as mean fluorescence intensity for 6E10 (n=4 mice per group; 2-tailed unpaired *t*-test). B) Representative confocal images of extracellular Aβ plaques immunostaining (6E10, green) in the hippocampus of 12-month-old Tg2576 mice receiving Sham or tDCS stimulation (scale bar: 50 μm) and plots reporting the number of Aβ plaque (*left*) and plaque area (*right*) (*Plaque number*: Tg Sham: n=5; Tg tDCS: n=4; *p=0.0453 with 2-tailed unpaired *t*-test; *Plaque area*: Tg Sham: n=30 plaques/5 mice; Tg tDCS: n=22 plaques/4 mice).

7. DISCUSSION

The present study represents a significant step forward in understanding the potential of prefrontal tDCS for modulating subcortical and deep brain targets like the dopaminergic VTA, highlighting the usefulness of this technique for AD therapy. Particularly, our results demonstrate the effectiveness of this tool in enhancing dopaminergic activity in the Tg2576 mouse model of AD, selectively by activating VTA TH^+ neurons. The acute assessment of c-Fos labelling allowed the immediate effects of neuronal activation to be captured, thereby providing valuable insights into the initial responsiveness of the dopaminergic system to neuromodulation. The lack of activation in LC TH^+ neurons shows that prefrontal tDCS does not broadly stimulate all catecholaminergic systems, but rather exerts a targeted effect specifically on DA neurons of the VTA. The observed activation was associated with a consequent increase in hippocampal DA outflow and an upregulation of DAT expression in the NAcc.

These findings are consistent with previous literature, particularly regarding the striatum, where tDCS has been shown to modulate dopaminergic pathways and enhance DA availability, both in healthy animal models and human subjects (245,246,252–254,256,257). However, our results are particularly noteworthy due to the early and progressive dopaminergic neurodegeneration present in the Tg2576 model, characterized by the loss of VTA dopaminergic neurons since early experimental ages. This underscores the capability of tDCS to effectively bypass extensive neurodegenerative barriers and drive the release of DA from the remaining dopaminergic neurons. Our findings align with previous evidence demonstrating that tDCS can enhance dopaminergic signalling even in the context of a pathology characterized by DA deficiency, such as PD (256). However, the differences in the underlying pathologies and mechanisms of action between the two studies merit careful consideration. In fact, in the MPTP-induced PD model, tDCS was reported to exert a neuroprotective effect, preventing the loss of TH^+ neurons in the striatum and thereby preserving dopaminergic signalling. This suggests that tDCS in this context acts to protect neurons from chemical toxicity and sustain DA production. By contrast, in our model, where significant dopaminergic neuron loss has already occurred by the time tDCS is applied, the evidence points to a distinct mechanism of action: tDCS appears to modulate the functional output of the surviving dopaminergic neurons in the VTA, even in the face of significant neurodegeneration. This suggests that tDCS has a dual therapeutic potential across different stages and types of neurodegenerative pathology, offering potential benefits through both neuroprotection and functional modulation depending on the timing and context of intervention. Notably, this raises an intriguing question: *had tDCS been applied at earlier time points in the Tg2576 model, before substantial degeneration had occurred (e.g., prior to 6 months of age),*

would it have also demonstrated neuroprotective effects? It is plausible that applying tDCS at earlier stages in the Tg2576 model might reveal neuroprotective effects akin to those described in the MPTP model, representing an intriguing avenue for future investigation.

The observed effects of prefrontal tDCS can be coherently explained within the framework of the mesocorticolimbic circuit's anatomy and physiology, within which the PFC exerts significant influence over the VTA and NAcc through its extensive projections. Anatomically, PFC terminals synapse on both DA and non-DA neurons in the VTA, selectively targeting GABA-containing mesoaccumbens neurons and DA-containing mesocortical neurons (298,299). This connectivity enables the PFC to modulate VTA neuron firing and DA release in forebrain regions, such as the hippocampus and NAcc (300). Functional coupling between the PFC and VTA has been observed, with DA neuron activity covarying with PFC cell activity on a subsecond timescale (301). This suggests a dynamic and bidirectional exchange of information, involving both excitatory glutamatergic projections and inhibitory pathways mediated by local INs. The observed effects of prefrontal tDCS in the present study can be coherently integrated within this framework. Moreover, this understanding is consistent with the broader consensus in the field, as multiple studies investigating the dopaminergic modulation induced by tDCS attribute its effects to the engagement of the mesocorticolimbic circuit. Within this framework, prefrontal tDCS likely enhances excitatory input to the VTA, predominantly *via* glutamatergic projections from the PFC. Simultaneously, tDCS may modulate local GABAergic INs in the VTA, resulting in a dual mechanism of increased excitation and reduced inhibition that elevates subcortical VTA dopaminergic output. This heightened dopaminergic activity subsequently activates downstream targets, including the hippocampus and NAcc, regions crucial for reward processing, memory and regulation of behaviour.

In the hippocampus, a region integral to learning and memory, basal DA levels remained unchanged following prefrontal tDCS. However, a significant increase in DA release was observed after KCl-induced depolarization, suggesting that dopaminergic terminals in this region become "more responsive" to stimulation. This heightened reactivity may result from tDCS-induced priming of synaptic mechanisms, such as enhanced vesicular DA storage or a lowered threshold for vesicular release, which mobilizes synaptic DA reserves in response to neural activation. These mechanisms could prime the system for more efficient release of neurotransmitters under specific conditions and are potentially mediated by changes in synaptic plasticity and receptor sensitivity. These observations correlate with the increase in hippocampal synaptic plasticity as well as improvements in cognitive performance that we observed in our mouse model, suggesting an enhancement of the dopaminergic responsiveness specifically under conditions of demand-dependent neurotransmitter release—such as

during HFS-induced synaptic potentiation. By selectively activating dopaminergic signalling in response to synaptic activity, tDCS could facilitate the restoration of LTP in the CA3-CA1 synapse in Tg2576 mice to levels comparable to WT controls. DA plays a critical role in these processes by modulating synaptic strength, facilitating the induction of LTP and enhancing the encoding and consolidation of memory (284,302). Thus, the selective increase in post-KCl DA levels, without altering basal DA levels, underscores the dynamic nature of the dopaminergic signalling, where baseline activity remains tightly regulated while the system's capacity for activity-dependent release is optimized. This dual effect—priming of hippocampal dopaminergic terminals and the resultant enhancement of plasticity and cognition—further supports the pivotal role of DA as a mediator of the beneficial effects of prefrontal tDCS on hippocampal-dependent functions. Additionally, the increased LTP observed in Tg2576 mice not only reflects an enhanced responsiveness of the dopaminergic system to specific stimuli but may also involve mechanisms previously documented to be modulated by tDCS, encompassing the modulation of NMDA receptor activity, promotion of BDNF expression and balancing of excitatory and inhibitory neurotransmission (223,303–305)—all mechanisms known to be modulated by DA (138,306,307). These synergistic effects provide a robust mechanistic framework for the restoration of LTP and highlight the multifaceted role of tDCS in engaging both dopaminergic and non-dopaminergic pathways critical for synaptic and cognitive function.

Numerous lines of evidence suggest that tDCS holds significant potential to enhance cognitive functions across a variety of contexts, with demonstrated efficacy in healthy human populations (308–310) and in animal models without underlying neuropathology (305,311). However, in the field of neurodegenerative diseases such as AD, most findings have emerged primarily from clinical studies: while the results obtained are promising—showing that tDCS may mitigate cognitive deficits and improve global cognition in AD patients—a substantial portion of the studies report inconclusive or relatively weak findings, particularly in MCI cases; moreover, these outcomes are often challenging to interpret due to variability in stimulation protocols, cognitive assessment tools and patient-specific factors (274,312–317). This inconsistency highlights the urgent need for a more robust preclinical research framework to refine and optimize tDCS protocols. Establishing standardized, targeted approaches within controlled experimental settings could pave the way for more consistent and interpretable clinical outcomes in AD research. To the best of our knowledge, this study represents the first investigation of tDCS effects in the Tg2576 mouse model of AD. Our findings demonstrate that prefrontal tDCS restores object recognition memory in Tg2576 mice during the NORT, reflecting enhanced declarative memory associated with hippocampal integrity.

In addition, prefrontal tDCS demonstrates broad neuromodulatory effects that extend beyond cognitive improvements, influencing non-cognitive behaviours such as locomotion and affective responses. In the OF test, prefrontal tDCS normalized the hyperlocomotion observed in Tg2576 mice, reflecting its ability to modulate activity levels associated with mesocorticolimbic circuit function. This reduction in locomotion aligns with the established role of the VTA in regulating motivated behaviour. Notably, the VTA's involvement in locomotor control is further corroborated by findings from optogenetic studies on the NAcc medial (NAcMed) inputs to the VTA: in this study it has been demonstrated that optogenetic stimulation of NAcMed terminals did not induce effects on anxiety or locomotion, while there was a notable post-stimulation reduction in general locomotor activity, suggesting that NAcMed inputs to the VTA may contribute to modulating locomotor behaviour indirectly through mechanisms that involve motivational states and post-stimulation responses (318). These findings, in conjunction with prefrontal tDCS-induced modulation of locomotor activity, suggest that tDCS may engage the mesocorticolimbic circuit, potentially activating the PFC-NAcc-VTA pathway. Similarly, in the TST, tDCS-treated mice exhibited reduced immobility and increased active behaviours, indicative of antidepressant-like effects. These findings are consistent with prior research highlighting the central role of DA in motivation and depression (319,320), further underscoring the influence of tDCS on dopaminergic pathways. The observed increase in DAT levels in the NAcc core and shell provides critical mechanistic insights into these behavioural effects. The NAcc serves as a key integration center within the mesocorticolimbic circuit, with the core subregion primarily involved in goal-directed behaviours and the shell in reward and affective processing.

The selective activation of the VTA and its associated pathways by prefrontal tDCS may also involve indirect, retrograde mechanisms. For instance, enhanced dopaminergic signalling from the VTA to the PFC could amplify excitatory feedback to the VTA, forming a bidirectional loop that reinforces mesocorticolimbic circuit engagement. This precise targeting likely reflects the dual mechanism of tDCS in modulating both excitatory and inhibitory inputs, allowing for the enhanced dopaminergic activity observed in the hippocampus and NAcc.

Furthermore, we found that prefrontal tDCS significantly reduced microglial reactivity in the hippocampus, as evidenced by decreased $Iba1^{+}$ cell numbers and less activated microglial morphology. These effects were observed both at the pre-plaque stage (7 months) and more severely also during advanced stages of amyloid pathology (12 months), demonstrating the anti-inflammatory potential of tDCS in both early and late disease contexts. The reduction in neuroinflammation observed in our study provides additional insight into the therapeutic effects of tDCS. Existing literature supports tDCS as a modulator of neuroinflammation, with reported reductions in pro-

inflammatory cytokines and activation of glial cells (236,239,321). Given the known role of DA in exerting anti-inflammatory effects (124–127), it might be reasonable to assume that the ability of tDCS to ameliorate the neuroinflammatory states in Tg2576 mice is driven by the enhanced increase of hippocampal DA outflow.

Finally, our study demonstrated a significant reduction in hippocampal Aβ plaque burden at 12 months following prefrontal tDCS treatment, whereas intracellular oligomer levels at 7 months remained unaffected. This divergence likely reflects distinct mechanisms underlying intracellular and extracellular Aβ regulation. Dopaminergic modulation, which is enhanced by prefrontal tDCS, may play a pivotal role in this process. Indeed, it has been shown that DA facilitates extracellular Aβ clearance by upregulating neprilysin, an Aβ-degrading enzyme (297). Furthermore, DA and L-DOPA have been reported to disrupt amyloid fibril aggregation, reducing the formation of neurotoxic plaques (295,296). These findings suggest that tDCS-driven dopaminergic enhancements may preferentially target extracellular amyloid pathology, particularly during later disease stages when plaque deposition becomes more prominent. The observed reduction in plaque burden aligns with this hypothesis, indicating that prefrontal tDCS may exert its therapeutic effects by leveraging dopaminergic pathways to accelerate the clearance or disaggregation of extracellular amyloid deposits. Further investigation is warranted to delineate the temporal dynamics and molecular mediators involved in these processes, which could have profound implications for understanding the progression of amyloid pathology and the optimization of tDCS-based interventions in AD.

Actually, Deep Brain Stimulation (DBS) remains the gold standard for specific conditions requiring high-precision modulation of deep brain structures. This technique has remarkable effectiveness, achieving significant therapeutic outcomes in conditions such as PD, dystonia and essential tremor (322–325). However, despite its proven efficacy, DBS remains an invasive and costly procedure, necessitating neurosurgical implantation of electrodes into the brain, coupled with the associated risks of infection, haemorrhage and hardware-related complications (326–328). Moreover, it requires extensive preoperative planning and post-operative programming, further increasing its complexity and economic burden. In parallel, non-invasive brain stimulation techniques like TMS have also demonstrated the ability to modulate neural activity, with significant depth of penetration and therapeutic applications in depression, anxiety, and even stroke rehabilitation (329). While TMS is non-invasive and does not require surgery, it relies on high-intensity magnetic pulses, which can cause discomfort or even pain during stimulation, limiting patient tolerance (330,331). Additionally, TMS equipment is often expensive and its precise targeting of subcortical structures depends on indirect pathways mediated through the cortex, leading to less focused activation compared to DBS

(332). In this context, tDCS stands out as a groundbreaking alternative, uniquely positioned at the intersection of efficacy and accessibility. Unlike DBS, tDCS is non-invasive, requiring no surgical intervention, and unlike TMS, it operates through low-intensity direct currents, resulting in a painless and well-tolerated experience for most users. Furthermore, tDCS is not only more cost-effective and portable but also offers the advantage of continuous stimulation over longer durations, allowing for extended neuromodulatory effects. Additionally, tDCS requires only basic equipment for administration, making it highly adaptable for use in various settings, including large-scale clinical applications and at-home interventions. Its safety profile, simplicity of use and the possibility of combining it with behavioural or pharmacological interventions further enhance its therapeutic potential.

Of note, this is the first study to apply tDCS in awake AD mice. The scientific literature extensively documents the impact of general anaesthesia on cerebral metabolism, neuronal activity and sensory stimulus responses, highlighting potential alterations in neuronal excitability and synaptic dynamics (333,334). These effects may introduce significant biases in the interpretation of experimental data, making it plausible that findings from previous studies were at least partially influenced by aesthetic-related effects. In our study, instead, the adoption of a stimulation protocol conducted in the absence of anaesthesia represents a critical methodological choice to ensure the accuracy and reliability of the results. This approach preserves the natural physiology of the brain, minimizing confounding factors introduced by pharmacological interference with CNS function. Consequently, the findings reported here can be considered an authentic representation of the neural and behavioural responses induced by stimulation, providing a robust foundation for the conclusions drawn. Thus, this methodological adaptation not only ensures physiological relevance but also aligns our findings more closely with potential clinical applications.

8. LIMITATIONS AND FUTURE PERSPECTIVES

While our study provides robust evidence for the therapeutic effects of tDCS, certain limitations warrant consideration. A critical aspect to consider is that, while our study corroborates and extends the findings of Peanlikit et al. (246) to AD animal models, thus strengthening the evidence base, it is essential to validate these outcomes in human patients to ensure their robustness and clinical translatability. The inherent interspecies differences highlight the pressing need for rigorous investigation into how variations in current amplitude, density and brain architecture modulate the neuromodulatory effects of tDCS in MCI and AD patients. Notably, as emphasized also by Peanlikit et al., current intensity is a pivotal parameter when comparing preclinical and clinical studies: although tDCS in the preclinical models effectively activated specific brain regions—demonstrating region-specific modulation even at high current densities—the absolute current density used in animal studies remains disproportionately low relative to that applicable in humans. This discrepancy raises the possibility that the outcomes observed in our study may not be directly replicable in humans without adjustments to the stimulation protocol. Future studies will need to explore whether higher current intensities, within the safety limits of clinical tDCS, can replicate the same targeted neuromodulatory effects observed in preclinical AD models.

Additionally, the precise mechanisms underlying how prefrontal tDCS can restore dopaminergic function remain to be fully elucidated. In particular, it is necessary to determine whether tDCS activates the mesocorticolimbic DA system through direct action on neuronal elements or indirectly *via* broader network modulation. tDCS-induced electric fields in the PFC could, in theory, directly depolarize cortical neurons projecting to subcortical regions, thereby directly driving dopaminergic circuits. On the other hand, tDCS might engage the DA system indirectly by inducing plastic changes in brain networks that secondarily enhance dopaminergic tone. The challenge for future research is to disentangle these mechanisms. Electrophysiological or imaging studies could be designed to monitor dopaminergic neuron activity in real time during tDCS, determining if these neurons are directly excited by the stimulation or if their activation is downstream of cortical network changes. Likewise, combining tDCS with pharmacological blockers or chemogenetic/optogenetic approaches could help parse out direct versus polysynaptic effects on the DA system. Clarifying how tDCS promotes dopaminergic activation at the mechanistic level will not only deepen our understanding of its therapeutic action in AD but also inform the rational design of stimulation protocols that specifically target the most effective pathways.

A notable limitation is the narrow scope of our assessment of neuroinflammatory. Our study primarily evaluated microglial activation, but did not characterize astrocytic responses. AD-related

neuroinflammation is driven by a complex interplay between microglia and astrocytes, with astrocyte reactivity amplifying neuronal damage and contributing to a deleterious feedback loop of neurodegeneration. While evidence from the literature indicates that tDCS can modulate central immune cells – altering microglial and astrocytic activity and shifting cytokine release toward an anti-inflammatory profile – our experiments did not directly examine these effects on astrocytes or the broader cytokine environment. A more comprehensive characterization of neuroinflammatory processes is therefore needed. Future studies should include markers of astrocyte activation (such as GFAP) and measure pro- and anti-inflammatory cytokines to determine how tDCS influences a broader spectrum of neuroinflammation. This approach would clarify the role of tDCS in modulating glial-mediated inflammatory pathways in AD and potentially help to identify additional mechanisms by which tDCS confers neuroprotection.

Another key question that remains unanswered is the long-term persistence of the beneficial effects observed. We assessed outcomes shortly after the stimulation period, but it is unclear whether the improvements in synaptic plasticity, neuroinflammation and cognition are transient or enduring. The long-term sustainability of tDCS-induced effects remains unexplored in our model. This represents a limitation because any potential therapy for AD should ideally produce lasting benefits. Future investigations should extend the post-stimulation observation window to determine how long the enhancements in behaviour and pathology persist. For example, follow-up assessments weeks or months after tDCS could reveal if cognitive and synaptic gains diminish or are maintained. If the benefits are found to be short-lived, it will be important to evaluate strategies like booster stimulation sessions or combined therapeutic approaches (e.g., pharmacotherapy or cognitive training) to sustain the improvements. Conversely, if benefits persist, that would strengthen the case for tDCS as a viable long-term therapeutic strategy for neurodegenerative conditions.

The use of a single transgenic mouse model is a further limitation that affects the generalizability of our findings. The Tg2576 line, which overexpresses mutant human APP, models a specific aspect of AD pathology and does not encompass the full complexity of the human disease. It will be important for future research to replicate and extend our results in other AD models that capture different facets of the disease, such as tau pathology or faster amyloid deposition. Using multiple models could also elucidate whether certain pathological features influence the treatment efficacy, as well as enhance the robustness and translational relevance of prefrontal tDCS as a potential therapy for AD. Indeed, the extent to which our findings translate to clinical populations remains uncertain, necessitating further validation in additional preclinical models and, ultimately, in human trials. In

future research it would be useful to evaluate higher current stimulation intensities and their effects on MCI and AD patients to ensure prefrontal tDCS application into the clinical practice.

Lastly, an important future direction is the exploration of synergistic interventions, such as combining tDCS with cognitive training or rehabilitation exercises. Given that tDCS can enhance neuroplasticity and cortical network connectivity, pairing stimulation with concurrent cognitive or behavioural training could leverage this heightened plasticity to produce greater cognitive benefits. In clinical contexts, there is growing interest in such combination approaches. Addressing these limitations and pursuing the outlined future directions will be crucial for advancing prefrontal tDCS from an experimental therapy towards a clinically relevant intervention for AD. By building on the foundation laid in this thesis, future research can better determine the potential of tDCS to modify the course of AD and related disorders in a meaningful and sustained manner.

Despite these limitations, our study provides compelling evidence of the non-invasive therapeutic potential of tDCS in AD and highlights its broader implications for treating neurodegenerative disorders. The observed effects on dopaminergic modulation, synaptic plasticity and behaviour, as well as reductions in neuroinflammation and amyloid pathology—demonstrating the multi-targeted therapeutic potential of this tool—pave the way for future research and clinical applications, potentially transforming the landscape of non-invasive treatments for AD.

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