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Modulation of BPSD phenotypes in Alzheimer's disease by cognitive stress, voluntary physical activity, and social isolation: Studies in 3xTg-AD mice and non-transgenic C57BL/6J counterparts

Daniel Alveal Mellado

Doctoral Thesis

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Author	*Director and Supervisor*
Daniel Alveal Mellado	***Dra. Lydia Giménez Llort***

"Happiness is only real when shared."

—Christopher McCandless

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Abstract

Behavioral and Psychological Symptoms of Dementia (BPSD) are a significant challenge throughout the progression of Alzheimer's Disease (AD), imposing substantial strain on both patients and their families. These symptoms are often underexamined due to their complex and heterogeneous nature. In this context, animal models replicating these symptoms provide a valuable tool for isolating specific behaviors and studying their evolution across age and environmental conditions. This doctoral thesis investigates BPSD-like phenotypes using a triple-transgenic mouse model of AD (3xTg-AD) and non-transgenic C57BL/6J mice, across three experimental paradigms that model cognitive stress, voluntary physical activity, and social isolation.

In the first study, mice underwent the Morris Water Maze (MWM) test at two distinct ages (12 and 16 months). Their performance, in what is considered a stressful environment for mice, was assessed by analyzing the swimming strategies employed to locate a submerged platform. Employing robust statistical methods to account for interindividual and baseline variations, circling behavior was identified as a unique BPSD-like pattern within the transgenic group. The second study allowed mice access to a running wheel, and their circadian activity patterns were recorded for one month. Results indicated sex-dependent differences in behavioral regulation, with transgenic males displaying patterns consistent with disruptions reported in human AD. In the third study, the impact of social isolation was evaluated in 13-month-old mice. A distinctive digging pattern, mimicking obsessive-compulsive behavior, was identified exclusively in isolated animals, representing a novel non-cognitive marker of BPSD-like disruption.

Together, these findings highlight the subtle, context-dependent nature of BPSD-like behaviors and underscore the importance of environmental and individual factors in shaping their expression. This work contributes to the refinement of translational models for AD and enhances the understanding of BPSD under experimentally controlled conditions.

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Abbreviations

- Alzheimer's Disease (AD)
- Amyloid-beta plaques (Aβ)
- Amyloid precursor protein (APP)
- Apolipoprotein E (APOE)
- Behavioral and psychological symptoms of dementia (BPSD)
- Elevated plus maze (EPM)
- Generalized Linear Models (GLMs)
- Generalized Linear Mixed Effects Models (GLMMs)
- Hypothalamic-pituitary-adrenal axis (HPA axis)
- Linear Mixed Effects Models (LMMs)
- Marble burying test (MB test)
- Mild Cognitive Impairment (MCI)
- Mixed Effects Models (MEMs)
- Morris Water Maze test (MWM)
- Non-transgenic mice (NTg)
- Novelty-induced behavioral inhibition (NIBI)
- Nucleus accumbens (NAc)
- Obsessive-compulsive disorders (OCD)
- Open field test (OF)
- Physical activity (PA)
- Presenilin genes 1 and 2 (PS1 and PS2)
- Social isolation (SI)
- Triple-transgenic mouse model of Alzheimer's Disease (3xTg-AD)
- Ventral tegmental area (VTA)
- Voluntary wheel running (VWR)

1 INTRODUCTION

1.1 Aging, Dementia, and Alzheimer's Disease

The natural aging process is marked by a complex interplay of elements including molecular, structural, and social changes that significantly impact an individual's overall health (Hou et al., 2019). At the molecular level, factors such as DNA damage, telomere shortening, and chromosomal alterations contribute to compromised cellular function and eventual cell death (López-Otín et al., 2013). Structural changes, particularly in the hippocampus and prefrontal cortex, play a critical role in memory and spatial navigation, contributing to cognitive decline (Moffat, 2009). Additionally, aging often leads to social changes, including reduced interactions due to retirement, bereavement, and limited mobility (Schrack et al., 2014) . Collectively, these molecular, structural, and social variations increase the susceptibility to chronic diseases, including neurodegenerative disorders like dementia, which is particularly prevalent in older populations (Wyss-Coray, 2016).

Dementia is a syndrome characterized by a progressive decline in memory, thinking, and personality, caused by dysfunction in cortical and subcortical brain regions (Ritchie & Lovestone, 2002). This condition encompasses a range of diseases, each distinguished by specific underlying causes and clinical manifestations. For instance, vascular dementia results from impaired cerebral blood flow, while Lewy body dementia is defined by the presence of abnormal alpha-synuclein protein deposits. Similarly, frontotemporal dementia predominantly affects the frontal and temporal lobes, leading to marked changes in personality and language abilities. Among these, Alzheimer's Disease (AD) stands out as the most prevalent form of dementia, accounting for 60-80% of all cases ("2020 Alzheimer's Disease Facts and Figures," 2020; Ritchie & Lovestone, 2002). Given its prevalence and profound impact, AD is the primary focus of this doctoral thesis.

Dementia, and specifically AD, poses a significant global health challenge, placing a heavy burden on patients, their families, and healthcare systems (Lane et al., 2018). Currently, approximately 5% of individuals over 65 are affected, with this prevalence doubling every four years to reach about 30% by age 80 (Ritchie & Lovestone, 2002). Global estimates indicate that 57 million people were living with dementia in 2019, a number projected to surge to 150 million by 2050 (Nichols et al., 2022) (Figure 1). This substantial increase is largely attributed to population growth and ageing. However, a notable

heterogeneity in incidence exists between high-income and low-income countries. While incidence rates in high-income nations have remained relatively stable, low-income countries are expected to experience the most significant increases ("2025 Alzheimer's Disease Facts and Figures," 2025; Nichols et al., 2022).

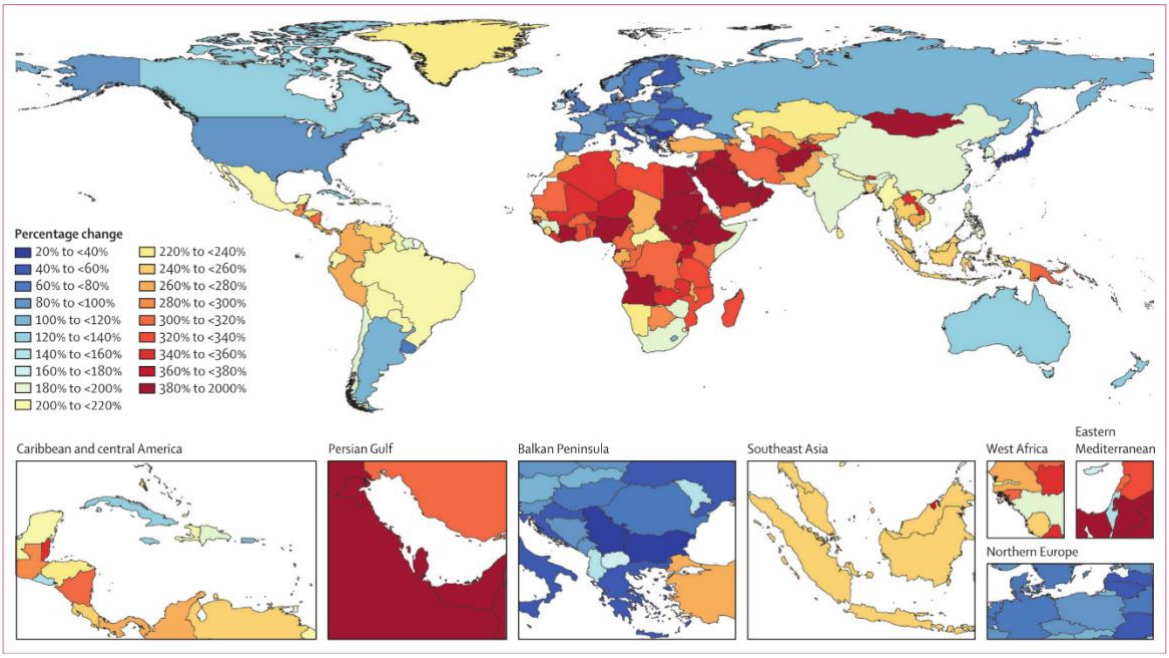

Figure 1. Percentage change between 2019 and 2050 in all-age number of individuals with dementia by country. Extracted from Nichols et al., 2022.

AD is a chronic neurodegenerative disorder that begins with memory loss, learning difficulties, and speech impairments. As the disease progresses, it leads to severe cognitive decline, behavioral disturbances, and loss of independence, with an average survival time of 8 to 10 years post-diagnosis (Jost & Grossberg, 1995; Lam et al., 2013; Masters et al., 2015).

In AD, the formation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) constitutes the hallmark of the condition (Lane et al., 2018). Aβ plaques arise from the aggregation of peptides derived from the amyloid precursor protein (APP) following enzymatic cleavage by beta-secretase and gamma-secretase. As a result, an overproduction or impaired clearance of Aβ, particularly the Aβ42 isoform, leads to the extracellular deposition of plaques (Schneider et al., 2009). These plaques interfere with neuronal communication and elicit immune responses, resulting in inflammation and neuronal damage (Hardy & Selkoe, 2002). Conversely, NFTs form intracellularly as a result of

hyperphosphorylated tau protein, which normally stabilizes microtubules within neurons. Hyperphosphorylation causes tau to detach and aggregate into paired helical filaments, disrupting neuronal function and ultimately leading to cell death (Serrano-Pozo et al., 2011). Together, Aβ plaques and NFTs drive synaptic dysfunction, neuronal loss, and the progressive cognitive decline characteristic of AD, with the severity of NFTs correlating strongly with the clinical manifestations of dementia (Lane et al., 2018; Schaap et al., 2024).

Post-mortem analyses have illuminated the temporal progression and distribution of biological markers in the brain, revealing distinct processes (Braak & Braak, 1991; Thal et al., 2002). Landmark studies, such as that by Braak and Braak (1991), have shown progressive biomarker accumulation and classified Aβ plaques into three stages (A-C) and NFTs into six stages (I-VI) (Figure 2).

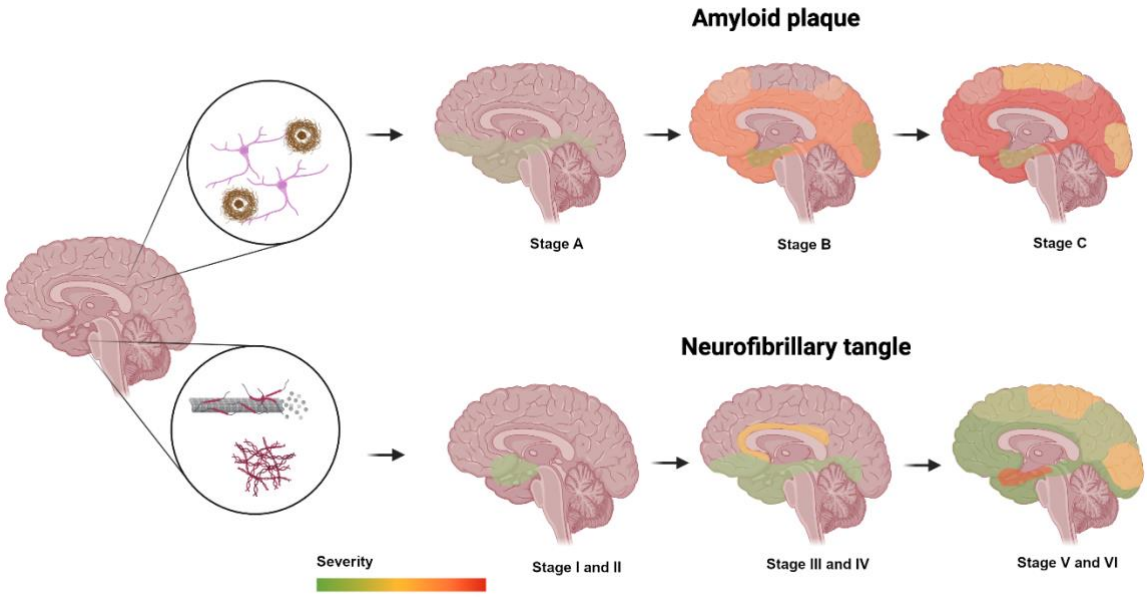

Figure 2. Pathological evolution of Alzheimer's Disease (AD) biomarkers in the human brain. The figure illustrates the extracellular accumulation of amyloid plaques (upper circle) and the intraneuronal accumulation of neurofibrillary tangles (bottom circle). Image adapted from Masters et al., 2015. Created with Biorender.com.

Amyloid deposition initially accumulates in the isocortex (the outermost layer of the cerebral hemispheres), particularly in the precuneus, posterior cingulate gyrus, and medial orbital frontal cortex (Lane et al., 2018) . Subsequently, subcortical regions such as the accumbens, putamen, and caudate show significantly increased accumulation. In contrast, NFT pathology typically originates in the allocortex (an older, more primitive cortical

region), affecting areas like the perirhinal and entorhinal cortex, followed by the CA1 region of the hippocampus (Serrano-Pozo et al., 2011).

Modern in-vivo imaging techniques, including positron emission tomography (PET) using ligand detection and cerebrospinal fluid (CSF) analysis in longitudinal studies, have enabled the detection and evaluation of biomarkers over time. These studies indicate that Aβ plaque accumulation precedes NFT accumulation and it begins decades before the onset of clinical symptoms (Gordon et al., 2018; Masters et al., 2015). Similarly, research has demonstrated a significant correlation between the presence of NFTs and cognitive decline (Macedo et al., 2024).

The clinical diagnosis of AD remains challenging due to symptom overlap with other dementias (Brenowitz et al., 2017; Kapasi et al., 2017). This underscores the need for a comprehensive clinical evaluation, biomarker analysis, and neuroimaging to distinguish AD from related conditions. Moreover, AD progression can be understood as a continuum, moving through distinct stages ("2020 Alzheimer's Disease Facts and Figures," 2020): (i) a preclinical or prodromal stage marked by brain changes without evident symptoms, (ii) mild cognitive impairment (MCI) due to AD, where early memory symptoms emerge as the brain's compensatory mechanisms begin to fail, and (iii) dementia due to AD, which is further divided into mild, moderate, and severe phases (Figure 3). These stages correspond to increasing levels of functional impairment in daily life.

The disease can be classified into two main categories based on its etiology and risk factors: familial and sporadic forms (Masters et al., 2015). Familial AD is a rare condition, accounting for only 1-5% of cases, and typically manifests before the age of 65. It is caused by genetic mutations in specific genes involved in Aβ processing, such as the APP and presenilin genes (PS1 and PS2) (Zhang et al., 2013). In contrast, sporadic AD is the most prevalent form, representing over 95% of cases, and usually develops after the age of 65.

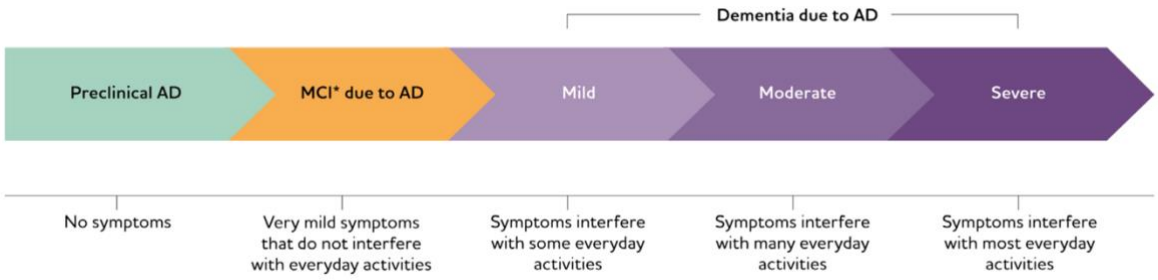

Figure 3. Alzheimer's Disease continuum. Extracted from 2020 Alzheimer's disease facts and figures (2020)

The risk factors for sporadic AD are broadly categorized into three groups: (i) age, as the incidence of AD increases significantly in individuals over 65 years old (Masters et al., 2015); (ii) genetic, primarily linked to the presence of the apolipoprotein E (APOE) gene, particularly the epsilon 4 (ε4) allele. APOE plays a key role in lipid metabolism, and the ε4 variant is associated with disrupted cholesterol homeostasis, which may impair brain myelination and contribute to cognitive decline (Blanchard et al., 2022; Evans et al., 2003); and (iii) environmental and lifestyle factors, such as physical inactivity, midlife hypertension, midlife obesity, smoking, diabetes, depression, and low educational attainment (Barnes & Yaffe, 2011; Norton et al., 2014).

1.1.1 Behavioral and Psychological Symptoms of Dementia

All forms of dementia encompass both cognitive and non-cognitive symptoms. While cognitive deficits often dominate clinical focus and public concern, non-cognitive symptoms impose a significant burden on patients and caregivers (Lyketsos et al., 2002; Piccininni et al., 2005). Historically referred to as "neuropsychiatric symptoms," these manifestations are now widely recognized as behavioral and psychological symptoms of dementia (BPSD) (Cerejeira et al., 2012).

BPSD comprise a wide range of manifestations, including disturbances in emotion, mood, perception, thought, motor activity, and personality traits (Finkel et al., 1997). Although highly heterogeneous, Cerejeira et al. (2012), followed by Kales et al. (2015), proposed a classification framework for BPSD, categorizing symptoms into affective clusters, such as depression and anxiety, and psychotic clusters, including delusions and hallucinations. Additional symptoms, such as agitation, aggression, and aberrant motor behaviors, have been described using terms like hyperactivity and frontal lobe syndromes. However, other symptoms, including apathy, sleep disturbances, and eating disorders, lack consistent classification. This classification framework is particularly valuable for drawing parallels with animal behavior, which will be explored further in subsequent sections.

Regarding their prevalence, BPSD are reported in 45% to 80% of patients with AD (Cerejeira et al., 2012). Notably, multiple disturbances often co-occur (Monastero et al., 2009). Among the various symptoms, apathy, depression, and agitation have been identified as the most frequently observed (Laganà et al., 2022; Lyketsos et al., 2002). Additionally, the prevalence of specific symptoms appears to vary along the disease continuum: apathy and depression are more common in the early stages of AD, while hallucinations and delusions tend to manifest more frequently in later stages (Piccininni et al., 2005).

An important characteristic of BPSD is the variation in symptom intensity throughout the day. For example, many patients experience heightened confusion, agitation, and behavioral disturbances during the late afternoon and evening—a phenomenon known as sundowning behavior, which is associated with disruptions in circadian rhythms (Frisoni et al., 1999). This phenomenon is particularly distressing for family members and caregivers,

often leading to institutionalization and representing a significant healthcare burden (De Vugt et al., 2003; Volicer et al., 2001).

Research to date has not yet identified the specific causes of BPSD. However, these symptoms are believed to arise from a dynamic interplay among patient-related factors, caregiver well-being, and environmental conditions (Kales et al., 2015). At the patient level, disruptions in neurocircuitry within regions responsible for emotion and cognition—such as the temporal, frontal, and parietal lobes—are thought to play a significant role, often linked to the pathological accumulation of amyloid plaques and tau protein. The mental health of caregivers is equally critical, as studies suggest that depression and stress in caregivers can exacerbate BPSD in community-dwelling patients (De Vugt et al., 2003). Furthermore, environmental factors, including household composition, daily routines, and caregiving practices, can act as significant triggers (Smith et al., 2006).

In summary, addressing BPSD requires a holistic approach that considers the neurological underpinnings of the disease, the emotional resilience of caregivers, and the influence of environmental factors. Future research should focus on integrated interventions that target these interconnected domains to improve outcomes for patients and their families.

1.2 Preclinical Research in AD

Various animal models have been developed to replicate the pathological and clinical characteristics of human AD, aiding in the study of disease progression and the evaluation of potential treatments (Mullane & Williams, 2019) . Among these, genetically modified mice are commonly used, with specific genetic mutations introduced into their genome. These mutations typically involve genes implicated in AD pathogenesis, such as APP, PS1, and tau protein.

This thesis focuses on the triple transgenic mouse model of AD (3xTg-AD), which is particularly valuable due to its expression of both Aβ and tau pathologies. These pathologies result from three key genetic mutations: APPswe, PS1M146V, and tauP301L (Oddo et al., 2003). These mutations lead to a range of behavioral and cognitive deficits that closely mimic those observed in humans, including impairments in working and reference memory, heightened anxiety, and altered motor functions (Giménez-Llort et al., 2007). Furthermore, 3xTg-AD mice exhibit age-related changes in cognitive performance and stress responses (Clinton et al., 2007).

Importantly, this model serves as a translationally relevant platform for preclinical research, enabling the study of interventions targeting both cognitive and non-cognitive symptoms of AD.

1.2.1 BPSD in Animal Models of AD

Replicating BPSD in animal models poses a significant challenge due to the subjective nature of these symptoms in humans. To address this, researchers have developed specific behavioral assays, where impaired responses in animals are interpreted as resembling particular BPSD features (Baeta-Corral & Giménez-Llort, 2014; Kosel et al., 2020; Santana-Santana et al., 2021). Utilizing the classification mentioned in the previous section (Cerejeira et al., 2012; Kales et al., 2015), symptoms in the affective cluster are assessed through tests such as the forced swimming test (behavioral despair) and the sucrose preference test (anhedonia), which evaluate motivational and affective deficits associated with depression-like behaviors (Crowley et al., 2004; Ramírez-Boix & Giménez-Llort, 2019). Anxiety-like behavior, on the other hand, is examined using the elevated plus maze (EPM) and open field

(OF) tests, which measure exploratory behaviors in potentially threatening environments (Carola et al., 2002). Additionally, the corner test (CT) -another neurobehavioral assessment- is used in mice to evaluate their response to a novel environment and exploratory activity by measuring the number of corners visited in a clean test cage (horizontal activity) and the latency to perform the first rearing (vertical activity) (Giménez-Llort et al., 2007).

Hyperactivity is assessed through in-cage monitoring and observation of agitation episodes, while aggression is evaluated using the resident-intruder test, quantifying interactions between a test subject and an unfamiliar intruder (Crawley, 1999). Additionally, sleep and eating disorders can be evaluated with in-cage monitoring systems enhanced by advanced software and protocols (Sethi et al., 2015). However, effective methodologies for replicating symptoms in the psychotic cluster, such as hallucinations or delusions, remain elusive (Kosel et al., 2020).

Parallels with circadian rhythm disruptions have also been observed in animal models. For example, altered motor activity patterns in APP23 mice, monitored in their home cages, bear similarities to sundowning behaviors (Vloeberghs et al., 2004). Furthermore, circadian rhythm disturbances in other AD models, including the 3xTg-AD mice, emphasize the relationship between AD pathology and sleep disruptions (Duncan et al., 2012; Wu et al., 2018), paving the way for preclinical investigations into these symptoms.

1.2.2 Integrating Cognitive and Non-Cognitive Assessment

Incorporating both cognitive and non-cognitive evaluations in animal models provides a more translationally relevant approach to studying the disease. This can be seen in traditional behavioral tests, such as the Morris Water Maze (MWM) test, that has evolved to include non-cognitive elements, broadening their applicability (Baeta-Corral & Giménez-Llort, 2015).

Initially designed to assess spatial learning and memory, the MWM test requires animals to locate a submerged platform in a pool full of water (Morris, 1984). Over time, researchers have enhanced its utility by analyzing additional behavioral parameters, such as swimming strategies and the presence of stereotypies during the platform search (Dalm et al., 2000; Graziano et al., 2003; Wolfer & Lipp, 1992). These measures offer insights into

the animals' stress-coping mechanisms and adaptive behaviors, allowing for a more detailed characterization of models. The combination of cognitive and non-cognitive aspects establishes the test as a versatile tool for investigating animal deficits in AD.

Certainly, the detailed characterization and interpretation of both behaviors, particularly in 3xTg-AD mice, are critical for advancing our understanding of BPSD-like patterns, which are an integral part of AD progression and hold promise for informing therapeutic strategies aimed at addressing the broader impacts of the disease.

1.2.3 Statistical Models

In applied statistics, the term "statistical models" refers to a set of assumptions used to estimate and predict values from data (J. Fox, 2019). These models serve as essential tools in both clinical and preclinical research, enabling the identification and analysis of data patterns. Within the scope of this doctoral thesis, statistical models play a crucial role in analyzing experimental data, as will be demonstrated in the subsequent chapters. To provide context for their application, a brief overview of the methodologies employed is presented below.

Statistical models rely on explanatory variables, or predictors, to evaluate their effect on a response variable. In simple terms, they assess how changes in specific characteristics of an individual or population influence the outcome of interest. The response variable may be numerical (e.g., weight) or categorical (e.g., presence or absence of a behavior) and can follow either a normal or non-normal distribution. Based on these characteristics, statistical models can be broadly classified into three main categories: Linear Models, Generalized Linear Models, and Mixed Effects Models.

1.2.3.1 Linear Models

Linear models evaluate the relationship between explanatory variables and a numeric response variable that follows a normal distribution. These models test systematic differences and are commonly used in experimental research. A widely recognized example is one-way ANOVA, which assesses differences in group means and has gained popularity in applied sciences.

1.2.3.2 Generalized Linear Models (GLMs)

GLMs extend the functionality of linear models to handle categorical or discrete response variables. Unlike linear models, GLMs do not require the response variable to follow a normal distribution, making them better suited for real-world data. Logistic regression, for example, is a GLM frequently used to model binary outcomes, such as the presence or absence of a behavior.

1.2.3.3 Mixed Effects Models (MEMs)

Mixed effects models are designed to address more complex structures, such as dependent or hierarchical data. Dependent data refers to repeated measures from the same individual across different time points, while hierarchical data involves subgroups nested within larger populations. These models incorporate fixed effects, which capture consistent influences across groups, and random effects, which account for variability at the individual or subgroup level. MEMs are further divided into two types:

i) Linear Mixed Effects Models (LMMs): Extend linear models to evaluate dependent data.

ii) Generalized Linear Mixed Effects Models (GLMMs): Extend GLMs to accommodate dependent data with categorical or non-normally distributed responses.

While LMMs and GLMMs are widely used in social sciences and experimental psychology, their application in preclinical studies, particularly in animal research, remains less common (Singmann & Kellen, 2019). However, their adoption in preclinical settings offers significant advantages. These models improve the interpretation of behavioral data by accounting for individual variability and hierarchical structures, which are often overlooked by simpler statistical approaches. For instance, LMMs and GLMMs are particularly valuable in studying complex behaviors, such as those associated with BPSD in longitudinal research. By accommodating the complexities of dependent data, they provide a robust framework for uncovering meaningful associations and advancing our understanding of behavior in translational models.

1.3 AD and Physical Activity

In the context of understanding AD and its associated BPSD, it is essential to explore modifiable lifestyle factors that may mitigate disease progression. One such factor is physical activity (PA), which has gained recognition as a vital component in promoting cognitive health (Law et al., 2018; Paillard et al., 2015; Sewell et al., 2023). Consistent engagement in PA has shown considerable benefits, including delaying both the onset and progression of AD, highlighting the importance of movement in supporting overall well-being and cognitive resilience (Buchman et al., 2012; De Almeida et al., 2020; E. Santana-Sosa et al., 2008; Jiang et al., 2025; Rolland et al., 2008; Santos-Lozano et al., 2016). However, its counterpart, physical inactivity has emerged as a significant public health concern due to its association with cognitive worsening and augmented risk for neurodegeneration (Gogniat et al., 2025). Thus, sedentarism is currently acknowledged as one of the primary modifiable risk factors for AD (Barnes & Yaffe, 2011; Norton et al., 2014; Roemers et al., 2019).

To proceed with a comprehensive analysis, it is crucial to define key terms that are frequently used interchangeably but possess distinct meanings, these are PA and exercise. PA refers to any bodily movement produced by skeletal muscles that leads to energy expenditure, encompassing activities such as occupational tasks, household chores, and recreational pursuits that are not explicitly intended to enhance fitness (Caspersen et al., 2000; Dasso, 2019). Exercise, by contrast, is a structured subset of PA characterized by planned, repetitive movements aimed at improving or maintaining specific components of physical fitness, including strength, flexibility, and cardiovascular endurance. This thesis will focus on PA as a method of treatment and evaluation in animal models, investigating its potential as an intervention to reduce BPSD-like symptoms in AD.

1.3.1 PA patterns in Humans

Extensive research has examined PA patterns in older adults, both with and without clinical conditions. Various methodologies, including accelerometers, pedometers, and self-reported surveys, have been used to assess energy expenditure and PA levels. These studies consistently show an age-related decline in PA, which is even more pronounced in individuals with AD (Harvey et al., 2013; Varma & Watts, 2017; Watts et al., 2013). As people age, PA becomes less frequent and more fragmented (Schrack et al., 2014). This

decline is particularly severe in AD patients, who exhibit increased sedentary behavior and delayed initiation of daily activities (Lu et al., 2018) . The transition from healthy aging to AD-related inactivity is postulated to be driven by a combination of neurobiological, psychological, and environmental factors that collectively reduce motivation and participation.

From a neurobiological perspective, the age-related decline in dopamine receptors, particularly D2 receptors in the neostriatum, significantly contributes to reduced movement motivation. Given that the dopaminergic system regulates both motor activity and reward-seeking behavior, this decline directly affects PA engagement (Ingram, 2000). Further neural and systemic mechanisms contribute to this reduction, including apathy due to disruption of medial frontal/anterior cingulate–insula–ventral striatal networks and parietal hypometabolism, impairing initiation and effort-based decision making (Gatchel et al., 2017; Jones et al., 2019; Marshall et al., 2013). Additionally, degeneration of the circadian system affects the suprachiasmatic network, causing sleep–wake fragmentation, daytime somnolence, and reduced diurnal activity (Coogan et al., 2013; Leng et al., 2019). Autonomic and cardiovascular dysregulation, characterized by orthostatic hypotension, blunted chronotropic responses, reduced heart-rate variability, and impaired neurovascular coupling, leads to fatigue and dizziness, thereby discouraging exertion (Beishon et al., 2022; Cremer et al., 2017; Tulbă et al., 2020). Moreover, sarcopenia and low lean mass are highly prevalent in AD, limiting strength and endurance (Nazareth et al., 2025; Yang et al., 2023).

Psychological barriers further discourage PA participation. Discomfort from exertion is a common restraint (Bevan et al., 2020) , while personality traits also play a role—individuals with high neuroticism (prone to emotional instability) are less likely to engage in PA, whereas those with higher extraversion (sociable and energetic) tend to be more active (Rhodes & Smith, 2006). Additionally, mood disorders and depressive symptoms, which are prevalent among older adults, are strong predictors of reduced PA levels (Watts et al., 2018). These psychological challenges are compounded by chronic health conditions, fear of falling, and low self-efficacy—an individual's belief in their ability to successfully engage in PA (Bauman et al., 2012; Choi et al., 2017). In AD patients, cognitive impairments further exacerbate these difficulties, leading to poor adherence to PA routines(Lu et al., 2018).

Environmental factors also pose significant barriers to PA participation. Dissatisfaction with recreational facilities, perceived neighborhood unsafety, and the infrequent observation of others engaging in PA all contribute to reduced motivation for physical activity (Tortosa-Martinez et al., 2025; Trost et al., 2002).

1.3.2 PA in Animal Models of AD

Laboratory rodents, and transgenic mice in particular, are widely used to investigate the mechanisms underlying the beneficial effects of PA on brain function (Shepherd et al., 2018). Voluntary exercise, such as wheel running in home cages, is the preferred method for these studies (Novak et al., 2012). This approach enables the collection of key variables, including distance, duration, and speed, which serve as analogs to human PA metrics while aligning with rodents' natural behaviors. Consequently, voluntary exercise minimizes stress and enhances the reliability of experimental outcomes (De Bono et al., 2006).

In contrast, forced exercise protocols, such as treadmill running or swimming at prescribed intensities, may introduce stress, potentially confounding results and limiting the validity of findings (Svensson et al., 2016). Given these considerations, voluntary wheel running (VWR) will be employed here to assess PA patterns and their correlation with BPSD.

1.3.3 Animal and Human PA Parallels

Preclinical models have demonstrated that PA enhances cognitive function and reduces anxiety- and depression-like behaviors in both mice and rats (Adlard et al., 2005; Binder et al., 2004). Additionally, research has explored the role of dopamine receptors, particularly D1 and D2, in locomotion and exercise predisposition (Knab et al., 2009). Moreover, voluntary exercise has been linked to the activation of brain regions associated with motivation and reward, including the nucleus accumbens (NAc), ventral tegmental area (VTA), and striatum, which may underlie individual differences in PA engagement (Garland et al., 2011).

In AD models specifically, PA interventions have yielded mixed results, suggesting that outcomes may depend on factors such as the specific mouse model, age, and genetic background. Positive effects include attenuated cognitive decline in SAMP8 mice (J. Dong et al., 2018), increased resistance to stress-induce cell damage in APPsw mice (Um et al.,

2008), reduced Aβ burden in aged Tg2576 mice (Francis et al., 2020) , and decreased inflammatory markers alongside improved synaptic function in 3xTg-AD mice (Revilla et al., 2014) . Conversely, studies have reported neutral effects of PA interventions in middle-aged APP/PS1 mice (Wang et al., 2021) and the 5xFAD mouse model (Svensson et al., 2020).

Despite extensive research on PA in AD models, little is known about its effects on specific BPSD patterns. Therefore, experimental protocols aimed at evaluating these aspects are necessary to advance our understanding and improve reproducibility in preclinical studies.

1.4 AD and Social Isolation

The degree of social connectedness has a significant role in maintaining overall health and probably longevity (Garrido & De La Fuente, 2023) . Its interest dates back to the mid-20th century, when shifts in family structures in the United States led to an increase in older adults living alone, and the effects of social isolation (SI) on health and well-being were observed. In early studies, SI was associated with feelings of neglect, poor mental health and higher mortality rate (Brown, 1960; Duleep, 1989) . On the other hand, social support was reported to mitigate the negative health effects of SI by promoting adaptive behaviors that benefit cardiovascular, endocrine, and immune system function (Cassel, 1976; Cobb, 1976; Garrido et al., 2022; House et al., 1988).

1.4.1 Different forms of isolation

The aim of this section is to review the effects of SI on humans and animals, with special focus on AD. However, before proceeding to examine them, it is crucial to distinguish between related yet distinct concepts frequently mentioned in the literature. These are SI, loneliness and solitude. SI refers to the objective condition of living alone or having limited social contact, while loneliness is the subjective, aversive experience resulting from a discrepancy between desired and actual social interactions. In contrast, solitude represents a positive, self-chosen state of being alone (Cacioppo et al., 2015; Victor et al., 2000).

Both SI and loneliness have been linked to adverse health behaviors, including increased smoking, physical inactivity, and poor sleep quality (Cacioppo et al., 2002) . They are also associated with higher risks of all-cause morbidity and mortality, with impacts comparable to traditional risk factors such as obesity (Holt-Lunstad et al., 2015). In addition, indicators of SI, such as the size of one's social network and the frequency of social interactions, have been correlated with depression, anxiety, and cognitive decline in older adults (Hämmig, 2019; Mumtaz et al., 2018). On the other hand, not much focus has been put into solitude effects, but some researchers establish a relationship between less negative health outcomes in these individuals and more resilient characteristics, possibly associated with better mental health status (Chen & Liu, 2023).

Regarding AD, research indicates that quantity and quality of social attachments affects the risk of suffering it. SI and loneliness are significant risk factors for AD (Shafighi et al., 2023), but interestingly, the perception of being alone, rather than the physical state of being isolated, seems to play a crucial role. Thus, loneliness is linked to a more than doubled risk of AD, compared to not lonely people, independent of quantitative SI indicators (Wilson et al., 2007).

Nowadays, SI has become a critical problem in Western societies, amplified during the COVID-19 pandemic in 2021 when social distancing measures severely limited interpersonal interactions (Matthews & Tye, 2019). Vulnerable groups, such as older adults and individuals with AD, were particularly affected, with institutionalized AD patients showing accelerated cognitive and emotional decline (Borges-Machado et al., 2020; Iodice et al., 2021). Additionally, reduced social contact in these populations has been linked to worsening BPSD, including increased agitation, anxiety and obsessive-compulsive disorders (OCD) (Banerjee, 2020; Rivera & Carballea, 2020).

1.4.2 SI in Animal Models

In terms of animal research, due to the subjective qualities of loneliness and solitude, only SI can be replicated. In doing so, single housing is the most common experimental method employed; while it does not imitate complete isolation, as animals can still hear and vocalize with conspecifics, it effectively models its key features (Võikar et al., 2005). Importantly, animal response to SI depends upon the duration and life stage where social deprivation is applied. Hence, SI duration in rodents—whether acute or chronic—yields different behavioral outcomes. That is, acute SI often prompts prosocial behaviors (Tomova et al., 2020), whereas chronic isolation has been associated with increased apathy and signs of despair (Ma et al., 2011).

The effects of isolation at different ages seem to vary according to species. Studies involving primates have demonstrated that early-life isolation can lead to profound behavioral changes and enduring difficulties into adulthood (Harlow et al., 1965; Harry F. & Harlow, 1962), while middle-aged rodents have shown more susceptible to negative effects of SI, both behavioral and neurobiologically, compared to younger subjects who appear more resilient to such stressors (Magalhães et al., 2024).

1.4.3 Effects of SI

As a consequence of SI, biological and behavioral effects are experienced. Biological effects are manifested with an activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to elevated levels of stress hormones such as cortisol in humans and corticosterone in rodents (Cacioppo et al., 2015; Vitale & Smith, 2022). SI is also associated with altered neuroendocrine function (Mumtaz et al., 2018), altered inflammatory responses (Magalhães et al., 2024) and hippocampal changes that affect cognition. Behavioral effects in rodents can lead to anxiety-like and depressive-like behaviors (Cruces et al., 2014; Võikar et al., 2005), memory and learning impairments (Wilson et al., 2007), and altered exploratory behaviors (Muntsant & Giménez-Llort, 2020).

1.4.4 The social homeostasis theory

In an attempt to elucidate the neural mechanisms driving the effects of SI in the organism, Matthews et al. (2019) proposed the social homeostasis theory, and since its proposal, it has attracted considerable interest. This hypothesis emphasizes that social connections are biologically essential, similar to basic needs like food or sleep. Disruptions in these connections activate compensatory mechanisms aimed at restoring balance, as seen in animals that exhibit heightened vigilance and diminished interaction motivation when deprived of social contact.

Social homeostasis functions analogously to other homeostatic systems that regulate physiological necessities, such as energy or fluid balance. Just as hunger and thirst induce behaviors to replenish energy and hydration, deficits in social connection trigger adaptive mechanisms designed to restore emotional balance and encourage re-engagement with others. Neural circuits involving the amygdala, prefrontal cortex, and anterior cingulate cortex are integral to this process, playing critical roles in detecting social deficits and processing social cues. Evidence from the study by Lee et al. (2021) shows heightened activity in these brain regions during experiences of SI, underscoring their importance in maintaining social equilibrium.

1.4.5 SI and BPSD

As previously mentioned, SI is associated with worsening BPSD in AD patients. Within BPSD, OCD is of particular interest in this thesis. In humans, OCD manifests as repetitive behaviors or mental acts performed to alleviate anxiety caused by persistent obsessions. Similarly, in animal models, such behaviors can be evaluated through tasks like the marble burying (MB) test, where increased digging behavior is used as an indicator of compulsive-like actions (Deacon, 2006)

Besides the exclusive expression of compulsive-like symptoms, digging behavior is also a natural action that can be understood as a measure of general activity (Dixit et al., 2020). Thus, a definitive interpretation of this act is still unclear. Moreover, little attention has been paid to the role of SI on BPSD-like symptoms in animals. Hence, the 3xTg-AD mouse model utilized in our laboratory presents a unique opportunity to explore how chronic SI influences behavioral changes associated with those patterns, specifically in digging as a compulsive-like symptom.

In the following sections, attention is put on neuroethological changes that may arise after a SI period in the 3xTg-AD model and how these changes may be related with underlying biological processes.

2 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Distinguishing non-cognitive symptoms of AD, often manifesting as BPSD-like behaviors, presents a considerable challenge when comparing most animal models for Alzheimer's disease and their counterparts with normal aging. This inherent difficulty stems from the broad spectrum of behaviors that can be categorized as BPSD-like, many of which are also observed as part of the normal aging process in healthy individuals. The subtle line between pathological and age-related behavioral changes makes it arduous to definitively attribute specific manifestations to the disease itself, thereby complicating the precise identification and characterization of AD-related non-cognitive symptoms.

The 3xTg-AD mouse model for AD is unique because it exhibits a slow development of the two neuropathological hallmarks of the disease, mimicking advanced stages at 12 (beta-amyloid neuropathology) and 16 months of age (both beta-amyloid and tau pathologies). However, in the current thesis, our main interest in this model is because, as compared to others, it exhibits a conspicuous BPSD-like phenotype.

The central hypothesis of this PhD research project posits that the manifestation and subsequent identification of BPSD-like behaviors in 3xTg-AD mice can be significantly enhanced through progressively increasing stress exposure via a sequential battery of behavioral tests.

The rationale behind this approach is that controlled and incremental stress can unmask or exacerbate latent behavioral deficits that might not be apparent under baseline conditions. Subsequently, by exposing the mice to a series of environmental manipulations, differences in their behavioral responses are expected to be further amplified. These manipulations could include alterations in housing conditions, social interactions, or sensory stimuli, designed to elicit a wider range of behavioral expressions. Ultimately, a more accurate identification of these behaviors can be achieved by meticulously analyzing patterns or "signatures" of specific motor acts. Furthermore, the application of sophisticated statistical methodologies that account for interindividual variations, treating each mouse's basal state as a unique reference point, is anticipated to further facilitate this intricate process.

2.2 General Objective

The main objective of this doctoral thesis is to characterize specific BPSD-like behaviors in 3xTg-AD mice at advanced disease stages through a sequential battery of stress-inducing behavioral tests and environmental manipulations. Such BPSD-like manifestations include anxiety-like, compulsive-like, and circadian behavior. In doing so, three studies will be carried out. The first study adopts a longitudinal experimental design, providing insights into the progression of non-cognitive symptoms over time and their expression under a stressful experience for mice, such as the Morris water maze. The second study utilizes voluntary exercise as both an evaluation and intervention method to investigate the correlation between behavioral traits and patterns of PA engagement. Lastly, the third study examines the impact of social isolation on BPSD-like behaviors, highlighting how single-housing affects non-cognitive symptoms in the 3xTg-AD model. Specific Objectives

2.3 Specific Objectives

2.3.1 Study 1

1. To characterize the evolution of swimming strategies in 3xTg-AD mice during repeated MWM testing and determine their value as indicators of BPSD-like symptoms by comparing them to those of NTg controls.
2. To assess genotype-, sex-, and age-dependent differences in swimming strategies between 3xTg-AD and NTg mice using generalized linear mixed-effects models (GLMMs)

2.3.2 Study 2

1. To evaluate the effects of four weeks of VWR on anxiety-like behaviors in 14-month-old 3xTg-AD mice.
2. To analyze the temporal dynamics of voluntary PA in 14-month-old 3xTg-AD mice using MEMs, and to determine whether individual differences in neophobia influence activity patterns.

2.3.3 Study 3

1. To evaluate the effects of prolonged social isolation on neophobia—a core BPSD-like symptom—in 13-month-old 3xTg-AD male mice, using the corner test (CT) and open field (OF) paradigms.
2. To compare digging behavior—reflecting compulsive-like tendencies—between socially isolated and group-housed 3xTg-AD male mice during the CT and MB test.

3 MATERIAL AND METHODS

This thesis comprises three studies, each described in detail within its corresponding results section. This chapter briefly outlines the experimental procedures used across these studies.

3.1 Study 1: Cognitive stress in the Morris Water Maze

3.1.1 Experimental Design

A longitudinal MWM test protocol was conducted at 12 and 16 months of age to assess spatial learning and cognitive flexibility in 22 3xTg-AD and 15 NTg mice (both sexes). The task was divided into three phases:

i) Day 1 (Cue Stage): Platform visible, placed in the northeast quadrant.
ii) Days 2–5 (Place Task): Hidden platform in the southwest quadrant.
iii) Day 5 (Removal Test): Platform removed to assess memory retention and quadrant preference.

3.1.2 Behavioral Recording and Classification

Behavior was recorded with ANY-MAZE software. Classical measures (escape latency, path length, speed) were computed automatically. Additionally, swimming paths were visually classified into 10 predefined strategies, grouped as:

i) Search strategies: thigmotaxis, random search, scanning, chaining, focal search, focal wrong, perseverance, direct search
ii) Non-search strategies: circling, floating

3.1.3 Cognitive Flexibility Index

A novel index was developed to assess behavioral adaptation during learning phases, defined as the time spent in the first strategy relative to total escape time.

3.1.4 Statistical Analysis

Classical variables were analyzed via Mixed ANOVA. Strategy proportions and cognitive flexibility were analyzed GLMMs with Ordered Beta Regression. Circling and floating episodes were modeled using GLMMs with negative binomial distribution.

3.2 Study 2: Physical Activity in the Wheel Running

3.2.1 Experimental Design

A group of 14-month-old 3xTg-AD mice (n = 34; both sexes) was exposed to 30 days of VWR. Mice were randomly assigned to:

i) SED group: Standard housing, no wheel access
ii) VWR group: Individual housing with access to a running wheel 24/7

3.2.2 Activity Monitoring and Behavioral Testing

VWR activity was recorded continuously to assess circadian patterns. Behavioral assessments were conducted pre- and post-intervention using:

i) CT: Neophobia and horizontal/vertical exploration
ii) OF: Exploratory behavior and emotional reactivity

3.2.3 NIBI

Mice were behaviorally phenotyped into high- and low-NIBI based on reduced exploration in novel environments, used as a covariate in activity analysis.

3.2.4 Statistical Analysis

Circadian data were analyzed using Linear Mixed Effects Models. Pre-post differences and sex × treatment interactions were assessed via repeated-measures ANOVA and post hoc testing.

3.3 Study 3: Natural Social Isolation Paradigm

3.3.1 Experimental Design

This experiment included 13-month-old male mice: NTg (n = 15) and 3xTg-AD (n = 28). A subgroup of 3xTg-AD males (n = 7) was naturally isolated after the loss of cage mates due to age-related mortality. All groups had been raised socially until isolation occurred naturally over 2–3 months.

3.3.2 Behavioral Tests

Three behavioral paradigms were used:

i) CT: Response to novel cage with bedding
ii) OF: Exploratory activity and emotionality
iii) MB: Compulsive-like behavior (12 marbles, 30 min)

3.3.3 Digging Ethograms and Anxiety Measures

Detailed digging behavior and rearing frequency were manually scored. Defecation, urination, and activity zone preferences were recorded as indirect anxiety and stress markers.

3.3.4 Statistical Analysis

Group comparisons were conducted using One-Way ANOVA with post hoc tests (Duncan's). Categorical variables were analyzed using Fisher's exact test. Significance was set at $p < 0.05$.

3.4 Ethical Considerations

All procedures were approved by the Ethics Committee of Departament de Medi Ambient i Habitatge, Generalitat de Catalunya (CEEAH 3588/DMAH 9452) on 8 March 2019.

4 RESULTS

4.1 Study 1

Alveal-Mellado, D., & Giménez-Llort, L. (2024). **Use of Ordered Beta Regression Unveils Cognitive Flexibility Index and Longitudinal Cognitive Training Signatures in Normal and Alzheimer's Disease Pathological Aging.** Brain Sciences, 14(5), 501. https://doi.org/10.3390/brainsci14050501

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Use of Ordered Beta Regression Unveils Cognitive Flexibility Index and Longitudinal Cognitive Training Signatures in Normal and Alzheimer's Disease Pathological Aging

Daniel Alveal-Mellado [1,2] and Lydia Giménez-Llort [1,2,*]

[1] Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona, Spain; daniel.alveal@autonoma.cat
[2] Department of Psychiatry and Forensic Medicine, School of Medicine, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona, Spain
* Correspondence: lidia.gimenez@uab.cat

Abstract: Generalized linear mixed models (GLMMs) are a cornerstone data analysis strategy in behavioral research because of their robustness in handling non-normally distributed variables. Recently, their integration with ordered beta regression (OBR), a novel statistical tool for managing percentage data, has opened new avenues for analyzing continuous response data. Here, we applied this combined approach to investigate nuanced differences between the 3xTg-AD model of Alzheimer's disease (AD) and their C57BL/6 non-transgenic (NTg) counterparts with normal aging in a 5-day Morris Water Maze (MWM) test protocol. Our longitudinal study included 22 3xTg-AD mice and 15 NTg mice (both male and female) assessed at 12 and 16 months of age. By identifying and analyzing multiple swimming strategies during three different paradigms (cue, place task, and removal), we uncovered genotypic differences in all paradigms. Thus, the NTg group exhibited a higher percentage of direct search behaviors, while an association between circling episodes and 3xTg-AD animals was found. Furthermore, we also propose a novel metric—the "Cognitive Flexibility Index"—which proved sensitive in detecting sex-related differences. Overall, our integrated GLMMs-OBR approach provides a comprehensive insight into mouse behavior in the MWM test, shedding light on the effects of aging and AD pathology.

Keywords: behavioral studies; data analysis; Alzheimer's disease; aging; Morris Water Maze; search strategies; cognitive flexibility

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1. Introduction

Experimental research commonly requires sophisticated statistical methodologies capable of handling non-normally distributed data collected from dependently sampled observations, such as those clustered into groups or with repeated observations from the same individuals (i.e., longitudinal studies) [1,2]. As a result, there has been an increase in the use of innovative data analysis strategies including generalized linear mixed models (GLMMs). This methodology allows for the analysis of response variables from different distributions in longitudinal studies by including so-called random effects [3–7]. Although GLMMs have been used mainly to treat binary and categorical data [8], they have been recently applied in ecological and behavioral studies that gathered continuous response data such as percentages or ratios [9–11]. Previously, the beta regression model was proposed as an accurate method for handling percentage data [12]. Nevertheless, the model struggles with calculating degenerated data, wherein the range of values includes 0 or 1. In response, Kubinec [13] introduced the ordered beta regression model, which is capable of handling degenerated data. Nowadays, the continuous advancements in statistical software, such as R Software, offers the potential to amalgamate GLMMs and

ordered beta regression models [13,14]. In our laboratory, we consider that this novel approach has broad applications, including handling various tests for assessing animal behavior, such as the neuroethological ones used in our translational behavioral neuroscience research. Notably, we hypothesize that applying this methodology to the Morris Water Maze (MWM) test, a common method for evaluating spatial learning and memory in rodents, might be highly beneficial in solving the complexity of data analysis of search strategies.

Generally, the MWM test is used to evaluate spatial cognitive performance in rodents and measure positive or negative effects of pharmacological and non-pharmacological preventive and therapeutic interventions in cognitive function; it can also be used to track the progression of cognitive dysfunction in models of neurological and psychiatric diseases. Thus, test performance enhancements are are considered to be an indicator of therapeutic efficacy [15,16]. Despite several reported test protocols in the literature [17–19], a standard procedure typically involves three stages. The first stage, known as the visual perceptual learning or CUE stage, requires mice to swim in an opaque water-filled circular pool to reach a visible platform. The subsequent place task (PT) stage submerges and relocates the platform, requiring animals to search for it using external references. In the final probe trial or removal (RM) stage, the platform is removed, and the time animals spend in the previous platform location is analyzed.

Classical parameters such as escape latency, distance traveled, and mean speed are typically used to interpret an animal's performance and infer its cognitive state in these paradigms [20]. However, criticisms have been raised about relying solely on these parameters as they may not provide meaningful information on animal behavior [21–23]. Therefore, researchers have proposed incorporating the analysis of swimming strategies adopted by animals into the standard MWM test protocol [24,25]. This approach aims to reveal behavioral disparities and memory deficits, enhancing our understanding of subtle signs of cognitive impairments. Our studies [26,27] and several others [28–30] showed that animals adopt a variety of swimming strategies during the test. These include search (i.e., thigmotaxis, random search, scanning, chaining, focal search, focal wrong, perseverance, and direct search) and non-search strategies (i.e., circling and floating).

Non-search strategies were previously highlighted as atypical behaviors in the test [31–33], indicating reduced attention to the platform search. Therefore, these are seen as confounding elements in the test analysis [34]. In addition, a correlation has been established between search strategies and different learning stages of animals [26,27,30]. This suggests that the spatial learning process evolves from an initial stage of self-centered or "egocentric" navigation, relying on sensorimotor information, to a later stage of "allocentric" navigation. In the latter stage, the animals' trajectory is based on a non-self-centered cognitive map, where the hippocampus plays a significant role [30,35].

Cognitive flexibility, another potential aspect to evaluate in the test, involves the adaptation of behavior to new circumstances based on prior knowledge [36]. This concept has been proposed as a relevant measure to consider during the PT stage where animals are required to learn a new platform location [37,38]. Then, the inclusion of a swimming strategies analysis provides an opportunity to further investigate cognitive flexibility by examining the transition among different strategies.

The effect of prior experience in the test is another factor to consider when undertaking animal model assessment. A retest can impact performance, fluctuating based on mouse strain characteristics [39]. Longitudinal studies involving models of Alzheimer's Disease (AD) [40,41] observed remarkable test performance stability, suggesting a potential training influence from the repeated test battery administration.

Based on our own experience in animal behavior analysis, we acknowledge the difficulty in differentiating AD and age-related impairments in mice in the MWM test. Challenges are due to the complex interplay of age, sex, and behavioral variability. Previously, our research suggested that the inclusion of multiple strategies within a single trial in the test may be an effective method to identify behavioral disparities in mice models of

accelerated aging and in the triple-transgenic (3xTg-AD) model [26,27]. The 3xTg-AD model was created at the University of California, Irvine [42] , and has been shown to have high face and construct validity [43,44].

Recent studies have expanded the multi-strategy approach by incorporating the classification of swimming strategies in other AD-like models [45,46]. Despite these advancements, the field lacks statistical methods to uncover the factors influencing these strategies and their evolution in longitudinal designs. Therefore, the aim of this study is to explore whether the application of GLMMs to a multi-strategy approach can identify differences by sex, genotype, or age in a group of normal (gold standard wild-type C57BL/6strain) and AD-pathologically aged mice (3xTg-AD mice) that may have been overlooked in traditional variable analysis.

2. Materials and Methods

2.1. Animals

Fifty-nine mice were used in the longitudinal experimental design. Twenty-two died during the follow-up period from 12 to 16 months of age, and finally, 37 were considered in the pre–post analysis including 22 3xTg-AD animals (n = 14 males and n = 8 females) from the Spanish colony established at the Universitat Autònoma de Barcelona, Barcelona, Spain, in a C57BL/6 background strain [47] and their 15 non-transgenic (NTg) counterparts (n = 8 males and n = 7 females). All groups were assessed at 12 and 16 months of age.

All animals were kept under standard laboratory conditions in macrolon cages (35 cm × 35 cm × 25 cm) with ad libitum food and water, at a temperature of 22 ± 2 °C and 50–70% humidity on a 12/12 h light/dark cycle starting at 8 a.m.

2.2. Morris Water Maze Protocol

The MWM test protocol was adapted from a previous study [48] and consisted of one day of the CUE stage, four consecutive days of the PT stage, and an RM stage on day 5.

Day 1 (CUE stage): Animals were trained to locate a visible platform (1 cm above the opaque water surface) situated in the northeast (NE) quadrant, which was marked with a black striped flag. External cues were not provided, and each animal completed four trials.

Days 2–5 (PT1-PT4 stage): The platform was submerged 1 cm below the water level and repositioned in the opposite quadrant (southwest, SW). External cues were present, and four consecutive trials were conducted daily. Mice were introduced into the pool from different starting points (north, south, west, and east) for each trial.

Day 5 (RM stage): The platform was removed 2.5 h after the last trial on PT4, and a single trial was performed.

Throughout the experiment, mice were gently placed into the pool facing the wall and allowed to swim for a maximum of 60 s per trial. When animals failed to find the platform in 60 s, they were gently guided to it and remained standing there for 10 s.

2.2.1. Classical MWM Test Analysis

Classic measurements (namely, escape latency, distance traveled, and mean speed) were automatically calculated at all stages using the video tracking software ANY-MAZE version 6.33. Additionally, during the RM stage, the time spent in each pool quadrant and in a zone surrounding 1.5 cm of the previous platform location was recorded.

2.2.2. Swimming Strategy Classification

Swimming patterns were visually identified based on the track plots recorded by ANY-MAZE. Patter identification was undertaken by an observer trained for such a task and blind to the animal's age, sex, and genotype. The initial and final trials (T1 and T4) were considered for the analysis since they represent the main changes in the animals at each stage [48]. Swimming strategies were classified according to previous reports [30,48] (see Figure 1):

Non-search Behavior:

(a) Circling: Animals turn around their own axis in short loops without clear directionality.
(b) Floating: The animal remains inactive, not swimming in a forward motion.

Search Behavior:

Search behavior was further divided into eight distinct swimming strategies as follows:

(a) Thigmotaxis: A swim pattern performed near the pool walls or within an external ring accounting for 10% of the pool surface.
(b) Random search: The animal covers all four pool quadrants in a pattern with frequent direction changes.
(c) Scanning: Characterized by direction changes, but the pattern is limited to a couple of quadrants or a central area of the pool.
(d) Chaining: The swimming pattern is executed at a fixed distance from the wall but closer to the pool's center than in the thigmotaxis strategy.
(e) Focal search: The search pattern is confined to the target quadrant, characterized by a dense concentration of overlapping loops and turns.
(f) Focal wrong: A focal search performed in an incorrect quadrant.
(g) Perseverance: The animal persists in searching in the target quadrant of the CUE stage after the platform has been moved to the PT position .
(h) Direct search: A straight swim toward the platform location.

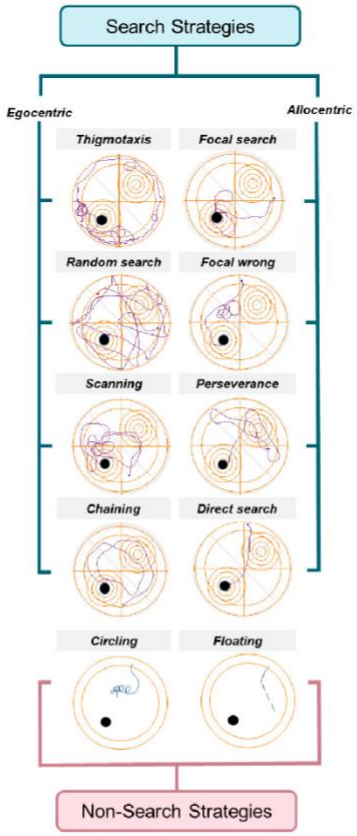

Figure 1. Swimming strategy classification. The swimming strategies performed by the animals in the MWM test were categorized into search and non-search strategies. Search strategies, based on

hippocampal involvement, were further divided into two types including egocentric dominance and allocentric dominance.

2.2.3. Variables Associated with Swimming Strategies

Percentage of Swimming Strategies

The video analysis approach facilitated the identification of multiple strategies within a single trial. Thus, the percentage of time spent on each was calculated as follows:

$$\text{Search Strategy} = \text{Time spent in swimming strategy (s)}/\text{Escape Latency (s)}$$

Cognitive Flexibility

In the present work, we propose a measurement of cognitive flexibility, which was calculated as the percentage of time spent until the first strategy used to find the platform changed.

$$\text{Cognitive Flexibility} = \text{Time spent in first strategy (s)}/\text{Escape Latency (s)}$$

Non-Search Strategy Episodes

The number of circling and floating episodes in each trial was recorded.

2.3. Statistical Analysis

All analyses were performed using R software (version 4.3.2). We used the afex package (version 1.3.0) to analyze classic parameters via a mixed model analysis of variance (Mixed ANOVA). Genotype and sex were treated as between-subject factors, while days, trials, and ages were considered within-subject factors.

An ordered beta regression model (glmmTMB package version 1.1.5) [13,49] was used to analyze proportion-dependent variables such as the proportion of swimming strategies and cognitive flexibility. A set of multiple models was generated from simplest to most complex to examine the effects of sex, genotype, trial, and age on the dependent variables. Animal ID was included as a random effect to account for repeated measures and individual variability. An AICc model selection (bbmle package version 1.0.25.1) was then applied to identify the top models.

A generalized linear mixed model (GLMM) with a negative binomial distribution was conducted to determine whether the number of non-search strategies (namely, circling and floating episodes) was influenced by the trial, sex, genotype, or age of the animals.

The DHARMa package (version 0.4.6) was used to examine the model fit and residual diagnostics.

Post hoc analyses with multiple comparisons were performed using the emmeans package (version 1.8.4.1). The *p*-value was then adjusted using the Tukey method for multiple comparisons.

Data were considered statistically significant when the *p*-value was less than 0.05.

3. Results

3.1. Survival Analysis

During the follow-up period, there was a 37.28% mortality rate, with a loss of 22 animals, with no genotype or sex differences (Fisher exact test, $p = 1.000$) since about half of them were NTg mice (9 females, 3 males) and the other were 3xTg-AD mice (5 females, 5 males).

3.2. Classical MWM Test Analysis

3.2.1. CUE Stage

In the CUE stage, the mixed ANOVA test for escape latency revealed a significant age effect (Figure 2) [$F(1,33) = 24.37$, $p < 0.001$], indicating a decrease in escape latency for all animals ($n = 37$) at 16 months of age [16 m vs. 12 m: $t(33) = -11.4$, $p < 0.001$]. Upon analyzing the distance covered, significant sex [$F(1,33) = 10.68$, $p = 0.003$] and age effects [$F(1,33) = 29.38$, $p < 0.001$] were found (Figure 2); thus, females outperformed males [females vs. males: $t(33) = -1.27$, $p = 0.003$], and all animals decreased the distance swam with age [16 m vs. 12 m: $t(33) = -1.79$, $p < 0.001$].

Regarding mean speed, a significant sex effect was detected [$F(1,33) = 5.24$, $p = 0.029$], with females swimming slower than males [females v/s males: $t(33) = -0.024$, $p = 0.029$].

3.2.2. PT Stage

A significant "stage" effect [$F(2.87,94.73) = 4.23$, $p = 0.008$] and a "stage × genotype" interaction effect [$F(2.87,94.73) = 2.77$, $p = 0.48$] were found in escape latency during the PT stage (Figure 2). Further analysis revealed that the "stage" effect accounted for differences between the PT1 and PT4 [$t(33) = 6.38$, $p = 0.029$] stages and the PT2 and PT4 [$t(33) = 5.30$, $p = 0.031$] stages in all animals ($n = 37$). The "stage × genotype" interaction can be explained by genotypical differences only on the fourth day of the task (PT4), where the 3xTg-Ad animals performed worse than the NTg animals [$t(33) = 8.54$, $p = 0.022$].

When the distance covered was analyzed, a significant stage effect [$F(2.81,92.75) = 5.22$, $p = 0.003$] indicated differences in the average values of the PT1 and PT4 [$t(33) = 1.59$, $p = 0.011$] stages and the PT2 and PT4 [$t(33) = 1.07$, $p = 0.025$] stages for all animals at both ages ($n = 37$).

In the mean swimming speed, a "stage" effect [$F(1.94,63.87) = 5.46$, $p = 0.007$] and a "sex × age" interaction effect [$F(1,33) = 4.22$, $p = 0.048$] were observed. The post hoc analysis revealed differences between the PT1 and PT3 [$t(33) = 0.01$, $p = 0.018$] stages and sex differences at 16 months of age only, with females swimming slower than males ($t(33) = -0.03$, $p = 0.002$).

3.2.3. RM Stage

Time and distance parameters were analyzed in each of the pool's quadrants (Figure 2). A significant "quadrant" effect was observed for both time [$F(2.18, 72.03) = 4.08$, $p = 0.18$] and distance [$F(2.30, 75.95) = 6.77$, $p = 0.001$]. Further analysis revealed significant differences between the time spent in the adjacent left and opposite quadrants (AL vs. O: $t(33) = -4.26$, $p = 0.002$), the distance covered in the adjacent left and opposite quadrants (AL vs. O: $t(33) = -4.547$, $p < 0.001$), and the distance covered in the opposite and previous platform location quadrants (O vs. P: $t(33) = -0.59$, $p = 0.025$).

Additionally, a "sex × age" interaction effect was detected in the time spent [$F(1,33) = 7.7$, $p = 0.009$] within a zone surrounding the previous platform location by 1.5 cm. Consequently, females spent more time in this zone than males at 12 months of age ($t(33) = 4.56$, $p = 0.014$).

No differences were found in the mean swimming speed of the animals at any age.

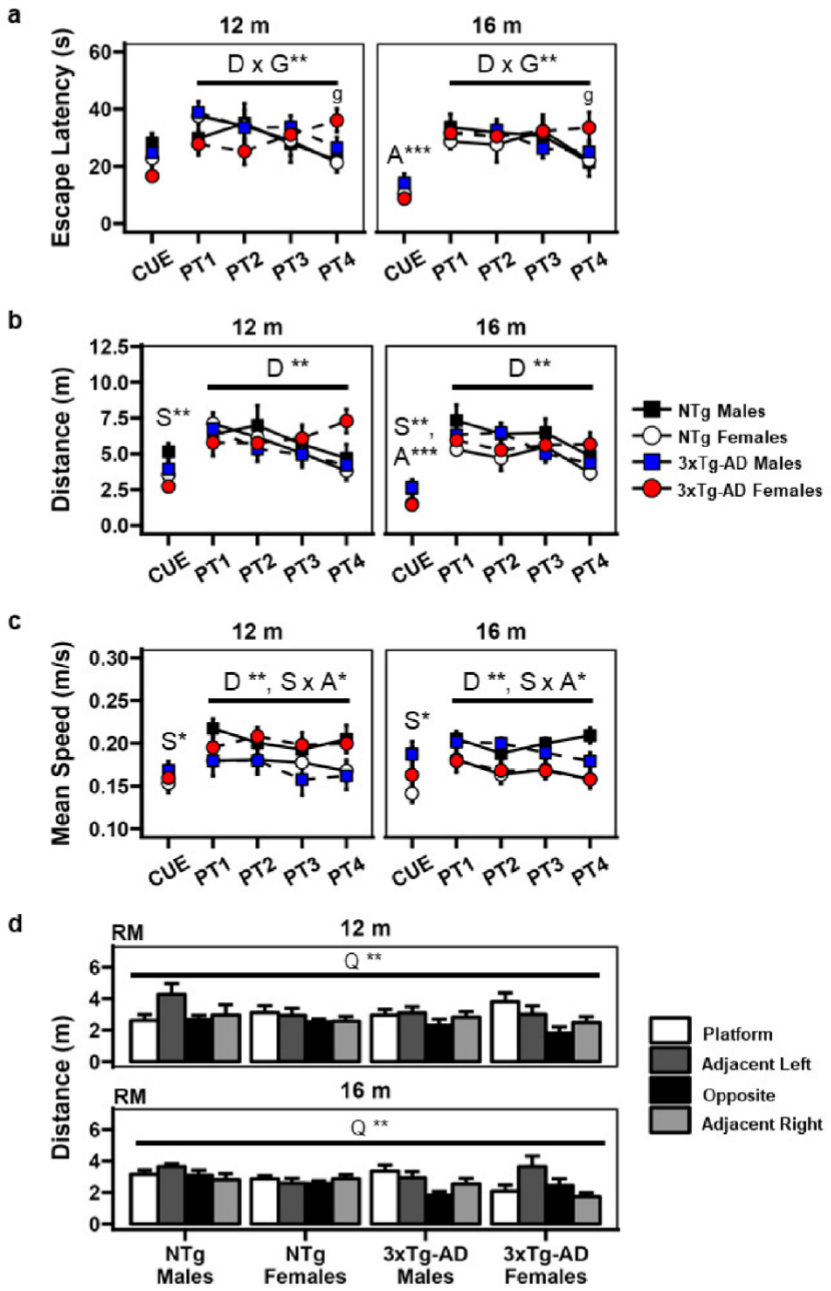

Figure 2. Classic parameters in the MWM test. (**a**) Escape latency. (**b**) Distance traveled. (**c**) Mean speed. (**d**) Distance traveled in the different quadrants during the removal stage. Mixed ANOVA: A, age effect; D, day effect; G, genotype effect; Q, quadrant effect; S, sex effect; g, genotype effect in stage PT4. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3. Swimming Strategy Analysis

A total of 814 trials were analyzed during both MWM tests at 12 and 16 months of age. The distribution of strategies in each trial was analyzed (Figure 3). We found that the animals used up to four strategies per trial to find the platform (1.36 ± 0.56). A mixed-regression Poisson analysis showed a significant trial effect ($p = 0.036$), with fewer strategies used in T4 (1.25 ± 0.06) compared with T1 (1.43 ± 0.06), regardless of age, sex, genotype, or stage.

3.3.1. Search Strategies

CUE Stage

The impact of sex, genotype, trials, and age on each strategy was evaluated. The optimal model varied depending on the strategy used as the dependent variable. Significant responses to predictors were observed in the random search, scanning, chaining, focal wrong, and direct search strategies (Table 1).

Table 1. Mixed-effects beta regression model coefficients for the top models describing the proportion of time spent on different swimming strategies. The significant influence of independent variables and pairwise comparisons is indicated in bold.

MWM Stage	Dependent Variable	Top Model	Independent Variables	Estimate	Predictor *p*-Value	Pairwise Comparison (Estimated Marginal Mean ± Standard Error)	*p*-Value Pairwise Comparison
CUE	Random search	~Sex + Genotype + Trial + Age + (1|animal ID)	Sex	−0.119	0.961		
			Genotype	−0.659	**<0.001**	**3xTg-AD (1.20×10^{-6} ± 0.0002) < NTg (2.32×10^{-6} ± 0.0004)**	**<0.001**
			Trial	−0.122	0.960		
			Age	−1.151	0.384		
	Scanning	~Trial + Age + Age: Trial + (1|animal ID)	**Trial**	−1.958	**<0.001**	**Trial 4 (0.27 ± 0.07) < Trial 1 (0.58 ± 0.04)**	**<0.001**
			Age	−0.579	0.028	16 m (0.43 ± 0.06) > 12 m (0.41 ± 0.07)	0.821
			Age × Trial	1.294	**0.031**	**12 m Trial 4 (0.21 ± 0.08) < 12 m Trial 1 (0.65 ± 0.04)**	**0.0011**
						16 m Trial 1 (0.51 ± 0.06) < 12 m Trial 1 (0.65 ± 0.04)	0.1267
						16 m Trial 4 (0.35 ± 0.09) < 12 m Trial 1 (0.65 ± 0.04)	**0.0078**
						16 m Trial 1 (0.51 ± 0.06) > 12 m Trial 4 (0.21 ± 0.08)	**0.0391**
						16 m Trial 4 (0.35 ± 0.09) > 12 m Trial 4 (0.21 ± 0.08)	0.5466
						16 m Trial 4 (0.35 ± 0.09) > 16 m Trial 1 (0.51 ± 0.06)	0.3127
	Chaining	~Age + (1|animal ID)	**Age**	0.601	**0.002**	**16 m (0.53 ± 0.02) > 12 m (0.39 ± 0.04)**	**0.002**
	Focal wrong	~Trial + Genotype + (1|animal ID)	**Trial**	−0.994	**0.015**	**Trial 4 (0.24 ± 0.10) < Trial 1 (0.46 ± 0.09)**	**0.015**
			Genotype	−0.857	0.062		
	Direct search	~Trial + Age + (1|animal ID)	**Trial**	1.414	**<0.001**	**Trial 4 (0.49 ± 0.07) > Trial 1 (0.19 ± 0.03)**	**<0.001**
			Age	0.638	**<0.001**	**16 m (0.40 ± 0.06) > 12 m (0.26 ± 0.04)**	**0.008**
PT	Thigmotaxis	~Age+ (1|animal ID)	**Age**	−1.543	**<0.001**	**16 m (0.03 ± 0.02) < 12 m (0.13 ± 0.08)**	**<0.001**
	Random search	~Age + (1|animal ID)	**Age**	−0.546	**0.008**	**16 m (0.40 ± 0.05) < 12 m (0.54 ± 0.05)**	**0.008**
	Direct search	~Trial + Genotype + Trial: Genotype+ (1|animal ID)	Trial	0.465	0.019	Trial 4 (0.21 ± 0.02) > Trial1 (0.21 ± 0.02)	0.858
			Genotype	0.290	0.154		

			Genotype × Trial	−0.881	**0.002**	NTg Trial 4 (0.26 ± 0.03) > NTg Trial 1 (0.18 ± 0.03)	0.0894
						3xTg-AD Trial 1 (0.23 ± 0.02) > NTg Trial 1 (0.18 ± 0.03)	0.4844
						3xTg-AD Trial 4 (0.17 ± 0.03) < NTg Trial 1(0.18 ± 0.03)	0.9437
						3xTg-AD Trial 1 (0.23 ± 0.02) < NTg Trial 4 (0.26 ± 0.03)	0.8111
						3xTg-AD Trial 4 (0.17 ± 0.03) < NTg Trial 4 (0.26 ± 0.03)	**0.0358**
						3xTg-AD Trial 4 (0.17 ± 0.03) < 3xTg-AD Trial 1 (0.23 ± 0.02)	0.1732
RM	Scanning	~Age + (1|animal ID)	**Age**	−0.615	**0.031**	**12 m (0.61 ± 0.05) > 16 m (0.46 ±0.06)**	**0.031**
	Focal wrong	~Sex + Genotype + Age + (1|animal ID)	**Sex**	−0.350	**<0.001**	**Female (0.34 ± 1.01 × 10^{-6}) < Male (0.43 ± 1.09 × 10^{-6})**	**<0.001**
			Genotype	−0.213	**<0.001**	**3xTg-AD (0.36 ± 7.37 × 10^{-6}) < NTg (0.41 ± 1.32 × 10^{-6})**	**<0.001**
			Age	0.278	**<0.001**	**16 m (0.42 ± 7.6 × 10^{-7}) > 12 m (0.35 ± 1.25 × 10^{-6})**	**<0.001**
	Direct	~Genotype + Age + (1|animal ID)	**Genotype**	−0.580	**<0.001**	**3xTg-AD (1.11 × 10^{-8} ± 0.02) < NTg (1.99 × 10^{-8} ± 0.03)**	**<0.001**
			Age	−0.310	1		

The genotype of the animals significantly influenced the proportion of random search, after adjusting for sex, age, and trials ($p < 0.001$). A pairwise test revealed that the 3xTg-AD mice were less likely to employ this strategy compared with their NTg counterparts (NTg vs. 3xTg-AD, odds ratio = 1.93, z = 18.646, $p < 0.001$).

An interaction effect between age and trial ($p = 0.032$) was detected in the scanning strategy. Specifically, a decrease in the likelihood of employing this strategy was observed in T4 compared with T1 at 12 months of age (odds ratio: 7.08, z = 3.73, $p = 0.001$).

An age effect ($p = 0.002$) was observed in the percentage of chaining, with an increase in its likelihood by 16 months of age (odds ratio: 0.55, z = −3.04, $p = 0.002$).

In the focal wrong strategy, a trial effect ($p = 0.015$) was found when adjusted by genotype, indicating that the likelihood of observing this strategy decreased by T4 compared with T1 (odds ratio = 2.70, z = 2.42, $p = 0.015$).

Regarding the direct search strategy, a model including age ($p = 0.008$) and trials ($p < 0.001$) was the top raked. Pairwise comparisons showed a significant increase in the likelihood of employing it by 16 months of age (odds ratio = 0.53, z = −2.65, $p = 0.008$) and by T4 (odds ratio = 0.23, z = −4.57, $p < 0.001$).

PT Stages

To evaluate the general changes in platform search patterns during this stage, data from PT1 to PT4 were pooled and analyzed, with a specific focus on T1 and T4. The response variables for each model included sex, genotype, trial, and age as predictors. Significant responses to some predictors were observed in the thigmotaxis, random search, and direct search strategies (Table 1).

A significant influence of age was found in the percentage of thigmotaxis used by the animals ($p < 0.001$). Specifically, a reduction was observed at 16 months of age compared with 12 months (odds ratio = 4.68, z = 4.40, $p < 0.001$). Additionally, age significantly influenced the percentage of random search ($p = 0.008$), with a reduction observed at 16 months (odds ratio = 1.73, z = 2.641, $p = 0.008$).

In the case of direct search, the top model included genotype and trial. A significant interaction effect was found ($p = 0.002$), with the NTg animals showing higher percentages than the 3xTg-AD animals in T4 (odds ratio: 1.81, z = 2.70, $p = 0.035$), regardless of sex and age.

RM Stage

During this stage, models that included sex, genotype, and age as predictors were evaluated. A significant influence of these predictors was found in the scanning, focal wrong, and direct search strategies (Table 1).

The age of the animals significantly influenced the percentage of scanning ($p = 0.03$), with a reduction observed at 16 months (odds ratio = 1.85, z = 2.16, $p = 0.031$).

For the focal wrong strategy, sex ($p < 0.001$), genotype ($p < 0.001$), and age ($p < 0.001$) significantly influenced this strategy. Specifically, an increase was observed at 16 months of age (odds ratio = 0.76, z = −43,924, $p < 0.001$), a higher percentage was observed in males (odds ratio = 1.42, z = 55,110, $p < 0.001$), and a higher percentage was observed in the non-transgenic (NTg) animals (odds ratio = 1.23, z = 33,454, $p < 0.001$).

Finally, the percentage of direct search was significantly influenced by genotype ($p < 0.001$), adjusted by age. Thus, the NTg animals showed a higher probability of using this strategy than the 3xTg-AD animals (odds ratio: 1.79, z = 4.451, $p < 0.001$).

3.3.2. Non-Search Strategies

The effects of age, genotype, sex, and trial were examined on the response variables circling and floating in the different MWM stages (Table 2).

Table 2. Analysis of non-search strategies and cognitive flexibility. Significant influences of independent variables and results of pairwise comparisons are highlighted in bold.

Dependent Variable	MWM Stage	Top Model	Independent Variables	Estimate	Predictor *p*-Value	Pairwise Comparison (Estimated Marginal Mean ± Standard Error)	*p*-Value of Pairwise Comparison
Circling episodes	CUE	~ Trial + Age + (1| 1|animal ID)	**Trial**	−1.54	**<0.001**	**T4 (0.67 ± 0.14) < T1 (3.12 ± 0.41)**	**<0.001**
			Age	−0.82	**<0.001**	**16 m (0.96 ± 0.17) < 12 m (2.17 ± 0.32)**	**<0.001**
	PT	~ Trial + Age + (1|animal ID)	**Trial**	−0.39	**<0.001**	**T4 (0.99 ± 0.13) < T1 (1.48 ± 0.18)**	**<0.001**
			Age	0.23	0.056		
	RM	~Sex + Genotype + Age + (Genotype × Age)+ (1|animal ID)	Sex	0.125	0.713		
			Genotype	−0.743	0.171		
			Age	−0.007	0.986		
			Genotype × age	1.419	**0.026**	3xTg-AD 12 m (0.60 ± 0.27) < NTg 12 m (1.26 ± 0.50)	0.519
						NTg 16 m (1.25 ± 0.53) < NTg 12 m (1.26 ± 0.50)	1
						3xTg-AD 16 m (2.46 ± 0.71) > NTg 12 m (1.26 ± 0.50)	0.402
						3xTg-AD 12 m (0.60 ± 0.27) < NTg 16 m (1.25 ± 0.53)	0.524
						3xTg-AD 12 m (0.60 ± 0.27) < 3xTg-AD 16 m (2.46 ± 0.71)	**0.008**
						3xTg-AD 16 m (2.46 ± 0.71) > NTg 16 m (1.25 ± 0.53)	0.383
Floating episodes	CUE	~Trial + (1|animal ID)	**Trial**	−1.704	**0.027**	**T4 (0.02 ± 0.02) < T1 (0.11 ± 0.03)**	**0.027**
	PT	~Trial + Age + (1| animal ID)	Trial	0.091	0.602		
			Age	−0.368	**0.038**	**16 m (0.075 ± 0.02) < 12 m (0.11 ± 0.04)**	**0.038**
	RM	~1 + (1| animal ID)					
Cognitive flexibility	CUE	~Trial + Sex + (1| animal ID)	**Trial**	0.803	**0.004**	**T4 (0.72 ± 0.05) > T1 (0.53 ± 0.03)**	**0.004**
			Sex	0.441	**0.032**	**Female (0.68 ± 0.04) < Male (0.58 ± 0.04)**	**0.032**
	PT	~1 + (1|animal ID)					
	RM	~1 + (1|animal ID)					

Circling Behavior

Circling episodes were observed in 49.88% of the trials (406 out of 814), with an average duration of 3.23 s (SD: 6.37 s). A mixed-effect Poisson regression analysis during the CUE stage revealed significant influences from both trial ($p < 0.001$) and age ($p < 0.001$) on the number of circling episodes. Specifically, a decrease was noted by T4 (odds ratio = 4.67, z = 8.02, $p < 0.001$) and at 16 months of age (odds ratio = 2.26, z = 5.06, $p < 0.001$). In the PT stages, the number of circling episodes was significantly influenced by trial ($p = 0.007$), with a decrease observed in T4 compared with T1 (odds ratio = 1.44, z = 2.67, $p = 0.007$), and age ($p = 0.013$), with an increase noted at 16 months of age compared with 12 months (odds ratio = 0.70, z = −2.47, $p = 0.013$). During the RM stage, a significant interaction effect was found between genotype and age ($p = 0.026$). The post hoc analysis revealed a significant increase in the number of circling episodes only for the 3xTg-AD animals when comparing 12 and 16 months of age (odds ratio = 0.51, z = 0.24, $p = 0.008$)

Floating Behavior

Floating behavior was observed in 18.18% of the trials (148 out of 814), with an average duration of 1.92 s (SD: 6.28). During the CUE stage, the top model for the mixed-effect Poisson regression included only trial as a predictor, revealing a significant influence ($p = 0.027$). Specifically, a reduction was observed by T4 compared with T1 (odds ratio = 5.5, z = 2.28, $p = 0.027$). For the PT stages, the top model included both trial ($p = 0.60$) and age ($p = 0.038$) as predictors. The post hoc analysis showed a decrease in floating episodes by 16 months of age (odds ratio = 1.44, z = 2.07, $p = 0.038$). Finally, during the RM stage, no significant influences of sex, genotype, or age were found.

3.3.3. Cognitive Flexibility

Multiple mixed-model ordered beta regressions were built for each stage of the MWM test (Table 2). In the CUE stage, the top model included trial and sex as predictors, both of which showed a significant effect on the response variable (trial: $p = 0.003$, sex: $p = 0.032$). The post hoc analysis revealed an increase in the time taken to change the initial strategy across trials (Trial 1/Trial 4: odds ratio = 0.45, z= −2.91, $p = 0.003$) and that females required more time than males to change it (male/female: odds ratio = 0.64, z = −2.15, $p = 0.032$).

For the PT and RM stages, the null models, which included only the random effect of animal ID, were the top models. Thus, we assumed no influence of sex, genotype, age, or trials on cognitive flexibility during these stages.

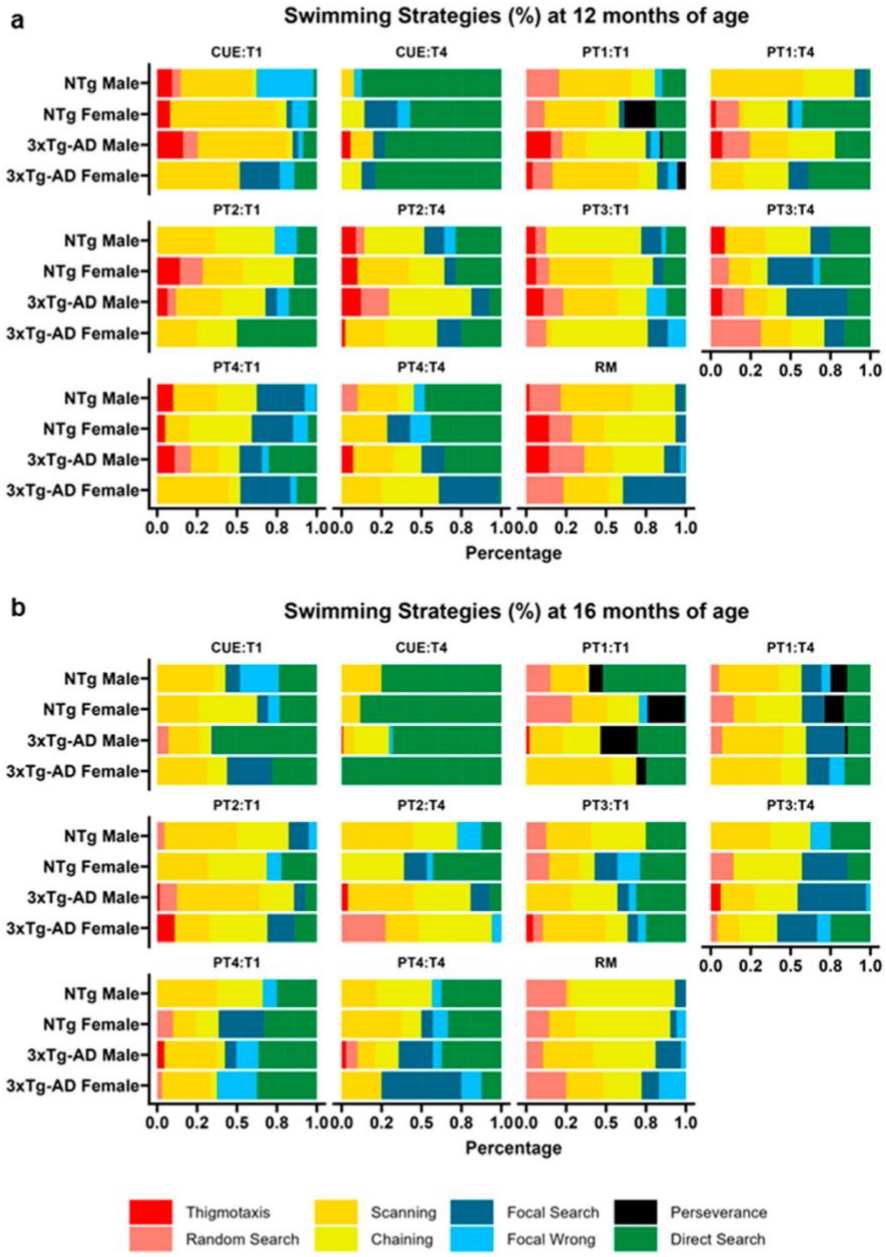

Figure 3. Comparative analysis of swimming strategies by group. Distribution of swimming strategies among different groups during the initial (T1) and final (T4) trials. Panels (**a**,**b**) represent the data collected at 12 and 16 months of age, respectively, showcasing the evolution in strategy adoption over time.

4. Discussion

Our findings harnessed state-of-the-art statistical tools, including ordered beta regression, to sensitively detect sex and genotype cognitive nuances between normal and pathologically aged mice in a multi-strategy identification approach in different stages/paradigms of the MWM test. The present work also advocates for a comprehensive

perspective that integrates classical variables—such as escape latency, distance covered, and swimming speed—with search and non-search strategies analysis to enhance our understanding of animal behavior under normal and pathological scenarios.

4.1. Classical MWM Test Analysis

In our analysis, quantitative variables gained significance primarily during the CUE stage, where the animals were introduced to the fundamental principles of the test. This phase is thus considered a period of training [50]. Importantly, females covered shorter distances than males before finding the platform, which may indicate a better habituation in this group. Furthermore, females exhibited slower swimming speeds than males.

Genotypical differences were detected when escape latency was analyzed during the last day of PT stages (PT4). Thus, 3xTg-AD mice required a longer time to find the platform at both 12 and 16 months of age. Nevertheless, we expected these differences in the acquisition curve to be more evident and detectable between both groups sooner, i.e., already at PT3 [51]. This absence of clear differences supports the inclusion of other variables for a more thorough interpretation of the test when aged animals are evaluated.

4.2. Relevant Swimming Strategies

Previous studies underscored the significance of categorizing swimming strategies during the MWM test in mouse models of AD [45,46]. Others were probably discouraged because of statistical limitations in handling the percentage of multi-strategic performances. Therefore, in most studies, the focus was primarily centered on the predominant strategy. In our previous investigations, we proposed an approach considering multiple strategies in each single trial [26]. In the present work, we further develop this approach and include statistical tools to deal with the challenges that such data can cause. This allowed us to identify genotypic differences at all stages of the test (CUE, PT, and RM). The main differences were observed during the PT stages, where NTg animals employed a direct search strategy more frequently than their 3xTg-AD counterparts. Such differences extend beyond traditional analysis, highlighting the importance of the use of statistical tools in a multi-strategy classification.

Although genotypic differences in random search and direct search were observed during the CUE and RM stages, respectively, the magnitude of these differences was minimal and thus not considered relevant.

Strategies associated with lower hippocampal involvement declined with age, while those more reliant on hippocampal function increased. These findings align with prior research [32,52,53], suggesting a transition in rodent swimming patterns from wall proximity (thigmotaxis) to proactive search strategies (direct search) following training. In fact, our laboratory has proposed that batteries of tests, mainly in longitudinal designs should be considered a "behavioral" cognitive training [54]. As further discussed below (Section 4.5), the current data support this understanding and provide a comprehensive analysis with further evidence on the functional aspects of this cognitive training.

4.3. Importance of Non-Search Strategies

Circling behavior was more prevalent than floating. As animals became accustomed to the test, the occurrence of both behaviors decreased, both across trials and upon retesting after a four-month interval. Notably, only the 3xTg-AD group exhibited an increase in circling behavior during the RM stage. This might be interpreted as an intensification of neuropsychiatric-like symptoms in the transgenic group when confronted with a non-escapable paradigm, potentially impacting the animals' engagement in an active platform search.

Our observation of an association between circling episodes and 3xTg-AD animals aligns with previous research [27,48]. Despite this, in the 3xTg-AD animal set assessed in the present study, built on a C57BL/6 pure genetic background, we did not observe the

persistent hyperactive phenotype often associated with this animal model when based in their original hybrid C57BL/6 × 129 background [26,42], as typically evidenced by a higher mean speed in the test. This would also explain other differences between this and the previous report with respect to the higher incidence of floating episodes previously reported in their NTg (C57BL/6 × 129) counterparts [48], which is not observed under this background strain. In this respect, variations in the stress response profiles among these animals because of their "survival bias" (see Section 4.6) could explain these differences in the floating behavior, a natural characteristic of mice swimming patterns that depends on basal anxiety levels and the behavioral profile [55,56].

4.4. Cognitive Flexibility

We noted sex variations in the transition between initial and subsequent swimming strategies during the CUE test. Specifically, females transitioned slower from their first strategy used to locate the platform compared with males. We propose that the CUE test challenges the cognitive flexibility of the animals. In the first experience, the test may be perceived as non-escapable, while learning opportunities make escape possible in subsequent trials. This observation suggests that males exhibit enhanced adaptability when navigating a novel environment, which could be a potential manifestation of cognitive flexibility.

While previous research has not specifically examined the transition of swimming strategies as a method for evaluating cognitive flexibility, the present work provides further evidence of the importance of assessing it in the test.

4.5. Retest Effect

Our investigation explored the role of prior experience in the test, specifically considering mouse strain characteristics. Thus, according to the classical and multi-strategy approach, animals seemed to improve their performance when retested. Previous longitudinal assessments involving Tg2576 [40] and APP/PS1 [41], which are AD-like models, consistently reveal stable performance upon retesting, similar to control groups. Zhang [57] underscores the protective effect of prior experience against non-cognitive decline in AD-like models. Notably, genotypic differences in 3xTg-AD mice manifest early but attenuate at advanced ages [43,54,58,59]. This reduction may stem from training effects, mortality bias, and age-related declines observed across both the transgenic and non-transgenic cohorts [41,60].

4.6. Limitations

Only animals that survived were included throughout the follow-up period. This "mortality" selection process skews our sample toward animals with distinct behavioral profiles, mainly regarding their stress response characteristics. As recently reported in our laboratory in 3xTg-AD and APP/swe mice, this "survival bias" renders a new window of observation in the experimental scenario [54,60,61].

Our methodology, although comprehensive, has certain limitations. There were two potential issues in the strategy identification i.e., it can be labor-intensive and may lack of reliability. However, we addressed this latter concern by ensuring inter-evaluator agreement. Moreover, it is important to highlight that current track analysis software may not be fully accurate for advanced feature analysis. As such, visual analysis continues to be a practical approach for intricate tasks.

4.7. Future Directions

One significant aspect for future research involves contrasting our findings by integrating the analysis of swimming strategies in other AD-like models within longitudinal studies. This approach will not only strengthen inter-study and inter-laboratory reliability but also offer a more detailed understanding of the observed behavioral patterns.

Previous reports from our laboratory [60] have depicted a neuropathological progression in 3xTg-AD mice of similar ages, with extracellular A-beta plaques present in 12-month-old females and 16-month-old males across various brain regions. Consequently, delving deeper into the neuroanatomical aspects contributing to the behavioral signatures observed in the MWM test can shed light on the mechanisms propelling these behaviors. Most importantly, as shown here, the longitudinal design that scrutinizes the effects of prior training in the test presents an intriguing avenue for research.

In agreement with our prior work on normal and AD-pathological aging [15,27,62,63], we consider that employing swimming strategies adds stronger methodological sensitivity to assess the effects of new compounds or interventions. It is essential to pay close attention to any alterations in the variables, such as those outlined here. This approach holds promise for the development of more effective preventive and/or therapeutic interventions for AD and normal aging.

5. Conclusions

We have successfully integrated GLMMs and ordered beta regression into the MWM test analysis to interpret the swimming patterns of the animals. This integration has allowed us to gain a more comprehensive understanding of mouse behavior in normal and AD-pathological aging. The results of our study underscore the effectiveness of this methodology. We believe that future research, particularly those focusing on the evaluation of normal and pathological aging in animals under intrinsic (i.e. sex-perspective) and extrinsic factors (i.e. social and environmental conditions, non-pharmacological and pharmacological interventions), will consider this approach as an indicator of the performance in the test.

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4.2 Study 2

Alveal-Mellado, D., Castillo-Mariqueo, L., & Giménez-Llort, L. (2022). **Sex- and Neuropsychiatric-Dependent Circadian Alterations in Daily Voluntary Physical Activity Engagement and Patterns in Aged 3xTg-AD Mice.** International Journal of Molecular Sciences, 23(22), 13671. https://doi.org/10.3390/ijms232213671

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Sex- and Neuropsychiatric-Dependent Circadian Alterations in Daily Voluntary Physical Activity Engagement and Patterns in Aged 3xTg-AD Mice

Daniel Alveal-Mellado [1,2], Lidia Castillo-Mariqueo [1,2] and Lydia Giménez-Llort [1,2,*]

[1] Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
[2] Department of Psychiatry and Forensic Medicine, School of Medicine, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
* Correspondence: lidia.gimenez@uab.cat; Tel.: +34-93-5812378

Abstract: Alzheimer's disease (AD) patients suffer from circadian rhythm alterations affecting their daily physical activity patterns with less willingness to perform a voluntary exercise. In preclinical studies, there is no clarity on whether animal models of AD can replicate these impairments. Here, we provide a proof of concept of the performance and behavioral effects of four weeks of voluntary wheel running (VWR) in a group of 14-month-old male and female 3xTg-AD mice at advanced stages of AD and the daily variance (behavioral circadian rhythmicity) of VWR associated with sex and their neuropsychiatric-like phenotype. Higher levels of horizontal exploration in the open field (OF) test were found in mice submitted to exercise. A linear mixed effect model showed significant sex-dependent differences in the VWR activity performed on the first night of follow-up, with high-NIBI males running less than high-NIBI females. Thus, an influence of NPS-like symptoms on the circadian patterns of VWR may account for such differences. In addition, males remained more active than females during diurnal periods. We hypothesize that this increment in energy expenditure during resting periods may be related to hyperactive behavior, similar to that observed in humans' exacerbated agitation or sundowning behavior. These findings support the usage of the 3xTg-AD mouse as a reliable model for studying circadian rhythm alterations in AD and, at the translational level, the importance of tailored and individualized physical activity programs in clinical settings.

Keywords: Alzheimer's disease; animal model; sex differences; rehabilitation; exercise; physical activity; circadian rhythms

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1. Introduction

An insidious cognitive decline comprising anterograde memory and executive function impairments are the main diagnostic criteria for dementia caused by Alzheimer's disease (AD) [1]. In addition, a broad spectrum of neuropsychiatric symptoms (NPS) may accompany the instauration of the disease [2], including affective disorders (anxiety and depression), behavioral and emotional difficulties (apathy and mood fluctuation), psychotic symptoms (hallucinations and delusions), and sundowning behavior (circadian rhythm alterations) [3]. Indeed, when severe, such dysfunctions represent the leading cause of institutionalization [4,5]. Because of the heterogeneity of the clinical phenomena, NPS are also referred to as behavioral and psychological symptoms of dementia (BPSD) [6,7].

Circadian rhythm dysfunctions (CRD) are also reported in the early stages of the disease, and it comprises sleep, thermoregulation, and movement disorders [8]. CRD are also observed in the healthy aging process due to changes in afferent pathways to the circadian pacemaker of the organism, the suprachiasmatic nucleus (SNC) [9]. However, AD patients suffer more pronounced changes, and degeneration affects the SCN even before the cognitive decline begins [10]. Furthermore, CRD have been proposed as a preclinical sign

of the disease [11]. Therefore, nonpharmacological strategies that can be implemented in individuals at risk or be part of the individuals' lifestyle can play an important role as prevention strategies during asymptomatic and prodromal stages to hamper or counteract the underlying detrimental processes.

Environmental factors such as maintaining moderate levels of physical activity (PA) may prevent cognitive decline [12,13] and modify neuropathological changes that occur in the early stages of AD [14]. However, clinical evidence warns that NPS/BPSD and CRD negatively influence routine exercise and physical rehabilitation engagement in these patients [15,16]. Thus, low levels of PA have been reported in free-living and institutionalized AD patients, with a marked reduction in women [16–18]. At the translational level, the effects of PA have also been studied in animal models of AD. Thus, improvements in cognitive and BPSD-like symptoms are reported at the early and moderate stages of the disease [19,20], whereas the effects of PA at more advanced stages remain unclear. We have also shown that, despite the benefits, a forced exercise paradigm may also exert some minor adverse effects on females [21]. In the present work, we addressed the animal's engagement in the voluntary wheel-running paradigm for the first time.

Equally important have been the study of CRD in aging and AD using rodent models. Daily movement activity variations, measured as "in cage" activity or running wheel (RW) activity, are frequently reported with a general age-dependent decline [21] and greater levels of PA in females [22,23], probably attributable to a protector effect of estrous cycle hormones [24].

The triple transgenic model for AD (3xTg-AD) developed by Frank LaFerla's laboratory [25] has shown good face and construct validity [26], replicating the extracellular beta-amyloid plaques and intracellular neurofibrillary tangles accumulation within specific brain areas, such as the hippocampus and neocortex, considered the neuropathological hallmarks of the disease [25,27,28].

Cognitive symptoms arise in animals' early ages and can be detected in tests evaluating spatial orientation and working memory (i.e., Morris water maze and T-maze) [29]. On the other side, tests involving novel environments (i.e., corner test and open-field test) have shown sensible to detect noncognitive symptoms (NPS-like symptoms) through the induction of higher freezing behavior and reduced horizontal exploration. These findings have been previously referred to as novelty-induced behavioral inhibition (NIBI) [30,31].

Regarding CRD, the model exhibits circadian alterations [29] and reproduces sleep and movement activity disorders at 6 months old [32,33]. Nevertheless, the temporal progression of such impairments at older ages has not been fully depicted.

The present work aims were (1) to model the pattern of behavioral circadian rhythmicity of PA in a group of 3xTg-AD animals at advanced ages submitted to daily voluntary wheel running (VWR) and (2) to evaluate the effects of 30 days of PA in noncognitive symptoms of AD.

2. Results

Body weight was monitored from the day before the start of the behavioral battery of tests until the week after the end of the 30-day follow-up (see Figure 1). Weight variation differences were analyzed through a 2 × 2 × 2 (sex × treatment × days) ANOVA test showing a treatment effect [T, $F(1,30) = 11.325$, $p = 0.002$] with a significant decrease in weight after 30 days in the VWR group [SED vs VWR, $t(18,16) = 2.97$, $p = 0.006$, *t*-test], mostly due to statistically significant differences observed in males [SED males vs VWR males, $t(10,7) = 4.16$, $p = 0.001$, *t*-test]. Indeed, the sex × treatment interaction effect [$F(1,30) = 5.70$, $p = 0.023$] shows that SED females also showeda negative weight variation.

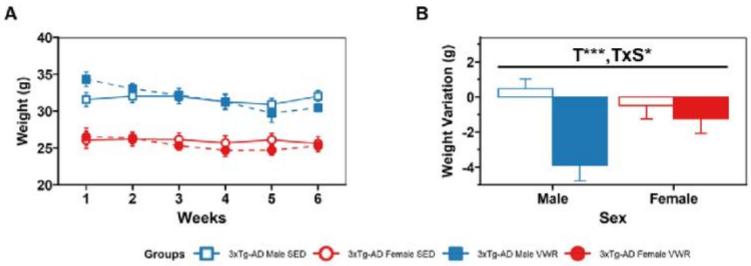

Figure 1. Body weight (**A**) at baseline (week 1) and after 30 days follow-up (week 6). The intervention took place between weeks 2 and 5. (**B**) Weight variation per group (weight at week 6 -week 1). Factorial analysis: D, days; T, treatment; S, sex. * $p < 0.05$; *** $p < 0.001$.

2.1. Corner (CT) and Open Field (OF) Tests

In the CT, no baseline differences between groups were found (Figure 2). By contrast, a 2 × 2 × 2 (sex × treatment × days) mixed (split-plot) ANOVA test yielded a days effect in almost all variables evaluated (D, all Fs(1,30) > 14.09, $p < 0.001$), indicating a reduction in exploratory activity and an increment in neophobia in the pre-post analysis. Thus, all animals ($n = 34$) reduced the number of corners at 30 s (t(34) = 6.35, $p < 0.001$, paired t-test) and 60 s (t(34) = 6.51, $p < 0.001$, paired t-test); the number of rearings at 30 s (t(34) = 5.94, $p < 0.001$, paired t-test) and 60 s (t(34) = 5.62, $p < 0.001$, paired *t*-test), and the number of corners until first rearing (t(34) = 3.65, $p < 0.001$, paired t-test). Conversely, an increase in the latency of first rearing when re-tested was found (t(34) = −4.44, $p < 0.001$, paired t-test). In addition, a treatment × days interaction effect was observed in the ratio of visited corners/rearings in 60 s (CTratio60) (T × D, F(1,30) = 7.14, $p = 0.012$) with an increase in the VWR animals (re-test vs. baseline, t(16) = −2.51, $p = 0.024$, paired *t*-test) and higher values compared with the SED group (t(18,16) = −2.21, $p = 0.041$, *t*-test).

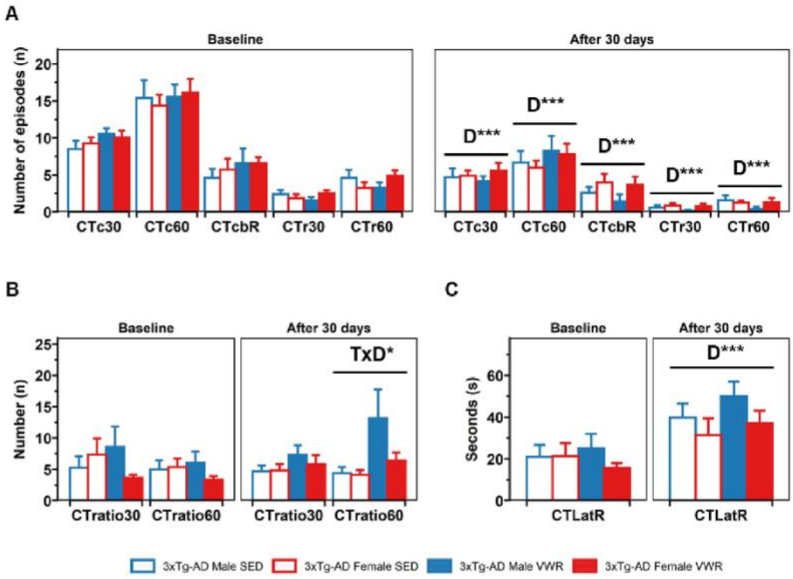

Figure 2. Corner test. (**A**) Horizontal activity: number of corners visited at 30 s (CTc30) and 60 s (CTc60), number of corners before rearing (CTcbR), rearing at 30 s (CTr30) and 60 s (CTr60) (**B**) ratios of horizontal/vertical activity: corners visited/n rearings at 30 s (CTratio30) and 60 s (CTratio60) and

(**C**) vertical activity: rearing latency (CTLatR). Factorial analysis: D, days; T, treatment; S, sex. * $p < 0.05$; *** $p < 0.001$.

In the OF test, a 2 × 2 (sex × treatment) ANOVA showed a treatment effect after 30 days in the latency of movement (movement) [T, $F(1,30) = 5.72$, $p = 0.023$] and in the latency to start exploring the central zone (Zcentral) [T, $F(1,30) = 7.25$, $p = 0.011$] (Figure 3). A post hoc analysis yielded a reduction of these parameters in the group submitted to VWR compared with SED animals [VWR vs. SED, all $t(16,18) > 56.6$, $p < 0.015$, t-test]. Regarding vertical activity, a sex × treatment × days interaction was found [S × T × D, $F(1,30) = 5.07$, $p = 0.032$, Mixed ANOVA]. A further analysis showed that significance could be explained by sex differences in the VWR group at baseline [3xTg-AD male VWR vs. 3xTg-AD female VWR, $t(7,9) = 2.4$, $p = 0.042$]. No differences were found in the latency of rearing, the latency of grooming, or emotionality (urination and defecation boli) (data not shown). Finally, when the distance covered by the animals was analyzed, a days effect was found in the central zone [D, $F(1,30) = 12.60$, $p = 0.001$] with a decrease in all groups when retested [retest vs. baseline, $t(34) = 3.66$, $p < 0.001$, paired t-test]. Moreover, sex effects were found in the periphery [S, $F(1,30) = 7.98$, $p = 0.008$] and total field [S, $F(1,30) = 6.02$, $p = 0.02$] after 30 days with males covering more distance.

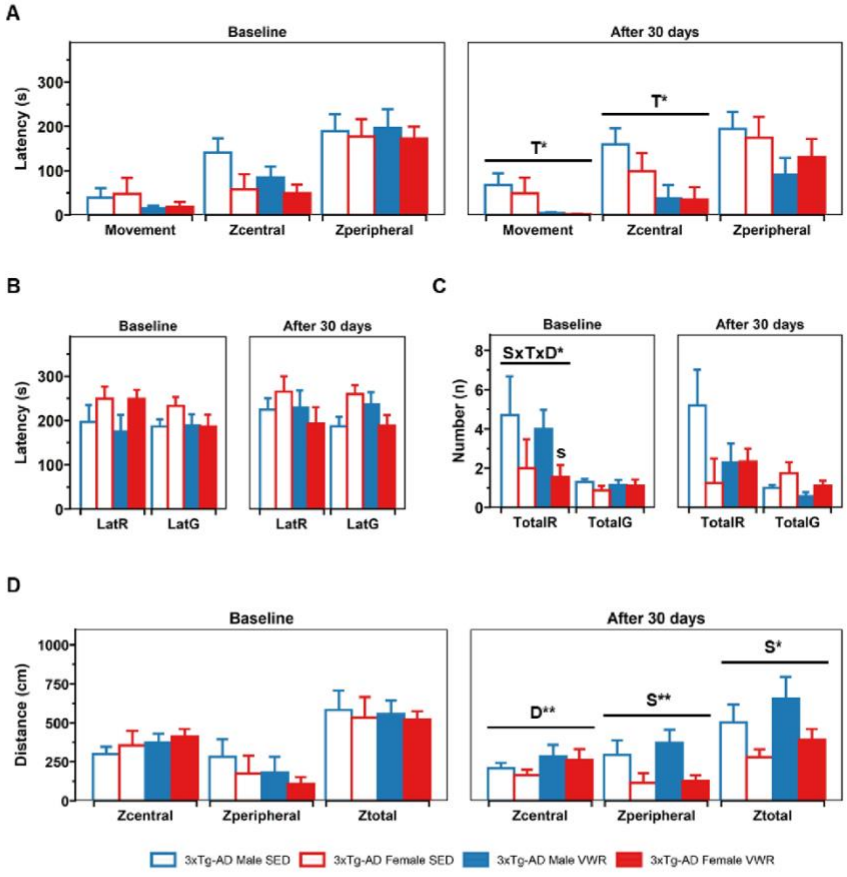

Figure 3. Open field test. (**A**) Horizontal activity latencies: movement latency (movement), latency to start exploring in the central zone (Zcentral), latency to reach the periphery (Zperiphery). (**B**) and (**C**). Vertical activity: rearing latency (LatR), grooming latency (LatG), total rearing episodes in 5 min (TotalR), total grooming episodes in 5 min (TotalG) (**D**) distance covered in the OF in the central

zone (Zcentral), peripheral zone (Zperiphery), and total field (Ztotal). Factorial analysis: D, days; T, treatment; S, sex; s < 0.05 vs. male counterpart in the same treatment group. * $p < 0.05$; ** $p < 0.01$.

2.2. T-Maze Spontaneous Alternation (TMSA)

The TMSA (Figure 4 test showed no differences in the latencies related to freezing behavior (to move and turn) and exploration; namely, the time until start to explore (Explore), reach the intersection (LatT), and the time spent until reaching the first (Arm1) and second (Arm2) arm of the maze, and the test completion criteria (End) [All Fs(1,29) < 3.49, $p > 0.05$, mixed ANOVA]. However, a sex × treatment × days interaction was found in the time elapsed until crossing the intersection with their four paws (LatT4) [F(1,29) = 4.80, $p = 0.037$, mixed ANOVA] after 30 days, with 3xTg-AD males VWR reaching this goal in a shorter time compared with 3xTg-AD females VWR [t(7,8) = −2.20, $p = 0.048$, t-test]. Moreover, considering the goals in the TMSA ethogram as a whole (pooled data), a trend is observable with better performance of the 3xTg-AD males VWR compared with all other groups [S × T × D, F(1,29) = 3.90, $p = 0.058$] in the retest. Instead, no significant differences were found after 30 days in the number of errors and vertical activity (rearings and grooming) in the test (data not shown).

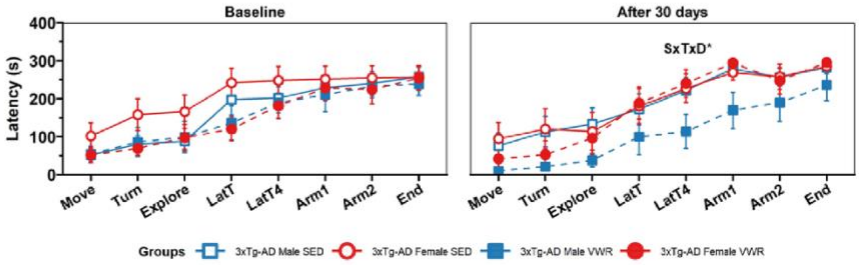

Figure 4. TMSA test at baseline (**left**) and after 30 days follow-up (**right**). Latency to move (Move), latency to turn (Turn), latency to reach the maze intersection (LatT) and cross the intersection with the four paws (LatT4), latency to explore the first (Arm1) and second (Arm2) arms and latency to complete the test completion criteria (End). Factorial analysis: D, days; T, treatment; S, sex. * $p < 0.05$.

2.3. Sensorimotor Assessment

The sensorimotor assessment comprised (1) the muscular strength (5 s) and muscular endurance (60 s) grip tests evaluating forelimbs strength and (2) geotaxis, focused on the response to gravity assessment (Figure 5). In the muscular strength trial, a days effect [D, F(1,29) = 19.95, $p < 0.001$], sex × day [S×D, F(1,29) = 5.23, $p = 0.03$] and a sex × treatment × day interaction effects [S × T × D, F(1,29) = 5.57, $p = 0.025$] were found. A post hoc analysis showed that all groups increased their latency after 30 days [t(34) = −4.30, $p < 0.001$, paired t-test], females last longer than males after 30 days (3.21 vs. 2.77 s) [t(17,17) = −2.66, $p = 0.01$], and 3xTg-AD VWR females had better performance after 30 days compared with their values at baseline [t(9) = −7.79, $p < 0.001$, paired t-test] vs. 3xTg-AD SED females [t(8,9) = −2.42, $p = 0.046$] and vs. 3xTg-AD VWR males [t(7,8) = −3.14, $p = 0.021$]. In the muscular endurance trial a days effect [D, F(1,30) = 13.98, $p < 0.001$] and a sex × days interaction effect [F(1,30) = 10.51, $p = 0.003$, mixed ANOVA] were found with females improving their performance when retested [t(17) = −4.78, $p < 0.001$, paired t-test] and resisting more than males after 30 days [t(17,17) = −3.06, $p = 0.005$]. In a post hoc, 3xTg-AD SED females and 3xTg-AD VWR females improved after 30 days and differences were found between the 3xTg-AD VWR females and 3xTg-AD VWR males [t(7,9) = −3.92, $p = 0.003$, t-test]. Respecting geotaxis, no treatment effect nor differences between groups were found.

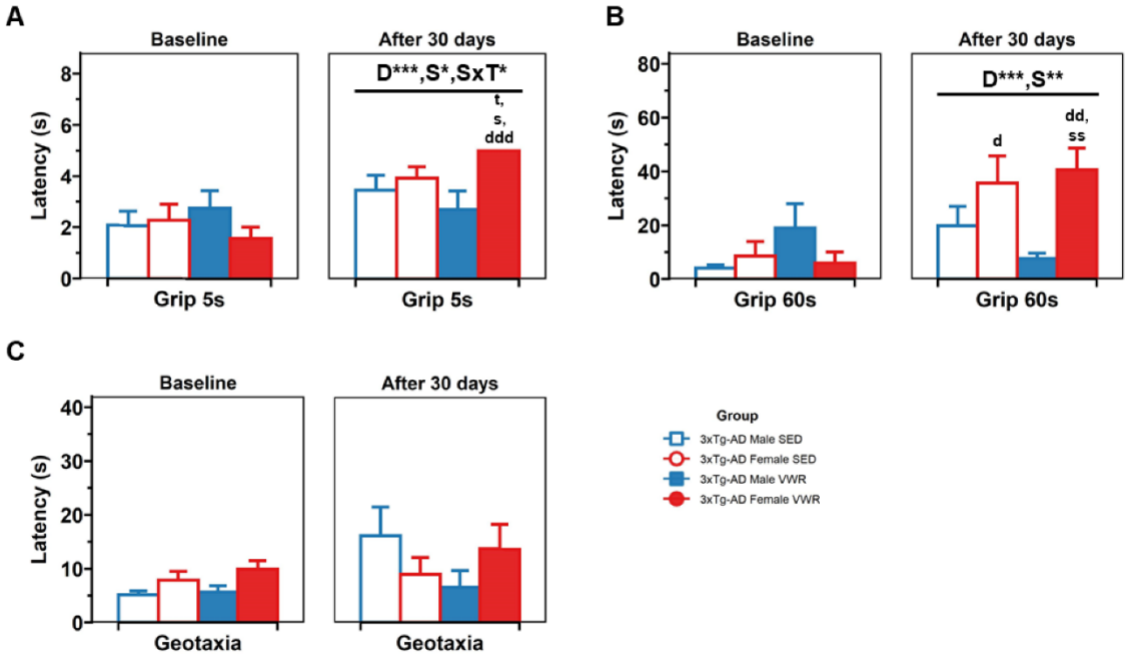

Figure 5. Sensorimotor assessment. (**A**) Muscular strength at baseline and after 30 days. (**B**) Muscular endurance at baseline and after 30 days. (**C**) Geotaxia at baseline and after 30 days. Factorial analysis: D, days; T, treatment; S, sex. t < 0.05. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; d < 0.05, dd < 0.01 and ddd < 0.001 vs. the same group at baseline; s < 0.05 and ss < 0.01 vs. male counterpart in the same treatment group.

2.4. Rotarod

In the constant mode of the rotarod (Figure 6), a days effect was found [$F(1,30) = 6.10$, $p = 0.019$, mixed ANOVA], with all animals learning faster when retested [$t(34) = 2.43$, $p = 0.021$, paired t-test]. Then, in the accelerated mode, a days effect [D, $F(1,30) = 20.528$, $p < 0.001$] and a treatment × days interaction [T × D, $F(1,30) = 4.21$, $p = 0.049$] effects were found. Further analysis showed that 3xTg-AD VWR males last longer than 3xTg-AD SED males (210.35 s vs. 182.1 s) [$t(42,60) = -2.05$, $p = 0.04$] and 3xTg-AD VWR females last longer than 3xTg-AD SED females (207.70 s vs. 152.83 s) [$t(54,48) = -5.76$, $p < 0.001$]. Then, when coordination was evaluated in the rocking mode, a general days effect was found [D, $F(1,30) = 10.531$, $p = 0.003$], in a post hoc analysis differences were found in the retest, with 3xTg-AD VWR males performing better than 3xTg-AD SED males [VWR vs. SED, $t(10,7) = -2.44$, $p = 0.03$, t-test].

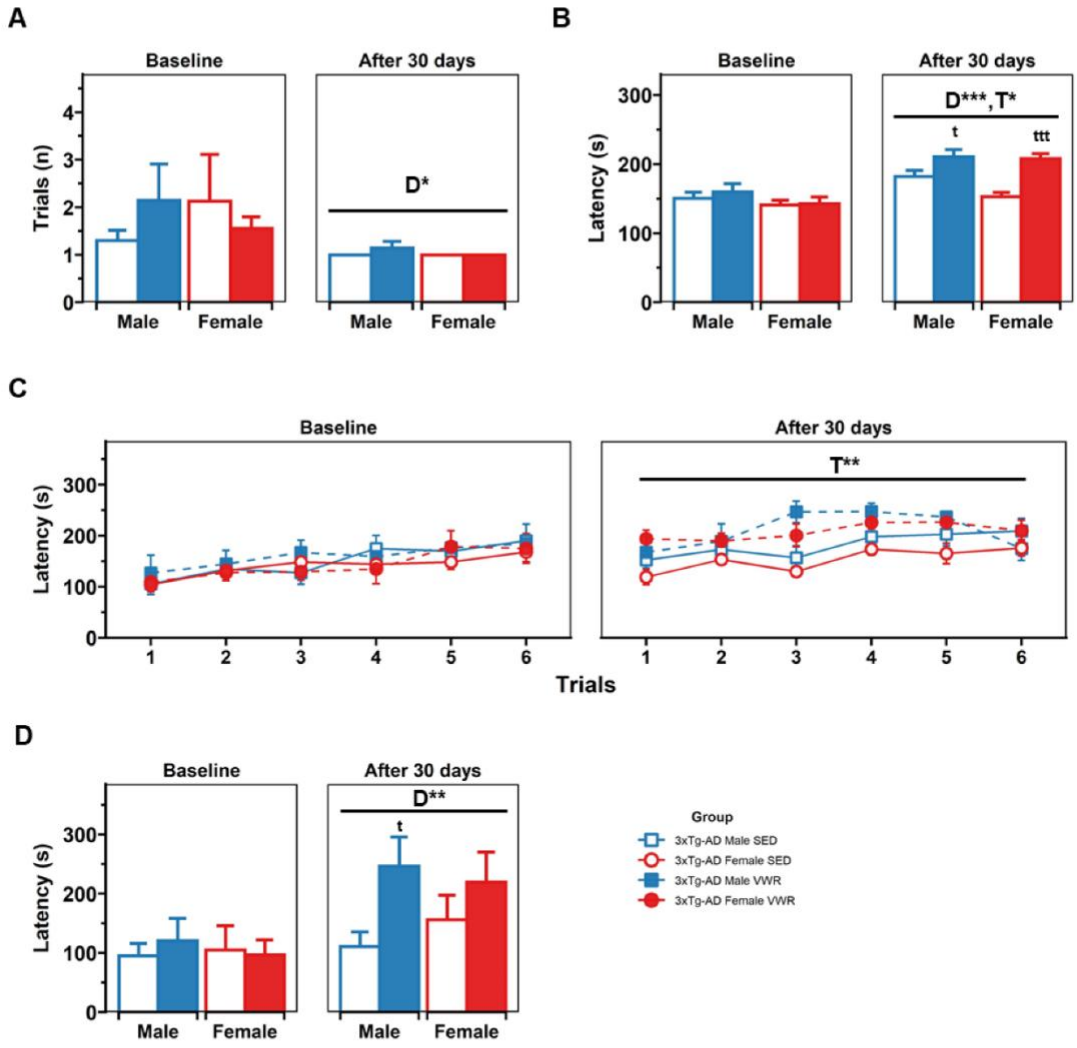

Figure 6. Rotarod test. (**A**). Learning phase. (**B**). Accelerated phase (pooled values). (**C**). Accelerated phase (trial by trial). (**D**). Rocking phase. Factorial analysis: D, days; T, treatment; S, sex. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. t < 0.05 and ttt < 0.001 vs. sedentary counterpart in the same sex.

2.5. Daily Patterns of Voluntary Wheel Running (VWR)

The pattern of behavioral circadian rhythmicity in the RW was evaluated in the animals allocated in the VWR group through two linear mixed effect models to assess the changes by sex in the trajectories of activity along 30 days and the influence of NIBI behavior in the CT at baseline (Figure 7A,D and Table 1). The main findings in model 1 were: (1) the average level of exercise increases with days in male cages during the nocturnal period (β_{Days}: $p < 0.001$), (2) the difference in the average level of exercise between nocturnal and diurnal periods in male cages was significant at day 1 (β_{Period}: $p < 0.001$), and (3) sex differences varies depending on the period at day 1 ($\beta_{Sex,Period}$: $p < 0.01$); thus; VWR males performed less exercise than females in the first night of follow-up. Respecting model 2, the main findings showed that (1) the differences by sex were higher in the nocturnal period for high NIBI animals at day 1 ($\beta_{Sex,NIBI,Period}$: $p = 0.025$) and (2) the slope of

the VWR trajectory along nocturnal periods varies depending on the sex and the initial NIBI behavior ($\beta_{Days,Sex,NIBI}$: $p = 0.018$).

In addition, a two-way ANOVA test was applied in the total levels of VWR pooled by weeks (Figure 7B). Here, a "Weeks" effect was found in the nocturnal period only [$F(3,18) = 5.08$, $p = 0.01$], explained by a significant decrease in the mean total activity by the fourth week compared to the third one [week 3 vs. week 4: $t(8) = 4.40$, $p = 0.019$, paired t-test].

When the mean total activity per week was considered (Figure 7C), significant sex differences were found during diurnal periods, with males performing higher level than females [males vs. females: $t(16,16) = 2.30$, $p = 0.031$, t-test].

Table 2 depicts the significant correlations in VWR group based on the aggregated values performed at baseline in the 8 cages equipped with the RW.

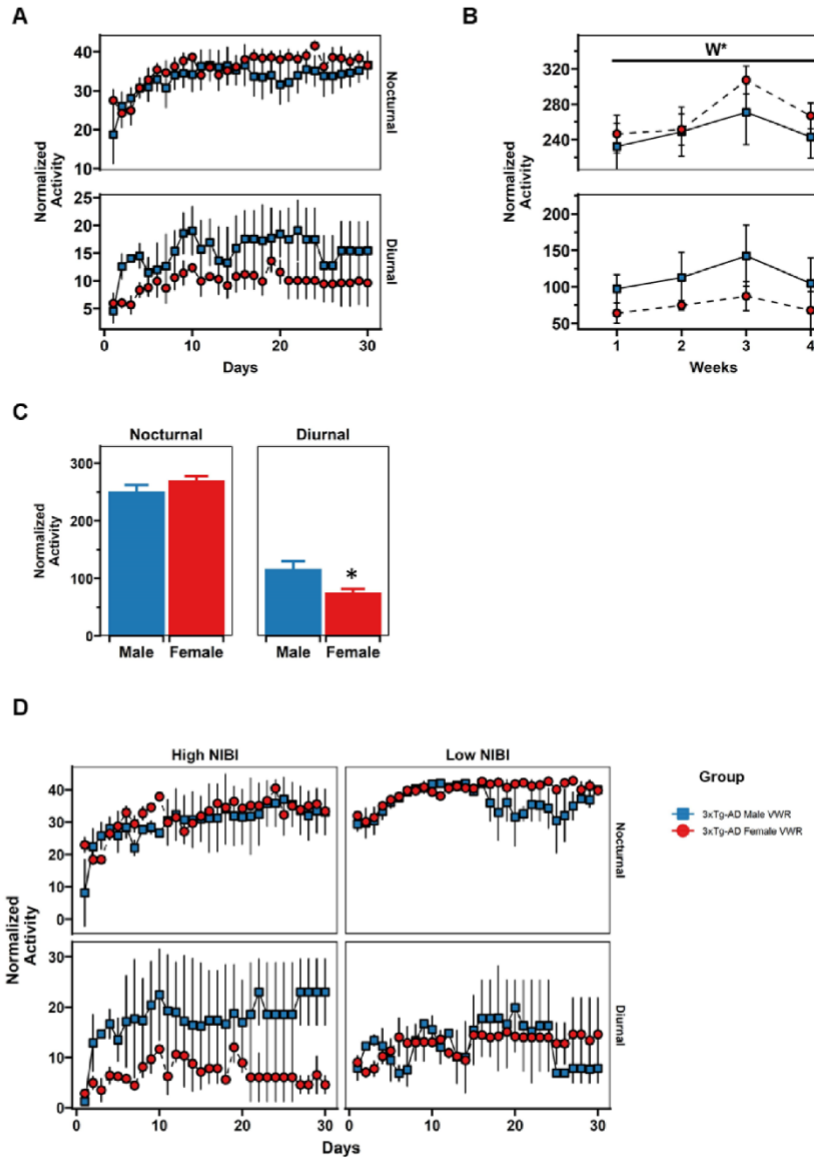

Figure 7. Patterns of voluntary wheel running (VWR). (**A**) Daily nocturnal (upper box) and diurnal (lower box) VWR activity. (**B**) Mean total activity in the RW week by week during nocturnal (upper box) and diurnal (lower box) periods. (**C**) Mean total activity per week during nocturnal and diurnal periods. (**D**) Activity in the RW adjusted by high and low novelty-induced behavioral inhibition (NIBI) in the corner test (CT) at baseline. W, weeks effect; * $p < 0.05$ and *t*-test vs. sex counterpart in the same period.

Table 1. Results for the linear mixed-effects models assessing the activity in the RW by days (Model 1) and adjusted by previous NIBI behavior in the CT (Model 2).

Variable	Estimate	SE	Z Value	*p*-Value
Model 1	Revolutions~I (Days-1) ×Sex × Period + (1|Cage)			
β_0	29.71	2.68	11.09	<0.001 (***)
β_{Days}	0.24	0.06	4.05	<0.001 (***)
β_{Sex}	1.11	3.79	0.29	*n.s.*
β_{Period}	−16.59	1.40	−11.81	<0.001 (***)
$\beta_{Days,Sex}$	0.10	0.08	1.20	*n.s.*
$\beta_{Days,Period}$	−0.09	0.08	−1.12	*n.s.*
$\beta_{Sex,Period}$	−5.61	1.99	−2.82	0.004 (**)
$\beta_{Days,Sex,Period}$	−0.17	0.12	−1.41	*n.s.*
Model 2	Revolutions~I (Days-1) × Sex × NIBI × Period + (1|Cage)			
β_0	22.97	3.83	5.99	<0.001 (***)
β_{Days}	0.48	0.07	6.47	<0.001 (***)
β_{Sex}	3.12	5.42	0.58	*n.s.*
β_{NIBI}	13.48	5.42	2.49	0.013 (*)
β_{Period}	−9.57	1.78	−5.38	<0.001 (***)
$\beta_{Days,Sex}$	−0.08	0.11	−0.73	*n.s.*
$\beta_{Days,NIBI}$	−0.49	0.11	−4.62	<0.001 (***)
$\beta_{Sex,NIBI}$	−4.02	7.67	−0.52	*n.s.*
$\beta_{Days,Period}$	−0.17	0.11	−1.63	*n.s.*
$\beta_{Sex,Period}$	−9.59	2.52	−3.81	<0.001 (***)
$\beta_{NIBI,Period}$	−14.04	2.52	−5.58	<0.001 (***)
$\beta_{Days,Sex,NIBI}$	0.35	0.15	2.38	0.018 (*)
$\beta_{Days,Sex,Period}$	−0.24	0.15	−1.60	*n.s.*
$\beta_{Days,NIBI,Period}$	0.16	0.15	1.05	*n.s.*
$\beta_{Sex,NIBI,Period}$	7.96	3.56	2.24	0.025 (*)
$\beta_{Days,Sex,NIBI,Period}$	0.15	0.21	0.69	*n.s.*

p-Value significance level: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0.01$.

Table 2. Significant correlations in VWR group ($n = 8$ cages).

	Rearing Latency (CT)	Center Latency (OF)	Total Grooming (OF)	Rocking (RR)	Grip 60	Initial Weight
Total activity (in 30 days)	0.511	**0.737** *	0.669	**0.719** *	0.371	**0.712** *
Mean nocturnal activity	0.354	0.583	**0.714** *	0.635	0.482	**0.746** *
Mean diurnal activity	**0.745** *	0.644	−0.101	0.557	0.007	0.308
Mean daily activity	0.491	0.681	0.642	**0.711** *	0.450	**0.760** *
Mean nocturnal activity (days 1–10)	−0.114	0.229	0.605	0.101	**0.721** *	**0.730** *
Mean diurnal activity (days 1–10)	**0.781** *	0.592	−0.013	0.688	−0.038	0.215
Ratio days 1–10	**−0.800** *	−0.174	−0.582	−0.674	−0.048	−0.051

Data showing Pearson's correlation between variables of VWR and the initial battery of tests (CT, OF, RR, sensorimotor assessment, and initial body weight) of animals in the VWR group. * $p < 0.05$.

2.6. Survival

During the month of intervention, mortality rate was 5/39 (12.8%) and restricted to males (Chi-square = 4.432, 1 degrees of freedom, two-tailed $p = 0.0353$ vs. females), with 1/11 (9.09%) in the SED group and 4/11 (36.36%) in the VWR group (*n.s.*).

3. Discussion

An age-dependent decline in PA levels has been reported in healthy humans [34], and most of the species used in preclinical studies focusing on aging progression [21,22,35]. In clinical scenarios, PA engagement is found to decrease at higher rates in AD patients [17], pointing out the presence of NPS (i.e., apathy or depressive behavior) as a barrier to incorporating in recreational/social activities of higher energy consumption and further impeding the protective benefits from its regular practice [15]. Moreover, compared to healthy controls, AD patients show lower levels of PA during daylight periods, with a delay in the onset of their daily life activities during the morning, being more active during the night [8,17,36]. In addition, gender differences have been found in the circadian rhythms of movement activity in these patients, with males expending significantly more total energy than women during a 24 h period [18]. The causes of incremented activity overnight seem to be related to agitation or the "sundowning" condition in this population [37–39].

Here, we present for the first time the behavioral circadian rhythm pattern of PA in the animals submitted to VWR. Our main results showed sex-dependent differences in the mean total levels of VWR performed during diurnal periods. Thus, males remained more active than females during the daylight cycle.

Mice are nocturnal animals and their activity decreases during daylight hours [22]; therefore, we hypothesize this increment in energy expenditure during resting periods may be a sign related to hyperactive behavior, similar to that observed in the exacerbated agitation or "sundowning" behavior in humans. Interestingly, a second finding is that we also observed that the PA levels increased in the VWR animals along the days with a plateau after the third week of follow-up, attributable to a "learning phase" necessary for the animal's adaptation or acclimation. A process previously reported occurring in rodent animal models under similar conditions of VWR [22,40–42].

In this proof-of-concept analysis of the animals in the VWR group, we found a mixture of meaningful behavioral correlations between the battery of tests performed at baseline and the VWR activity. Hence, in line with human studies [43], motor/body composition variables help predict the levels of PA performed during diurnal and nocturnal cycles. Specifically, better coordinated (according to the rocking phase of RR test) and stronger animals (based on grip 60 s results) positively correlated with total activity and mean nocturnal activity of the first 10 days of VWR follow-up. Furthermore, the weight at baseline positively correlated with the VWR activity. However, this correlation could be influenced by the sex differences found during the light-phase cycle (sexual dimorphism in body weight). On the other hand, variables of behavioral profiles such as neophobia in the CT and the exploratory activity in the OF test were also positively correlated with the VWR. Indeed, animals lasting longer to perform the first rearing in the CT (CTLaR) and to start the exploration in the OF central zone (Zcentral) run more in the wheel.

Interestingly, both linear mixed-effect models showed a significant sex-dependent difference in the VWR activity performed the first night of follow-up (day 1, nocturnal period) with male high-NIBI animals running less than females. These findings point out an influence of NPS-like symptoms (i.e., neophobia) on the circadian patterns of VWR of the 3xTg-AD animal model and/or different strategies to scope with the stress associated

with novel environments. Nevertheless, further experiments are required to clarify the involvement and interaction of behavioral dimensions in VWR.

Taken together, the enhanced diurnal activity of males in the RW cannot be supported by sex motor differences since limb strength, endurance in the grip test, and coordination in the RR were identical for the SED and VWR groups at baseline and after 30 days. Therefore, we propose that there are factors beyond physical characteristics that influence the hyperactive diurnal pattern.

We have previously shown that at advanced stages of the disease, mortality rates are higher in 3xTg-AD males [44]. In the present work, sexual differences were also found with survival in all 17 females but mortality in 5 of the 22 males. In addition, the VWR male group showed a higher mortality ratio than the SED male group, albeit it did not reach statistical significance. This would agree with a previous work at the same stage of the disease, where only females survived in a VWR protocol, whereas NTg and 3xTg-AD males did not [45].

Considering all animals evaluated, an increase in neophobia with lower exploratory activity was detected in the CT after 30 days. These changes support a previous association of worsening NPS-like symptoms in the 3xTg-AD model, caused by the pathological aging process [46–48]. Furthermore, here, we tested the effect of PA starting at the late stages of AD, once cognitive and neuropsychiatric symptoms have been already established [29].

In our previous longitudinal study [49], including mice at 12 and 16 months of age at normal and AD-pathological stages, we found a reduction in the CTratio60 with age. Now, an increment in the CTratio60 was observed in the VWR group. We believe the increment was due to a general reduction in the CTrearings episodes, more than the potential beneficial effects of exercise. Thus, in experiments carried out in mice at earlier stages of AD [19], four weeks of voluntary running did not attenuate the reduction of horizontal (CTc60) or vertical activity (CTr60) in the test.

In the OF test, the exploratory activity is commonly used to analyze the animal's response to stressful/anxiogenic events [50]. Indeed, we have shown that in the 3xTg-AD mice the usage of repeated of tests can help monitor the development of AD [51]. Interestingly, in a study with 20 different mouse strains [52], a positive correlation between the distance covered on the test and the distance run in several days of VRW follow-up has been described. However, in the present study, we found no correlation between such variables.

Animals submitted to VWR reduced the latency to move (Movement) and to start exploration (Zcentral) in the OF, indicating a possible reduction in the test-induced anxiety or an improvement in the habituation process to the test, which is also one of the simplest types of memory [48].

The behavior of all animals in the TMSA was similar, with most failing to achieve the completion criteria of the test. This can be related to the advanced aged status of the animals and lack of motivation [44]. Moreover, differences in TMSA performance have been linked to impaired immune function and shorter lifespans in slower animals [53]. Here, the 3xTg-AD Male VWR group tended to complete the exploratory goals in a shorter time. Indeed, they crossed the intersection of the maze significantly faster than all other groups. This would agree with their faster performance in exploratory variables in the OF test mentioned above.

Improvement in muscular strength and endurance in the VWR group are presumably associated with the benefits of exercise. Nevertheless, sedentary animals also improved after 30 days. This increment can be related to the effect of a previous experience because the animals performed better when reassessed [48]. Another factor affecting the increase in forelimb strength involves the natural activities of the animals in their home cages, such as grid climbing [54]. Because the RW, as an object, may represent an enrichment in the environment for the VWR group, the optimal SED groups should include such an environmental cage condition (a blocked wheel). Still, in previous experiments [19,45]

using this kind of SED control group, we have demonstrated that the benefits of the exercise can be clearly discriminated compared to those 3xTg-AD mice housed in a cage with a blocked wheel.

Finally, in the RR test, VWR animals outperformed the SED group in the accelerated and coordination protocols, probably due to muscle memory and the similarity of RR apparatus and the RW used as treatment.

In summary, the main findings of the present work are: (1) The 3xTg-AD male model seems to replicate some physical activity circadian rhythm dysfunctions, with enhanced diurnal activity compared to females. (2) The increased activity in males seems to be associated with NPS-like symptoms. (3) Despite advanced age and AD pathology, VWR improved some anxiety-related NPS-like symptoms, such as freezing behavior in the OF and TM. However, cognitive improvement was not observed. These findings support the importance of tailored and individualized physical activity programs in clinical settings (walking, daily life activities) that should be designed and implemented considering sex/gender, NPS profile, and circadian rhythms.

4. Methods

4.1. Animals

The 3xTg-AD model was previously generated [25] by microinjecting (1) a human cDNA harboring the APP transgene, with a translocation in amino acids, named the Sweden mutation (APPSWE), and (2) the human P301L tau transgene (tauP301L), which impairs binding of tau from microtubules, into single-cell embryos of homozygous knockin mice (hybrid 129/C57BL6 background), with mutations in presenilin 1 (PS1M146V), and a protease forming one of the domains of the β-secretase protein.

An initial sample of thirty-nine 14-months-old 3xTg-AD animals (22 males and 17 females) from the Spanish colony of homozygous 3xTg-AD mice, established at the Autonomous University of Barcelona, were included in the study and assigned to the sedentary (SED: 11 males and 8 females) or voluntary wheel-running (VWR: 11 males and 9 females) group in a counterbalanced manner after considering the number of visited corners in the corner test at 60 s (CTc60) in the baseline assessment.

All animals were kept in 12 h light–dark cycles starting at 8 a.m., with vivarium temperature at 22 ± 2 °C and free access to food and water.

During the month of intervention, the mortality rate was monitored daily. The data of animals completing the 30 days of intervention were gathered and analyzed with the following animals per group composition: thirty-four animals (17 males and 17 females) allocated in the sedentary (SED: 10 males and 8 females) and voluntary wheel running (VWR: 7 males and 9 females) groups.

4.2. Behavioral Assessment

4.2.1. Corner Test (CT)

Neophobia response was evaluated in the CT by direct observation of the animal's behavior in a new home cage for 1 min. The test started with the mouse in the center of the cage; then, the horizontal (number of corners at 30 s and 60 s and number of corners until the first rearing) and vertical (number of rearings at 30 and 60 s) activity were recorded. The ratio of these variables was also calculated (ratio corners/rearings at 30 s and 60 s).

4.2.2. Open Field (OF) Test

The exploratory activity was evaluated in an OF (metalwork, beige, 44 × 38 × 10 cm height) for 5 min. Animals were placed in the central area, and vertical (number and latency of rearings and groomings) and horizontal (latencies until the first movement and

reaching the periphery) behavior was registered. In addition, the total distance was recorded using the VideoTrack analysis system (ViewPoint Behavior Technology, Lyon, France). Finally, the number of defecation boli and urination episodes were also noted.

4.2.3. T-Maze Spontaneous Alternation (TMSA)

The spontaneous alternation task paradigm in the T-shaped maze (two short arms of 30 × 10 cm2 and a single long arm of 50 × 10 cm^2) was used to evaluate coping with stress strategies, risk assessment, and working memory. Thus, animals started the test facing the end wall of the long arm and were free to explore for a maximum of 5 min. Latencies of horizontal activity involved the time until the first move and turn, reaching the intersection of the three arms, crossing the intersection with their 4 paws, and exploring the two short arms (test completion criteria). Latencies of vertical activity comprised the number of rearings and groomings. Defecation and urination were considered too.

4.2.4. Sensorimotor Assessment

The muscle strength of the animals was evaluated in the muscular strength (grip 5 s) and muscular endurance (grip 60 s) paradigms of the grip test. Here, two trials (5 s and 60 s) were carried out, and the duration of grasping a bar using their forepaws while suspended was registered. Next, response to gravity was assessed in a single trial in the 90° Geotaxis test. Thus, animals were placed in a grid (10 cm × 12 cm) facing downwards and the latency until reaching a vertical position (facing upwards) was recorded.

4.2.5. Rotarod

The constant, accelerated, and rocking protocols of rotarod (Ugo Basile, mouse rotarod NG) were utilized. The apparatus consists of five rods (3 cm diameter) located at 16 cm height, and six 25 cm dividers forming five lanes, where animals are placed with their back to the experimenter. Steel "Trip boxes" are placed below each rod to receive mice after falling off.

Firstly, in the constant mode, mice run over the rods at a continuous 10 rpm and the number of trials needed to reach 60 s without falling was reported. A maximum of 10 trials were performed.

Then, the accelerated mode was configured to an acceleration of 0–48 rpm for six trials with a maximum of 6 min each (resting time between trials: 1 min). A trial was finished when the animal fell from the rod.

Finally, in the single rocking mode, coordination was assessed by changing the direction of rod rotation after reaching 20 rpm.

4.3. Intervention Protocol

Mice in the VWR group were housed in groups of 2–3 in cages equipped with an RW (36 × 20 × 14 cm, activity wheel cage system for mice, Techniplast, Buguggiate, Italy). Those in the SED group were in similar cages without RW.

VWR 3xTg-AD mice were free to perform voluntary PA for 30 continuous days. The system allowed assessment of circadian motor activity by recording revolutions on the wheel, which were registered at 8 h (Nocturnal activity) and 20 h (Diurnal activity).

4.4. Statistical Analysis

Most behavioral data were analyzed using mixed models analyses of variance (ANOVAs), with sex and treatment as between factors, and days as within factor. Pairwise t-test comparisons were used as a post hoc method.

In the circadian behavioral analysis of VWR, a linear mixed-effect model was used to calculate the association of VWR activity in two different models. In Model 1, the "fixed effect" comprised the interaction between a dependent variable (normalized VWR activity) and independent variables days (30 days of follow-up), sex (male and female) and

periods (diurnal and nocturnal). In Model 2, normalized VWR activity was adjusted by the previous performance of the animals in the CT. Thus, animals were classified as presenting high (below the 33rd percentile in the CTc60) or low (above the 33rd percentile in CTc60) novelty-induced behavioral inhibition (NIBI) [30].

Results were expressed as the mean ± standard error of the mean (SEM). All the analyses were made using the open-source programming language R software, version 4.1.3.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Departament de Medi Ambient i Habitatge, Generalitat de Catalunya (CEEAH 3588/DMAH 9452) on the 8 March 2019.

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4.3 Study 3

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Digging Signatures in 13-Month-Old 3xTg-AD Mice for Alzheimer's Disease and Its Disruption by Isolation Despite Social Life Since They Were Born

Lydia Gimenez-Llort [1,2*] *and Daniel Alveal-Mellado* [1,2]

[1] *Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain,* [2] *Department of Psychiatry and Forensic Medicine, School of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain*

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***Correspondence:**
Lydia Gimenez-Llort
lidia.gimenez@uab.cat

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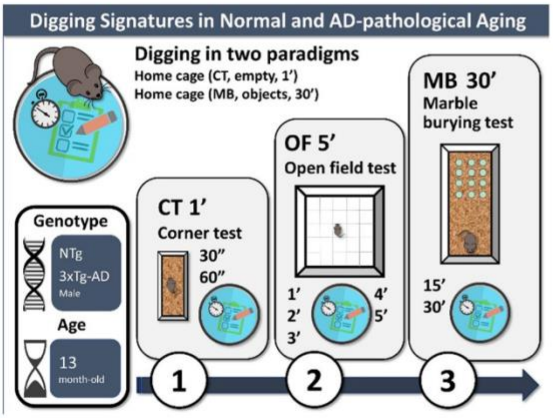
The severity of this pandemic's scenarios will leave significant psychological traces in low resistant and resilient individuals. Increased incidence of depression, anxiety, obsessive-compulsive disorder (OCD), and post-traumatic stress disorder has already been reported. The loss of human lives and the implementation of physical distance measures in the pandemic and post-COVID scenarios may have a greater impact on the elderly, mostly in those with dementia, as OCD and other neuropsychiatric symptoms (NPS) are quite prevalent in this population. Modeling NPS in animals relies in neuroethological perspectives since the response to new situations and traumatic events, critical for survival and adaptation to the environment, is strongly preserved in the phylogeny. In the laboratory, mice dig vigorously in deep bedding to bury food pellets or small objects they may find. This behavior, initially used to screen anxiolytic activity, was later proposed to model better meaningless repetitive and perseverative behaviors characteristic of OCD or autism spectrum disorders. Other authors found that digging can also be understood as part of the expression of the animals' general activity. In the present brief report, we studied the digging ethograms in 13-month-old non-transgenic and 3xTg-AD mice modeling normal aging and advanced Alzheimer's disease (AD), respectively. This genetic model presents AD-like cognitive dysfunction and NPS-like phenotype, with high mortality rates at this age, mostly in males. This allowed us to observe the digging pattern's disruption in a subgroup of 3xTg-AD mice that survived to their cage mates. Two digging paradigms involving different anxiogenic and contextual situations were used to investigate their behavior. The temporal course and intensity of digging were found to increase in those 3xTg-AD mice that had lost their "room partners" despite having lived in social structures since they were born. However, when tested under neophobia conditions, this behavior's incidence was low (delayed), and the temporal pattern was disrupted, suggesting worsening of this NPS-like profile. The outcomes showed that this combined behavioral paradigm unveiled distinct features

of digging signatures that can be useful to study these perseverative behaviors and their interplay with anxiety states already present in the AD scenario and their worsening by naturalistic/forced isolation.

Keywords: OCD, 3xTg-AD, BPSD, Translational neuroscience, Neurospsychiatric symptoms, Loneliness, isolation, COVID-19



GRAPHICAL ABSTRACT | Digging signatures in normal and AD-pathological aging. Experimental design: 3-days battery of tests to assess digging in two home cage paradigms (CT, corner test, 1 min; MB, marble burying test with 12 marbles), also including an open-field test to control for exploration, anxiety, and emotionality. In the present work, 13-month-old NTg (normal aging) and 3xTg-AD mice (advanced stages of disease) were assessed.

INTRODUCTION

On the day that the COVID-19 pandemic took one million people's life, there is no doubt that the severity of the COVID-19 pandemic will leave important psychological traces in low resistant and/or resilient individuals (Farhan and Llopis, 2020). The first clinical reports already reported increased incidence of sleep disorders, depression, anxiety, as well as obsessive-compulsive disorder (OCD), and post-traumatic stress disorder (Banerjee, 2020; Rivera and Carballea, 2020). Since then, the number of reports on OCD, a complex disorder due to its diagnostic classification and clinical heterogeneity (Ruscio et al., 2010), has fast increased (Kumar and Somani, 2020; Shafran et al., 2020; Sulaimani and Bagadood, 2020). The American Psychiatry Association[1] defines this mental health disorder as a series of recurring unwanted and distressing thoughts, ideas or sensations (obsessions) and compulsive repetitive behaviors (such as hand washing, cleaning) or mental acts aimed to decrease the resulting distress or according to rules. Pre-pandemic descriptive epidemiology of OCD referred to a prevalence of 3.1% of the general population and the association of this disorder with significant interference of social interactions, daily activities, and reduced quality of life (Fontenelle et al., 2006; Ruscio et al., 2010). In the current situation, the clinical relevance of OCD has increased, and the consequent worsening of daily life (Fontenelle and Miguel, 2020) has urged the elaboration of guides with international consensus for the management of OCD during COVID-19 and post-COVID-19 scenarios (Fineberg et al., 2020).

Among older people, the impact of COVID-19 is devastating (Van Loon et al., 2020), mainly among the frailest living in long-term care homes (Commas-Herrera et al., 2020). In these nursery care settings, most residents are affected by dementia (Seitz et al., 2010), a population that already has higher mortality rates than aged-matched control populations, with males showing deranged neuro-immuno-endocrine system and worse survival than females despite not as bad neuropathological status (van Dijk et al., 1991; Mitchell et al., 2010). On the other hand, despite the current social and working roles can make female workers more exposed to COVID-19, the first study on gender differences in COVID-19 found both genders having the same prevalence of the disease, but males being more at risk for worse outcomes and death independently of the age (Jin et al., 2020). Dementia care during COVID-19 is a challenge (Wang et al., 2020). In most home-care centers, the confinement and implementation of physical distance measures, with patients being segregated and relocated in new rooms to protect them from the virus, is resulting in social isolation. It is well-known that social vulnerability, frailty, and mortality in older adults is a worrisome triad (Andrew et al., 2008). Social isolation increases the risk of dementia (Wilson et al., 2007) and enhances not only its hallmark cognitive decline but neuropsychiatric disorders, which are already quite prevalent in these patients (Cummings, 1997; Seitz et al., 2010; Zhao et al., 2016). The management of their neuropsychiatric symptoms (NPS) is considered a major issue for the patients' and caregivers' quality of life (Kamiya et al., 2014).

At the translational level, modeling NPS, also known as behavioral and psychological symptoms associated with dementia (BPSD), in rodents relies in neuroethological approaches since the response to different kind of situations and traumatic events, critical for survival and adaptation to the environment, are strongly preserved in the phylogeny (Giménez-Llort et al., 2007). In the wild, burying was first described as a defensive response in rats and considered as reflecting the anxiety state of animals (Pinel and Treit, 1978). In the laboratory, mice dig vigorously in deep bedding to bury food pellets or small objects such as marbles (Gyertyan, 1995). Thus, digging and marble burying have been proposed as simple methods for *in vivo* identification of biological impacts in mice (Broekkamp et al., 1986; Deacon, 2006). This behavior, initially used to screen anxiolytic activity, was later proposed

[1]APA Obsessive-compulsive disorder https://www.psychiatry.org/patients-families/ocd/what-is-obsessive-compulsive-disorder.

to model meaningless repetitive and perseverative behaviors as compulsive-like characteristics of OCD or autism spectrum disorders in rodents (de Brouwer et al., 2020; Mahmood et al., 2020). Also, the animal's general activity can be a confounding factor and, conversely, digging can also be understood as a measure of general activity rather than a measure of repetitive or anxiety-related behavior (Greene-Schloesser et al., 2011; Mitra et al., 2017; de Brouwer et al., 2019; Dixit et al., 2020). The controversy around the digging behavior and what the paradigm might measure is open.

Spontaneous (non-induced) compulsive-like mice based on bidirectional selection for excessive, repetitive, and perseverant digging behaviors are considered mouse models of OCD exhibiting good face, predictive, and construct validity (Greene-Schloesser et al., 2011; Mitra et al., 2017). In our precedent studies with the 3xTg-AD mice, a genetic model of Alzheimer's disease (Oddo et al., 2003) that presents AD-cognitive dysfunction but also a conspicuous BPSD-like phenotype (Giménez-Llort et al., 2006, 2007), we showed increased marble-burying in middle-aged (12-month-old) male 3xTg-AD mice as compared to age-matched non-transgenic (NTg) mice with normal aging (Torres-Lista et al., 2015). Their marble-burying response was related to neophobia and shown to be modulated by repeated handling, reversed by chronic treatment with neuroleptic risperidone (Torres-Lista et al., 2015), and modified by chronic caffeine (Baeta-Corral et al., 2018). In a social paradigm, elicitation of digging behavior was similar among males independently of their genotype but early expressed in female 3xTg-AD mice (Torres-Lista and Giménez-Llort, 2019). In our most recent work, in male 3xTg-AD mice under a long-term isolation (Muntsant and Giménez-Llort, 2020a) increased gross and fine motor activity was recorded, with enhanced nest-building. This is also a species-specific natural behavior that, when disrupted, some authors consider a homologous to hoarding in humans with OCD (Warneke, 1993) and modeling compulsive-like behavior in mice (Greene-Schloesser et al., 2011). Recently, during housing routines, we observed that the spontaneous digging behavior elicited when an animal is transferred to a new home cage with clean beddings was disrupted in a subgroup of old male 3xTg-AD mice that had recently lost their cage-mates. This observation was restricted to males and AD-genotype since the increased mortality rates of male 3xTg-AD mice (Giménez-Llort et al., 2008) often lead to some males living alone for such natural reasons.

Given the above studies in 3xTg-AD mice and the current clinical reports of increased incidence of OCD and anxiety disorders (Banerjee, 2020; Rivera and Carballea, 2020) due to strict physical distance measures, a brief translational study was designed. The work aimed to confirm the observations of derangement of digging patterns in the subgroup of male 3xTg-AD mice that, after 10 months of social life, lost their partners and lived alone during the last 2–3 months. We aimed to define the nuances of their digging patterns and their expression under different anxiogenic conditions, as they are also serving as a behavioral tool for current investigations. Therefore, digging patterns were assessed in 13-months-old NTg and the two subgroups of 3xTg-AD male mice, an age that corresponds to normal aging and advanced stages of the disease, respectively (Belfiore et al., 2019). The expression of digging behavior was recorded in experimental scenarios where a home-cage with beddings is used, such as the corner test (change to a new and clean cage) and the marble tests, also involving different anxiogenic and contextual situations (home-cage with beddings, and without/with objects, respectively). The open-field test was included to monitor the animal's general activity in a classical anxiogenic environment without beddings.

MATERIALS AND METHODS

Animals

A total number of 43 13-month-old male homozygous 3xTg-AD ($n = 28$) and non-transgenic (NTg, $n = 15$) mice on a C57BL/6J background (after embryonic transfer and backcrossing at least 10 generations) established in the Universitat Autònoma de Barcelona (Baeta-Corral and Giménez-Llort, 2014) were used in this study. The 3xTg-AD mice harboring transgenes were genetically engineered at the University of California Irvine, as previously described (Oddo et al., 2003). Animals were maintained in groups of 3–4 mice per cage (Macrolon, 35 × 35 × 25 cm) filled with 5 cm of clean wood cuttings (Ecopure, Chips6, DateSand, UK; Uniform cross-cut wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 × 20 cm, White). In the current work, seven of the 28 3xTg-AD mice had lost their cage-mates and lived alone in their cage for 2–3 months. In all the cases, the standard home cages covered with a metallic grid allow the perception of olfactory and auditory stimuli from the rest of the colony. All the animals were maintained under standard laboratory conditions of food and water ad lib, 22 ± 2°C, 12 h light: dark cycle with lights on at 8:00 am, and relative humidity 50–60%.

Behavioral Assessment

Digging and other behaviors were measured in the corner test, open-field test, and marble burying test under dim white light (20 lx) during their light phase of the light: dark cycle (from 10 am to 1 pm) (see **Graphical abstract**).

Behavioral assessments were performed by direct observation by two independent observers blind to the genotype, in a counterbalanced manner and with the support of a computerized video system. All procedures followed the Spanish legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" and the EU Directive (2010/63/UE) on this subject. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used (Kilkenny et al., 2010).

Day 1. Digging in a new home cage only with clean wood cuttings was assessed in a corner test (CT). The animal was introduced in the cage's center, and the neophobia response was observed for 1 min. The number of visited corners before the elicitation of the first rearing (CTcbR) and the latency of rearing (CTlatR) were noted. Then, the number of visited corners (CTc) and rearings (CTr) were recorded at 30 and 60 s. The ratio of these variables (Ratio CTc/r) was calculated. The number of digging episodes (>2 s, in order to avoid false-positive

observations) elicited during 30 and 60 s were noted. Prevalence of digging was recorded at the end of the test.

Day 3. The animals were placed in the center of an open-field (metalwork, beige, 44 × 38 × 10 cm height), and their general and vertical activity (R, rearings) was recorded for 5 min. The time and distance covered in the center and periphery during the test were recorded using the VideoTrack analysis system (ViewPoint Behavior Technology, Lyon, France) (Giménez-Llort et al., 1995). Walking speed was calculated as the distance covered per unit of time. In those animals where distance and time in the periphery were zero, zero was given to the walking speed. The number of defecations boli (Def) and presence/absence of urination (Ur) were also noted. Prevalence of rearing, defecation boli, and urination were recorded at the end of the test.

Day 12. One week later, animals were assessed in the marble-burying test (MB). The animal was introduced facing the wall of a home-cage. Twelve glass marbles (1.5 cm diameter, 8 g) were evenly spaced (four rows in the cage's upper area) on a 5 cm thick layer of clean wood cuttings. The mice were left in the cage with marbles for a 30 min period. The evaluation was done twice to record the temporal course at 15 and 30 min. (Santana-Santana and Giménez-Llort, Submitted). Digging in the new home cage was measured by the level of marbles' burying: "Buried" (B, 100% buried), those left "Intact" (I), and the remaining subset considered as those "Moved or partially buried" (M).

Statistics

Results are expressed as mean ± SEM. SPSS 20.0 software was used. One way ANOVA followed by *post-hoc* Duncan's test was used to compare the three independent groups. Differences within the same group were analyzed with a Paired *t*-test. For categorical variables, Fisher's exact test with 2 × 2 was used. In all the tests, $p < 0.05$ was considered statistically significant.

RESULTS

As a first step, we investigated the genotype differences, those due to the three human transgenes. **Table 1** summarizes the results of the 30 variables studied and point at vertical exploratory activity as the most sensitive variable to show genotype differences, with a severe drop of rearing behavior in the periphery. Also, a reduced number of moved marbles in favor of an increase in those buried lead to genotype differences.

To verify our hypothesis that the 3xTg-AD mice that recently lost their home-cage partners exhibited different digging patterns, the data of 3xTg-AD mice was depicted in two subgroups, according to their most recent housing conditions. The results clearly showed that while NTg ($n = 15$) and 3xTg-AD mice ($n = 21$) were quite alike in most behaviors, genotype differences emerged in the subset of 3xTg-AD mice ($n = 7$) that recently lost their partners. The following paragraphs, illustrated in **Figures 1–3**, show the nuances in the digging patterns elicited in two distinct home-cage settings with beddings.

As illustrated in **Figure 1A**, in all the groups, the ethogram of neophobia in the corner test started with an inspection of the cage ($n = 43$, 4.18 ± 0.39 visited corners) followed by a first rearing ($n = 43$, latency: 12.30 ± 1.02 s). At 30 s, NTg and 3xTg-AD mice permanently living in social conditions showed similar values in the total number of visited corners and rearings. However, the behavior of isolated 3xTg-AD mice was significantly reduced to 62% of corners and 48% of rearings, reaching statistical significance the drop in vertical activity [$F(2, 40) = 3.765$, $p = 0.0318$; *post-hoc*, $p < 0.05$ vs. each of the other two groups]. The ratio between horizontal and vertical activity was maintained equal in all the groups ($n = 43$, 2.87 ± 0.24). At the end of the test (60 s), the drop of vertical activity [$F(2, 40) = 8.034$, $p = 0.0017$] was now observable in both subgroups of 3xTg-AD compared to NTg mice. However, it was more intense in the isolated 3xTg-AD mice (*post-hoc*, $p < 0.05$, 3xTg-AD vs. NTg; but $p < 0.001$, isolated 3xTg-AD vs. NTg) that also differed from those permanently living in social groups ($p < 0.05$, isolated 3xTg-AD vs. 3xTg-AD). The horizontal activity also showed a group effect [$F(2, 40) = 4.565$, $p = 0.0164$], but it was due to a drop of total visited corners in isolated 3xTg-AD mice ($p < 0.01$, isolated 3xTg-AD vs. the other two groups). The ratio CTc/r of NTg dropped to 64% while that of both subgroups of 3xTg-AD mice was maintained ($n = 28$, 2.63 ± 0.26), albeit these differences did not reach statistical significance.

Digging behavior at 30 and 60 s in the corner test is illustrated in **Figure 1B**. Prevalence of digging behavior in the new home cage at 30 s was scarce, as it was only shown by 1/15 (6.7%) NTg mice, 4/21(19%) 3xTg-AD mice, and 1/7 (14%) in the subgroup of isolated 3xTg-AD mice. Thereafter, the behavior increased in NTg (prevalence 7/15, 47%; incidence 6/15 40%) and 3xTg-AD (prevalence 12/21, 57%; incidence 8/21, 33%) mice. In both groups, the ethogram measured as the number of digging episodes showed scarce emergence during the first 30 s and increased at 60 s (accumulated counts). This temporal pattern was statistically significant in both groups (NTg mice, D60 vs. D30, $p < 0.001$; 3xTg-AD mice, D60 vs. D30, $p < 0.01$). In contrast, no new digging behavior episodes were observable among isolated 3xTg-AD mice.

Figures 2A–E, illustrate the different variables assessed in the open-field test. Except for the distance covered in the central area of the field where all the groups covered a similarly short distance [$F(2, 212) = 1.894$, $p = 0.153$], all the other variables showed that the pattern of NTg mice was distinct from that of the two subgroups of 3xTg-AD mice [all *F*'s$(2, 212) > 9.327$, $p < 0.000131$]. In particular, thigmotaxis was different among groups as shown by preference (in time spent, $p = 1.16$ e-21, and distance covered $p = 1.89$ e-19) for the periphery shown by NTg mice, but lost in both groups of 3xTg-AD mice for distance and inverted in terms of time (3xTg-AD mice, $p = 0.00628$; isolated 3xTg-AD mice, $p = 0.00214$) (**Figures 2A,B**). This was due to increased freezing behaviors in the center. The total distance covered by both subgroups of 3xTg-AD mice in the periphery was as scarce as in the center and drastically lower than in NTg mice. The distance and time spent allowed to calculate the animals' averaged walking speed (**Figure 2C**). The walking speed was different among groups since both 3xTg-AD groups walked slower than NTg mice and independently of the open-field area. The paired analysis indicated that NTg mice walked faster in the center than in the periphery ($p < 0.001$). This was confirmed by their sustained rearing behavior levels

TABLE 1 | Genotype differences between 13-month-old male 3xTg-AD mice and NTg mice in the corner, open-field, and marble tests.

	NTg mice $n = 15$ (Mean ± SEM)	3xTg-AD mice $n = 28$ (Mean ± SEM)	Genotype differences
1.Corner test			
CTcbR (*n*)	4.64 ± 0.58	4.00 ± 0.53	*n.s.*
CTlatR (*n*)	13.40 ± 1.90	11.64 ± 1.23	*n.s.*
CTc30 (*n*)	9.93 ± 0.71	9.36 ± 0.89	*n.s.*
CTr30 (*n*)	4.33 ± 0.42	3.64 ± 0.44	*n.s.*
Ratio CTc/r30 (index)	2.74 ± 0.55	2.95 ± 0.24	*n.s.*
CTc60 (*n*)	15.13 ± 0.89	13.14 ± 1.18	*n.s.*
CTr60 (*n*)	9.60 ± 0.86	5.93 ± 0.66	**
Ratio CTc/r60 (index)	1.77 ± 0.19	2.66 ± 0.26	**
Digging 30" (*n* of episodes)	0.07 ± 0.07	0.25 ± 0.11	*n.s.*
Digging 60" (*n* of episodes)	0.80 ± 0.24	0.89 ± 0.26	*n.s.*
2. Open field test			
Distance covered in the center (cm)	86.49 ± 10.59	72.07 ± 6.09	*n.s.*
Distance covered in the periphery (cm)	266.57 ± 10.99	78.13 ± 9.17	***
Time spent in the center (s)	15.43 ± 2.31	27.98 ± 1.84	***
Time spent in the periphery (s)	49.84 ± 1.14	16.70 ± 1.76	***
Walking speed in the center (cm/s)	8.81 ± 0.71	3.07 ± 0.23	***
Walking speed in the periphery (cm/s)	5.50 ± 0.23	3.02 ± 0.42	***
R1 (*n*)	3.20 ± 0.83	0.04 ± 0.01	***
R2 (*n*)	4.20 ± 1.08	0.43 ± 0.08	**
R3 (*n*)	4.07 ± 1.05	0.46 ± 0.09	***
R4 (*n*)	5.00 ± 1.29	0.93 ± 0.18	***
R5 (*n*)	3.80 ± 0.98	1.00 ± 0.19	***
Total rearings (*n*)	20.27 ± 5.23	2.86 ± 0.54	***
Defecation (*n* of boli)	0.33 ± 0.09	4.11 ± 0.78	***
Urination (presence/absence)	0.00	0.74 ± 0.14	***
3. Marble test			
I15 (*n*)	7.20 ± 0.87	6.64 ± 0.73	*n.s.*
M15 (*n*)	1.33 ± 0.43	1.07 ± 0.37	*n.s.*
B15 (*n*)	3.47 ± 0.83	4.21 ± 0.67	*n.s.*
I30 (*n*)	2.80 ± 0.63	3.64 ± 0.71	*n.s.*
M30 (*n*)	2.20 ± 0.61	0.75 ± 0.23	*
B30 (*n*)	7.00 ± 0.74	7.64 ± 0.75	*n.s.*

Student's *t*-test, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. $p > 0.05$ vs. NTg mice.

Corner test: CTcbR, visited corners before the first rearing; CTlatR, latency of rearings in seconds; CTcn, visited corners at n s; CTrn, rearings at n s; Ratio CTc/r n, ratio visited corners divided by rearings at n s of the test; Digging in the corner test at 30 (D30) and 60 (D60) s. Open-field test: Rn, rearings at minute n; Marble test: marbles left intact (I15, I30), half-buried or moved (M15, M30) or completely buried (B15, B30) at 15 and 30 min, respectively.

(**Figure 2D**) through the 5 min of the test. Only 12/21 (57%) group-housed 3xTg-AD and 5/7 (71%) isolated 3xTg-AD mice performed rearings, and their number was small. Statistically significant differences in the prevalence were shown between 3xTg-AD vs. NTg mice ($p < 0.05$).

Emotionality, as measured by defecation and urination, was found increased in both subgroups of 3xTg-AD mice as compared to NTg mice [both F's(2, 40) > 16.4, $p <$ 6.78 e-06; *post-hoc* $p < 0.001$ vs. NTg]. Also, 19/21 (90%) grouped and 7/7 (100%) isolated 3xTg-AD defecated during the test, while only 3/15 (20%) NTg mice did so (both, $p < 0.001$ vs. to NTg mice). The presence of urination was only shown in 3xTg-AD mice ($n = 24$, 0.72 ± 0.09, absent in NTg, $p < 0.001$), with a prevalence of 17/21 (81%) in group-housed 3xTg-AD mice and 6/7 (86%) in those isolated (both, $p < 0.001$ vs. NTg mice).

As illustrated in **Figure 3**, digging in the marble test was different among groups as measured by the number of marbles left intact at 15 min [$F(2, 40) = 4.283$ $p = 0.0206$]. NTg and 3xTg-AD mice showed similar patterns. However, digging behavior in the subgroup of isolated 3xTg-AD mice was increased as measured by a reduced number of marbles left intact (*post-hoc* isolated 3xTg-AD vs. NTg $p = 0.0202$; vs. 3xTg-AD $p = 0.0086$).

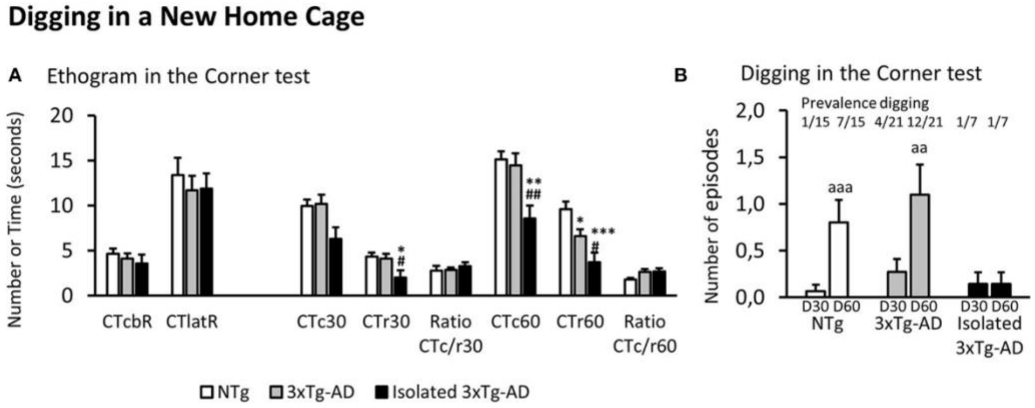


FIGURE 1 | Ethogram and Digging in a new home cage (Corner test). Results are expressed as mean ± SEM. **(A)** Ethogram in the corner test: CTcbR, visited corners before the first rearing; CTlatR, latency of rearings in seconds; CTc*n*, visited corners at *n* s; CTr*n*, rearings at *n* s; Ratio CTc/r n, ratio visited corners divided by rearings at *n* s of the test. **(B)** Digging in the corner test at 30 (D30) and 60 (D60) s. Statistics: One-way ANOVA followed by *post-hoc* Duncan's test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. the NTg group; #$p < 0.05$ and ##$p < 0.01$ vs. the 3xTg-AD mice. Paired *t*-test, aa $p < 0.01$ and aaa $p < 0.001$ vs. the same group at 30 s.

At the end of the test, differences in the number of marbles half-buried o moved were found among groups [$F(2, 40) = 3.551$, $p = 0.0381$, *post-hoc,* 3xTg-AD mice vs. NTg $p = 0.0211$]. *Post-hoc* analysis also detected differences between isolated 3xTg-AD mice and 3xTg-AD in the number of marbles left intact at the end of the test [$F(2, 40) = 3.180$, $p = 0.0523$, but *post-hoc,* isolated 3xTg-AD mice vs. 3xTg-AD $p = 0.0284$] and buried [$F(2, 40) = 2.938$, $p = 0.0645$, but *post-hoc,* isolated 3xTg-AD mice vs. NTg $p = 0.0429$ and vs. 3xTg-AD $p = 0.0305$].

DISCUSSION

Species-typical behaviors can provide neuroethological tools to follow up the animal's well-being, age/aging and disease processes, and modulation by treatments and environmental factors (Oddo et al., 2003; Greene-Schloesser et al., 2011). Digging can be spontaneously elicited when animals are transferred into a new home-cage with clean beddings during housing routines but can also be reproduced in experimental scenarios such as the corner and the marble tests. In the present brief report, two digging paradigms involving different anxiogenic and contextual situations were used to investigate the digging patterns in the 13-month-old males with normal and genetically induced AD-pathological aging. We confirmed the disruptive effects of short isolation (2–3 months) observed in a subgroup of 3xTg-AD that recently lost their cage-mates after 10 months living in a standard social environment. These 3xTg-AD mice that survived to their partners indicated that their current social housing conditions modified the patterns, achieving divergence with NTg mice but also 3xTg-AD mice still living in social groups.

Elicitation of digging behavior can be more or less conspicuous, depending on many intrinsic and extrinsic factors, and it is usually under-recorded. Slight methodological adaptations of the corner test and the marble test protocols were made to enhance the chances to record digging behavior. The changes were related to the windows and frames of observation. The corner test, performed at 30 s, was prolonged until 60 s. In the marble test, measures were taken at 15 min, and the end of the test to record the temporal progression (Santana-Santana and Giménez-Llort, Submitted).

The results show the consistency of some behavioral patterns, independently of the genotype or social condition. However, the short battery of tests also allowed to confirm the emergence of disruptions in digging in the 3xTg-AD mice and, among them, in those that lost their cage-mates. This "natural isolation" subgroup performed worse than expected. As described in the present work, for the first time, the analysis of the segregated data unveiled distinct modulation of digging signatures in terms of prevalence, intensity, and patterns, depending on the context. When tested for neophobia (immediate fear of a new place), the prevalence was lower, and the digging pattern was mostly absent. However, the activity patterns, thigmotaxis, and emotionality of both 3xTg-AD mice subgroups did not differ in the open-field test. In this classical test for anxiety, the phenotype of all the 3xTg-AD mice was different from that shown by NTg mice with normal aging. According to the action program by Lát (1973), describing the immediate fearful response and the subsequent actions developed by the animal confronted to a new environment, the open-field elicited a severe anxiety/fear-like pattern in both subgroups of 3xTg-AD mice. It was characterized by drastically reduced general activity, as measured in terms of time, distance covered, and rearing in the walls. Low performance, with long-lasting periods of freezing in the center of the field, resulted in an inverted pattern for thigmotaxis. Walking speed patterns were also found disrupted. Emotionality, as measured by defecation and urination, was increased.

Spontaneous animal models with face validity for OCD, such as the "BIG mice" (Greene-Schloesser et al., 2011; Mitra et al., 2017) are based on their divergent nesting and marble-burying

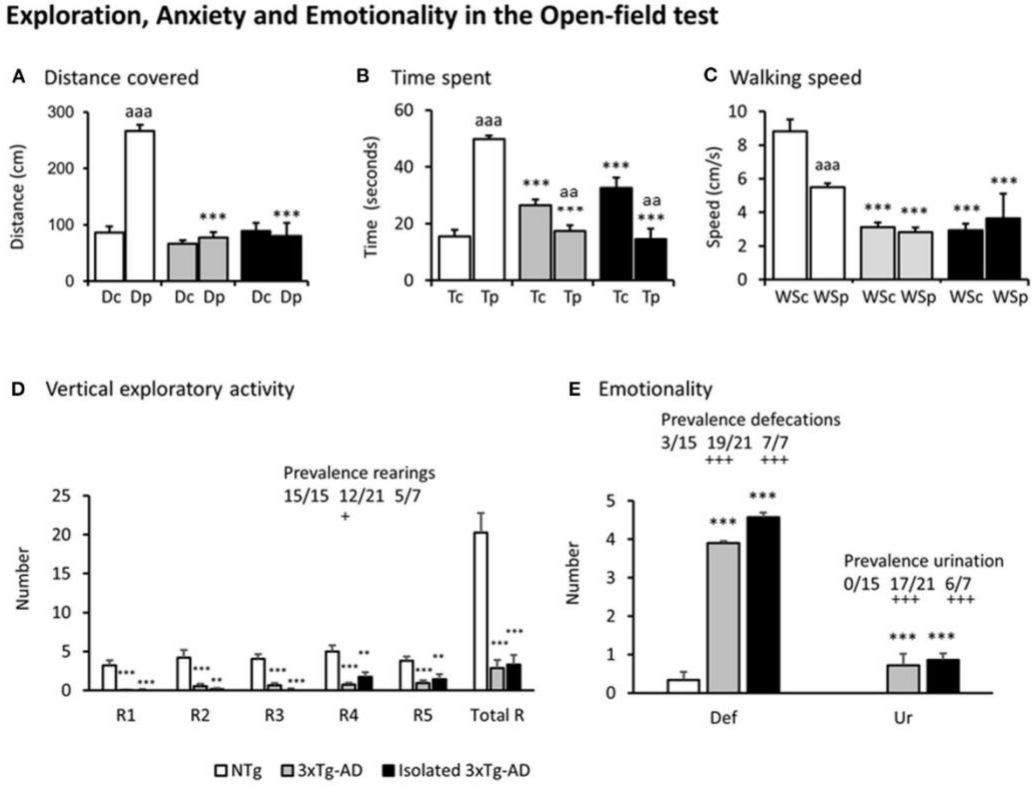


FIGURE 2 | Exploration, Anxiety, and Emotionality in the Open-field test. Results are expressed as mean ± SEM. **(A)** Distance covered, **(B)** Time spent, **(C)** Walking speed, **(D)** Vertical exploratory activity, **(E)** Emotionality. Abbreviations: Dc, distance in the center; Dp, distance in the periphery; Tc, time in the center; Tp, time in the periphery; WSc, Walking speed in the center; WSp, walking speed in the periphery; Rn, rearings at minute n; Total R, total number of rearings; Def, defecation boli; Ur, urination. Statistics: One-way ANOVA followed by *post-hoc* Duncan's test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the NTg mice; $\#p < 0.05$. vs. the 3xTg-AD mice, Paired *t*-test, aa $p < 0.01$, aaa $p < 0.001$ vs. the same variable in the center of the open-field test. Fisher's exact test, $+p < 0.05$ and $+++p < 0.001$ vs. the NTg mice.

behaviors, increased compulsive-like digging, with respect to "SMALL mice" counterparts that exhibit risk assessment and inhibition of movement in the open-field and the elevated plus-maze. These animals also diverge in the thigmotaxis, and the rearing frequency in the periphery is three-fold higher in the "BIG mice" compared to the "SMALL." In the present work in 3xTg-AD mice, the temporal course and intensity of digging behavior in the marble test were found increased in the isolated mice. Here, the marble-test experimental setup was similar to the one used in the corner test except for marble pieces and the starting position facing the wall. As compared to other laboratory protocols, the number of marble pieces was reduced to 12, and they were allocated in one half of the test cage to reduce the chance that differences in buried pieces be due to differences in locomotor activity (Nicolas et al., 2006; Thomas et al., 2009). Still, as shown in the open-field test, the NTg mice were the active animals, similar to "BIG mice," and both 3xTg-AD mice subgroups exhibited the same anxiety/fear-like behavior. Therefore, the increased number of pieces buried in isolated 3xTg-AD mice could not result from locomotor activity since this was drastically reduced, whereas it was normal in NTg mice. Both subgroups of 3xTg-AD mice showed similar anxiety levels but differed in the corner and marble tests' digging signatures. In this regard, some pharmacological studies have noted that while the dose-dependent reduction in marble-burying could be due to several drugs, this effect was not always related to their locomotor effects (Jimenez-Gomez et al., 2011). Due to this controversy, the open-field or other anxiety tests that also monitor the general activity are a must for interpretations and discard confounding factors.

The genetic background is determinant for the expression of behaviors (Albelda and Joel, 2012). Also, animal models based on overexpression of human transgenes from familial forms of the disease are not exempted from limitations and shortcomings. The main translational gap, but also a challenge, is the difficulty to fully recapitulate the complexity of neurological and psychiatric patterns observed at the clinical level in the human patient (Giménez-Llort et al., 2006, 2007; Kosel et al.,

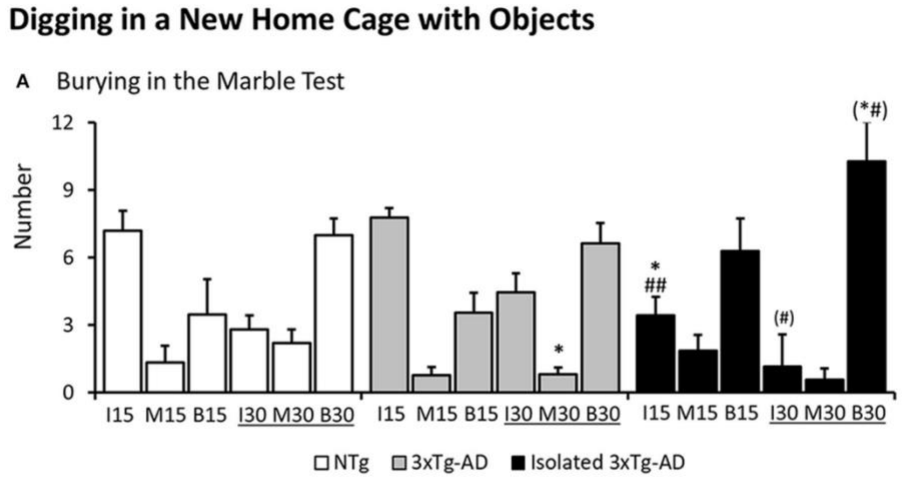


FIGURE 3 | Digging in a new home cage with small objects (Marble test). Results are expressed as mean ± SEM **(A)** At 15 and 30 min of the test, the number of marbles left intact (I15, I30), half-buried or moved (M15, M30) or completely buried (B15, B30) is indicated. Statistics: One-way ANOVA followed by *post-hoc* Duncan's test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. the NTg mice; $\#p < 0.05$. vs. the 3xTg-AD mice.

2020). Other critical issues refer to genetic engineering since transgene insertions can have phenotypic consequences and could confound some experiments (Goodwin et al., 2019). The present results used the new Spanish colonies of 3xTg-AD mice established at Universitat Autònoma de Barcelona after embryonic transfer on a congenic C57BL/6J background from progenitors with a hybrid C57BL/6J x 129 Sv genetic background kindly provided by Prof. Frank M LaFerla in 2011. This animal model, also provided by The Jackson Lab as Stock #004807, is adapting this genetic strategy, with animals on congenic C57BL/6J or 129S4 expected to be available beginning last January 2019[2] Concerning our work, we can compare the present results with those already published in our laboratory using the first colonies of NTg and 3xTg-AD mice in the C57BL/6J x 129 Sv genetic background. Those results showed that, at this old age, 3xTg-AD mice's performance was already disrupted in the marble test (Torres-Lista et al., 2015). Their pattern was similar to that exhibited here by 3xTg-AD mice that lost their partners. Thus, it seems that in the present work, the C57BL/6J genetic background induces a delay in the expression of the disruption, but the pattern emerges in the animals that have confronted social isolation in the last 2 months. This would agree with the extended window of observation in the corner test, allowing to detect of reduced rearing in both subgroups of 3xTg-AD mice, with different degrees of statistical significance (worse in isolated). Furthermore, we recently reported that in the social interaction test (Torres-Lista and Giménez-Llort, 2019), the ethogram of 14-month-old male and female 3xTg-AD mice points at digging as a robust and consistent non-social interaction behavior. In that context, digging emerged on minute three of the test together with self-grooming. In females, the behavior was elicited earlier in the ethogram (on average, at 90 s), in agreement with their increased anxious-like profile and shrinking the action program described by Lát (1973). Thus, although the repetitive and compulsive behaviors that characterize the OCD are defined as "meaningless," they are also considered to decrease the resulting distress.

In other animal models of AD such as the APP/PS1, Tg2576, and 5xFAD mice, several authors had reported a social isolation-induced increase in amyloid pathology (Hsiao et al., 2011; Huang et al., 2011; Peterman et al., 2020). In contrast, in 3xTg-AD mice, limited impact was found when long-term isolation was implemented from postweaning to adulthood (Pietropaolo et al., 2009). However, in our most recent work with these new Spanish colonies of 3xTg-AD mice, and also studying naturally occurring long-term isolation due to loss of partners in an aged scenario, we found that tau pathology of 3xTg-AD mice was enhanced, albeit did not reach the statistical significance, probably due to a celing effect (Muntsant and Giménez-Llort, 2020a). Nevertheless, the hippocampal atrophy asymmetry found in human patients with AD (Wachinger et al., 2016) was modeled for the first time in mice and allowed us to show its worsening after long-term isolation. Further analysis has also found brain differences in the cortical areas (Muntsant and Giménez-Llort, 2020b). In the present work, the effects of a short-isolation did not modify the general motor activity pattern nor the anxiety-like profile of 3xTg-AD mice. However, as we have shown recently (Muntsant and Giménez-Llort, 2020a), a longer isolation regime induced re-structured negative valence system with the emergence of bizarre behaviors and flight copying-with-stress strategies were found. More importantly, with regards of the present work, the long-term period induced a prominent hyperactive pattern in both gross (general motor activity in most tests) and fine-motor functions (increased nesting-behavior)

[2] Alzforum.org (2020) https://www.alzforum.org/research-models/3xtg.

that were correlated to hippocampal tau pathology (Muntsant and Giménez-Llort, 2020a). Activation of cortical and striatal regions during the expression of a naturalistic compulsive-like behavior in the rabbit have been described (Cano-Ramírez and Hoffman, 2018), so these are also our target areas in our current investigations. Excessive nest building has been described as a unique behavioral phenotype in the deer mouse model of obsessive-compulsive disorder (Wolmarans et al., 2016). As mentioned before, psychogenetic bidirectional selection of mice for nesting behavior is used as an animal model for compulsive-like behaviors, and it is also leading to distinct marble-burying (Greene-Schloesser et al., 2011; Mitra et al., 2017).

Thus, despite the current brief report could be considered preliminary, our precedent data describing the consistency of digging in males studied in a social paradigm (Torres-Lista and Giménez-Llort, 2019) and of increased nest-building in animals under a long-term isolation (Muntsant and Giménez-Llort, 2020a) support the consistent observations during housing routines that lead to this work and the present results showing disruptions in the subgroup under isolation. Still, the limitations of the current work must be noted and are mainly due to the naturalistic scenario, since the natural death of home-cage partners at this age only affects to 3xTg-AD mice (Giménez-Llort et al., 2008). This fact also limits the sample size of the animals under a "naturalistic isolation." To solve it, in the ongoing investigations, a forced isolation paradigm is being used for both genotypes and including both sexes. Studying digging signatures in other paradigms and the impact of isolation at different ages/stages of disease is also a matter of current investigations (Marin-Pardo and Gimenez-Llort, 2020).

Among our different reports on non-pharmacological preventive/therapeutic interventions in this mouse model, handling was able to modify marble-burying (Torres-Lista et al., 2015) and the long-lasting effects of early-life interventions (postnatal handling and environmental enrichment) were also observable in other perseverative behaviors (Torres-Lista and Giménez-Llort, 2015). However, in another model, the 5xTg-AD mice, Petermam et al. showed that prolonged isolation stress accelerated the onset of disease-related pathology despite running wheels and environmental enrichment (Peterman et al., 2020). Still, at the clinical level, the beneficial effects of these interventions foreseen in the context of the pandemic are supported by the literature (Davim et al., 2020). Our preliminary results, with regards to the implementation of a stimulation program to improve both psychological and motor functions in a long-term care center, are encouraging (Castillo-Mariqueo et al., 2020), but also indicate that the time frames for intervention seem to be critical and depend on the frailty of aging/AD scenarios (Giménez-Llort, 2010).

In summary, as a translational neuroscience approach, the present brief report in the 3xTg-AD mice can be useful to estimate the impact of a short-isolation regime with regards to the emergence or enhancement of compulsive behaviors in Alzheimer's disease where NPS/BPSD-like symptomatology is already present. The prevalence and incidence, the temporal course, and the intensity of this behavior were found to increase in the 3xTg-AD mice that had lost their "room partner" despite having lived in social structures for more than 10 months, since they were born. When they were tested under neophobia conditions, the incidence of this behavior was smaller (delayed), and the pattern of digging was disrupted, resembling that previously reported for worse stages of the disease. Despite the limitations of a naturalistic isolation (small sample and only affecting one genotype), together with precedent results of long-term isolation enhancing nesting-behavior, we present this combined paradigm unveiling distinct features of digging signatures as it can be useful to study these perseverative behaviors, their complex interplay with anxiety states already present in the AD scenarios and its worsening by naturalistic or forced isolation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by CEEAH Universitat Autònoma de Barcelona and DMAH Generalitat de Catalunya.

AUTHOR CONTRIBUTIONS

LG-L: concept and draft manuscript. Both authors equally contributed to the performance and analysis of the experiments. Both authors revised and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5 GENERAL DISCUSSION

This thesis explored the influence of biological factors (sex, genotype and age) and environmental contexts (task type, test repetition, enrichment, and deprivation) on the expression of BPSD-like symptoms in 3xTg-AD mice at advanced stages of the disease. To enhance translational relevance, the findings were interpreted in light of existing literature on symptomatology in human AD. The work brings together three interconnected studies. The first followed the progression of behavioral changes and bizarre behaviors in the MWM test across two time points under stable housing conditions. The second looked at how novelty-related inhibition affected VWR, reflecting alterations in motivation and engagement similar to those observed in people with AD. The third study examined the behavioral consequences of social isolation, particularly its resemblance to obsessive-compulsive symptoms presented in humans. Overall, the results show that BPSD-like behaviors in this animal model vary depending on the context and do not follow a fixed trajectory across tests. To better capture the patterns observed, statistical techniques that are well-suited to complex designs—but not yet common practice in the field—were employed, underscoring the importance of methodological refinement in this type of research.

5.1 Study 1

Based on the longitudinal design adopted in this study, we found that circling behavior increased significantly in 3xTg-AD mice during the RM stage of the MWM test at 16 months of age compared to their behavior at 12 months. Notably, this age-related increase was not observed in NTg mice, suggesting a distinctive genotypic characteristic. The causes of circling behavior in AD models have been attributed to various factors, including retinal impairments (Chintapaludi et al., 2020), false recognition of the platform (Romberg et al., 2012) , and topographical disorientation resulting from hippocampal and striatal dysfunction (Karunakaran, 2020). Although our study did not include tissue analyses to directly investigate these mechanisms, the comparable performance between transgenic and control animals during the visual CUE and PT stages makes it unlikely that these biological alterations alone account for the increased circling episodes.

Instead, our findings may be better understood through the lens of stress-related behavioral responses. Previous work from our laboratory (Baeta-Corral & Giménez-Llort, 2015; Castillo-Mariqueo & Giménez-Llort, 2020) has suggested that circling may reflect

maladaptive coping strategies under stress. In line with this interpretation, the progressive increase in circling behavior observed in 3xTg-AD mice, but not in their NTg counterparts, may point to an age-dependent decline in the ability to regulate stress. The preserved circling levels in NTg mice across time further reinforce the notion that this behavioral deterioration is specific to the AD model and may reflect a worsening of stress regulation mechanisms.

It remains challenging to definitively establish parallels between circling behavior in the MWM test and specific AD manifestation in humans. Nonetheless, we propose that it may be considered a BPSD-like pattern analogous to stereotyped behaviors in AD patients. This is supported by the observation that circling, a non-search strategy, increased in the absence of the platform, a condition that maximized the animals' stress levels.

Another important aspect of this study was the analysis of multiple swimming strategies using MEMs, a method that allowed for a more comprehensive assessment of both cognitive and non-cognitive performance, most of them with a predominant qualitative nature but small quantitative representation. By capturing a broader range of behavioral patterns, this approach provided novel insights into the animals' decision-making processes across trials, testing days, and aging—insights that traditional methods could not achieve.

The application of MEMs in preclinical research has facilitated the implementation of complex experimental designs and advanced data collection strategies. For instance, Soto et al. (2023) demonstrated a methodology requiring sophisticated statistical analyses, reflecting the growing trend toward more intricate approaches. This progress is particularly evident in AD studies, where longitudinal designs are increasingly used to track symptom progression and evaluate the effects of interventions over time (Ferguson et al., 2013; Matzel et al., 2011). By incorporating repeated measures within individuals, these approaches offer a more detailed understanding of AD pathology and behavioral manifestations.

5.2 Study 2

In this study, we observed that (i) four weeks of VWR reduced anxiety-related behavior in 3xTg-AD mice, as assessed in the OF test, and (ii) altered circadian patterns of VWR in 3xTg-AD males were associated with BPSD-like symptoms, as evaluated in the CT.

The anxiolytic effect of VWR was supported by a reduction in latencies to move and explore the center of the arena in the OF test. Similar effects of exercise on reducing anxiety and depressive symptoms have been widely reported in humans (Carek et al., 2011; Ströhle, 2009); however, findings in preclinical research have been inconsistent, with some studies showing agreement (Duman et al., 2008; J. H. Fox et al., 2008) and others reporting contradictory results (Svensson et al., 2020; Wang et al., 2021). These discrepancies are often attributed to differences in mouse strains and the specific behavioral test batteries employed. Regarding AD models, and the 3xTg-AD model specifically, there appears to be greater consensus on the beneficial effects of exercise on emotional phenotypes (García-Mesa et al., 2011, 2012; J. Rodriguez et al., 2011; Revilla et al., 2014), further supporting our current findings.

The exploratory activity in the OF and EPM tests is commonly used to assess anxiety in rodents (Komada et al., 2008; Prut & Belzung, 2003). These tests measure the active choice to take risks while exploring, where higher latencies are typically interpreted as greater anxiety. In the present work, we analyzed movement latencies in the OF, which we interpret as representing a comparable paradigm. The EPM test was excluded from our experimental protocol due to its highly stressful nature for 3xTg-AD mice, which are typically more hesitant to explore (Muntsant & Giménez-Llort, 2022). Anticipating limited sensitivity in detecting within-group differences using this test, we instead employed the CT. This less stressful environment, resembling the animals' home-cage conditions, provided a more suitable setting to assess exploratory activity within a risk assessment framework.

Using this framework, we identified that animals displaying less exploratory behavior in the CT (indicative of higher anxiety to novelty) were more active during periods expected to correspond to resting in the 24-hour VWR recordings. This observation suggests a link between heightened anxiety-related behaviors and disrupted circadian patterns in 3xTg-AD males, contributing to our understanding of BPSD-like symptoms in this model. To evaluate this association, we utilized LMMs. These tools allowed us to explore the relationship between high and low NIBI animals and their VWR activity slopes across the 30-day intervention, providing a nuanced understanding of these dynamics.

The promising results reported here underscore the importance of adopting statistical techniques commonly used in social sciences to uncover complex interactions in preclinical research. We strongly encourage researchers to integrate such methodologies into their experimental designs to enhance the reliability and depth of behavioral analyses.

5.3 Study 3

The primary finding of this study is a distinct, context-dependent digging phenotype in socially isolated 3xTg-AD male mice. To understand this, one must first consider the ethological purpose of the CT, which evaluates an animal's response to a novel environment (Giménez-Llort et al., 2007). Typically, animals start by engaging in low-risk assessment behaviors, such as horizontal exploration and sniffing. Only after this initial period, if no threats are perceived, does a wider behavioral repertoire emerge, including acts like rearing, grooming, and digging (Janus & Westaway, 2001). While digging in the MB test is often interpreted as an OCD-like repetitive action, its appearance in the CT remains unclear but can be associated with a post-habituation mechanism. Consequently, the observed increase in digging during the second half of the test in both group-housed cohorts likely represents a successful habituation process. In contrast, the static digging pattern in the isolated 3xTg-AD animals points to a failure to habituate, likely driven by heightened neophobia that prevents the emergence of this more complex, late-stage behavior.

Building on this interpretation, the behavior in the MB test completes the picture. In this subsequent test, the same isolated animals engaged in significantly more digging episodes than either control groups. Taken together, the divergence between the tests—a failure to initiate digging in a novel open space (CT) and a potentiation of repetitive digging in a task-oriented setting (MB)—suggests a specific disruption in the species-typical behavioral repertoire. This disruption manifests as both an anxiety-like response to novelty and an exacerbation of compulsive-like behavior following social isolation.

These results are consistent with extensive evidence that social isolation exacerbates BPSD-like symptoms in animal models (Bartolomucci et al., 2003; Magalhães et al., 2024; Võikar et al., 2005). The unique contribution of our study, however, lies in revealing this nuanced behavioral phenotype by analyzing a single motor act across different contexts. This divergence presents a compelling puzzle: why do genetically identical animals behave so

differently based solely on their social condition? To explain this, a framework that links social need to brain function is required.

Drawing upon the social homeostasis theory, it can be hypothesized that chronic social deprivation induced a profound homeostatic imbalance in the single-housed mice. This imbalance likely acted as a persistent, low-grade stressor, promoting functional alterations within key neural circuits such as the brain's reward system–which includes the VTA, NAc and ventromedial prefrontal cortex – and circuits for emotional regulation and stress response. The resulting neural dysfunction could then manifest behaviorally as the precise patterns we observed. Consequently, in this view, the divergent digging patterns are not contradictory but are two symptoms of the same underlying issue that involves a brain state fundamentally altered by the disruption of social equilibrium.

5.4 Environmental modifications

The three studies systematically examined the influence of environmental modifications, ranging from enriched conditions that promote physical activity to the impoverished state of social isolation. An intermediate context was represented by the MWM test, which inherently combines two opposing forces (i) the enriching potential of repeated cognitive training and (ii) the stress-inducing aspects of the task itself.

Across all studies, environmental manipulations yielded subtle effects. These were not immediately evident but emerged after an in-depth analysis including the detailed characterization of swimming strategies in the MWM (Study 1), the identification of NIBI animals in the CT (Study 2), and the quantification of specific digging episodes (Study 3). These nuanced outcomes align with existing literature, where the effects of environmental enrichment in AD models remain ambiguous. While some studies report improvements in amyloid or tau pathology and reductions in BPSD-like symptoms (Liu et al., 2022; Maliszewska-Cyna et al., 2016), others show no significant changes or even detrimental outcomes (Hansson et al., 2019; Jankowsky et al., 2003, 2005). In contrast, the adverse impact of SI is more consistently reported, though often influenced by variables such as strain, sex, litter origin, or experimental design (Dong et al., 2004; Peterman et al., 2020). These inconsistencies indicate that a simplistic dichotomy—enrichment as beneficial, impoverishment as harmful—is inadequate.

To better account for this complexity, this thesis supports a dynamic, bidirectional model in which the organism and its environment are constantly interacting. The environment modulates behavioral expression, while the organism's phenotype shapes its responsiveness to environmental input. This guided the decision to assess BPSD-like behaviors in tasks beyond their conventional scope, reflecting the interconnectedness of cognitive and non-cognitive symptoms.

5.5 Importance of sex in AD research

Significant sex-dependent differences appeared in Studies 1 and 2, indicating a subtle, context-dependent pattern across experimental paradigms. In the MWM test, females showed signs of impaired cognitive flexibility compared to males, while males demonstrated slightly superior engagement in VWR. Although caution is necessary when comparing studies with different experimental conditions, littermates, and genotypes, these results collectively emphasize the complexity and context-specific nature of sex-related behavioral differences.

Historically, females have been underrepresented in clinical and preclinical research, driven primarily by assumptions that hormonal fluctuations would introduce excessive variability, thereby complicating data interpretation (Bierer et al., 2022; Uhl et al., 2007). Such exclusion has limited the generalizability and translational value of findings, particularly in conditions such as AD, where sex differences profoundly influence disease trajectory (Ferretti et al., 2018). In addition, analysis blind to sex can lead to false positive or negative results that can even hide distinctive male and female patterns in the behavioral outcomes (Cañete & Giménez-Llort, 2021). However, advancements in statistical methodologies now enable researchers to address variability effectively, thus facilitating deeper, sex-specific insights into disease pathophysiology and treatment responses.

Furthermore, male and female sexes are viewed as two distinct biological contexts when studying biological, psychological, social, and environmental factors and their interactions (Baeta-Corral et al., 2018). Therefore, recognizing and systematically addressing sex-dependent differences in preclinical models is fundamental to developing more effective, personalized therapeutic interventions. Given the higher prevalence of AD in women and potentially faster disease progression in men, incorporating sex as a biological variable is critical to achieving broader applicability and clinical relevance in neuroscience research.

5.6 Limitations and Future Perspectives

Despite its contributions, this thesis presents limitations that warrant consideration. First, the visual classification of swimming strategies in the MWM test, although highly detailed, was time-intensive and subject to observer bias. This constraint may hamper broader adoption and replication by other research groups. Future implementation of automated classification tools—such as artificial intelligence-based trajectory analysis—would significantly enhance reproducibility, scalability, and analytical objectivity.

Second, the absence of histological and molecular analyses across all three studies limited the capacity to identify underlying neurobiological mechanisms. Without correlating behavioral findings with markers of neuroinflammation, synaptic plasticity, or neurodegeneration, interpretations remain largely speculative. For instance, behavioral phenotypes associated with BPSD-like symptoms could be meaningfully complemented by assessing activity in the prefrontal cortex, amygdala, hippocampus, and ventral striatum, along with quantification of stress hormones and neurotransmitter-related markers.

Study 2 was further limited by an unbalanced experimental design since only transgenic animals were included due to the logistical impossibility of adding NTg mice. Similarly, the absence of combined SI+PA group restricted the evaluation of genotype- and interaction-dependent effects. Additionally, although the connection with social homeostasis theory provides a compelling interpretive framework, this link remains hypothetical in the absence of neurobiological validation. Specifically, the proposed involvement of the prefrontal cortex and amygdala in response to chronic isolation was not directly tested. The behavioral scope of the study, while informative, would benefit from integration with longitudinal neurobiological profiling.

Moreover, the cross-sectional design of Study 3, though ecologically valid—modeling naturally occurring isolation due to cage-mate mortality—prevents dynamic tracking of how social disconnection unfolds over time. A critical next step would be the implementation of a longitudinal design that monitors both behavioral and neurobiological changes from the onset of isolation. This should include both sexes, given the sex-dependent

patterns identified, and assess temporal alterations in social behavior, neurocircuit activity, and peripheral stress biomarkers.

While the direct clinical translation of these findings to human AD necessitates further investigation, the methodological innovations presented, particularly the advanced statistical approaches, offer substantial promise. Their capacity to elucidate subtle, longitudinal behavioral changes in animal models provides a crucial framework for future basic science studies of AD. This approach can facilitate a more routine integration of detailed behavioral analyses, thereby enhancing our ability to discern fine responses to interventions, such as anxiolytics, in late-stage AD models. The tracking of such responses through repetitive measures like the MWM or changes in PA engagement offers a vital lens for interpreting complex behavioral phenotypes, ultimately guiding more effective preclinical research strategies for AD.

In summary, although it presents certain limitations, this work advances our understanding of how environmental modifications interact with AD pathology to influence BPSD-like symptoms in the 3xTg-AD mouse model. By integrating advanced statistical methodologies with ecologically relevant behavioral paradigms, this thesis contributes a valuable framework for investigating the non-cognitive dimensions of AD. Once the outlined limitations are addressed, we hope this line of research will enhance the translational value of preclinical models and support the development of targeted, sex-sensitive therapeutic strategies.

6 CONCLUSIONS

1. In the MWM test, circling behavior was identified as a distinctive characteristic of BPS-like behavior in 3xTg-AD mice. This strategy became more prevalent with age in this group, suggesting a breakdown in their ability to cope with the stressful nature of the test.

2. GLMMs proved effective in analyzing MWM test swimming strategies, revealing subtle genotypical and sexual variations. Notably, 3xTg-AD animals demonstrated a preference for less efficient strategies in reaching the platform.

3. Differences observed through GLMMs were context- and sexual-dependent with variations in the strategy adopted during their first MWM test experience. Overall, females tended to exhibit less cognitive flexibility.

4. A voluntary PA protocol lasting four weeks reduced the expression of anxiety-like behaviors in the OF in 3xTg-AD mice at advanced stages of the disease.

5. MEMs proved to be a valuable statistical technique for behavioral analysis, revealing sexual differences in the circadian patterns of PA engagement in 3xTg-AD mice.

6. Interindividual differences, specifically fearful response assessed by the CT test, were a significant predictor of later response to PA engagement. This effect on the circadian activity of voluntary exercise was observed only in males.

7. The effects of natural social isolation in 3xTg-AD male mice showed a possible alteration in the habituation process as they showed a distinctive pattern of static digging behavior in the CT.

8. Socially isolated 3xTg-AD male mice exhibited a distinctive behavioral signature: low digging activity in the second half of the CT and high digging episodes in the MB test. This pattern was exclusive to the isolated group. While novel and intriguing, this behavior has not yet been definitively established as a marker of social isolation effects.

9. Environmental modifications elicited BPSD-like responses in 3xTg-AD mice; those responses were consistent across experimental designs and occurred probably as an inability of the animal to properly cope with the stress triggered by the test and setting.

10. Specific BPSD-like symptoms manifested in 3xTg-AD mice increased circling behavior in the MWM test, altered circadian rhythm activity when exposed to VWR and disrupted patterns of digging behavior when exposed to social isolation.

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