Primary and secondary signatures of psychomotor dysfunction in experimental models of normal aging and Alzheimer's disease

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Doctoral Thesis

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Nada te turbe, nada te espante todo se pasa,

Dios no se muda, la paciencia todo lo alcanza,

quien a Dios tiene nada le falta sólo Dios basta.

(Santa Teresa de Ávila)

Agradecimientos

Agradezco el apoyo de mi familia, padres y hermanas, quienes me han acompañado con mucha paciencia durante estos años pese a la distancia. También, agradecer a mi esposo Sebastián, quien me ha acompañado en cada momento, hemos compartido muchas experiencias y vivencias que han sido significativas en nuestras vidas desde todo ámbito.

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ABSTRACT

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder traditionally described through cognitive and neuropsychiatric/behavioural symptomatology. However, psychomotor motor dysfunctions and motor impairment remain under-explored.

The 3xTg-AD model shows the neuropathological and cognitive alterations that characterise the disease similar to what occurs in humans, presenting β-amyloid plaques in the cortex and hippocampus at 12 months of age spread throughout the cortex. Subsequently, at 15 months, intraneuronal Tau tangles accompany this spread in the cerebral cortex.

This research is based on the International Classification of Functioning and Disability, which is used worldwide in the rehabilitation of neurodegenerative pathologies, including AD, and from a life course approach associated with normal, accelerated and pathological ageing reproducible in different translational research contexts.

For the study of AD, two research phases were included. In the first phase, the primary and secondary motor signatures of psychomotor dysfunction in normal ageing and Alzheimer's disease were characterised. Male 3xTg-AD and NTg mice aged 6 and 12 months were included in phase 1. In the second phase, the primary and secondary signatures were modulated and integrated under the exploration of external factors. Male 3xTg-AD and C57BL/6 mice were included between 13 and 16 months of age, and male and female 3xTg-AD and NTg mice aged 12 and 16 months were also included.

Psychomotor tests assessed performance from spontaneous gait, exploratory activity, muscle strength, and physical endurance. In addition, frailty phenotype and specific phenotypes such as clasping reflex and geotaxis were included. Additionally, sarcopenia studies and HPA axis analysis were performed.

For the first time, the results report the classification of primary and secondary signatures of psychomotor dysfunctions in the 3xTg-AD mice model. They were detected as primary signatures present in gait and exploratory activity from the study of quantitative and qualitative variables of psychomotor performance. They show a decrease in stride length, speed and cadence, modified by postural alterations such as structural kyphosis in 3xTg-AD mice. In addition, kyphosis is age-sensitive, changing from postural to structural in the ageing process. Also, the clasping reflex indicates the severity of AD, a primary signature like kyphosis. It includes the frailty phenotype that accompanies general psychomotor impairment in mice and increases with age.

On the other hand, bizarre behaviour patterns that were evidenced in dry and water tests showed greater severity of affectation among 3xTg-AD mice. In addition, neophobia (freezing) alters performance, particularly exploration and locomotion. Also, bizarre patterns and kyphosis modify the performance of spontaneous gait and exploratory activity, which is preserved in normal ageing. Even secondary dysfunctions can be modulated by external factors such as isolation and a re-test of the

behavioural battery, where 3xTg-AD mice improve motor performance in females and isolation in males. Also, physical endurance measured in rotarod was sex-sensitive, with 3xTg-AD and non-transgenic females performing better. However, they showed the highest indicators of frailty, sarcopenia and alteration of the HPA axis.

Finally, functional impairments and alterations in 3xTg-AD mice are related to Alzheimer's disease stages, providing a scenario to understand the heterogeneity of non-cognitive symptoms of motor performance.

ABBREVIATIONS

3xTg-AD **:** Triple transgenic mice

Aβ **:** β-amyloid

AD **:** Alzheimer's Disease

ADRDG **:** Alzheimer's Disease and Related Disorders Group

APP **:** Amyloid precursor protein

BPSD **:** Behavioural psychologist symptoms of dementia

EEG **:** Electroencephalogram

EOAD **:** Early-onset AD

LOAD **:** Late-onset AD

MCI **:** Mild cognitive impairment

MWM **:** Morris Water Maze

NINCDS **:** National Institute of Neurological and Communication Disorders and Stroke

NFTs **:** Neurofibrillary tangles

NPS **:** Neuropsychiatric symptoms

NTg : Non-transgenic mice

PS1 : Presenilin 1

PS2 : Presenilin 2

WHO : World Health Organization

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CHAPTER 1. INTRODUCTION

1. ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder (Piaceri et al., 2013) that leads to a progressive decline in the brain functionally and morphologically (Deture and Dickson, 2019; Dugger and Dickson, 2017; Long and Holtzman, 2019). Its presentation is progressive and chronic, with subsequent loss of cognitive function (Long and Holtzman, 2019). AD is the most common form of dementia and may contribute to 60-70% of cases (WHO 2017). Most cases occur after the age of 65, comprising late-onset AD (LOAD), while cases occurring before the age of 65 are considerably rarer, constituting less than 5% of all cases and are termed early-onset AD (EOAD) (Alzheimer's Association 2020; Long and Holtzman 2019; Piaceri et al., 2013). In addition, the risk of AD is 60% to 80% dependent on hereditary factors, with more than 40 genetic risk loci associated with AD already identified (Tanzi, 2012). In more than half of patients with inherited AD, they have mutations in one of three different genes (APP; presenilin 1, PS1; encoded by PSEN1, and PS2; encoded by PSEN2) (Bekris et al., 2010; Clark et al., 1996; Vetrivel et al., 2006). Furthermore, APOE alleles have the strongest association with the disease (Scheltens et al., 2021; Serrano-Pozo et al., 2021). Moreover, in recent years, it is rapidly becoming one of the most costly, deadly, and burdensome diseases of this century (Scheltens et al. 2021; Alzheimer's Association 2020). The most recent data indicate that, by 2050, the prevalence of dementia will double in Europe and triple worldwide, and this estimate is three times higher when based on a biological definition of the disease (Scheltens et al. 2021).

Biologically, AD is defined by the presence of a specific neuropathological profile (Duyckaerts et al., 2009) that includes extracellular deposition of β-amyloid (Aβ) arising from proteolytic cleavage of amyloid precursor protein (APP) in the form of diffuse; neuritic plaques and the presence of intraneuronal neurofibrillary tangles (NFTs); and neuropil threads within dystrophic neurites consisting of aggregated hyperphosphorylated tau protein (Deture and Dickson, 2019; Duyckaerts et al., 2009; Serrano-Pozo et al., 2011). Most mutations result in the overproduction of Aβ, specifically, the 42-amino acid Aβ isoform (Aβ42), which has amyloidogenic characteristics and is more prone to aggregation (Golde et al., 2000; Masters et al., 2015). On the other hand, most mutations in APP modify APP processing such that the ratio of Aβ42 to Aβ40 increases in the plasma of affected patients (O'Brien and Wong, 2011; Scheuner et al., 1996). In addition, mutations in PSEN1 and PSEN2 result in increased Aβ42/Aβ40 ratios (Kumar-Singh et al., 2006; Weggen and Beher, 2012). The type of mutation and the associated Aβ42/Aβ40 ratio predict the average age of onset of dementia (Graff-Radford et al., 2007).

Braak and Braak described the pathological evolution of the disease in 1991, 1995 (Braak and Braak, 1995, 1991); in their report, they mapped the movement of both β-amyloid and hyperphosphorylated tau in the brain during disease progression (Braak and Braak, 1995, 1991). Thus, the movement of amyloid was divided into three stages: A, B, and C; and tau in six: I to VI (Braak and Braak 1991). In typical cases of Alzheimer's disease, Aβ deposition precedes neurofibrillary and neuritic changes with an apparent origin in the frontal and temporal lobes, hippocampus and limbic system (Masters et al.,

2015). Less frequently, the disease appears to arise from other regions of the cerebral neocortex, with relative sparing of the hippocampus (Masters et al., 2015; Swarbrick et al., 2019). Amyloid deposition develops before the appearance of tau (Braak and Braak, 1991; Scheltens et al., 2016). However, the presence of amyloid does not mean that tau pathology will develop (Braak and Braak 1991).

In addition, neurofibrillary tangles and neuritic degeneration begin in the medial temporal lobes and hippocampus and progressively spread to other areas of the neocortex (Braak and Braak, 1995, 1991, 2018; Swarbrick et al., 2019). Thus, as illustrated in Figure 1, during stage A, amyloid is found in the base layer of the frontal, temporal, and occipital lobes; in stage B, amyloid progresses to almost all areas of the isocortex; and during stage C, amyloid becomes densely packed (Swarbrick et al. 2019). Braak stages I and II are centred in the transentorhinal region; in stage II the tau pathology is more densely packed than in stage I. In stage III, pathology shifts to the entorhinal region with low levels of tau observed in CA1 of the hippocampus and mild or absent changes in the isocortex (Swarbrick et al. 2019; Masters et al. 2015; Braak and Braak 1991).

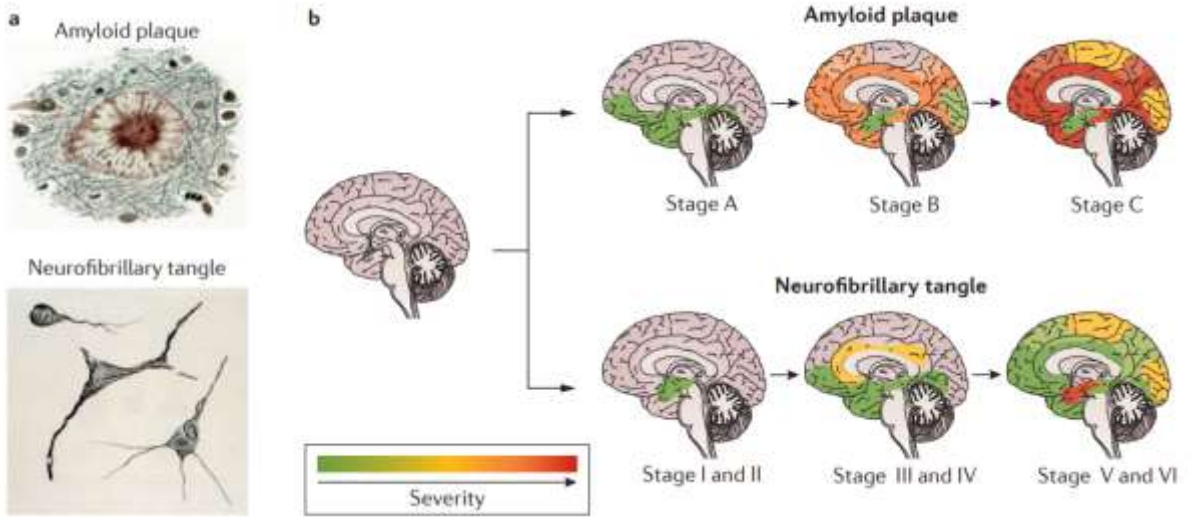


Figure 1. The pathological evolution of Alzheimer's disease (Masters et al., 2015). a) Amyloid plaques and neurofibrillary tangles spread throughout the brain as the disease progresses. b) Typical cases of Alzheimer's disease, Aβ deposition, neurofibrillary and neuritic changes.

Clinically, AD presents with progressive loss of short-term memory, long-term memory and abstract thinking (MacDonald, 2007; Tarawneh and Holtzman, 2012). Symptoms of the disease have been classified according to criteria published in 1984 by the National Institute of Neurological and Communication Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Group (ADRDG) (Knopman et al., 2019; McKhann et al., 1984). In typical amnesic cases, there are usually early impairments in learning and memory, followed by later impairments in complex attention, executive function, language, visuospatial function, praxis, gnosis and social behaviour and conduct (Hugo and Ganguli, 2014; McKhann et al., 2011). The first symptoms of the disease are observed in episodic memory loss and correspond to mild cognitive impairment (MCI) (Gold and Budson, 2008;

McKhann et al., 2011). Patients who fit this definition are 3-5 times more likely to develop dementia within 3-5 years (Petersen et al., 1999). They may also present atypical clinical syndromes with early impairment in non-memory domains (Galton et al., 2000). Also, posterior cortical atrophy presents early deficits in visuospatial function, praxis, and gnosis (Sperling et al., 2011; Tang-Wai et al., 2004). In addition, the behavioural/dysexecutive variant of AD may present with early executive dysfunction or behavioural impairment, especially apathy, hyperorality and perseveration (Ossenkoppele et al., 2015). The severity of clinical dementia can be rated using standardised instruments such as the Clinical Dementia Rating, which rates disease severity based on the composite level of dysfunction in the domains of memory, orientation, judgment and problem solving, participation in community affairs, functioning at home and hobbies, and self-care (Long and Holtzman, 2019).

Figure 2 shows the onset and progression of clinical symptoms according to biomarkers measuring Aβ deposition, tau or brain neurodegeneration (Toniolo et al., 2020). Amyloid load, measured by cerebrospinal fluid (CSF) or positron emission tomography (PET) of Pittsburgh compound B (PiB) amyloid ligand, is the first to increase (Scheltens et al., 2016; Toniolo et al., 2020). A prolonged preclinical phase of the disease is characterised by the early onset of amyloid deposition (Dubois et al., 2016). At the same time, there are early neuroinflammatory changes (Long and Holtzman, 2019). Disease progression continues with the spread of NFT tau pathology from the medial temporal lobes to the neocortex (Braak and Tredici, 2018). Synaptic dysfunction, synapse loss and neurodegeneration accumulate with the pathologic spread of tau aggregates (Tai et al., 2012). Progression and onset of cognitive impairment correlate with tau accumulation and hippocampal volume loss but not with amyloid deposition (Long and Holtzman 2019). Electroencephalogram (EEG) abnormalities increase longitudinally as the disease progresses, with suboptimal detection rates (Toniolo, Sen, and Husain 2020). The combined effect of Aβ amyloid and tau induces hyperexcitability in the early stages and hyperexcitability in the late stages of the disease, as shown by hippocampal activation by fMRI (Toniolo et al., 2020; Long and Holtzman 2019). Inter-individual differences exist, and some older individuals with preclinical evidence of pathophysiological changes may not become symptomatic during their lifetime, possibly due to slower progressing disease or death due to competing mortality (Long and Holtzman 2019; Toniolo et al., 2020). These inter-individual differences are attributable to environmental and genetic factors, including brain reserve, cognitive reserve, genetic polymorphisms, and coexisting pathologies, such as age-related brain diseases and medical comorbidities (Toniolo et al., 2020). Furthermore, lifestyle factors do not directly affect the pathology of Alzheimer's disease, but may contribute to a positive outcome in people with Alzheimer's disease (Scheltens et al. 2021).

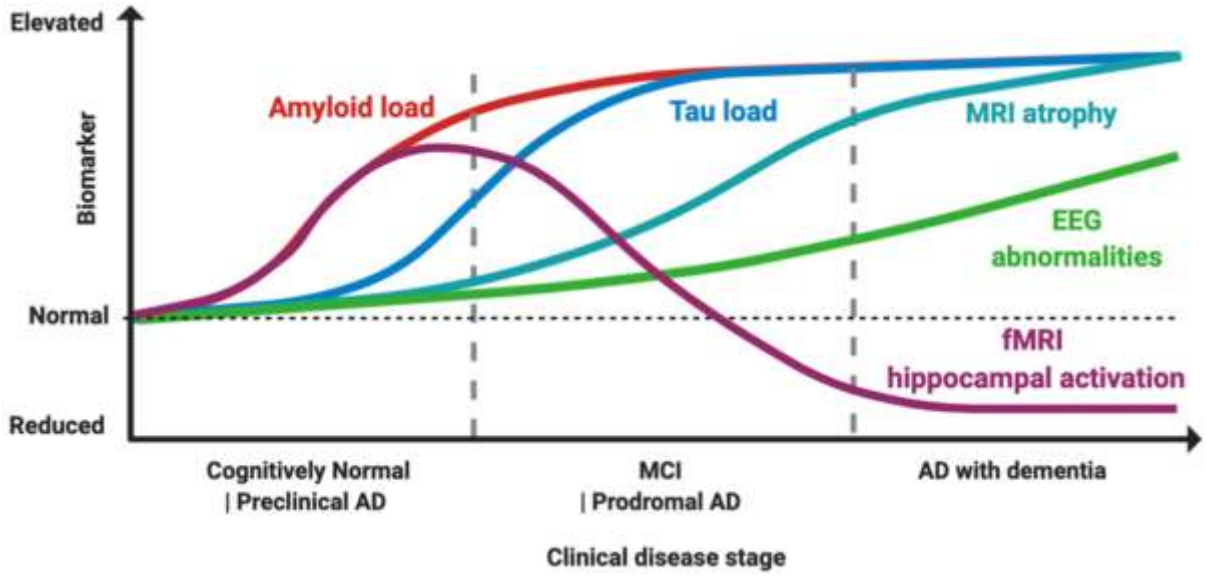


Figure 2. Biomarker dynamics model of AD hyperexcitability in humans (Toniolo et al., 2020).

Currently, four drugs are approved by the Food and Drug Administration (FDA) to treat cognitive impairment and global activities dysfunction in symptomatic AD (Long and Holtzman, 2019). These include three cholinesterase inhibitors (ChEIs): donepezil, rivastigmine and galantamine; and memantine, a non-competitive NMDA receptor modulator (Long and Holtzman, 2019; Patel and Grossberg, 2011). However, some drugs are in advanced stages of clinical trials and include anti-amyloid β, anti-tau and anti-inflammatory strategies (Scheltens et al., 2021). The evidence underlying the amyloid cascade hypothesis remains strong, and findings should continually inform and update our understanding of the pathobiology of the disease, ultimately leading to the development of new treatment approaches (Long and Holtzman, 2019).

2. MOTOR AND NON-MOTOR SYMPTOMS OF AD

AD is traditionally described through cognitive and behavioural symptomatology (Hsieh et al., 2016). However, motor dysfunctions and impairment remain under-explored (Koppelmans et al., 2020). Consequently, comorbidity of functional and cognitive impairment is a warning sign of increasing disability (Liou et al., 2020), a growing public health problem (Buchman and Bennett, 2011), and is already present in the preclinical stages of Alzheimer's disease (Beeri et al., 2021).

Motor and sensory impairments are less frequent at the onset of the disease but may appear in later stages (Zidan et al., 2012). Although the main signs of AD are defined around cognitive impairment, motor impairments such as bradykinesia, rigidity and gait disorders are of great importance due to the limitations and functional impairments they cause in the disease (Montero-Odasso and Perry, 2019). Several studies have shown different motor impairments during the last two decades, particularly those

associated with gait in AD (Beauchet et al., 2014; Montero-Odasso et al., 2018; Muir et al., 2012). For example, it is longitudinally associated with cognitive impairment, dementia and falls in older adults (Dyer et al., 2020; You et al., 2021), and slow gait is associated with an increased risk of falls and poor baseline cognition (Beeri et al., 2021). Thus, gait disorders in AD patients have been described within the group of impairments known as “frontal gait” and, in particular, gait in AD has been defined as *“*cautious gait*”* (Baker, 2018; Pirker and Katzenschlager, 2017).

In this way, patients with this type of disorder who seem to have forgotten how to perform the act of walking are defined as frontal gait (Pirker and Katzenschlager, 2017). Patients have difficulties standing and postural misalignments that prevent them from changing to different positions in a coordinated manner with segments such as the arms and legs, leading to difficulties in achieving a stable position (Muňoz et al., 2010; Pirker and Katzenschlager, 2017). In execution, the gait has a broad base of support, with a short stride length, the arms may be extended laterally or may reduce their swing, and the trunk posture may be bent, upright or even hyperextended (Pirker and Katzenschlager, 2017). The onset of walking is affected, as in Parkinson's disease, with bradykinesia. Some patients begin to walk by swaying the trunk laterally or making exaggerated arm movements (Magrinelli et al., 2016). There is shuffling ("magnetic feet"), but gait usually improves after walking a few steps (Pirker and Katzenschlager, 2017; Sanders and Gillig, 2010). In addition, freezing episodes can occur, especially when turning and facing obstacles (Pirker and Katzenschlager, 2017). Balance and postural stability are also affected (Lee et al., 2017; Mesbah et al., 2017). In some cases, retropulsion occurs, which can lead to backward falls (Baker, 2018; Jahn et al., 2019; Montero-Odasso et al., 2018; Pieruccini-Faria et al., 2021; Pirker and Katzenschlager, 2017). These impairments are associated with grey matter atrophy in the midbrain and are caused by a dysfunction in a network linking the primary motor cortex to the locomotor region of the midbrain (Pirker and Katzenschlager, 2017).

On the other hand, cautious gait is more frequent in patients with mild dementia (Clinical Dementia Rating Scale: Hughes CDR, stage 1), and frontal gait disorder becomes prominent in more advanced AD (CDR stage 2 and 3) (Beauchet et al., 2016). This gait pattern is similar to that observed in ageing, and may present: a decrease in gait speed, step length and postural gait stability, manifested more specifically in static and dynamic balance, with an enlarged base of support (Beauchet et al., 2008; Montero-Odasso et al., 2012). Dynamic instability has also been observed in mild and moderate AD (Beauchet et al., 2016; Pirker and Katzenschlager, 2017). Furthermore, AD patients show progressive deterioration of the visual system, with severe neuropathology in visual association areas in advanced stages, although primary sensory areas remain relatively unchanged (Thompson et al., 2003). Thus, patients tend to make greater use of the relatively unaffected somatosensory system (resource reallocation) once the visual system has deteriorated too much as a compensatory measure to maintain their autonomy and functionality (Pirker and Katzenschlager, 2017). In addition to cautious gait, gait-related motor activity disturbances such as rigidity and bradykinesia have been observed, which show rapid progression throughout the course of the disease and are considered extrapyramidal signs (Beauchet et al., 2016, 2014). However, in AD, rigidity is paratonic and the gait disturbances are possibly due to apraxia (Fuller and Manford, 2010). Paratonic rigidity and gait apraxia are not indicative

of parkinsonism. Furthermore, bradykinesia in AD is different from the slowness of movement seen in normal ageing (Beauchet et al., 2016; Caranasos and Israel, 1991; Cohen et al., 2016; Zwergal et al., 2012).

Finally, Table 1 differentiates functional gait impairment according to the variables that quantitatively describe gait. Normal ageing, Parkinson's and Alzheimer's disease have been included to visualise the differences in each parameter.

Table 1. Differentiation of impairments and deficits in spatial, temporal, biomechanical and postural parameters of gait in normal and pathological ageing in humans (Castillo-Mariqueo, 2018; Castillo Mariqueo and Giménez Llort, 2020).

Parameters of gait		**Ageing**	**Alzheimer's disease**	**Parkinson's disease**
Spatial	*Stride length, step height and width*	Gradual decline	Initial stages, similar to ageing	Stage 1 and Stage 2 (Hoehn and Yahr), similar to ageing
	Base and support area	Increase	Advanced stages, decreases	Advanced stages, decreases
Temporal	*Step and stride time, and frequency*	Increase	Initial stages, similar to ageing	Stage 1 and Stage 2 (Hoehn and Yahr), similar to ageing
	Gait phases	Increases contact time with the ground	Advanced stages, apraxia/ataxia	Advanced stages, Bradykinesia/Festination
Spatial-Temporal	*Speed*		Initial stages, decrease	Stage 1 and Stage 2 (Hoehn and Yahr), similar to ageing
	Cadence	Gradual decline	Initial stages, decrease	Advanced stages may increase
	Automatic turn reaction		Decreases considerably	Decreases considerably
Biomechanical	*Joint range*	Gradual decline	Initial stages, similar to ageing	Stage 1 and Stage 2 (Hoehn and Yahr), similar to ageing
	Muscular strength		Advanced stages run with fragility	Advanced stages run with fragility
Postural	*Postural changes in a static or resting position, and dynamic stability*	Gradual instability. Joint stiffness due to cartilage wear	Lost stability in a static position. In advanced stages, loss of dynamic stability with paratonic rigidity.	Involuntary movements: rest tremor, cogwheel rigidity. Increases severity from Stage 3 and Stage 4 (Hoehn and Yahr)

On the other hand, behavioural and psychological symptoms of dementia (BPSD), also known as neuropsychiatric symptoms (NPS), represent a heterogeneous group of non-cognitive symptoms and behaviours that occur in subjects with dementia (Cerejeira et al., 2012). BPSDs include agitation, aberrant motor behaviour, anxiety, elation, irritability, depression, apathy, disinhibition, delusions, hallucinations, and changes in sleep or appetite (Cerejeira et al., 2012). There is a consensus that greater symptom severity predicts faster cognitive decline, loss of independence and even poorer survival (Li et al., 2014). Their presence has been described as the result of a complex interaction of neurobiological, psychosocial and environmental factors such that, although there is significant variability from patient to patient, their frequency and severity tend to increase progressively as the disease's cognitive and functional impairment progresses (Mintzer et al., 2000). Furthermore, different anatomical-clinical entities are accompanied by types or combinations of NPS to the extent of showing a preference for different brain regions (Cerejeira et al., 2012). For example, frontal damage caused by the disease is associated with behavioural changes such as apathy, hyperactivity, disinhibition, poor judgment or contrast with reality, such as thought disturbances (Mintzer et al., 2000). In particular, apathy has been associated with dysfunction in the areas that make up the frontal-subcortical circuits, showing hypoperfusion and hypometabolism in the anterior cingulate gyrus (AGG), orbitofrontal cortex (OFC), nucleus basalis of Meynert and hippocampus (García Morúa et al., 2010; Levy and Dubois, 2006). While depression has been associated with dysfunction in frontal-subcortical and subcortical limbic circuits (locus coeruleus, substantia nigra, hippocampus and hypothalamus), finding hypoperfusion in the GCA and dorsolateral prefrontal cortex (DLPC) as well as frontal and prefrontal hypometabolism (Bonelli and Cummings, 2007).

Similarly, psychotic symptoms have been associated with increased neurofibrillary tangles in the neocortex, while agitation has been related to a greater accumulation of neurofibrillary tangles in the OFC (Farber et al., 2000). BPSDs could be linked to changes in neurotransmitter systems involved in AD, especially those involving glutamate, acetylcholine, serotonin, norepinephrine or dopamine (Cerejeira et al., 2012; Kristensen, 1990), and their dysfunction could be related to the presence of mood swings (serotonin and norepinephrine), movement disorders (dopamine), aggression (serotonin) and apathy (acetylcholine) (Cerejeira et al., 2012; Mintzer et al., 2000). Unfortunately, the success of treatment of these psychiatric symptoms may be lower when AD is comorbid, underlining the importance of future research into their pathobiology and treatment (Li et al., 2014).

3. FUNCTIONING AND DISABILITY IN AD

Functioning is conceptualised as a person's ability to perform the activities necessary to achieve well-being through the interrelation of biological, psychological (cognitive and affective) and social domains (Hopper, 2007). Under this definition, the International Classification of Functioning, Disability and Health (ICF) proposes a conceptual framework that establishes a standard language to describe health and its dimensions (WHO 2001). It was adopted by the WHO in 2001 and has since had wide applicability in the field of health rehabilitation (Hopper 2007). Figure 3 lists the three components of this model: body functions and structures, activity, and participation. The first component relates to physiological and psychological functions and anatomical elements; the second component refers to the individual performance of tasks and activities; the third component relates to the development of social situations (WHO 2001). Functional impairment is a central symptom of AD (Arrighi et al., 2013; Gauthier et al., 1997). The most accurate indicator of functional impairment is a decline in the performance of activities of daily living (ADL) (Arrighi et al., 2013; Muò et al., 2009; Yeh et al., 2011). A report using the ICF has pointed out that activity and participation are restricted to domestic life, self-care and mobility, communication, interaction and social relationships (Muò et al., 2009). Furthermore, subjects who appeared more compromised on the Mini-Mental State Examination (MMSE) and the Global Deterioration Scale (GDS) showed greater impairment of function, limitation of activity and restriction of participation (Muò et al., 2009). Executive dysfunction and declines in general measures of cognitive functioning have also been reported to be associated with decreased ability to perform instrumental ADLs (Pereira et al., 2008). ADLs are affected in a progressive and hierarchical manner associated with cognitive impairment, but substantial variability persists between individuals and the relative order of affected items (Arrighi et al., 2013). It has also been noted that there are disability profiles with restricted patterns of time use in a variety of domains spanning both obligatory and discretionary activity, accompanied by a significant increase in a passive activity, such as sleeping during the day or sitting in front of the television (Lomax et al., 2004). Restrictions in the social and environmental contexts of the patient's life and diminished levels of subjective enjoyment have also been associated with their daily use of time (Hopper, 2007; Lomax et al., 2004).

The ICF currently consists of 1,424 mutually exclusive categories that together cover a complete and comprehensive spectrum of human experience and are organised as a hierarchical structure of 4 levels differentiated from least to most accurate (WHO, 2013). The ICF categories are indicated by alphanumeric codes with which it is possible to classify functioning and disability, both at individual and population levels. According to this hierarchical structure, the highest-level category (4th) shares the attributes of the lowest level category (1st) to which it belongs. In addition, ICF rates quantify the magnitude of a problem in the different ICF categories, which are mathematically weighted quality descriptors that record the presence or severity of a problem at the bodily, personal or societal level (WHO, 2001). Thus, a problem may involve an impairment, limitation or restriction that can be graded from 0 (no problem: 0-4%), 1 (mild problem: 5-24%), 2 (moderate problem: 25-49%), 3 (severe problem:

50-95%) to 4 (total problem: 96-100%). Environmental factors are quantified with a negative or positive scale indicating how an environmental factor acts as a barrier or facilitator (WHO, 2013).

The ICF is a useful tool for describing the health condition of AD patients, as it highlights important aspects of daily life that are not usually considered in activities of daily living scales, such as communication, social relationships, and recreation and leisure (Badarunisa et al., 2015; Gauthier et al., 1997; Hopper, 2007).

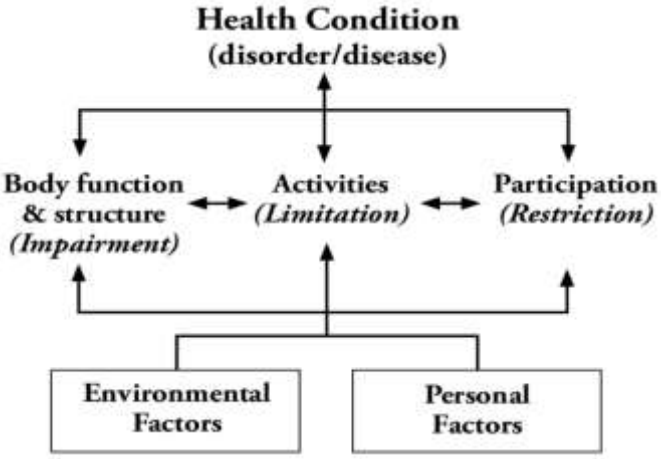


Figure 3. International Classification of Functioning, Disability, and Health (ICF*)* (WHO 2001).

4. FRAILTY AND SARCOPENIA IN AD

Frailty is a pre-disability syndrome in which an older person can be identified as being at risk when exposed to stressors associated with a high risk of disability or requiring hospitalisation (Cesari et al., 2016; Gómez-Gómez and Zapico, 2019; Morley, 2016). In turn, sarcopenia, decreased function with low muscle mass, is a major cause of frailty (Lo et al., 2020; Morley, 2016). Therefore, motor function is the main driver of associations between sarcopenia and physical frailty with several adverse health outcomes (Buchman et al., 2021; Suryadevara et al., 2020). In chronic and progressive diseases, such as AD, early identification of changes in parameters, such as muscle mass and strength and reductions in physical performance, is essential to identify and take precautions in the early stages considering the limitations of the preventive effects of treatment applied after AD diagnosis (Suryadevara et al., 2020; Yazar and Yazar, 2019).

Thus, lower grip strength has been reported to be associated with an increased risk of cognitive decline and dementia (Cui et al., 2021). People with sarcopenia are more likely to have a single but dual decline in cognitive and physical function (Tolea and Galvin, 2015). Furthermore, it has been noted that changes in muscle strength are detectable in the early stages of the disease, where women have decreased muscle strength without loss of muscle mass in the upper and lower extremities and men in the lower extremities with decreased muscle mass and muscle strength in intermediate or advanced

stages of AD (Ogawa et al. 2018). It has also been mentioned that the prevalence of sarcopenia is higher in the early stages of AD (Ogawa et al. 2018; Bai et al. 2021). Particularly in women with mild cognitive impairment, sarcopenia and physical frailty have been associated with cognitive and affective functions (Ohta et al., 2019), with a higher prevalence associated with dementia in old age (Ruan et al., 2017b).

Emerging biomarkers of sarcopenia and physical frailty could be the basis for establishing clinical and research criteria for AD and thus be useful for a new clinical vision (Ruan et al., 2017a).

5. 3XTG-AD MICE MODEL FOR THE STUDY OF AD

The triple transgenic 3xTg-AD mouse model was first described in 2003 (Oddo, et al. 2003a). This model was created by microinjecting the APPSwe and tauP301L transgenes into the pronucleus of an embryonic mouse cell-induced (knock-in) for the PS1M126V gene; as a result, it carries the human PS1M146V, APPSwe, tauP301L transgenes (Oddo, et al. 2003a; Oddo, et al. 2003b).

The model progressively develops amyloid-beta and tau in brain regions such as the cortex, hippocampus and amygdala, which are relevant to AD pathology (Oddo et al. 2003b). The temporal and anatomical specific profile of the model reproduces a similar pattern observed in humans (Oddo et al. 2003a; Mesulam 2000). In addition, the model exhibits behavioural and cognitive deficits that increase with age (Giménez-Llort et al., 2007).

Intraneuronal Aβ pathology correlates with the appearance of cognitive deficits in the CA1 region of the hippocampus at 4 months but is also visible in the cortex and amygdala (Oddo et al. 2003b; Oddo et al. 2003a; Clinton et al. 2007). However, Aβ immunoreactivity has been observed in the hippocampus at 2 months (Mastrangelo and Bowers, 2008). Extracellular deposits of Aβ in 3xTg-AD mice are evident at 6 months, predominantly in layers 4 and 5 of the frontal cortex (Oddo et al. 2003a). At 12 months, they continue to spread into the hippocampus and cortical regions. (Giménez-Llort et al., 2007). However, tau immunoreactivity is first evident at 12 months in the pyramidal neurons of area CA1 (Oddo et al. 2003a; Oddo et al. 2003b).

In addition, behavioural characterisation of mice has revealed a reduction in exploratory behaviour and an increase in ambulation, which is observed prior to Aβ formation, learning and memory deficits and the development of neophobia at 6 months (Giménez-Llort et al., 2007; Sterniczuk et al., 2010a). A decrease in the amplitude of locomotor activity and arrhythmic behaviour has also been observed prior to the development of AD pathology (Sterniczuk et al., 2010b). In addition, 3xTgAD mice have reduced accuracy to short, spatially unpredictable stimuli when the attentional demand of the task is high, accompanied by a general tendency to make more perseverative responses (Romberg et al., 2011).

On the other hand, the AD mouse model shows marked differences in frailty phenotypes, where genotype- and sex-dependent life expectancy determines the frailty status of 3xTg-AD mice (Kane et al., 2018). Thus, frailty increases with age in males and females (Kane et al., 2018; Muntsant et al., 2021; Torres-Lista et al., 2017). In addition, signs of frailty accompany functional decline in these animals (Castillo-Mariqueo et al., 2021). Furthermore, signs of sarcopenia are present at an advanced stage of AD, with differences in fibre distribution, number of cell nuclei and presence of adipose tissue in male mice at 6, 12 and 16 months (Castillo-Mariqueo et al., 2021), a value that is also reproduced in females at 16 months (Castillo-Mariqueo and Giménez-Llort, 2022). Thus, the frailty index indicates the risk of mortality in the 3xTg-AD model (Kane et al. 2018), so the frailty assessment could indicate a measure of lifespan to facilitate comparisons between different AD mouse models (Kane et al., 2016). Table 2 shows the different mouse models that have been proposed for the study of frailty according to clinical indicators in humans, where we could place the 3xTg-AD model in a model of biological age and frailty, sarcopenia in similar aged mice such as in C57BL/6J mice.

Table 2. Different models of frailty in mice and their comparison with clinical indicators of frailty in humans (Castillo-Mariqueo and Gimenez-Llort 2019)

Clinical basis	Animal model concept	Experimental subject	Study	Parameters of frailty assessment and their applications
Biological Age and frailty in ageing mice	*Biological age*	C57BL/6J (male mice)	(Reynolds et al., 1985)	To assess biological age using a battery of psychomotor tests: rotarod (balance on a rotating rod), grip strength, exploratory behaviour and wheel running tasks. This study is not specific to frailty, but is useful for measuring general health or biological age in animal experiments on ageing.
Frailty in Genetically manipulated mice	*IL-10 knock-out mice*	Female IL-$10^{tm/tm}$ mice on a C57BL/6J background	(Walston et al., 2008)	Based on the characterisation of the genetically modified IL-$10^{tm/tm}$ model. Exploring the biological mechanisms of frailty. Model of inflammation and multi-systemic decline.
Biological Age and frailty in ageing mice	*Sarcopenia in frailty*	C57BL/6J (male breeder mice) and *Sprague Dawley male Rat*	(Weber et al., 2012)	Characterisation of skeletal muscle ageing in preclinical mammalian models. Measurement of muscle performance, size and architecture using micro-X-ray computed tomography (micro-CT) imaging and muscle histology.
Based on Rockwood's Frailty Index	*Mouse frailty index*	C57BL/6J (male and female mice)	(Parks et al., 2012)	To assess different health parameters: activity levels, haemodynamic measures, body composition and baseline metabolic status. The mouse frailty index can be used to quantify frailty in ageing mice.
Biological Age and frailty in ageing mice	*C57BL/6J neuromuscular healthspan scoring*	C57BL/6J (male mice)	(Graber et al., 2013)	The neuromuscular healthspan scoring system provides a score for each animal from three individual scores obtained from functional assessment: rotarod, grip strength and maximal isometric force. It also provides information on muscle contractility in vitro.
Based on Fried's Frailty Phenotype	*Frailty phenotype index*	C57BL/6J (male mice)	(Liu et al., 2014)	To assess physical performance levels: grip strength, walking speed (rotarod), physical activity (voluntary running on wheels), endurance (average of grip strength and walking speed test).
Based on Rockwood's Frailty Index	*Mouse clinical frailty index*	C57BL/6J (male and female mice)	(Whitehead et al., 2014)	To assess parameters of potential age-related deficits primarily through visual inspection by the assessor: Integument, physical - musculoskeletal, vestibulocochlear - auditory, ocular/nasal, digestive/urogenital, discomfort and body weight and temperature. This model is based on the accumulation

				of deficits over a lifetime and has features observed in human clinical studies.
Frailty in Genetically manipulated mice	*Cu/Zn superoxide dismutase knockout mouse*	Sod1KO mice	(Deepa et al., 2017)	The model shows alterations similar to the defining features of human frailty: weight loss, weakness, low physical activity and exhaustion. Sod1KO mice show increased inflammation and sarcopenia. Useful for studying the aetiology of frailty.
Based on Fried's Frailty Phenotype	*Inactivity as a model of frailty (Valencia Score)*	C57BL/6J (male mice)	(Gomez-Cabrera et al., 2017)	The frailty score is based on Fried's five criteria for frailty in humans: they propose a score of Valencia (frailty in rodents): unintentional weight loss, weakness, grip strength, low endurance and energy, slowness and low level of physical activity (tightrope test). The study divided the animals into two groups: sedentary mice and spontaneous wheel runners.

Currently, several studies report cognitive and emotional deficits in the different stages of AD (Giménez-Llort et al., 2007). However, results in the motor and psychomotor domains are still scarce. In humans, motor coordination of gait is an important aspect of AD, as bradykinesia and gait disturbances are part of the motor deficits in AD (Scarmeas et al., 2004). Thus, this gait disorder is also reproduced in 3xTg-AD mice, where male mice at different stages of AD show functional impairment (Castillo-Mariqueo, Pérez-García, and Giménez-Llort 2021). In addition, they are also accompanied by low exploratory activity, with a range of bizarre behaviours that account for the severity of functional impairment (Baeta-Corral and Giménez-Llort, 2014). Other mouse models have shown decreased stride in gait. The P301S model has reported a decrease starting at 6 months, increasing up to 12 months (Samaey et al., 2019). In the 5xFAD model, a decreased stride length has also been detected in 12-month-old females, accompanied by leg dragging (O'Leary et al., 2018).

Consequently, deficits in learning and motor performance have also been detected in the 3xTg-AD model; thus, at 6.5 months of age, mice show mild deficits in learning and spatial memory (Stover et al., 2015a), with reduced grip strength in suspension tests in both sexes (Stover et al., 2015b). In the 5xFAD model, reduced locomotor activity has been demonstrated in the open field test and balance beam with decreased grip strength at 15-16 months in both sexes (O'Leary et al., 2020). Similarly, TgCRND8 mice have also shown reduced motor skills from 3 to 9 months (Yuan et al., 2017). Likewise, in P301S mice, motor deficits are apparent as early as 3 or 4 months and progress rapidly up to 5.5 months (Koivisto et al., 2019).

On the contrary, the physical parameters: strength and physical performance in rotarod, 13-month-old male 3xTg-AD mice showed a coincidence with hyperactivity (Castillo-Mariqueo and Giménez-Llort, 2021a), similar to what occurs in the P301S model at 2 to 3 months (Koivisto et al., 2019) in the THY-Tau22 male model at 12 months (Van der Jeugd et al., 2013) and in the APP/PS1 model at 9 months (Lok et al., 2013). Particularly, rotarod performance, expressed as physical performance, 3xTg-AD females have the highest performance, even above wild-type males and females at 12 months, and this is replicated at 16 months when females have been previously trained on the behavioural battery (Castillo-Mariqueo and Giménez-Llort 2022). In contrast, males of similar age show poor performance even with previous training (Castillo-Mariqueo and Giménez-Llort, 2022). Similarly, in males of the

TPR50 model at 8.5 months, they present deficits in rotarod performance (Onishi et al., 2014) and males and females of the P301S models at 7 and 16 months of age (Lathuilière et al., 2017), Tau58-2/B at 10-11 months (Van der Jeugd et al. 2016). Also, aged female Tg4-42 mice perform poorly in rotarod, but general locomotor activity and muscle strength are not affected at 7 months (Wagner et al., 2019).

Similarly, females in the 5xFAD model perform less well in the rotarod at 12 months (O'Leary et al., 2018). Other studies of the 3xTg-AD model have reported that female mice have lower rotarod performance, possibly due to differences in protocols (Filali et al., 2012), but it is also suggested that tau P301L might be responsible for the better motor performance in the case of females (Gould, 2012; Stover et al., 2015b; Wagner et al., 2019). These findings are quite heterogeneous, but the behaviours are similar among male models. Table 3 lists the main findings of motor performance in different mouse models of AD.

Table 3. Motor impairment, frailty, and clasping reflex in different mice models of AD

Mouse model	Age tested	Sex tested	Motor impairments, frailty and clasping reflex	References
3xTg-AD	6 months	Males Females	(1) 3xTg-AD mice have a lower grip strength on the grid suspension compared to controls; (2) In gait analysis, 3xTg-AD mice had a longer stride length and performed more foot slips on the balance beam than wild-type mice; (3) 3xTg-AD mice had greater motor performance in the rotarod; (4) In the rotarod, females appeared to perform better than males.	(Stover et al. 2015)
	6 months	Males Females	(1) Bizarre behaviours as early-BPSD-like symptoms in 6-month-old male and female 3xTg-AD mice detected in open field test.	(Baeta-Corral and Giménez-Llort, 2014)
	11 months	Males	(1) 3xTg-AD isolated mice showed a prominent hyperactive pattern as shown in gross motor function in all the tests (open field, corner test, spontaneous activity test, T-maze). (2) 3xTg-AD showed the bizarre behaviours, and intensified nesting behaviour (fine-motor function) in the isolated group.	(Muntsant-Soria and Gimenez-Llort, 2020)
	12, 14 months	Females	(1) Mutants showed hypoactivity in two open-field tests and in the elevated plus-maze; (2) Hind paw clasping was observed in 10% of mice; (3) 3xTg-AD mice had a high performance in the rotarod.	(Filali et al., 2012)
	14 months	Males Females	(1) The frail male 3xTg-AD exhibited sustained activity in open field test, mostly as a thigmotaxis response and slower habituation pattern. (2) The repetition of the open field test elicited reduced activity, in all the groups.	(Muntsant et al., 2021)
	16 months	Females	(1) No significant differences between genotype and sex in wire hang or grid suspension tasks, stride length or stride width, but 3xTg-AD mice performed worse than WT mice on the balance beam; (2) Age-related decline in most aspects of motor behaviour; (3) 3xTg-AD mice showed better motor coordination and learning than WT mice in the rotarod.	(Garvock-de Montbrun et al., 2019)
5xFAD	3, 11 months	Males Females	(1) 5xFAD females are less frail than 5xFAD males.	(Todorovic et al., 2020)
	3 to 12 months	Females	(1) After 9 months, mice had reduced locomotor activity with gait, balance and coordination dysfunctions; (2) 5xFAD mice had significantly higher frailty scores than WT mice and a shorter lifespan than WT mice.	(Gendron et al., 2021)
	3 to 16 months	Males Females	(1) At 12-13 months, mice develop reduced locomotor activity in the OF, which may be due to gait impairment; (2) Grid and wire suspension tests show fore- and hind-limb grip strength impairments; (3) At 15-16 months, motor deficits suggest global dysfunction in multiple domains of motor function; (4) However, after 12 months of age, 5xFAD mice frequently showed abnormal hindlimb clasping when suspended by the tail; (5) At 9 and 12 months of age, heavier 5xFAD mice performed better in the rotarod; (6) From 6 months of age onwards, they showed less activity in the home cage and had higher frailty scores.	(O'Leary et al., 2020)
	11, 13 months	Females	(1) 5xFAD mice had a short, shuffling gait with a shorter stride length than WT mice and had a slower swim speed; (2) 5xFAD mice fell faster than WT mice in the balance beam, wire suspension and grid suspension tasks, indicating balance and grip strength dysfunctions; (3) 5xFAD mice showed hindlimb clasping, weighed less and had a slower righting reflex than WT mice; (4) 5xFAD mice exhibited deficits in motor coordination and motor learning.	(O'Leary et al., 2018)

Tg4-42	3, 7 months	Females	(1) Tg4-42 mice at 7 months have impaired balance, and motor coordination, strength and locomotor activity are unaffected; (2) Mice develop motor deficits before memory deficits; (3) Mice did not show impairment of muscle strength; (4) Mice did not show a limb clasping phenotype; Impairment in the rotarod test was observed in young Tg4-42 mice and worsened with age.	(Wagner et al. 2019)
Tg2576	10 months	Males Females	(1) Decreased stride length and stride time at 10 months, base of support, stride time variability, stride length variability, cadence, phase dispersions and gait symmetry indices were not altered.	(Nyul-Toth et al., 2021)
TgCRND8	3, 9 months		(1) Early onset of motor deficits in TgCRND8 mice at the age of 3 months; Hindlimb extension reflex test and abnormal for 3 months.	(Yuan et al., 2017)
THY-Tau22	12 months	Males	(1) Hyperactivity in activity exploration, locomotor activity preserved; (2) Clasping, present in males at 12 months of age.	(Van der Jeugd et al., 2013)
Tau 58/4 P301S	3, 6, 9, 12 months	Males	(1) At 6, 9 and 12 months of age, the gait shows a decrease in stride length; (2) At 9 months of age, shows hindlimb clasping; (3) Motor dysfunction in the rotarod at 3 months of age increases with age and is severe at 12 months of age; (4) In Tau 58/4 mice, developmental denervation of the neuromuscular junction is accompanied by progressive muscle hypotrophy, indicating denervation-mediated atrophy.	(Yin et al., 2017)
P301S	1.5 to 5.5 months	Females	(1) From 2-3 months of age, the mice showed marked hyperactivity. These behavioural impairments did not progress with age; (2) Gait impairment, stride length and slowing of swing time of both limbs, as well as widening of the forepaws base of support; (3) Progressive development of hindlimb clasping. It started consistently in all mice around 4 months of age and reached its maximum extension at 5 months of age.	(Koivisto et al., 2019)
APP/PS1	3, 9 months	Males Females	(1) Hyperactivity and normal locomotion at 3 months and increased at 9 months of age; (2) APP/PS1 mice showed impaired motor and spatial memory and increased locomotor activity at 9 months of age.	(Lok et al., 2013)
	6, 12 months	Males Females	(1) Motor impairments at 12 months of age; (2) Significant motor deficits in the rotarod at 12 months of age.	(Leroy et al., 2012)
	7 to 15 months	Males	(1) From 9 months of age onwards, open-field activity decreases; (2 Motor coordination was normal in APP/PS1 mice and did not seem to decrease with age.	(Ferguson et al., 2013)
APP	12 months	Males Females	(1) Motor learning and coordination were impaired in APP mice.	(Manczak et al., 2018)
	5, 6 months	Males Females	(1) Increased spontaneous locomotor activity and increased locomotion in open-field test.	(Klevanski et al., 2015)
APLP2-KO	3, 11 months	Males Females	(1) Females show motor function impairments; (2) APLP2-KO males showed a similar reduction in the rotarod performance as WT males, while females performed better; (3) In males at 12 weeks, a significant reduction in α-motor neurons was detected, but APLP2-KO locomotor function does not appear to be affected; (4) There are sex differences related to the distribution of motor neurons and muscle fibres that may explain the improved motor performance observed in female APLP2-KO mice during ageing.	(Truong et al., 2019)

CHAPTER 2. HYPOTHESIS, OBJECTIVES

HYPOTHESIS:

- Primary structural dysfunctions, such as kyphosis, modify and limit the exploratory activity in 3xTg-AD mice.
- Bizarre behaviours constitute secondary dysfunctions that accompany dry and wet test performance and alter the development of MWM tests and exploratory activity.
- Primary and secondary dysfunctions can be modulated by external factors such as isolation and retesting of psychomotor batteries.

OBJECTIVE:

The main aim of this work is to establish primary and secondary motor signatures and their integration in psychomotor dysfunction in experimental models of normal ageing and Alzheimer's disease.

SPECIFIC OBJECTIVES:

PHASE 1: CHARACTERISATION OF PRIMARY AND SECONDARY MOTOR SIGNATURES OF PSYCHOMOTOR DYSFUNCTION IN NORMAL AGEING AND ALZHEIMER'S DISEASE

1. With respect to flotation and bizarre circling in the MWM:
   1.1. To identify the distinctive expression patterns of flotation and bizarre circling in 6-month-old male 3xTg-AD mice mimicking early disease stages and in age-matched non-transgenic counterparts with normal ageing.
   1.2. To assess whether flotation and circling patterns are also sensitive to the accelerated neurobiology of ageing, by chronic administration of D-galactose.
2. To differentiate dysfunctions, gait disorders and exploration in the 3xTg-AD model at different stages of Alzheimer's disease progression (early, middle and advanced) compared to NTg with normal ageing.
3. To characterise the motor dysfunction of the male 3xTg-AD mouse model from early (6 months of age) to advanced stages of the disease (12 and 16 months of age) in different motor tasks, focusing on the abnormal clasping reflex and coordination impairments measured through the Phenotype Scoring System.
4. To identify the most useful mouse models used in research based on the biological hypothesis of human frailty syndrome.

PHASE 2: MODULATION AND INTEGRATION OF PRIMARY AND SECONDARY PSYCHOMOTOR SIGNATURES OF ALZHEIMER'S DISEASE DYSFUNCTION AND EXTRINSIC FACTORS

5. Isolation scenario:

    5.1. To explore the psychomotor performance of 13-month-old male NTg and 3xTg-AD mice, corresponding to normal ageing and advanced stages of Alzheimer's disease, respectively.

    5.2. To assess the impact of isolation in a subgroup of male 3xTg-AD mice that lost their partners and lived alone for the last 2-3 months after ten months of social life.

6. In an end-of-life scenario:

    6.1. To identify and characterise kyphosis and bizarre gait patterns associated with functional limitations of gait and exploratory activity in male 3xTg-AD and NTg mice at the age of 16 months, corresponding to advanced stages of disease or ageing, respectively.

7. To determine the effects of retesting on the behavioural performance of animals tested in two scenarios:

    7.1. In a longitudinal design, with within-subjects analysis of a set of 12-month-old animals retested four months later, at 16 months of age;

    7.2. In a cross-sectional design, comparing 16-month-old animals that had (retested) or had not (naïve) experienced the test battery.

CHAPTER 3. METHODOLOGY

METHODOLOGY

Phase 1, 6, and 12-month-old 3xTg-AD and NTg male mice were included. A battery of psychomotor tests was used that had: 1) Morris Water Maze (MWM); 2) Phenotype Scoring System; 3) Physical Frailty Phenotype, 4) Spontaneous Gait Phenotype - Exploratory Activity; 5) Quantitative parameters of gait; 6) Muscular Strength - grip strength of the forelimbs and Muscular Endurance - suspension test, 7) Motor performance: learning, physical endurance and coordination - Rotarod, and 8) Geotaxis.

In the second phase, male 3xTg-AD and C57BL/6 mice aged 13 and 16 months were included. Male and female 3xTg-AD and NTg mice aged 12 and 16 months were also included. The battery of psychomotor tests similar to phase 1 was applied, except for the MWM, which was not included. Also, an analysis of the HPA axis and sarcopenia measured in the quadriceps and triceps surae muscles was incorporated.

All animals were housed at the Autonomous University of Barcelona (Baeta-Corral and Giménez-Llort, 2014). 3xTg-AD mice harbouring transgenes were genetically modified at the University of California, Irvine, as previously described (Oddo, et al. 2003a). Mice were kept in groups of 3-4 mice per cage (Macrolon, 35 × 35 × 25 cm) filled with 5 cm of clean wood cuttings (Ecopure, Chips6, Date Sand, UK; wood pellets of uniform cross-section). with 2.8-1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm x 20 cm, White). Evaluations were performed under dim white light (20 lx) during the light cycle of the light: dark cycle (10 am to 1 pm). Behavioural evaluations were performed between 1 to 5 days, depending on the experiment. The experimental groups were counterbalanced by the observation of two independent observers blinded to the genotype. The tests were carried out during the morning; Animals were allotted 30 minutes to habituate in the testing room before starting measurements. All procedures followed the Spanish legislation on "Protection of animals used for experimentation and other scientific purposes" and the EU Directive (2010/63/EU) 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). For each study, the specific methodology used is described.

CHAPTER 4. EXPERIMENTAL RESEARCH

PHASE 1: CHARACTERISATION OF PRIMARY AND SECONDARY MOTOR SIGNATURES OF PSYCHOMOTOR DYSFUNCTION IN NORMAL AGEING AND ALZHEIMER'S DISEASE

Research articles:

A. Indices for flotation and circling, two non-search behaviours in the water maze, sensitive to D-galactose-induced accelerated ageing and Alzheimer's disease
B. Modelling Functional Limitations, Gait Impairments, and Muscle Pathology in Alzheimer's Disease: Studies in the 3xTg-AD Mice
C. Clasping and ledge-score coordination impairment as primary behavioural markers of functional impairment in Alzheimer's disease (accepted)

Review article:

D. Frailty, from Humans to Mouse Models

Proceeding:

Annex 1: Bizarre behaviours limit exploratory activity and impair spontaneous gait performance in aged mice with AD pathology. 2nd International Electronic Conference on Brain Sciences (2021) MPDI, doi:10.3390/IECBS2021-10671.

Poster presentation:

Annex 2: Bizarre circling behaviour and floating in the MWM and the effects of D-galactose-induced accelerated aging in 6-months-old 3xTg-AD mice and NTg mice. VI Scientific conferences of the INc-UAB (2018)

Annex 3: Fragilidad física y sistémica, situaciones de novedad y supervivencia en el envejecimiento y la enfermedad de Alzheimer: una aproximación traslacional a las residencias de larga estadía. XXIV Congreso de Geriatría y Gerontología de Chile (2020).

Annex 4: Gait impairments and functional limitations in the exploratory activity in an animal model of Alzheimer's disease. EUROPEAN JOURNAL OF NEUROLOGY (2021).

Annex 5: Bizarre behaviours limit exploratory activity and impair spontaneous gait performance in aged mice with AD pathology. 2nd International Electronic Conference on Brain Sciences (2021).

Annex 6: Hindlimb clasping, kyphosis and piloerection: Frailty markers from middle to very old ages in mice. EUROPEAN JOURNAL OF NEUROLOGY (2021).

INDEXES FOR FLOTATION AND CIRCLING, TWO NON-SEARCH BEHAVIOURS IN THE WATER MAZE, SENSITIVE TO D-GALACTOSE–INDUCED ACCELERATED AGING AND ALZHEIMER'S DISEASE

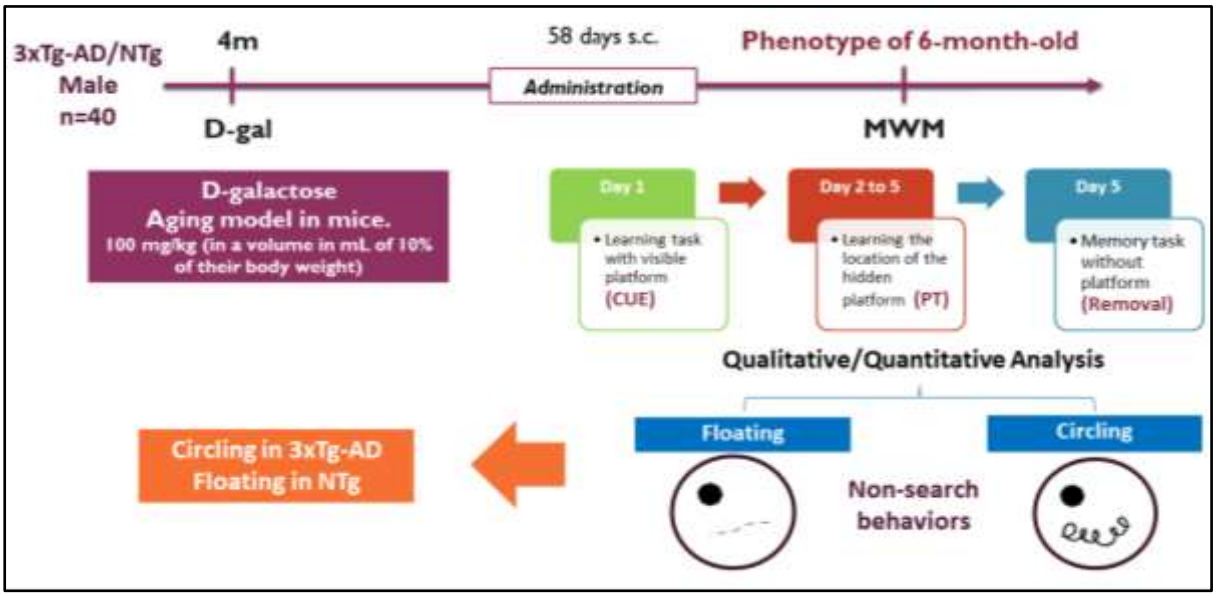
In this work, floating and circling behaviour was quantitatively and qualitatively investigated, the two most common non-search behaviours elicited in the Morris water maze. Male 3xTg-AD and non-transgenic 6-month-old mice were included. In addition, to assess whether these patterns are also sensitive to the accelerated neurobiology of ageing, D-galactose (D-gal) was chronically administered.

Specific objectives

1.1. To identify the distinctive expression patterns of flotation and bizarre circling in 6-month-old male 3xTg-AD mice mimicking early disease stages and in age-matched non-transgenic counterparts with normal ageing.

1.2. To assess whether floating and circling patterns are also sensitive to the accelerated neurobiology of ageing, by chronic administration of D-galactose.

Experimental design and D-galactose treatment



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Indexes for flotation and circling, two non-search behaviors in the water maze, sensitive to D-galactose–induced aging and Alzheimer's disease

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The existence of behavioral and psychological symptoms of dementia (BPSD) has been largely neglected in most experimental research on Alzheimer's disease (AD) classically focused on cognitive symptoms. The aquatic environment of the Morris water maze (MWM) implies a stressful condition for mice leading to lower cognitive performances and presence of other behaviors related to emotionality, that can be critical in animal models such as the 3xTg-AD mice that exhibit a noticeable profile resembling BPSD. The present work is aimed to provide a quantitative (number of episodes and duration) and qualitative (prevalence) analysis of flotation and circling, the most common 'non-searching behaviors' elicited in the MWM in order to study the expression of these behaviors in 6-month-old non-transgenic (C57BL/6) and 3xTg-AD mice with normal or submitted to chronic D-galactose induced accelerated aging. Thus, we recorded the elicitation of floating and circling during three standard MWM paradigms: visual perceptual learning, place task for spatial reference memory and a final probe trial for short-term memory. In view of the results, we propose a 'flotation index' as characteristic of non-transgenic performance, that is sensitive (reduction) to accelerated aging and AD. Conversely, circling behavior, more characteristic of 3xTg-AD mice, can be an additional tool for evaluating BPSD-like symptoms in AD models while its index unveils bizarre behavior induced by D-galactose induced aging. These results can be useful in relation to preventive and/or therapeutical strategies targeted at AD but they may also be suitable in the evaluation of the potential risk factors in normal animals.

Key words: Aging; Alzheimer; Flotation; Bizarre; Circling; BPSD; D-galactose

1. Introduction

Clinical and experimental research struggle to provide tools for identification and prevention of risk factors for accelerated age-related cognitive decline in the general population, their early diagnosis and treatment. As well, strong scientific efforts are aimed to reduce the burden that dementias, like Alzheimer's disease (AD), are predicted to cause on patients and caregivers in the next decades (Alzheimer's association, 2016; Cavedo *et al.*, 2014). In this scenario, apathy, depression, stereotyped behaviors, and anxiety are, among others, early signs of disease also refered as BPSD (behavioral and psychological symptoms of dementia), considered the most important source of distress that leads to institutionalization (Tan, Wong and Allen, 2005). In a general basis, at the translational level, the study of the expression of these common neuropsychiatric symptoms associated to aging but also quite prevalent among patients has been largely deserted as compared to cognition (reviewed by Giménez-Llort *et al.*, 2007).

In the Morris Water Maze (MWM) (Morris et al., 1984), several paradigms are used to assess hippocampal-dependent spatial learning and memory of the location of a platform (reviewed by D'Hooge and De Deyn, 2001). However, as compared to rats for whom the test was designed, the aquatic environment of the maze results in a stressful condition for mice, leading to lower cognitive performances and presence of other confunding behaviors mostly related to

emotionality (Lang et al., 2003; Janus, 2004; Brody and Holtzman, 2006). The mice usually start their navigation swimming along the walls before they develop a 'goal-directed' search strategy aimed to reach the platform which finally will be efficiently used during a probe trial. Others, just continue swiming along the walls or gradually adopt a chainning search strategy, which can be quite effective. During the acquisition of the task, mice also may exhibit flotation (inactivity without forward movement), which could be related to lack of motivation, even as a kind of behavioral despair (Porsolt *et al.*, 1977) or it may be attributable to the use of 'non-search' behavioral strategies assumed to be 'non-cognitive' (Janus, 2004).

In the case of murine models of Alzheimer's disease, most of the basic research using this water maze has being devoted to depict the distinct features of their impaired cognition and the effects of treatments. As in other cognitive tests, the presence of BPSD-like behaviors in the MWM are considered confounding factors that interfere in the analysis of cognition *per se*. However, in most cases, they are usually disregarded because of their low incidence and/or short duration, since experimental designs are adapted or animals are previously handled or trained to avoid/prevent their elicitation. Also, changes in emotionality or motivation can be specifically monitored by cue tasks or indirectly measured through swimming speed (Giménez-Llort et al., 2013). More translational research approaches, targeting the complex clinical scenario of cognitive and non-cognitive symptoms in the human patient, include on purpose the evaluation of BPSD-like behaviors in the so called 'non-search behaviors' (Janus, 2004; Baeta-Corral and Giménez-Llort, 2015).

Recently, the analysis and classification of floating and bizarre circling (swimming in closed circles) as 'non-search' behaviors exhibited in the MWM was proposed by our laboratory as a tool to better reflect both the AD-phenotype of 13-month-old 3xTg-AD mice (Oddo et al., 2003) mimicking advanced disease stages and that of mice with normal aging (Baeta-Corral and Giménez-Llort, 2015). Since the 3xTg-AD mice have a noticable BPSD-like profile (Giménez-Llort *et al.*, 2006; 2014) mimicking the high prevalence of BPSD among patients at early stages of disease, qualitative and quantitative analysis can be useful as a tool for their identification, assessment and monitoring in the different experimental paradigms used. On the other hand, the effort to define these profiles will also help to reduce the length of the behavioral battery animals are confronted to.

Therefore, the aim of the present work was to identify distinctive patterns of expression of flotation and bizarre circling, the two most common 'non-search' behaviors elicited in the MWM, in 6-month-old 3xTg-AD mice mimicking early stages of disease and age-matched non-transgenic counterparts with normal aging. Also, to evaluate if these patterns are also sensitive to accelerated neurobiology of aging, by the evaluation of the effects of chronic administration of D-galactose (D-gal) an intervention enhancing mitocondrial oxidative stress, that is also one of the cellular hallmarks of AD (Cabezas-Opazo et al., 2018).

2. Materials and methods

2.1. Animals

A total number of forty 6-month-old male mice from the Spanish colonies of homozygous 3xTg-AD and non-transgenic (NTg) mice in a C57BL/6 background were used. The 3xTg-AD mice were genetically engineered at the University of California Irvine as previously described (Oddo et al., 2003). Genotypes were confirmed by RT-PCR analysis. Animals were housed three or four per cage and maintained in Macrolon cages (35 × 35 × 25 cm) under standard laboratory conditions of food and water ad libitum, 22 ± 2 ºC, a 12 h light:dark cycle and relative humidity 50–60%. Behavioral assessments were performed blind to the experiment, in a counterbalanced manner under the approval of local policy 2481CEEAH/8700DMAH Generalitat de Catalunya, in accordance with Spanish legislation and the EU Directive (2010/63/UE) on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the EU Directive (2010/63/UE) on this subject, protocol CEEAH. The study complies with the ARRIVE guidelines developed by the NC3Rs and the aim to reduce the number of animals used.

2.2.Experimental design and D-galactose treatment

At 4 months of age, animals were randomly distributed in experimental groups: NTg mice treated with saline (NTg, Sal) or D-galactose (NTg D-gal), 3xTg-AD mice treated with saline (3xTg-AD Sal) or D-galactose (3xTg-AD D-gal). Thus, animals were subcutaneously administered with saline or D-gal, with a dose of 100 mg/kg (in a volume in mL of 10% of their body weight) during 58 consecutive days. The injection was rotationally applied on the back on the neck and the two sides of the body.

2.3. Behavioral assessment and quantitative/qualitative analysis

Flotation is defined as a state of inactivity without forward movement. Bizarre circling is defined as swimming in tight circles, often showing a general directional movement. The plotted path

is short and wide when tight circles are performed or has many visible loose loops when is performed with a random search strategy (Janus, 2004).

Behavioral features of flotation and circling in the MWM were studied using two paradigms of increasing difficulty, as previously described (Baeta-Corral and Giménez-Llort, 2015). Briefly, first, a 1-day task for cue learning of a visible platform (CUE, four 60s trials, every 20 min) followed, 24h later, by a 4 days place learning task using a hidden platform (PT1 to PT5, three 60 s trails, every 20 min). A final probe trial without the platform (Removal, 60s, 2h after the last PT5 trial) assessed their short-term memory. The prevalence and number of episodes of flotation and bizarre circling, as well as the number of animals per group exhibiting them was meticulously analyzed based on the video recordings of each MWM trial and their mean per day. In the first instance, the visual recognition of non-directed strategies "swim in circles" and "flotation" was carried out. Subsequently, the quantitative analysis was carried out with the Smart software. Finally, the manual records of the test performed by the examiner, blind to the experimental group, were compared and the analysis and assignment of the information to each experimental group was continued.

The index of flotation and index of circling were defined as the prevalence of each episode (floating and circling, respectively) for each experimental group with respect to the total of episodes of each task (CUE / PT / RM). The results were differentiated into (A) Test Paradigms, (B) Day by day. Total episodes and number of animals exhibiting episodes were illustrated for index and total episodes, and (D) trial by trial on the MWM. The floating time was defined as the average (mean ± SEM) of the time the animals remained floating in each of the groups. In addition, the floating time was differentiated according to the day and the trial.

2.4 Statistics

The statistical analysis was made in SPSS version 15. The factors studied were: Genotype (G), chronic treatment with D-galactose (T) and Day (D). Circling and Floating (presence / absence), were assessed by Chi square test or Fisher's F. The effect of the flotation time in each of the trial tests was obtained by the Friedman test. In all cases, $P<0.05$ was considered statistically significant.

3. Results

Figures 1, 2 and 3 illustrate the qualitative and quantitative analyses of floating and circling in the different paradigms of the Morris water maze.

In Figure 1 we summarize the main results related to prevalence and incidence of floating, measured for each of the three paradigms (Fig. 1A), detailed day-by-day for each of the four days of the place task (Fig. 1B), measured as total number of floating episodes (Fig. 1C) or as total number of animals exhibiting at least one episode of flotation in a trial (Fig. 1D). The results of the NTg groups show a high prevalence of floating in both CUE and PT acquisition tasks and the RM memory test, reaching 54% in the total of the trial tests (17 trials). In contrast, 3xTg-AD mice showed less than 20% of prevalence of floating along the three paradigms, and in each of the days and trials. The strongest between genotype difference was found in the CUE and the first (PT1) and second trial (PT2) of the place task, and it was smoothest in the next days (PT3,PT4). Chronic treatment with D-gal induced an overall lower incidence and total number of floating episodes in both groups mice, that reached statistical significance in PT, the place learning task. The effect of D-galactose in 3xTg-AD was so notorius that completely abolished the presence of this behavior during most of the trials.

In Figure 2, the mean floating time is represented for each of the three paradigms (Fig. 2A), detailed day-by-day for each of the four days of the place task (Fig. 2B) and for each trial (Fig. 2C). As shown, mean floating time was high in the NTg group reaching a maximum value of 48% in the PT, half of the 60 seconds of each trial. The low number of flotation time in the 3xTg-AD group resulted here in very scarce time. The effects of chronic treatment with D-gal are here also clearly shown, expressed in time, and emphatize the effect on NTg.

In Figure 3 for circling behavior, the results of the NTg group show a low prevalence in both CUE and PT acquisition tasks and that circling was completely absent in the memory test. In contrast, 3xTg-AD mice showed from 10 to 6% of prevalence of circling along the three paradigms, a difference that reached statistical difference as compared to NTg mice in the three paradigms. Chronic treatment with D-gal induced a number of circling episodes in NTg mice during the PT place task resembling the pattern shown in the 3xTg-AD group. This observation was restricted to the learning tasks, since circling was absent in the probe trial. The group of 3xTg-AD treated with D-gal presented a low prevalence of circling in all the tasks, as measured by the number of episodes and per the day of testing.

4. Discussion

The present study provides a detailed qualitative and quantitative analysis of floating and circling, two non-search behaviors elicited in the MWM that allowed to clearly differentiate 6-month-

old (onset of disease) 3xTg-AD mice from NTg mice with normal aging. Notoriously, NTg animals showed a high frequency of flotation and 3xTg-AD mice a higher frequency of circling. Thus, the present results confirm and extend our previous results were flotation in NTg mice and circling in 3xTg-AD mice were described as discriminative non-search behaviors in 13-month-old animals, corresponding to middle age and advanced stages of disease, respectively.

The age of 6 months was chosen because in the 3xTg-AD model of AD it corresponds to the onset of disease, with only intraneuronal beta-amyloid oligomers being detected in the hippocampus and the basolateral amygdala underlying their cognitive deficits and anxious-like profile (España *et al*., 2010). Besides, this adult age would help us to analyze these behaviors without confounding factors related to advanced age while providing an estable adult scenario where to observe the impact of an induced accelerated aging. Thus, the present work also describes the dysfunctional effects on these behaviors by increase of mitocondrial oxidative stress induced by D-galactose 100mg/kg, that reduced the flotation pattern and elicited circling in the NTg while worsen the low profiles of 3xTg-AD mice.

Among the different qualitative variables, the prevalence of flotation resulted the most sensitive to describe the NTg profile and differentiate the 3xTg-AD genotype, as well as to detect the effects of D-galactose induced accelerated aging, thus being proposed as a discriminative index. In contrast, the time of flotation was a sensitive variable to quantify the effects of D-galactose in flotation and circling, mostly in the NTg mice. Independently of the paradigm, sensitivity is expected to depend on the number of observations. Therefore PT (12 trials) would be better than CUE (4 trials) and RM (1 trial). Also the learning process (day effect) will imply a evolution in the Flotation Index, as it happens in the FST (increase of immobility in a non-escapable paradigm) but here it is the opposite because there is a chance to achieve the goal by learning in an escapable paradigm. The floating may be due to lack of motivation or behavioral despair, but the findings indicate that the young non-transgenic mice show the most floating behavior and accelerated aging reduced floating. Also, it is possible that some mice may float because they are able to get their bearings without exertion. It is still not clear whether a high flotation index is beneficial or detrimental in the context of cognitive performance.

We have previously shown, in the classical forced swimming test (FST) paradigm modeling behavioural despair in animals by loss of motivation to respond or the refusal to escape (Porsolt *et al*., 1977), that the 3xTg-AD mice showed persistance of swimming behaviours and scare immobility over time (Torres-Lista and Giménez-Llort, 2014).This paradoxical behavioural response was discussed as a lack of ability of 3xTg-AD mice to shift behaviour over time, poorest cognitive flexibility and copying with stress strategies. In agreement with that interpretation, we showed that it could be hampered by sensorial and environmental stimulation known to modulate emotional states and hamper aging processes (Torres-Lista and Giménez-Llort, 2015). Here, in the opposite scenario of accelerated aging, the flotation pattern of NTg mice was found reduced in animals treated with D-galactose being closer to the pattern expressed by 3xTg-AD mice. Similarly, in 3xTg-AD mice treated with D-galactose the low levels of flotation were reduced to null.

Stereotypies and other aberrant movements are scarcely studied in the literature, mostly because their low incidence and short duration, as well as the specificity of experimental conditions in which they are quantifiable. Observations of bizarre behaviors in the 3xTg-AD mice in anxiety tests and their reserval by early postnatal handling suggest that these behaviors are related to strategies to overcome the stress (Baeta-Corral and Giménez-Llort, 2014). Here, circling, which could be considered as a stereotyped behavior to cope with stress, appeared as a bizarre behavior, characteristic of 3xTg-AD mice. The evidence presented of D-galactose reducing the circling index in the 3xTg mice may highlight a positive effect of the approach (Figure 3A, RM data). The trajectory was far from the single loop that animals do to get oriented in the maze. In agreement with this bizarre consideration, it was induced by D-galactose in NTg mice that otherwise will scarcely show it. Besides, with D-galactose, the patterns of flotation and circling at 6 months of age become similar to those observed in 13-months-old animals (Baeta-Corral and Giménez-Llort, 2015). This would be in agreement with D-gal inducing oxidative stress *in-vivo*, which resembles the natural aging process in mice. In fact, it is widely reported that chronic administration of D-gal contributes to the progress of aging, mild neuronal damage, memory defects, and prominent changes in the first stage of AD (Ho et al., 2003; Qu et al., 2016). In 6-month-old males, we have recently shown that in the 3xTg-AD mice the accelerated aging effects of D-gal are exerted at the sensorimotor level and in the immunoendocrine system in males (Baeta-Corral *et al*., 2018). Therefore, based on these current and previous findings (Baeta-Corral and Giménez-Llort, 2018) it could be said that chronic D-gal, which is considered a good animal model for the study of memory impairment and the molecular mechanisms involved in aging and

neurodegeneration associated with age, can be also useful to study the modulation of emotional and bizarre behaviors.

5. Conclusions

In summary, the present study shows that the expression of floating and circling behaviors, the two non-search behaviors during three standard MWM paradigms, namely visual perceptual learning, place task for spatial reference memory and a final probe trial for short-term memory, are sensitive to the accelerated aging induced by chronic D-galactose and the AD-genotype. We propose a 'flotation index' as characteristic of non-transgenic performance, that is sensitive (reduction) to accelerated aging and AD. Conversely, a 'circling index' measuring this bizarre behavior characteristic of 3xTg-AD mice, can be an additional tool for evaluating BPSD-like symptoms in AD models while it unveils the bizarre behavior elicited by D-galactose-accelerated aging in normal mice.These results can be useful in relation to preventive and/or therapeutical strategies targeted at AD but it may also be suitable in the evaluation of the potential risk factors in normal animals.

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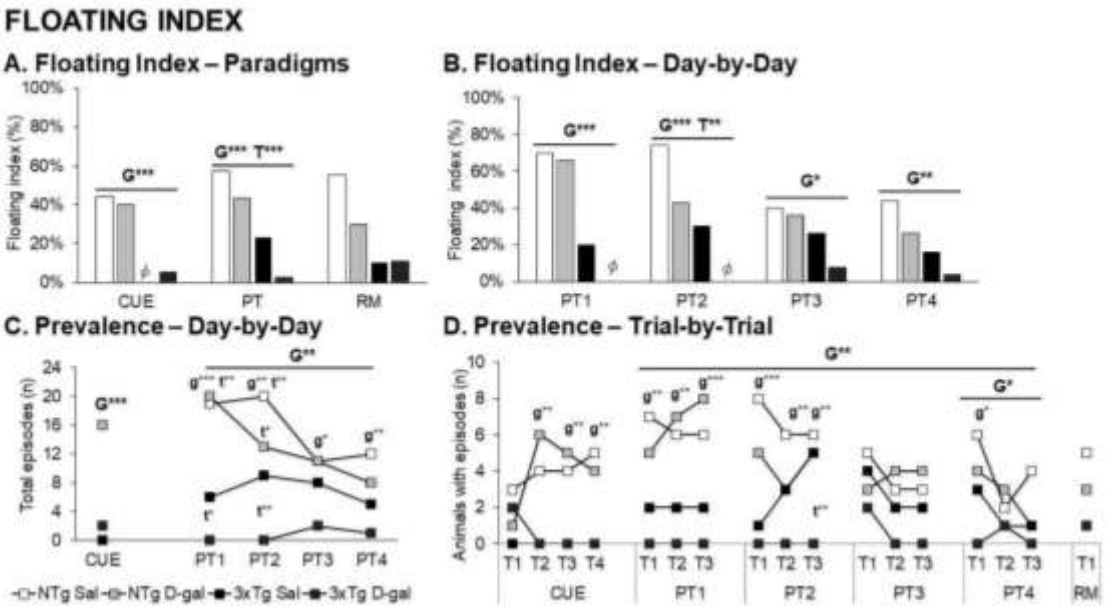


Fig 1. Flotation Index in 6-month-old (onset of disease) male 3xTg-AD mice in the different paradigms of the Morris water maze, as compared to age-matched NTg mice with normal aging and the effect of D-galactose on them. Results are expressed as percentage, number of episodes or animals. *n*=8-10 per group. A) Floating prevalence index: Prevalence of floating in the three paradigms for learning and memory: CUE, cue learning task; PT, place learning task; RM, probe trial for memory. B) Prevalence in the 4 days of the PT is illustrated, day by day. C) Total episodes of floating in the each day of the 5 days of test in the MWM. D) Total number of animals exhibiting at least one episode of flotation per trial, in a trial by trial analysis during the tests. * *P<0.05*,** *P<0.01*,*** *P<0.001*.

A) CUE: Fisher exact test (d.f.1) 34,098, G*** / PT: Fisher exact test (d.f.1) 69,661, G*** ; Chi-squared test (d.f.1) 12,387, T***.
B) PT1: Chi-squared test (d.f.1) 39,983, G*** / PT2: Chi-squared test (d.f.1) 21,714, G*** ; Chi-squared test (d.f.1) 9,651. T** / PT3: Chi-squared test (d.f.1) 6,256, G* / PT4: Chi-squared test (d.f.1) 9,766, G**.
C) CUE: U Mann Whitney test (d.f.1) 18,500, G*** / PT1-4: Friedman test (d.f.3) 16,527, G** / PT1: U Mann Whitney test (d.f.1) 9,000, g*** ; U Mann Whitney test (d.f.1) 42,500, t** ; U Mann Whitney test (d.f.1) 324,000, t* / PT2: U Mann Whitney test (d.f.1) 13,500, g**; U Mann Whitney test (d.f.1) 283,500, t** ; U Mann Whitney test (d.f.1) 280,500, t* / PT3: Kruskal Wallis test (d.f.3) 4,471, g* / PT4: Kruskal Wallis test (d.f.3) 8,440, g**.
D) CUE: T2 Kruskal Wallis test (d.f.3) 13,790, g** ; T3 Kruskal Wallis test (d.f.3) 11,562, g** ; T4 Kruskal Wallis test (d.f.3) 12,100, g** / PT1-4: Friedman test (d.f.11) 29,788, G** ; PT1-T1: Kruskal Wallis (d.f.3) 13,334, g** ; PT1-T2: Kruskal Wallis (d.f.3) 13,770, g** ; PT1-T3: Kruskal Wallis (d.f.3) 16,230, g*** / PT2-T1: Kruskal Wallis (d.f.3) 19,053, g*** ; PT2-T2: Kruskal Wallis (d.f.3) 9,060, g** ; PT2-T3: Kruskal Wallis (d.f. 3) 9,040, g** ; PT2-T3: U de Mann Whitney (d.f.1) 22,500, t* / PT4: Friedman test (d.f.2) 7,167, G* ; PT4-T1: Kruskal Wallis (d.f.3) 8,880, g*.

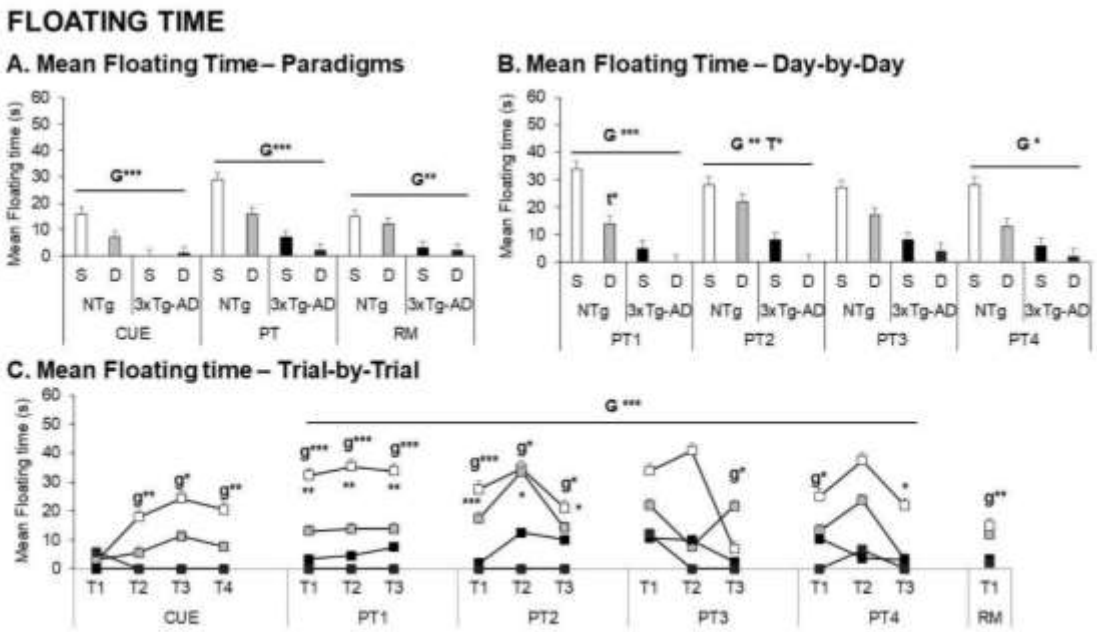


Fig 2. Floating time in 6-month-old (onset of disease) male 3xTg-AD mice in the different paradigms of the Morris water maze as compared to age-matched NTg mice with normal aging and the effect of D-galactose on them. Results are expressed as mean floating time in seconds. A) Mean floating time in the three paradigms for learning and memory: CUE, cue learning task; PT, place learning task; RM, probe trial for memory. B) Mean floating time in the 4 days of the PT is illustrated, day by day. C) Mean floating time in a trial by trial analysis during the tests. Results are expressed as mean floating time in seconds. *n*=8-10 per group. * *P<0.05,*** *P<0.01,**** *P<0.001.*

A) CUE: U de Mann Whitney (d.f.1) 59,500, G*** / PT: U de Mann Whitney (d.f.1) 50,000, G*** / U de Mann Whitney (d.f.1) 116,500, G**.
B) PT1: U de Mann Whitney (d.f.1) 24,000, G*** ; U de Mann Whitney (d.f.1) 18,500, t* / PT2: U de Mann Whitney (d.f.1) 73,000, G** ; U de Mann Whitney (d.f.1) 115,500, T* / PT4: U de Mann Whitney (d.f.1) 106,000, G*.
C) CUE: T2: Kruskal Wallis (d.f.3) 12,890, g** ; T3: Kruskal Wallis (d.f.3) 11,215, g* ; T4: Kruskal Wallis (d.f.3) 12,442, g** / PT1-4 Friedman (d.f.11) 33,711, G*** ; PT1-T1: Kruskal Wallis (d.f.3) 16,341, ** ; U de Mann Whitney (d.f.1) 73,500, g*** ; PT1-T2 Kruskal Wallis (d.f.3) 15,660, ** ; U de Mann Whitney (d.f. 1) 63,000, g*** ; PT1-T3: Kruskal Wallis (d.f.3) 16,215, ** ; U de Mann Whitney (d.f.1) 58,500, g*** / PT2-T1: Kruskal Wallis (d.f.3) 20,259, *** ; U de Mann Whitney (d.f.1) 60,000, g*** ; PT2-T2: Kruskal Wallis (d.f. 3) 10,015, * ; U de Mann Whitney (1) 113,000, g* ; PT2-T3: Kruskal Wallis (d.f. 3) 8,917, * ; U de Mann Whitney (d.f.1) 117,000, g* / PT3-T3: U de Mann Whitney (d.f.1) 127,000, g* / PT4-T1: U de Mann Whitney (d.f.1) 112,000, g* ; PT4-T3: Kruskal Wallis (d.f.3) 8,579, * / RM: U de Mann Whitney (d.f.1) 116500, g**

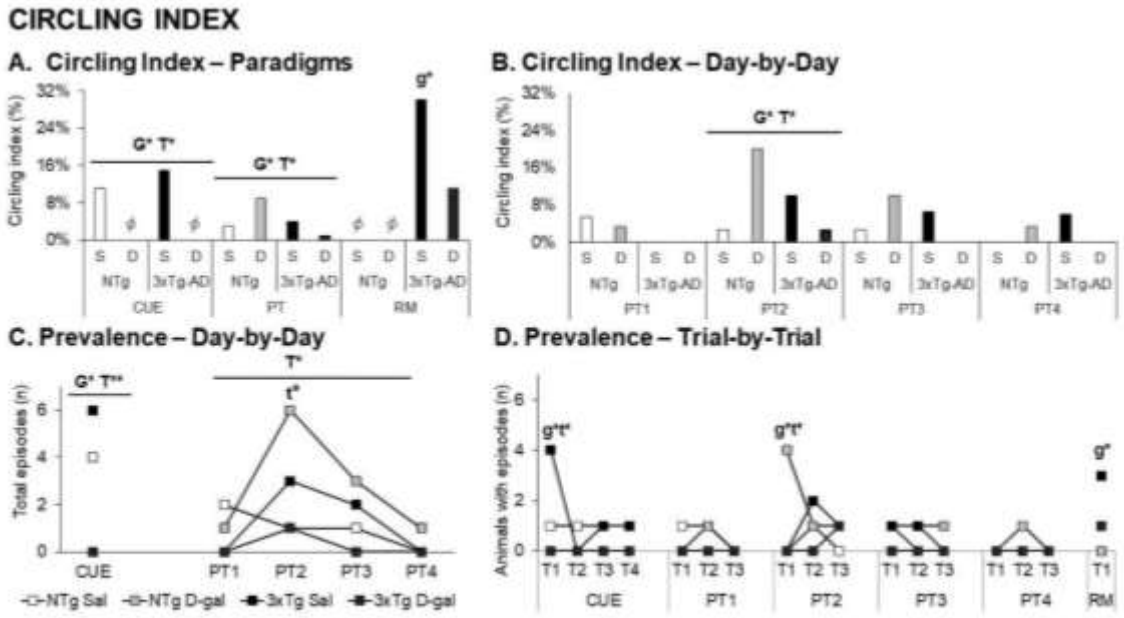


Fig 3. Circling Index in 6-month-old (onset of disease) male 3xTg-AD mice in the different paradigms of the Morris water maze as compared to age-matched NTg mice with normal aging and the effect of D-galactose on them. Results are expressed as percentage, number of episodes or animals. *n*=8-10 per group. A) Circling prevalence index: Prevalence of circling in the three paradigms for learning and memory: CUE, cue learning task; PT, place learning task; RM, probe trial for memory. B) Prevalence in the 4 days of the PT is illustrated, day by day. C) Total episodes of circling in the each day of the 5 days of test in the MWM. D) Total number of animals exhibiting at least one episode of circling per trial, in a trial by trial analysis during the tests.* *P<0.05,** P<0.01,*** P<0.001.*

A) CUE: Fisher exact test (d.f.1) 12,072, G*, T* ; PT: Fisher exact test (d.f.1) 69,661, G*** ; Chi-squared test (d.f.1) 12,387, T*** / RM: U de Mann Whitney (d.f.1) 142,500, g*.
B) PT2: Fisher exact test (d.f.3) 7,309 G*, T*.
C) CUE: Kruskal Wallis (d.f.3) 8,526, G* ; U de Mann Whitney (d.f.1) 254,600 T** / PT1-4: Friedman test (d.f.3) 10,222, T* ; PT2: U Mann Whitney test (d.f.1) 324,000, t*.
D) CUE: T1 Kruskal Wallis (d.f.3) 8,975, g* t* / PT2-T1: Kruskal Wallis (d.f.3) 12,188, g* t* / RM: U de Mann Whitney (d.f.1) 142,500, g*.

Conclusions

1) “Flotation and circling indices” discriminate 6-month-old 3xTg-AD mice from controls.
2) “Flotation index” is characteristic of non-transgenic performance at onset of disease.
3) “Flotation index” is sensitive (reduction) to accelerated ageing and AD genotype.
4) “Circling behaviour” is more characteristic of 3xTg-AD mice also at onset of disease.
5) “Circling index” reveals bizarre behaviour by D-galactose-induced ageing.

MODELLING FUNCTIONAL LIMITATIONS, GAIT IMPAIRMENTS, AND MUSCLE PATHOLOGY IN ALZHEIMER'S DISEASE: STUDIES IN 3XTG-AD MICE

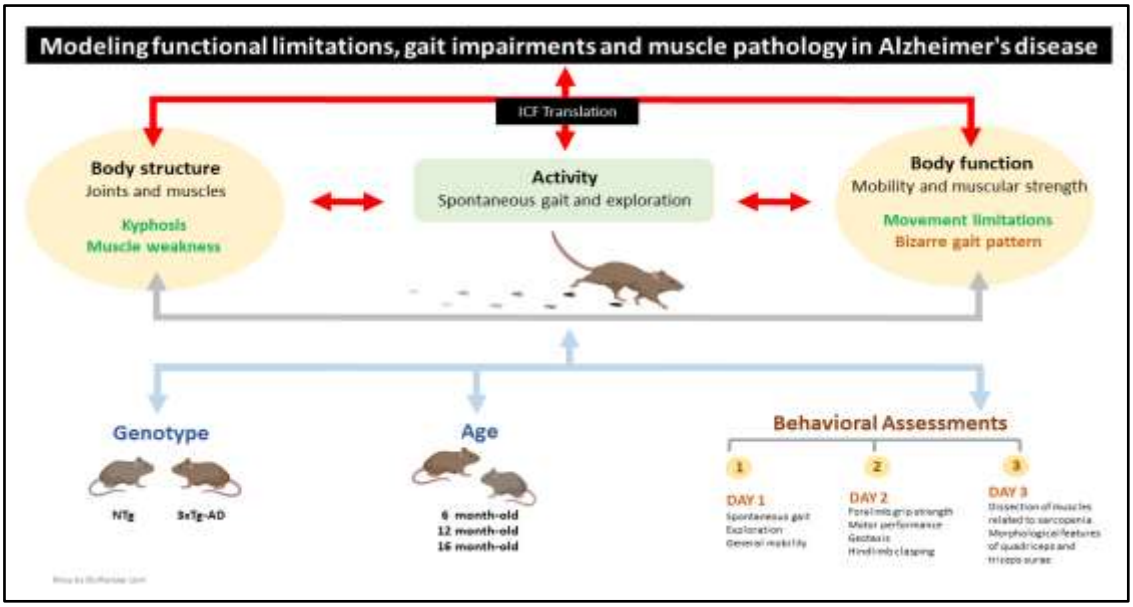
In this work, a behavioural observation method was developed to differentiate qualitative parameters of psychomotor performance in gait and exploratory activity of male 3xTg-AD mice and their non-transgenic (NTg) counterparts at different stages of disease progression in dichotomy with ageing, similar to behavioural patterns observed in humans.

From a translational point of view, the International Classification of Disability and Health Functioning (ICF) proposed the conceptual framework that has been used to classify and describe functioning and disability in gait and exploratory activity in the 3xTg-AD animal model.

Specific objective:

2. To differentiate dysfunctions, gait disorders and exploration in the 3xTg-AD model at different stages of Alzheimer's disease progression (early, middle and advanced) compared to NTg with normal ageing.

Experimental design:



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Modeling Functional Limitations, Gait Impairments, and Muscle Pathology in Alzheimer's Disease: Studies in the 3xTg-AD Mice

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Abstract: Gait impairments in Alzheimer's disease (AD) result from structural and functional deficiencies that generate limitations in the performance of activities and restrictions in individual's biopsychosocial participation. In a translational way, we have used the conceptual framework proposed by the International Classification of Disability and Health Functioning (ICF) to classify and describe the functioning and disability on gait and exploratory activity in the 3xTg-AD animal model. We developed a behavioral observation method that allows us to differentiate qualitative parameters of psychomotor performance in animals' gait, similar to the behavioral patterns observed in humans. The functional psychomotor evaluation allows measuring various dimensions of gait and exploratory activity at different stages of disease progression in dichotomy with aging. We included male 3xTg-AD mice and their non-transgenic counterpart (NTg) of 6, 12, and 16 months of age (n = 45). Here, we present the preliminary results. The 3xTg-AD mice show more significant functional impairment in gait and exploratory activity quantitative variables. The presence of movement limitations and muscle weakness mark the functional decline related to the disease severity stages that intensify with increasing age. Motor performance in 3xTg-AD is accompanied by a series of bizarre behaviors that interfere with the trajectory, which allows us to infer poor neurological control. Additionally, signs of physical frailty accompany the functional deterioration of these animals. The use of the ICF as a conceptual framework allows the functional status to be described, facilitating its interpretation and application in the rehabilitation of people with AD.

Keywords: translational neuroscience; Alzheimer's disease; gait; muscular strength; muscular endurance; motor performance; frailty

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1. Introduction

Alzheimer's disease (AD) is a complex and heterogeneous disorder with a distinctive clinical presentation [1,2]. Motor and sensory alterations are less frequent but can appear in intermediate and advanced stages of the disease [3]. Although the main signs of AD are cognitive impairment, motor disorders such as bradykinesia, rigidity, and gait disorders are of great importance due to the functional limitations and impairments that they cause during the disease [4,5]. In this sense, different studies have demonstrated different motor alterations during the last two decades, particularly those associated with walking and displacement [6–8]. Thus, gait disorders in AD patients have been described within the group of alterations known as "frontal gait" and, in particular, gait in AD has been defined as "cautious gait" [5,9,10]. This gait pattern is similar to that observed in the aging; there may be decrease in speed, stride length, and postural stability of gait, which is manifested more specifically in static and dynamic balance, with a widened base of support [11].

Walking (gait) constitutes a biological activity of the human being [12]. It is complex, learned, and begins with a voluntary act [13]. The mode of locomotion allows one to move in a vertical position without getting too tired; it is composed of three essential phases: support, double support, and swing [14,15]. It implies a dynamic balance, which is constantly lost and recovered each time a step is performed [9,14]. While the bodyweight is supported by one leg, the other swings forward to initiate the next support, this action being fluid, rhythmic, and automatically synchronized [16]. Achieving these steps makes it possible to reveal different patterns that can determine the healthy state of gait related to the physical health of individuals and, in particular, of older adults [17,18].

The gait pattern evolves through the different age groups; thus, in adolescents and young adults, it is characterized by a certain lightness, flexibility, and agility, qualities that will diminish as the years go by [19–21]. During aging, these parameters will change around 60 and 70 years of age, where the physiological process causes these changes to be progressive and of varying severity depending on the degree of alterations that may occur [20]. Even healthy people over the age of 65 show some decrease in performance on the timed walking test (TUG) and the 6 min walk test (6MWT) [22]. Lower mobility test scores in healthy older people have been shown to predict the development of future mobility limitations [23].

On the other hand, muscle strength is relevant to gait performance [24]. Some studies have reported that older adults' decreased muscle strength and gait speed were associated with poor cognition [25,26]. From the early stages of AD, a decrease in muscle strength can be observed even without loss of muscle mass, progressing to a loss of both in moderate stages [27]. However, optimal muscle strength and physical activity level are related to better performance on cognitive and learning tests in older adults with mild to moderate cognitive impairment who live in nursing homes [28].

In this way, sarcopenia is closely related to dementia, particularly AD, although few studies examine its prevalence and associated factors [27]. Poor muscle function, but not reduced lean muscle mass, drives the association of sarcopenia with cognitive decline in old age [29]. It needs more scientific studies that identify the characteristics of the muscular structure related to sarcopenia that identifies older adults at risk of cognitive deterioration in old age.

The present work proposes translating the International Classification of Functioning, Disability, and Health (ICF) conceptual framework to classify and describe functioning and disability in gait and exploratory activity in the 3xTg-AD animal model and its non-transgenic counterpart with normal aging. We developed a method for the characterization of qualitative parameters of psychomotor performance in the gait pattern of male mice in different stages of the disease: initial (6 months), advanced beta (12 months), and advanced beta-tau (16 months) in contrast with normal aging.

2. Materials and Methods

2.1. Animals

A total of forty-five homozygous 3xTg-AD (n = 24) and non-transgenic (NTg, n = 21) male mice of 6, 12, and 16 months of age in a C57BL/6J background (the transfer is accomplished by at least ten cycles of backcrossing) established at the Universitat Autònoma de Barcelona [30] were used in this study. As previously described, the 3xTg-AD mice harboring transgenes were genetically modified at the University of California at Irvine [31]. Animals were kept in groups of 3–4 mice per cage (Macrolon, 35 × 35 × 25 cm, Panlab, SL, Barcelona, Spain) filled with 5 cm of clean wood cuttings (Ecopure, Chips6, Date Sand, UK; uniform cross-sectional wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm × 20 cm, White). In all cases, standard home cages covered with a metal grid allow the perception of olfactory and auditory stimuli from the rest of the colony. All animals were kept under standard laboratory conditions of food and water ad libitum, 20 ± 2 °C, 12 h light cycle: dark with lights on at 8:00 a.m. and 50–60% relative humidity. All procedures followed the Spanish legislation on "Protection of animals used

for experimental and other scientific purposes" and the EU Directive (2010/63/EU) on this issue. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used [32].

2.2. Experimental Design

A cross-sectional study of 3 cohorts of 3xTg-AD and NTg male mice was carried out. The first group includes 6-month-old mice of both genotypes, the second 12-month-old, and the third 16-month-old. They were temporarily included in two batches once they reached the required study age. The evaluation of the gait and exploratory activity was carried out in two evaluation days.

2.3. Behavioral Assessments

In a translational approach, the concepts proposed in ICF have been incorporated to describe the factors that functionally intervene in spontaneous gait and exploratory activity. They have been included in this way: "Activity" describes the shape and quantification of the trajectory and displacement. "Body function" allows a description of the movement pattern and the associated muscular strength. Finally, "Body structure" gives an account of the state in which muscle groups and joints are found in our object of study. In the same way, we have included the concepts that account for disability in the assessed tasks, activity limitations, and body function and structure impairments (see Figure 1).

Behavioral evaluations were performed in two days. During the morning, the tests were carried out; 30 min were allowed to habituate the animals in the test room before starting the measurements. The evaluation protocol, bizarre behaviors registered, the physical phenotype of frailty, gait, and Rotarod used here were recently reported in Castillo-Mariqueo and Gimenez-Llort's 2021 study [33]. In addition, videos of gait were taken for posterior analysis with KINOVEA version 0.8.15 free software.

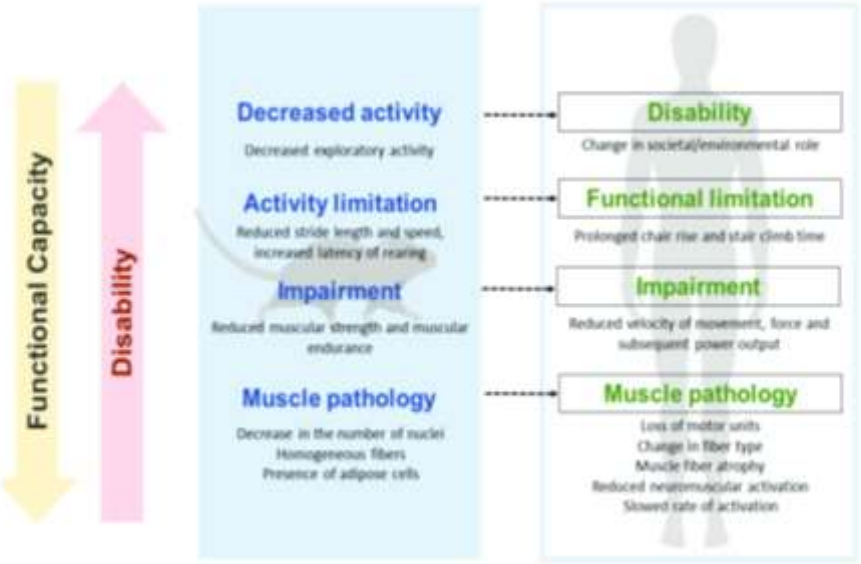

Figure 1. Proposal for a translational approach to motor dysfunction at different levels of disability. The figure details in a translational way the equivalence of the disability process from the 3xTg-AD mouse model to that expected in humans. It exemplifies a pathway that links pathology, deficiencies, functional limitations, and disability of gait and exploration (it has been adapted from Verbrugge and Jette, 1994; and Reid and Fielding, 2012) [34,35].

2.3.1. Activity—Spontaneous Gait and Exploration

The animals' spontaneous gait and exploratory activity were filmed from the undersurface [36] and a transverse plane that allowed the registration of the legs and the trajectory during the test. Each mouse was placed in a 27.5 × 9.5 cm transparent housing box, and gait performance was directly observed for one minute. The KINOVEA 8.15 software was used for the quantification and analysis of the gait trajectory.

Quantitative parameters of gait. The quantitative parameters were measured according to the Castillo-Mariqueo and Gimenez-Llort protocol 2021 [33]. Stride length, stride length variability, speed, and acceleration were included according to the methodology used by Wang et al. in 2017 [35].

Exploration includes body position, limb support, and moving around. The exploratory activity was recorded in parallel with spontaneous walking through video analysis and direct observation. For one minute, freezing or movement latency, the latency of the first rearing, the number of scans on the horizontal axis (corners visited), and scans on a vertical axis were recorded, taking the hind legs (rearings) as reference. During the tests, defecation and urination were also recorded.

2.3.2. Body Function—Mobility and Muscular Strength

General mobility includes bizarre gait patterns and freezing. For the discrimination of bizarre gait patterns, the trajectory of each route was differentiated according to the form of displacement, being able to be straight, scanning, backward, or circling. Then, the postural pattern of movement is differentiated: normal, shrink, or stretching. The movements were visually analyzed through videos and direct observation in the trajectory of the forward displacement in the transverse and sagittal planes. Their presence or absence was recorded for each one [33].

Forelimb grip strength-Hanger Test. The muscular strength of the forelimbs was measured using the hanger test, which is based on the tendency of a mouse to grasp a bar when suspended by the tail instinctively. We have replicated the methodology previously described by our group [33,37]. Muscle strength was measured on the second day of assessment.

2.3.3. Body Structure—Joints and Muscles

Joints to detect kyphosis. Kyphosis was differentiated into postural and structural, according to the analysis method established in previous research by our laboratory [38]. Kyphosis was measured during spontaneous locomotion and later confirmed in postural inspection (using joints and thoracic–lumbar structure as references), assigning a score from 0 to 2, where 0 indicates the absence of kyphosis, 1 indicates postural kyphosis, and 2 indicates structural kyphosis.

Muscles related to sarcopenia. The animals were sacrificed and subjected to necropsy to extract the quadriceps and triceps surae muscles, and these were subsequently weighed individually. The "sarcopenia index" [38] was applied to obtain an indirect measure of sarcopenia as a biological marker of frailty.

Morphological features of quadriceps and triceps surae. A qualitative evaluation of longitudinal sections of quadriceps and triceps surae stained with hematoxylin and eosin (H&E) was performed. The muscles were dissected and fixed with 10% formalin (Sigma-Aldrich, Saint Louis, MO, USA) for 24 h and then embedded in paraffin for further analysis. Histological sections of 5 µm were stained with a standard H&E method. We used the standard protocol of the Laboratory of Microscopic Anatomy of the Department of Morphological Sciences of the Autonomous University of Barcelona. Sections were first rehydrated by passing them through decreasing concentrations of Ethanol (EtOH) (absolute and 96°). The sections were incubated in Harris hematoxylin (Merck-Sigma Aldrich, St. Louis, MO, USA) for 10 min and washed under running water for 5 min. Because Mayer Hematoxylin was used, a de-differentiation process was needed, with consecutively Et-OH-HCl (2.5%) and ammonia water (0.3%) solutions. After this, sections were incubated

in Eosin Y (Merck-Sigma Aldrich) for 5 min, previously acidified with glacial acetic acid. Finally, the sections were dehydrated by passing them through increasing concentrations of EtOH and cleared in Xylol, and mounted with DPX (PanReac AppliChem, ITW reagents, IL, USA). The images were taken with 20X objective on a Nikon Eclipse 80i microscope, using a digital camera running on the control software ACT-1 (ver2.70) (Nikon Instruments Inc., Tokyo, JP). Characteristics of location and number of nuclei, fibers' distribution and shape, and adipose tissue were identified. A generic description and qualifier of intensity were given with five levels represented by (+) for the quantity graduation.

2.3.4. Motor Performance, Geotaxis, and Hindlimb Clasping

Additionally, motor learning, physical endurance, geotaxis, and hindlimb clasping were evaluated to show functional impairment related to gait and exploratory activity, according to the protocol developed by Castillo-Mariqueo, Giménez-Llort, 2021 [33]. In the present work, we have adopted the protocol combining learning and physical endurance. Thus, motor learning was evaluated in the constant mode and physical resistance in the accelerated mode of the Rotarod apparatus (Ugo basile®, Mouse RotaRod NG). We recorded the number of trials until reaching over 60 s of permanence on the wheel to measure learning. Subsequently, after 2 min of rest, we carried out a single trial in the accelerated mode to assess physical endurance.

2.4. Statistics

Statistical analyses were performed using SPSS 15.0 software. Results were expressed as the mean ± standard error of the mean (SEM) for each task and trial. Gait, exploration, forelimb grip strength, muscles (sarcopenia), motor performance, and geotaxis were analyzed with one-way ANOVA followed by post hoc Bonferroni. In addition, the effect of Genotype (G) and Age (A) in each of them was identified. The incidence and prevalence of body position, general mobility, kyphosis, and hindlimb clasping were analyzed using the Chi-square test or Fisher's exact test. Additionally, the relationship between activity limitation and restriction (presence/absence) with stride length, speed, and cadence was analyzed with the Point-Biserial Correlation. The horizontal and vertical exploration and rearing latency were also related to the deficiencies in exploration and gait. The survival curve of both genotypes was analyzed with the Kaplan–Meier test (Lon Rank). In all cases, statistical significance was considered at $p < 0.05$.

3. Results

As shown in Table 1, we have proposed a translational approach for the interpretation of the results obtained in the measurement of gait and exploratory activity in the 3xTg-AD mouse model in different stages of the disease and its counterpart NTg of normal aging, according to the analysis and quantification parameters proposed by ICF.

3.1. Activity—Spontaneous Gait and Exploration

As illustrated in Figure 2, the quantitative parameters of gait show a tendency to increase stride length in 3xTg-AD animals, although they are not statistically significant (stride length (cm), NTg 6 months: 4.48 ± 0.20; NTg 12 months: 4.24 ± 0.95; NTg 16 months: 3.84 ± 0.68; 3xTg-AD 6 months: 1.92 ± 0.57; 3xTg-AD 12 months: 3.09 ± 0.54; 3xTg-AD 16 months: 4.36 ± 0.48). Although the differences between NTg ages do not reach statistical significance, we can observe that stride length remains relatively stable as age increases, while in the 3xTg-AD group they present differences between the ages since the 95% confidence interval does not overlap between 6 months, 12 months, and 16 months (6 months, 1.92 + 0.57 = 2, 49; 12 months, 3.09 − 0.57 = 2.55; 16 months, 3.84 + 0.68 = 4.52).

Table 1. Proposal to functional analysis to mimics capacities and disabilities from humans in mice.

Translational Functioning and Disability (ICF)						
• **ACTIVITY—Spontaneous gait and exploration**	**NTg Mice**			**3xTg-AD Mice**		
1. Quantitative parameters of gait (see Figure 2)	**6 Months**	**12 Months**	**16 Months**	**6 Months**	**12 Months**	**16 Months**
A. Stride length (cm)	ABSENT	MILD	MILD	MODERATE	MODERATE	MILD
B. Variability of stride length (%)	ABSENT	ABSENT	MILD	SEVERE	MODERATE	MODERATE
C. Speed (cm/s)	ABSENT	ABSENT	MILD	SEVERE	SEVERE	MODERATE
D. Cadence (steps/s)	ABSENT	ABSENT	ABSENT	SEVERE	MODERATE	MODERATE
2. Body position (see Figure 2)						
E. Maintaining body position (%)	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	MILD
3. Limb support						
F. Base of support (cm)	ABSENT	ABSENT	MILD	ABSENT	ABSENT	ABSENT
4. Exploration (see Figure 2)						
G. Vertical and horizontal activity (n episodes)	MILD	MILD	MILD	MODERATE	SEVERE	MILD
H. Rearing latency (s)	MILD	MODERATE	SEVERE	COMPLETE	SEVERE	MODERATE
• **BODY FUNCTION—mobility and forelimb grip strength**						
1. General mobility (see Figure 3)						
A. Bizarre gait patterns (incidence %)	MILD	MODERATE	MILD	MODERATE	MODERATE	MILD
B. Freezing (movement latency)	ABSENT	MODERATE	MILD	MODERATE	MODERATE	MILD
C. Freezing—latency movement	ABSENT	MODERATE	MILD	MODERATE	MODERATE	MILD
2. Forelimb grip strength—Hanger Test (see Figure 3)						
D. Muscular Strength (latency)	ABSENT	MILD	MODERATE	MILD	MODERATE	MODERATE
E. Muscular Strength (distance)	ABSENT	MILD	SEVERE	MILD	SEVERE	MODERATE
F. Muscular Endurance (latency)	ABSENT	MODERATE	SEVERE	MILD	SEVERE	SEVERE
G. Muscular Endurance (distance)	ABSENT	MODERATE	SEVERE	MODERATE	SEVERE	SEVERE
• **BODY STRUCTURE—joints and muscles**						
1. Joints (see Figure 4)						
A. Kyphosis prevalence	ABSENT	MODERATE	MODERATE	MILD	MILD	MILD
2. Muscles (see Figure 4)						
B. Quadriceps muscle (weight)	MILD	MILD	MILD	ABSENT	MILD	MODERATE
C. Triceps surae muscle (weight)	MILD	MILD	MILD	MILD	MILD	MODERATE
D. Sarcopenia index—Quadriceps	MILD	MILD	MILD	ABSENT	MILD	MODERATE
E. Sarcopenia index—Triceps	MILD	MILD	MILD	MILD	MILD	MODERATE

Qualifiers:

Generic qualifier with the negative scale used to indicate the extent or magnitude of an impairment: NO impairment, (absent) 0–4%; MILD impairment, (slight, low) 5–24%; MODERATE impairment, (medium, fair) 25–49%; SEVERE impairment, (high, extreme), 50–95%; COMPLETE impairment, (total) 96–100%—not specified in the ICF for humans. Activity limitations are difficulties an individual may have in executing activities: NO difficulty, (absent) 0–4%; MILD difficulty, (slight, low) 5–24%; MODERATE difficulty, (medium, fair) 25–49%; SEVERE difficulty, (high, extreme) 50–95%; COMPLETE difficulty, (total) 96–100%—not specified in the ICF for humans.

Table 1. Outcome measures that link gait and exploration impairments and limitations of male 3xTg-AD mice at different stages of AD progression to describe the functioning, according to qualifiers of ICF. Translational Functioning and Disability: ACTIVITY—spontaneous gait and exploration, BODY FUNCTION—mobility and forelimb grip strength, and BODY STRUCTURE—joints and muscles. Absent (green), Mild (light green), Moderate (yellow), Severe (orange), and Complete (red).

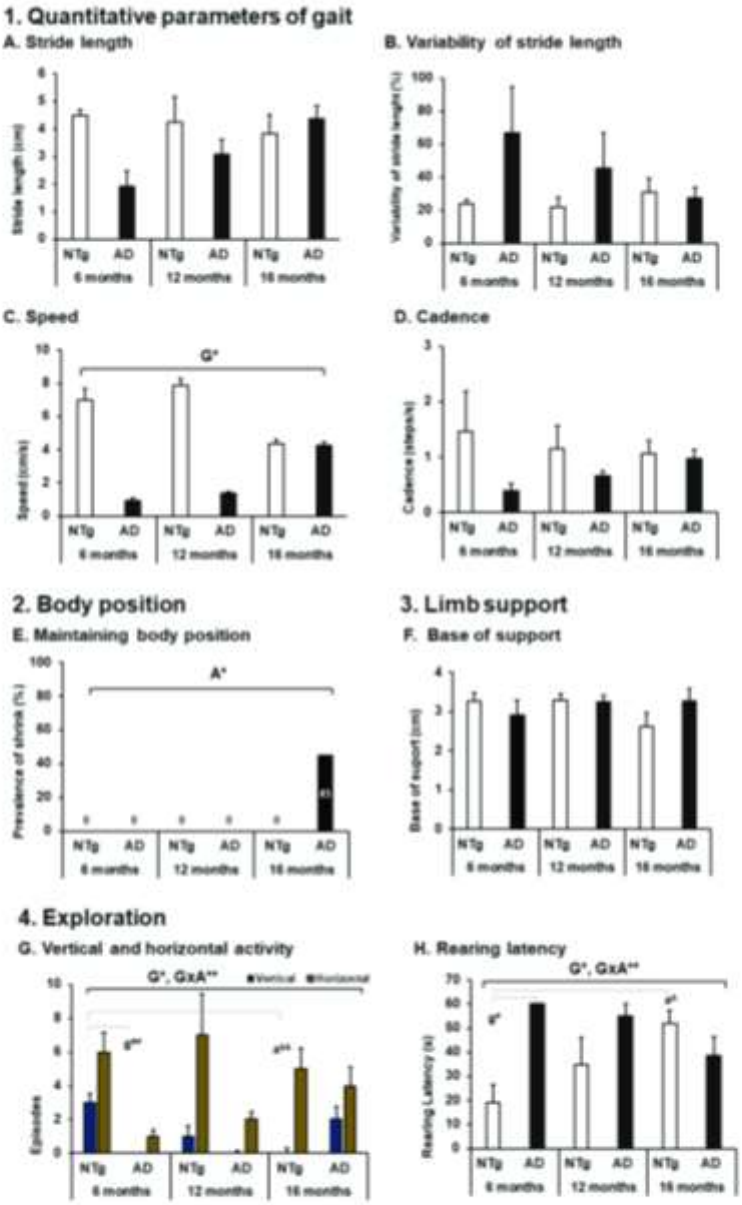

Figure 2. ACTIVITY: Spontaneous gait and exploration. 1. Quantitative parameters of gait; (**A**) stride length; (**B**) variability of stride length; (**C**) speed; (**D**) cadence. 3. Limb support. (**F**) Base of support. 4. Exploration. (**G**) Vertical and horizontal activity; (**H**) rearing latency. The results are expressed as mean ± SEM. Statistics: one-way ANOVA, Age effect expressed as (**A**); Genotype effect expressed as (**G**); Genotype and Age interaction effect is expressed as (GxA); * $p < 0.05$, ** $p < 0.01$ followed by post hoc Bonferroni test, * $p < 0.05$, ** $p < 0.01$,; differences between NTg vs. 3xTg-AD are expressed (g): ^{g} $p < 0.05$, ^{gg} $p < 0.01$; differences between age in each group are expressed (a): ^{4a} $p < 0.05$, ^{4a4a} $p < 0.01$. 2. Body position; (**E**) maintaining body position, the results are expressed as prevalence (%). Statistics: Fisher's exact test, Age effect are expressed as (**A**), Genotype effect are expressed as (**G**); Genotype and Age interaction effect are expressed as (GxA); * $p < 0.05$ and ** $p < 0.01$.

On the other hand, in the exploration, the animals differed in their performance in horizontal and vertical activity (horizontal activity, ANOVA F (5,39) = 2.427, $p = 0.050$; vertical activity, ANOVA F (5,39) = 4.600, $p = 0.002$). Genotype differences can be noted in each age group with a lower performance in 3xTg-AD animals (horizontal activity: genotype differences, ANOVA F (1.44) = 9.548, $p = 0.004$). Likewise, we can evidence a genotype difference in vertical activity (vertical activity: genotype differences, ANOVA F (1,44) = 7.209, $p = 0.011$) and a significant difference at the age of 6 months between the groups (Bonferroni post hoc: NTg 6 months vs. 3xTg-AD 6 months, $p = 0.004$). It was also detected that at an older age in the normal aging group there is a decrease in vertical activity (Bonferroni post hoc: NTg 6 months vs. NTg 16 months, $p = 0.012$). In turn, the genotype per age interaction (GxA) show the decrease in the vertical exploratory activity of NTg versus 3xTg-AD (GxA, ANOVA, F (2.43) = 8.519, $p = 0.001$), see Figure 2G (Exploration). The first time they perform vertical activity (latency of the first rearing) is also determined by the genotype and its interaction with age, highlighting that the group of 3xTg-AD mice at the age of 6 months did not register this activity and that in the animals NTg latency increases with age progressively (rearing latency, ANOVA F (5,39) = 4120, $p = 0.004$ post hoc NTg 6 months vs. 3xTg-AD 6 months, $p = 0.010$; NTg 6 months vs. NTg 16 months, $p = 0.026$. Genotype differences, ANOVA F (1.44) = 6.443, $p = 0.015$, GxA differences, F (2.43) = 8330 $p = 0.001$) (see Figure 2H (Eploration)).

3.2. *Body Function—Mobility and Muscular Strength*

As shown in Figure 3, the animals exhibited a series of bizarre behaviors called bizarre gait patterns. There is a high incidence of circling in 3xTg-AD animals (3xTg-AD: 6 months 3/6 (50%), 12 months 4/7 (57%), 16 months 3/11 (27%)); despite not being statistically significant, its presence can modify performance in gait and exploration, which are described later (see Figure 5). It can also be seen that this behavior appears in NTg animals with a lower incidence (NTg: 6 months 1/6 (17%), 16 months 2/9 (22%)). Stretching (NTg: 6 months 1/6 (17%), 12 months 2/6 (33%)—3xTg-AD: 6 months 3/6 (50%), 16 months 1/11 (9%)], and in animals 3xTg-AD 16 months backward movement [1/11 (17%)), see Figure 3A. In addition, a high incidence of freezing was evidenced in which as age increases, its incidence decreases regardless of genotype (Fisher exact test, $p = 0.032$). It is also appreciated that the time invested in this behavior varies with age, being less at 16 months without genotype effect and higher at 12 months (ANOVA, F (2.43) = 3.473 $p = 0.041$), (see Figure 3B,C).

Additionally, we have detected a correlation between the incidence of these behaviors and performance in gait and exploration, as shown in Figure 4. Thus, the variables stride length, speed, and cadence negatively correlate with the presence of bizarre gait pattern, causing limitation in the displacement and trajectory of gait (stride length, Pearson: r2 = (−) 0.294 $p < 0.0001$. Speed, Pearson: r2 = (−) 0.462 $p < 0.0001$. Cadence, Pearson: r2 = (−) 0.348 $p < 0.0001$), see Figure 4A–C. While the horizontal and vertical exploration variables correlate negatively, rearing latency does so positively with the presence of bizarre gait pattern, which leads to a restriction to the performance of these behaviors (horizontal activity, Pearson r2 = (−) 0.156 $p = 0.008$. Vertical activity, Pearson: r2 = (−) 0.118 $p = 0.021$. Rearing latency, Pearson: r2 = 0.098 $p = 0.035$) (see Figure 4D–F).

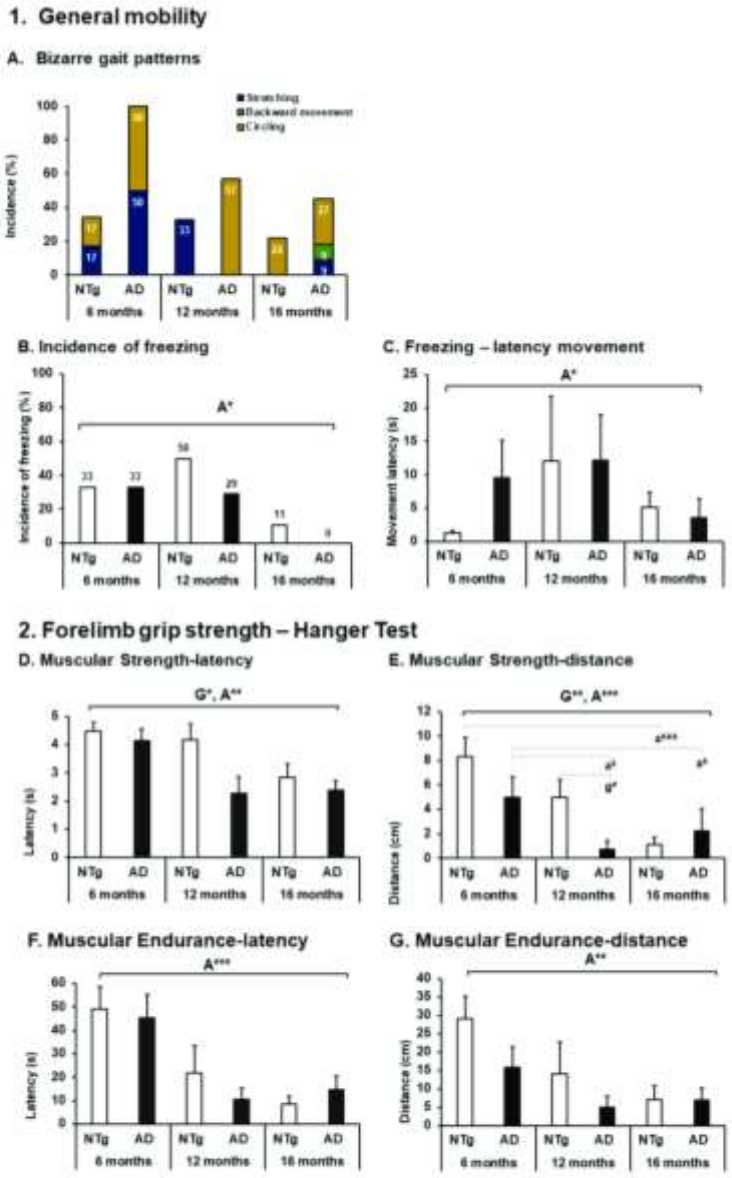

Figure 3. BODY FUNCTION—mobility and forelimb grip strength. 1. General mobility. (**A**) Bizarre gait patterns; (**B**) incidence of freezing; the results are expressed as incidence (%). Statistics: Fisher's exact test, Age effect is expressed as (**A**); Genotype effect is expressed as (**G**); Genotype and Age interaction effect is expressed as (GxA); * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (**C**) Freezing—latency movement; 2. Forelimb grip strength—Hanger Test. (**D**) Muscular Strength—latency; (**E**) Muscular Strength—distance; (**F**) Muscular Endurance—latency; (**G**) Muscular Endurance—distance, the results are expressed as mean ± SEM. Statistics: one-way ANOVA, Age effect expressed as (**A**); Genotype effect expressed as (**G**); Genotype and Age interaction effect is expressed as (GxA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ followed by post hoc Bonferroni test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; differences between NTg vs. 3xTg-AD are expressed (g): $^{\#}$ $p < 0.05$; differences between age in NTg group are expressed (a): $^{\&\&\&}$ $p < 0.001$, and for 3xTg-AD group are expressed (a): § $p < 0.01$.

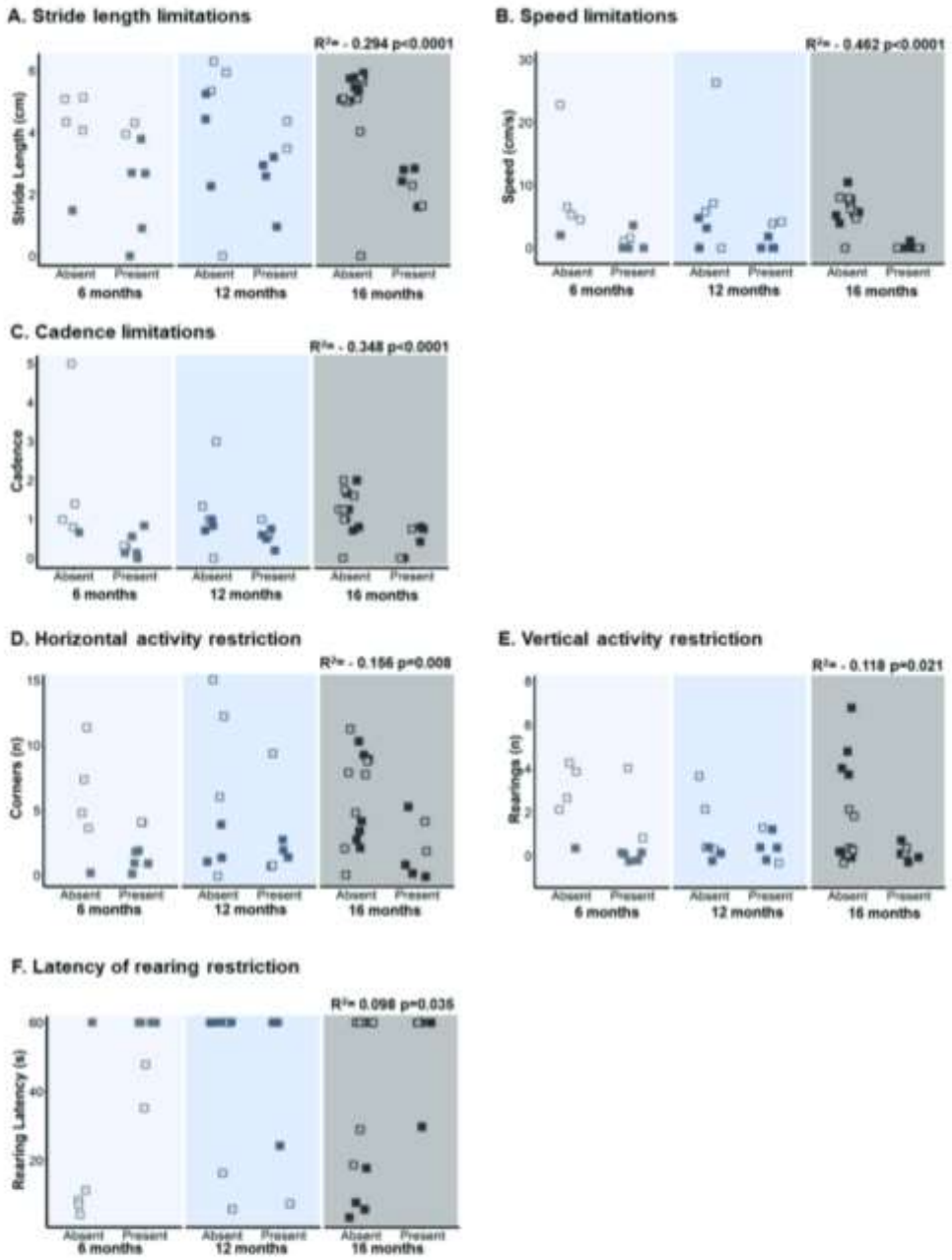

Figure 4. Activity limitations and restrictions of gait and exploration. (**A**) Stride length limitations, (**B**) speed limitations, (**C**) cadence limitations, (**D**) horizontal activity restriction, (**E**) vertical activity restriction, and (**F**) latency of rearing restriction. The NTg group has been represented by a white square and the 3xTg-AD group by a black square. According to the groups under study, it has been defined as "present/absent" the behaviors reported as bizarre gait patterns of each animal. The Point-Biserial Correlation has been applied to determine the relationship between the activity limitation and restriction (presence/absence) with stride length, speed, and cadence and exploration. Statistics: Pearson r2.

Muscle strength, on the other hand, was lower in older animals, and the transgenic genotype at each age was lower than the non-transgenic genotype at all ages (Muscular strength—latency: ANOVA F (5.39) = 4.385, $p = 0.003$; Age effect, F (2, 43) = 5.702, $p = 0.007$; Genotype effect, F (1,44) = 5.895, $p = 0.020$). In the same way, it can be noted that the distance reached when the animals move on the bar was less as age increases and the 3xTg-AD mice were lower than the NTg at 6 and 12 months; otherwise, it occurs at 16 months, but it is not statistically significant (muscular strength—distance: ANOVA F (5.39) = 9.847, $p < 0.0001$ post hoc NTg 6 months vs. NTg 16 months $p < 0.0001$; 3xTg-AD 6 months vs. 3xTg-AD 16 months $p = 0.023$; 3xTg-AD 6 months vs 3xTg-AD 12 months $p = 0.050$; NTg 12 months vs 3xTg-AD 12 months, $p = 0.045$). Age effect F (2.43) = 17,320 $p < 0.0001$. Genotype effect F (1.44) = 11.786 $p = 0.001$], see Figure 3D,E. At the same time, the muscular endurance and the distance of displacement was determined by the age of the animals decreasing as the age increases in both groups (muscular endurance—latency: ANOVA F (5,39) = 3.296, $p = 0.014$. Age effect, F (2,43) = 8,154, $p = 0.001$. Muscular endurance—distance ANOVA F (5.39) = 3.394, $p = 0.012$. Age effect, F (2.43) = 7.295, $p = 0.002$) (see Figure 3F,G).

3.3. *Body Structure—Joints and Muscles*

The most prevalent postural alteration was kyphosis, with structural kyphosis having the highest incidence in older animals regardless of genotype (Kyphosis prevalence, age differences Fisher exact test $p = 0.025$. Structural kyphosis incidence, age differences $p = 0.016$), see Figure 5A. This joint deformation was observed at the thoracolumbar level.

At the level of muscle tissue, the quadriceps presented variations in weight, with a tendency to decrease with age determined by the GxA interaction and a significant decrease between the 3xTg-AD of 6 months versus 16 months (quadriceps, ANOVA F (5, 39) = 4.314, $p = 0.003$, post hoc 3xTg-AD 6 months vs. 3xTg-AD 16 months, $p = 0.001$. Age effect, F (2.43) = 5.715, $p = 0.007$. GxA effect, F (2.43) = 4.291, $p = 0.021$), see Figure 5B. In the triceps surae muscle, no statistically significant differences were detected; it can be seen that all groups, regardless of age, seem to maintain a similar weight range, see Figure 5C. When applying the indirect measure of sarcopenia, the differences in quadriceps were maintained (sarcopenia Index—quadriceps: ANOVA F (5.39) = 6.705, $p < 0.0001$, post hoc 3xTg-AD 6 months vs. 3xTg-AD 16 months $p < 0.0001$. Age effect F (2.43) = 9.693, $p < 0.0001$. GxA effect F (2.43) = 5.623, $p = 0.007$), see Figure 5D. On the other hand, when applying this method in the triceps sural muscle, it was possible to distinguish a GxA interaction effect, where at six months, the 3xTg-AD mice present greater weight and decrease with age, and in the case of the NTg, this is maintained stable (sarcopenia index—triceps surae: ANOVA F (5.39) = 4.160 $p = 0.004$ post hoc 3xTg-AD 6 months vs. 3xTg-AD 16 months $p = 0.010$. NTg 16 months vs. 3xTg-AD 16 months $p = 0.020$. GxA effect, F (2.43) = 3.917 $p = 0.028$), see Figure 5E. Figure 6A,B illustrates the morphological characteristics of the quadriceps and triceps surae. Table 2 depicts the characteristics of the nucleus, fiber, and adipose tissue.

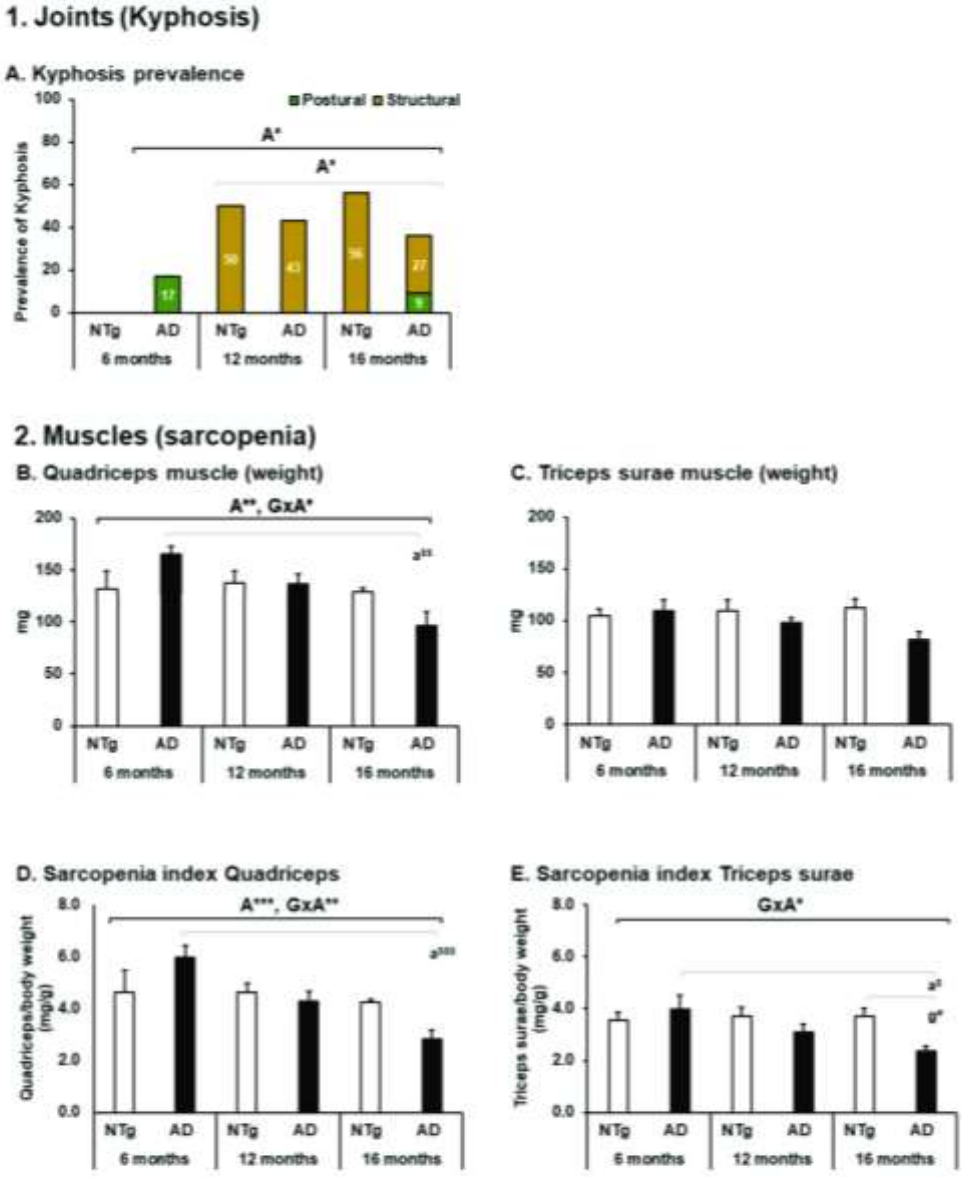

Figure 5. BODY STRUCTURE—joints and muscles. 1. Joints (Kyphosis), (**A**) kyphosis prevalence, the results are expressed as prevalence (%). Statistics: Fisher's exact test; Age effect is expressed as (**A**); * $p < 0.05$. 2. Muscles (sarcopenia), (**B**) quadriceps muscle (weight); (**C**) triceps surae muscle (weight); (**D**) Sarcopenia index Quadriceps; (**E**) Sarcopenia index Triceps surae; the results are expressed as mean ± SEM. Statistics: one-way ANOVA, Age effect expressed as (**A**); Genotype effect expressed as (**G**); Genotype and Age interaction effect is expressed as (GxA). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ followed by post-hoc Bonferroni test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; differences between NTg vs. 3xTg-AD are expressed (g): $^{\#}$ $p < 0.05$, differences between age in 3xTg-AD group are expressed (a): $^{\$}$ $p < 0.01$, $^{\$\$}$ $p < 0.01$, and $^{\$\$\$}$ $p < 0.001$.

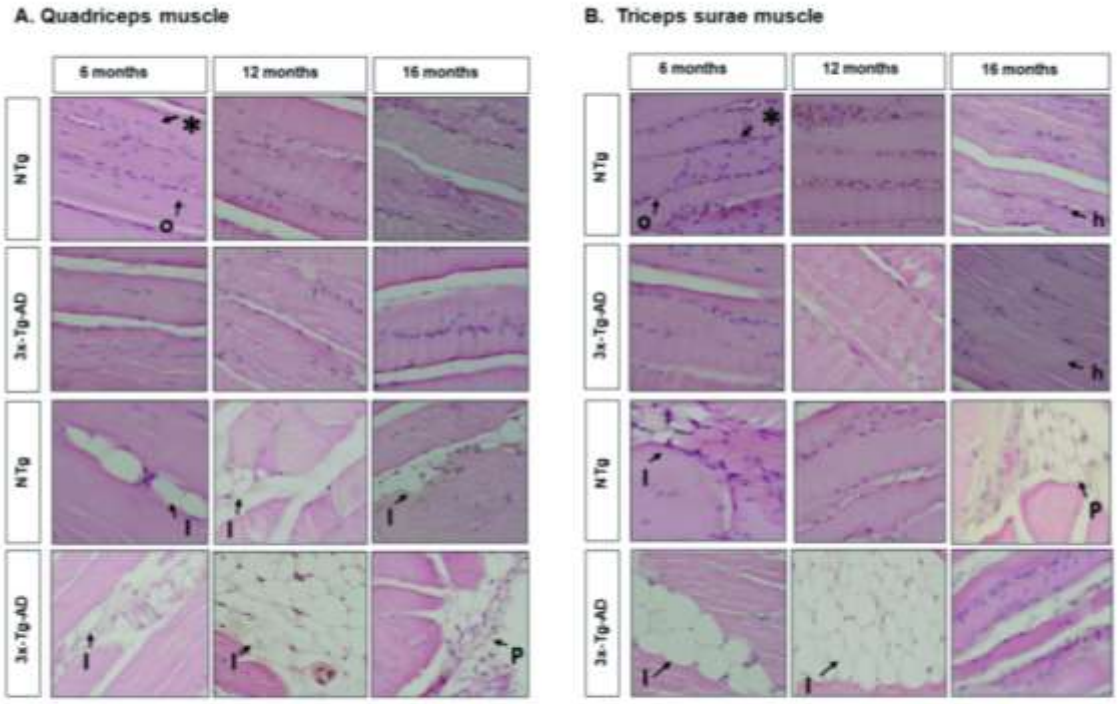

Figure 6. Morphological comparison of muscle tissue in normal and AD-pathological aging. Hematoxylin and eosin-stained horizontal sections of muscles. (**A**) Quadriceps muscle; (**B**) Triceps surae muscle. Representative H&E images of longitudinal skeletal muscle at 6, 12, and 16 months. Symbols indicate the morphological features, as follows: *—Peripheral nuclei; o—homogeneous fibre distribution; h—heterogeneous fibre distribution; I—intramuscular adipose tissue; P—peripheral adipose tissue. The images were taken with 20× objective lens; the scale bar represents 0.32 µm.

3.4. Motor Performance, Geotaxis, and Hindlimb Clasping

Motor performance and physical performance were evaluated in other tests to obtain a complete analysis regarding the psychomotor abilities of the animals. Thus, motor learning showed an interaction between the genotype factor and GxA, highlighting a low performance in 3xTg-AD mice at the age of 6 and 16 months, concerning NTg of the same age (motor learning—latency, ANOVA F (5.39) = 4.995, $p = 0.001$ post hoc NTg 16 months vs. 3xTg-AD 16 months $p = 0.026$. Genotype effect F (1.44) = 7.926, $p = 0.008$. GxA effect F (2, 43) = 5.184, $p = 0.010$. Trials, ANOVA F (5.39) = 3.953, $p = 0.005$. GxA F (2.43) = 5.454 $p = 0.008$) (see Figure 7A,B). On the other hand, physical endurance decreases with age, with 16 months being the age with the lowest performance in both groups, but statistically significant in 3xTg-AD mice (physical endurance, ANOVA F (5.39) = 5.189, $p = 0.001$ post hoc 3xTg-AD 6 months vs. 3xTg-AD 16 months, $p = 0.017$; 3xTg-AD 12 months vs. 3xTg-AD 16 months, $p = 0.006$. Age effect F (2.43) = 11.371, $p < 0.0001$) (see Figure 7C). Geotaxis did not show statistical differences, but a higher latency was observed in the 3xTg-AD animals in each age group (see Figure 7D). In the hindlimb clasping test, we can highlight a significant genotype difference in each age group with a higher incidence of this sign in each 3xTg-AD mice (hindlimb clasping, Fisher exact test genotype = 0.007).

Table 2. Morphological features of quadriceps and triceps surae.

Morphological Features	NTg Mice			3xTg-AD Mice		
	6 Months	12 Months	16 Months	6 Months	12 Months	16 Months
Quadriceps						
1. Nuclei						
Localization	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei
Number	++++	+++	+++	+++	++	+++
2. Fiber						
Distribution	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
3. Adipose tissue						
Localization	Intramuscular	Intramuscular	Intramuscular	Intramuscular	Intramuscular	Peripheral
Number	+	+	++	++	+	+
Triceps surae						
1. Nuclei						
Localization	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei	Peripheral nuclei
Number	++++	+++	+++	+++	++	+++
2. Fiber						
Distribution	Homogeneous	Homogeneous	Heterogeneous	Homogeneous	Homogeneous	Heterogeneous
3. Adipose tissue						
Localization	Intramuscular		Peripheral	Intramuscular	Intramuscular	
Number	+		++++	+	++++	

Qualifier: 75–100% = ++++. 50–75% = +++. 25–50% = ++. 0–25% = +. 0% = -.

Table 2. Morphological features of muscle tissue in 3xTg-AD mice: localization and number of nuclei, fiber distribution, and a number and localization adipose cells. Qualitative quantifier of intensity: (-) equal to 0%, (+) less to 25%, (++) less to 50%, (+++) less to 75%, and (++++) less or 100%.

3.5. Survival, Kyphosis, and Frailty Phenotype

Table 3 shows the survival, kyphosis, and frailty phenotype of the mice at each age. For the survival analysis, we carried out a follow-up from birth to 16-month-old of the siblings of the sample included in the study, completing a cohort of 115 male mice. Logarithmic rank analysis shows a significant genotype-dependent difference ($\chi 2$ (1) = 8.045, $p = 0.005$) with a higher mortality rate in NTg mice in each age group (6-month-old: NTg 3/15 (20%); 3xTg-AD 0/15. 12-month-old: NTg 3/9 (33.3%), 3xTg-AD 1/16 (6.2%). 16-month-old: NTg 20/40 (50%); 3xTg-AD 5/24 (20.8%)). On the other hand, kyphosis presents a higher incidence as age increases without genotype differences (Kyphosis (absent/present) Fisher exact test (5) = 10.694, $p = 0.052$. Age, Fisher exact test (2) = 10.070, $p = 0.007$. Genotype n.s). While postural kyphosis does not show significant differences between the groups, structural kyphosis increases its prevalence at ages 12 and 16 months of age independent of genotype (Fisher's exact test (2) = 8.464, $p = 0.016$). In the same way, body weight increases with age in the case of 3xTg-AD mice and is maintained in the case of NTg, with 3xTg-AD mice presenting greater weight at 16-month-old compared to NTg 16-month-old (Age effect, ANOVA F (2.44) = 3.268, $p = 0.049$; 3xTg-AD 12-month-old vs 16-month-old, $p = 0.037$). Regarding the physical conditions that the animals presented, no differences were detected in alopecia. On the other hand, body position, palpebral closure, and tail position were characteristics only present in the older group of 3xTg-AD mice (body position, Fisher's exact test (5) = 10.036, $p = 0.006$. Age effect, Fisher's exact test (2), $p = 0.046$. Palpebral closure, Fisher's exact test (5) = 7.493, $p = 0.037$. Tail position, Fisher's exact test (5) = 7.493, $p = 0.037$). Piloerection was present in NTg mice at the age of 12 and 16 months in contrast to 3xTg-AD mice, where its presentation appears at 16 months (Fisher (5) = 10.047, $p = 0.027$. Age effect, Fisher's exact test (2) = 8.338, $p = 0.010$).

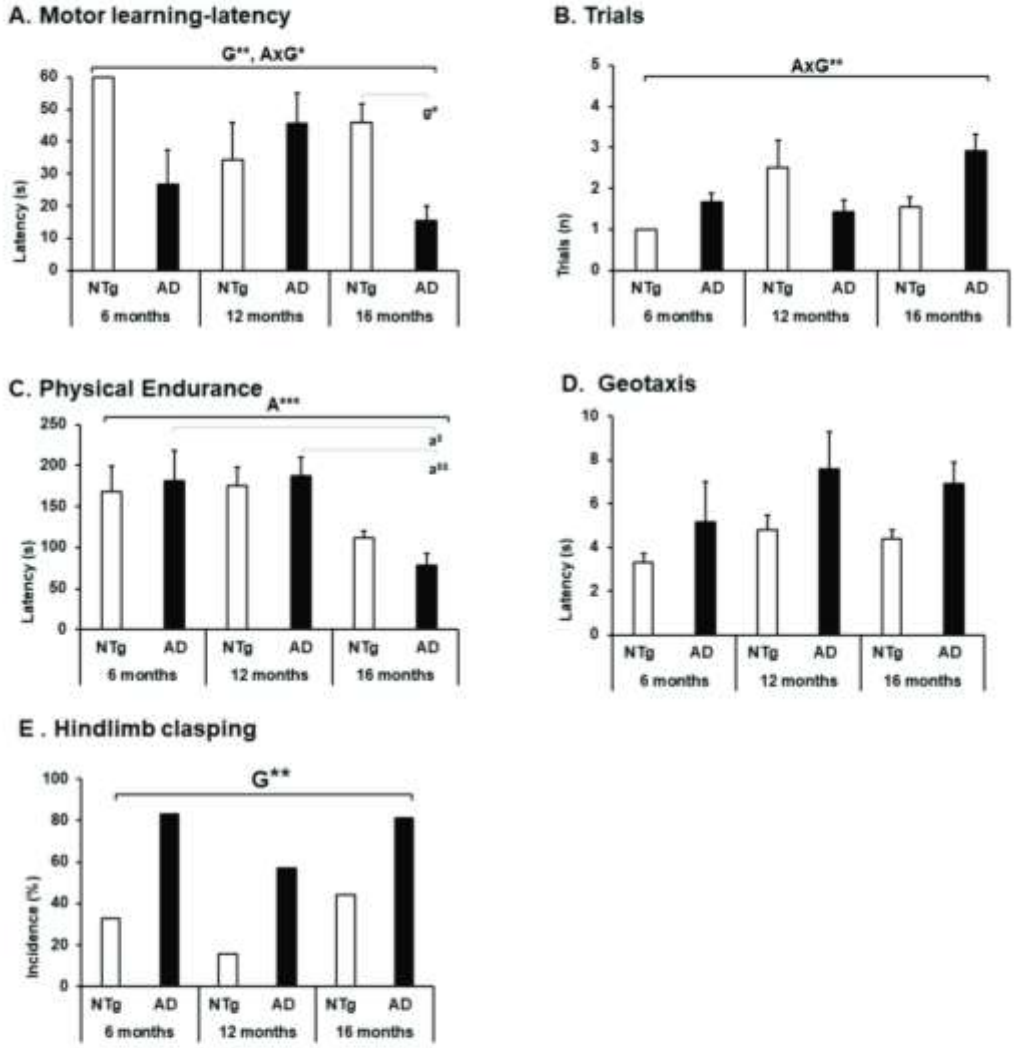

Figure 7. Motor performance, geotaxis and hindlimb clasping. (**A**) Motor learning—latency; (**B**) trials; (**C**) physical endurance; (**D**) geotaxis; the results are expressed as mean ± SEM. Statistics: one-way ANOVA, Age effect expressed as (**A**); Genotype effect expressed as (G); Genotype and Age interaction effect are expressed as (GxA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ followed by post hoc Bonferroni test, # $p < 0.05$; differences between age in 3xTg-AD group are expressed (a): $ $p < 0.01$, $$ $p < 0.01$.(**E**) Hindlimb clasping; the results are expressed as prevalence (%). Statistics: Fisher's exact test; Age effect is expressed as (A); Genotype effect is expressed as (G); Genotype,* $p < 0.05$.

Table 3. Survival, kyphosis, and frailty phenotype.

Conditions	NTg Mice			3xTg-AD Mice			Statistics
	6 Months	12 Months	16 Months	6 Months	12 Months	16 Months	
1. Survival (mean + SEM days) (Mortality ratio)	329 + 25.26 3/15 (20%)	337 + 29.09 3/9 (33.3%)	350 + 15.60 20/40 (50%)	208 + 1.26 0/15 (0%)	395 + 9.63 1/16 (6.2%)	481 + 25.31 5/24 (20.8%)	S &&
2. Kyphosis (animals, %)	-	3/6 (50%)	5/9 (56%)	1/6 (17%)	3/7 (43%)	4/11 (36%)	A **
Postural	-	-	-	1/6 (17%)	-	1/11 (9%)	n.s.
Structural	-	3/6 (50%)	5/9 (56%)	-	3/7 (43%)	3/11 (27%)	A*
3. Physical conditions (animals, %)							
Body weight	30 g.	30 g.	30 g.	28 g.	33 g.	34g.	A *, a #
Alopecia	2/6 (33%)	4/6 (67%)	5/9 (56%)	1/6 (17%)	4/7 (57%)	4/11 (36%)	n.s.
Body position	-	-	-	-	-	5/11 (45%)	a #
Palpebral closure	-	-	-	-	-	4/11 (36%)	a #
Piloerection	-	1/6 (17%)	2/9 (22%)	-	-	6/11 (55%)	A *
Tail position	-	-	-	-	-	4/11 (36%)	a #
Tremor	-	1/6 (17%)	-	-	-	9/11 (82%)	A **, G *

Kaplan–Meier, Log Rank: S && $p < 0.01$. X_2, A: age, ** $p < 0.01$ * $p < 0.05$, G: genotype, * $p < 0.05$. n.s. $p > 0.05$. # $p < 0.05$.

Table 3. Prevalence of physical conditions in male 3xTg-AD mice corresponding to the frailty phenotype. The progression of AD disease is contrasted with normal aging and the survival of the experimental lots included in the research.

Finally, tremor shows differences in genotype and age, presenting a high incidence at 16 months in 16-month-old mice (tremor, Fisher's exact test (5) = 23.346, $p < 0.0001$. Age effect, Fisher's exact test (2) = 10.170, $p = 0.005$. Genotype effect, $\chi 2$ (1) = 6.945, $p = 0.012$).

4. Discussion

In contrast to the huge literature on the AD-associated hallmark impairment in cognitive domains, gait disorders in Alzheimer's disease are an emerging field. They result from structural and functional deficiencies that generate limitations in the performance of activities and also imply restrictions in the biopsychosocial participation of individuals [39–43]. Evidence suggests that AD has a long preclinical phase, during which its characteristic pathology accumulates, and the patient's function diminishes considerably [42,44]. Motor problems have been described as occurring early in the AD process, rather than being a feature exclusively related to end-stage AD pathology [45,46].

At the translational level, in animal models, we have recently described alterations in the trajectory and displacement that interfere with gait and exploratory activity have recently been reported in middle-aged (13-month-old) and old (16-month-old) male C57BL/6 and 3xTg-AD mice, which in the mutant corresponds to ages mimicking advanced and very advanced stages of the disease [33]. Furthermore, these alterations increase their incidence in endpoint situations at different ages regardless of the studied genotype [38,47,48]. In this report, we have expanded the study of functionality and disability described for humans to provide a translational proposal, which allows us to differentiate dysfunctions, gait disorders, and exploration in the 3xTg-AD model at different stages of disease progression

and as compared to C57BL/6 with normal aging. As shown in Figure 1, the functional limitations that we have detected are equivalent to the difficulties that an older adult typically faces when carrying out their activities of daily living and that we can consider as markers of functional health deterioration.

4.1. Activity—Spontaneous Gait and Exploration

Particularly in gait, the variable speed, as in humans [43,49], seems to be the variable with the highest sensitivity to detect impairments of displacement and locomotion. Furthermore, when bizarre gait patterns (circling, backward movement, stretching) are present, stride length, speed, and cadence decrease in performance regardless of age and genotype. On the other hand, 6-month-old 3xTg-AD mice have a shorter stride length that increases with age. This result may be related to the novelty situation, where we have detected a higher incidence of freezing (no movement) and higher episodes of bizarre gait patterns in this group. Furthermore, it has previously been reported that in 3xTg-AD mice aged from 10 to 14 months, the stride length is greater than that of control mice [49]. The authors point out that a possible explanation for this difference is the differences between species, where quadruped locomotion seems to have compensatory mechanisms that intervene even after injury at the brain level [50], mediating that the kinematic parameters can be preserved. In contrast, it can be inferred that older animals present a favorable indicator in their gait performance, and this may be related to survival and individual characteristics.

A study conducted in 3- and 24-month-old male C57BL/6 mice found that aged mice exhibited significantly lower cadence and decreased stride time variability [50]. They also reported that aging tended to alter footstep patterns, for which they associated with aging the alterations that occur in gait [50]. There are also technological devices and software to make possible the equivalence of some human signatures in mice, highlighting those related to gait disorders in Parkinson's disease [41,51–53]. However, studying whole body gait and posture in rodent models requires specialized methods and remains a challenge if other motivational or emotional response behavioral factors are integrated, which is the case of some Alzheimer's disease models where a noticeable neuropsychiatric-like pattern is exhibited. In this sense, in the face of novelty situations, 3xTg-AD mice respond with neophobia and anxiety-like behaviors [54,55], whereas in humans, they have been reported from initials stages of the disease [56]. Neophobia modifies the exploratory activity as age increases, accentuating the symptoms [57]. However, we have described that there's a relationship between bizarre gait patterns and horizontal and vertical components of exploratory activity. Thus, bizarre gait patterns limiting locomotion in 3xTg-AD mice do the same in NTg mice, which tends to increase with age. In 3xTg-AD mice, these behaviors are mainly related to psychiatric and neurological disorders [30,37,58]. However, bizarre behaviors can be heterogeneous and have a low incidence in males compared to females, as described by Baeta-Corral and Giménez-Llort, 2014 [30]. Therefore, in this sex, these behaviors emerge at early stages and progress with the disease similar to that observed in the bizarre swimming patterns in the Morris water maze, where we have described the presence of circling appears at early stages (6 months of age) [59] and worsens with age [60,61].

4.2. Body Function—Mobility and Muscular Strength

General mobility was interfered with by periods of freezing. We can distinguish that the 12-month-old animals presented several freezing episodes in both genotypes. At the age of 6 months, the group of 3xTg-AD mice presented a long freezing behavior, taking longer to perform the first movement, which can also influence the decrease in exploration and the quantitative parameters of the gait, similar to what happens in scenarios of social isolation [33].

At the level of muscle strength, in humans, it has been described that the decrease in strength in the initial stages of AD does not imply changes at the muscle fiber level [61–64]. However, in intermediate stages, it could be accompanied by a decrease in the number of

muscle fibers that in advanced stages are reflected in sarcopenia associated with loss of muscle strength [35,63]. Our results showed that the decrease in muscle strength would be associated with aging, as occurs with muscular endurance. Nevertheless, at the age of 12 months, there is a drop in grip strength in 3xTg-AD mice. It has also been reported that at six months, 3xTg-AD mice have a deficit in grip strength [65], but at 16 months, these results are not reproduced [66]. In isolation, the 3xTg-AD mice show a conserved strength at the age of 13 months over the mice that lived in groups [33]. These findings may point to the heterogeneity of aging and the stage of AD in which muscle strength is measured.

4.3. Body Structure—Joints and Muscles

At the same time, postural patterns such as shrinkage and structural changes at the joint level of the thoracolumbar spine accompany 3xTg-AD mice with a high incidence of structural kyphosis [33,38,48]. Our results show that both 3xTg-AD and C57BL/6 mice show an increase in the incidence of structural kyphosis after 12 months of age, which could explain, from a postural point of view, the decrease in exploration in both groups as age increases.

As the weight of the quadriceps muscle shows, there is a progressive decrease in 3xTg-AD mice that, unlike the C57BL/6 controls in which it appears to be attenuated, a higher weight range is preserved even in older animals. In contrast, both groups maintain a similar weight of triceps muscle at 12 and 16 months of age. A study in C57BL/6J females reported a progressive weight loss from 15 months in the quadriceps muscle, which is considerably accentuated at 24 months [66]. Similarly, a decrease in muscle weight over 25 months has been reported in male C57BL/6J mice in the gastrocnemius and soleus muscle [67].

Furthermore, we have applied an indirect measure of sarcopenia to verify its presence to investigate these findings further. In the quadriceps muscle, aging is related to sarcopenia, while in the transgenic group, sarcopenia appears at 16 months. Interestingly, the triceps surae muscle also indicates sarcopenia in 16-month-old 3xTg-AD mice. Using this measure, a study conducted in female C57BL/6J mice concluded that sarcopenia would be present at around 24 months in the quadriceps muscle [68]. However, in male C57BL/6J mice, it could occur at earlier ages, reporting 20 months as the age of most significant change in the gastrocnemius muscle [69].

In natural aging models of the C57BL/6J strain, it has been reported that the primary phenotype of sarcopenia is a decrease in muscle mass and a decrease in the cross-sectional area of muscle fibers [70–72]. The optimal age of study would be 25 months [67,72]. Thus, we found the fibers are distributed homogeneously, with differences between them, but maintain a similar distribution. However, a difference is observed in the number of nuclei in NTg control animals that seems higher than in 3xTg-AD, especially at 12 months. We also found the presence of adipose cells, which exhibited a different distribution for each muscle type. Thus, adipose cells were present to a lesser extent in quadriceps, independently of genotype and age, with an intramuscular predominance. Oppositely, adipose cells exhibited a peripheral or intramuscular localization according to the genotype and age in the triceps. Thus, in the NTg control group, adipose cells were found in more peripheral areas, with a more significant proportion at 16 months.

In contrast, in the 3xTg-AD group, the adipose cells were more intramuscular, and a higher proportion was found at 12 months. Interestingly, the NTg control mice had a similar weight at each age, while the weight of 3xTg-AD mice increased with age.

4.4. Motor Performance, Geotaxis, and Hindlimb Clasping

On the other hand, we have measured the animals' motor learning and physical resistance to obtain a global vision regarding their psychomotor performance. We have shown that in the advanced stage of the disease, 3xTg-AD mice have a lower-than-expected performance that is replicated in motor learning and physical endurance. Similarly, in C57BL/6 control animals, the observed changes are more attenuated due to aging. In the

case of geotaxis, an increase in turning latency was found in the 3xTg-AD group, which, although it did not present significant differences, could indicate a poor use of postural and balance strategies to regain the verticality of their body on the grille in which they are located. For its part, hindlimb clasping showed a higher incidence in the 3xTg-AD group without being associated with the stages of disease progression. This particular sign can indicate the severity of the motor impairment that the mice present [73–75].

4.5. Survival, Kyphosis, and Frailty Phenotype

Finally, we can point out that C57BL/6 control animals have a higher mortality ratio in all age groups regarding survival, consistent with previous studies [76]. As the frailty phenotype shows, some signs of deterioration are related to one group or another. In the case of 3xTg-AD mice, physical and postural conditions appear to be the highest incidence, and in their NTg counterparts, piloerection and tremor, which in both groups, were found similarly increased with age. These variables indicate the general state of the mice without interfering with their functional performance of the gait and exploration that we have reported.

5. Conclusions

According to the literature, this is the first report that comprehensively presents the gait disturbances and functional limitations in the exploratory activity of the 3xTg-AD mouse model and, as compared to C57BL/6 with normal aging, uses a conceptual model that allows translation to humans. The use of the ICF as a conceptual framework allows describing the functional state, facilitating its interpretation and application in the rehabilitation of people with AD.

In summary, the main conclusions are:

(1) The 3xTg-AD mice show more significant functional impairment in gait and exploratory activity quantitative variables.
(2) The presence of movement limitations and muscle weakness mark the functional decline related to the disease severity stages that intensify with increasing age.
(3) Motor performance in 3xTg-AD is accompanied by a series of bizarre behaviors that interfere with the trajectory, which allows us to infer poor neurological control.
(4) Signs of physical frailty accompany the functional deterioration of these animals.
(5) Signs of sarcopenia are present in an advanced stage of AD, with differences in fibre distribution, number of cell nuclei, and presence of adipose tissue.

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Conclusion

1) 3xTg-AD mice show more significant functional impairment in quantitative variables of gait and exploratory activity.
2) The presence of movement limitations and muscle weakness mark functional decline related to disease severity stages that intensify with increasing age.
3) Motor performance in 3xTg-AD is accompanied by a series of bizarre behaviours that interfere with trajectory, inferring poor neurological control.
4) Signs of physical frailty accompany functional deterioration in these animals.
5) Signs of sarcopenia are present at an advanced stage of AD, with differences in fibre distribution, number of cell nuclei and presence of adipose tissue.

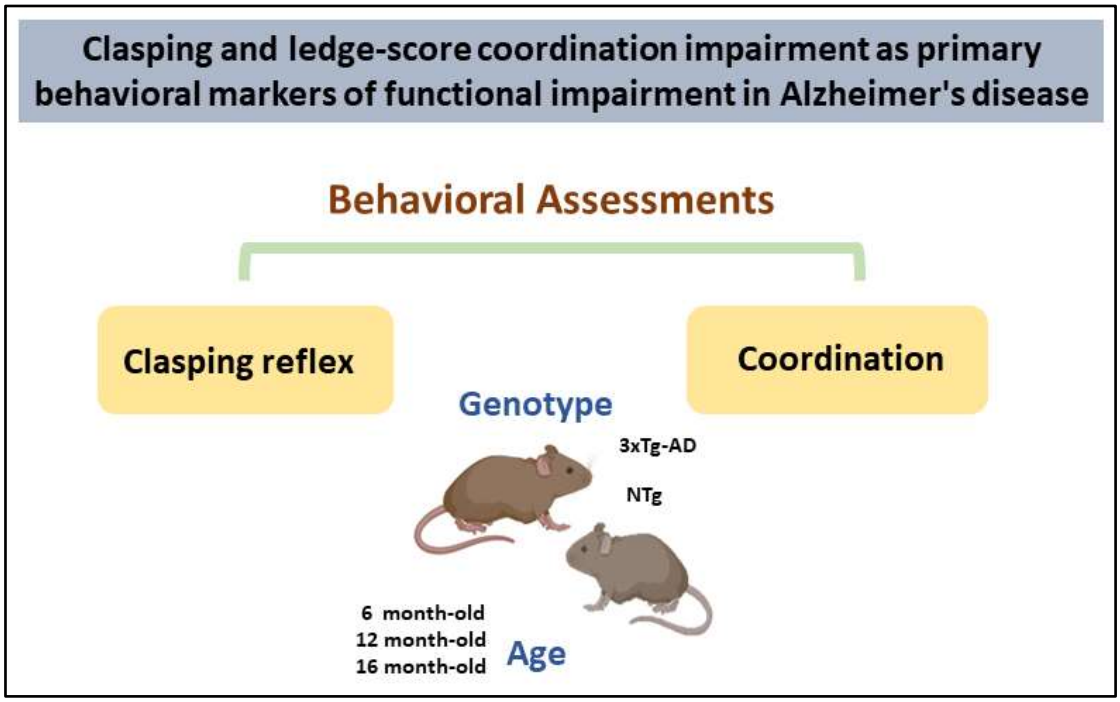
CLASPING AND LEDGE-SCORE COORDINATION IMPAIRMENT AS PRIMARY BEHAVIOURAL MARKERS OF FUNCTIONAL IMPAIRMENT IN ALZHEIMER'S DISEASE (CURRENT STATUS: ACCEPTED)

This work aimed to provide a behavioural characterisation of the main features of these motor impairments in male 3xTg-AD mice at three ages, mimicking the early (6 months of age), advanced (12 months of age) and late (16 months of age) stages of the disease and compared to age-matched mice with the same genetic background (C57BL/6J) and normal ageing.

Specific objective

3. To characterise the motor dysfunction of the male 3xTg-AD mouse model at 6, 12, and 16 months of age in different motor tasks, focusing on the abnormal clasping reflex and coordination impairments as measured by the Phenotype Scoring System.

Experimental design



Clasping and ledge-score coordination impairment as primary behavioural markers of functional impairment in Alzheimer's disease

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Abstract:
Motor performance is an element that facilitates the understanding of the functional state related to the progression of Alzheimer's disease. At the translational level, here we characterize the motor dysfunction of the 3xTg-AD mouse model in different motor tasks, focusing on the abnormal clasping reflex and coordination impairments measured through the Phenotype Scoring System, four items screening originally developed for models of ataxia. We studied male 3xTgAD mice (n=24) at 6, 12, and 16 months of age (mimicking the early, advanced, and late stages of the disease, respectively) and their age-matched non-transgenic counterparts (NTg, n=21) with normal aging. According to the score, incidence, or severity of the four items and the total score, the 3xTg-AD mice showed deficiencies in all score elements. Clasping was increased independently of age, and its severity worsened with repeated testing. In contrast, the impairment of coordination worsened with the progress of the disease. The gait score was sensitive to genotype, and the worse ledge score was evident at 16 months. Kyphosis and ledge scores were sensitive to age. The impairments and functional limitations of male 3xTg-AD mice related to the stages of Alzheimer's disease provide a scenario that allows understanding the heterogeneity of non-cognitive symptoms.

Keywords: Alzheimer's disease, ledge score, clasping score, coordination, functional impairment, behavioural markers.

1. Introduction

Motor impairments in Alzheimer's disease (AD) have been classified as late-onset symptoms and traditionally downplayed due to the severity of cognitive decline as the clinical hallmark [1]. However, it is currently known that motor dysfunction increases in the moderate and severe stages of dementia [2], while there is still no consensus on changes in mobility during its early stages [2]. Different laboratories have described significant motor impairment in transgenic mice modelling AD [3–5]. Specifically, we have recently reported movement limitations and muscle weakness in the 3xTg-AD model [3,4]. Both aspects mark functional deterioration, which was related to the severity stages of the disease and found intensified with aging. Besides, motor performance in 3xTg-AD males is accompanied by a series of bizarre behaviours that interfere with the trajectory, which could explain poor neurological control [4].

Interestingly, other mutant mice with lesions in the cerebellum, basal ganglia, neocortex, or the spinal cord pathologies have shown a flexural response, often characterized by clasping the hindlimbs [5–7]. The pathological clasping reflex is a hallmark phenotype described in several murine mutations with cerebellar atrophy [5,8,9], particularly in cerebellar ataxia and Huntington's disease [10]. Recently, it has been observed that transgenic mice with amyloid pathology caused by mutations of amyloid precursor protein (APP) show this reflex [6,11].

Previously, this laboratory described the loss of deep cerebellar nuclei neurons in the 3xTg-AD mice model of Alzheimer's disease at ages mimicking early stages of the disease [12]. We have also repeatedly observed the presence of clasping and coordination alterations during animal care routines when these animals walk on a beam in males 3xTg-AD [3,4], similar to that reported in models of cerebellar ataxia.

Therefore, the present work is aimed to provide a behavioural characterization of the main features of these motor alterations in the males 3xTg-AD mice at three ages, mimicking onset (6 months of age), advanced (12 months of age), and late (16 months of age) stages of the disease in contrast with NTg mice with normal aging.

2. Materials and methods

2.1. Animals

Forty-five homozygous triple-transgenic (3xTg-AD, $n = 24$) and non-transgenic (NTg, $n = 21$) male mice of 6, 12, and 16 months of age in a C57BL/6J background established at the Autonomous University of Barcelona were used. The 3xTg-AD mice harbouring human $PS1/_{M146V}$, APP_{Swe}, and tau_{P301L} transgenes were genetically modified at the University of California at Irvine [13]. The animals were kept in groups of 3-4 mice per cage (Macrolon, 35 × 35 × 25 cm, Panlab, SL, Barcelona, Spain) with 5 cm of clean wood cuttings in each cage (Ecopure, Chips6; chip size of 2.8-1.0 mm) and nesting materials (Kleenex, Art: 08834060, 21 × 20 cm). All animals were kept with food and water ad libitum, at a temperature of 20 ± 2 ° C, a 12 h light cycle (lights on at 8:00 a.m.), and 50-60% relative humidity. All procedures followed Spanish legislation on "Protection of animals used for experimental and other scientific purposes" and the EU Directive (2010/63 / EU) on this issue. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used [14].

2.2. Experimental Design and Behavioural Assessments

A cross-sectional study of 3 cohorts of 3xTg-AD and NTg male mice, at 6, 12, and 16 months of age, was carried out. All the animals were weighed and assessed with the Phenotype score system that includes four sub-tests and scores: ledge, clasping, gait, and kyphosis [10,11]. Each measure is recorded on a scale of 0-3, with a combined total of 0-12 for all four assessments. The measures can be analysed individually or combined into a composite phenotype score for increased statistical power [15]. Individual measures are scored from 0 to 3 [11]. The tests were carried out in one day. In addition, we have expanded the analysis of the results obtained in the clasping and ledge scores. Previously, the clasping score has been used as a marker of disease progression in several mouse models of neurodegeneration [9] and ledge score as a direct measure of coordination [15]. In this way, we differentiated the severity of the observed behaviours since, in both scores, the 3xTg-AD animals presented a more significant deficit. Thus, we assigned a score that reflects the degree of severity, 0: normal; 1: mild; 2: moderate; 3: severe.

2.3. Statistics

Statistical analyses were performed with SPSS 15.0 software. Results are expressed as the mean ± standard error of the mean (SEM) for each task and trial and frequency (animals and episodes). The total Phenotype score system and each sub-test were analysed with one-way ANOVA followed by post hoc Bonferroni. The incidence of clasping and ledge scores were analysed using the Chi-square test or Fisher's exact test. The effect of Genotype (G) and Age (A) in each test was identified. In all cases, statistical significance was considered at $p < 0.05$.

3. Results

Body weight increased with age in the 3xTg-AD mice, while it was maintained in the NTg group [6-months: NTg = 30 ± 1.7g; 3xTg-AD = 28 ± 1.6 g; 12-months: NTg = 30 ± 1.0 g; 3xTg-AD = 32 ± 2.4 g; 16-months: NTg = 30 ± 0.7 g; 3xTg-AD = 34 ± 1.5 g, Age effect, A, ANOVA $F(2,44) = 3.268$, $p = 0.049$; 3xTg-AD 16 months *vs.*12 months, $p = 0.037$].

In the phenotype scoring system, G and A differences were detected in the total score of the four tests, as depicted in table 1. In all ages, the 3xTg-AD animals showed significant deterioration that worsened in the older age [Genotype effect, G, ANOVA $F(2,44) = 6.981$, $p=0.015$, 16-months 3xTg-AD *vs.* NTg, $p=0.047$]. Although the increase in functional impairment occurred in both groups, this increase was higher in the 3xTg-AD group [Age effect, A, ANOVA $F(2,44) = 10.119$, $p=0.000$, 3xTg-AD: 16-months *vs.* 6-months, $p=0.000$; 16-months *vs.* 12-months, $p=0.033$]. The 3xTg-AD scored for gait impairment and kyphosis already at 6 months of age, mimicking the early stages of the disease.

Genotype differences were detected in the clasping and gait scores, with the 3xTg-AD group presenting greater deterioration in both scores [Clasping score: Genotype effect, G, ANOVA $F(2,44) = 6.941$ $p=0.012$; Gait score: Genotype effect, G, ANOVA $F(2,44) = 5.325$, $p=0.026$]. In the ledge score, the effect of age was detected on the performance of the 3xTg-AD animals, increasing their deterioration at 16 months [3xTg-AD, Age effect, A, ANOVA $F(2,44) = 7.110$ $p=0.002$, 16-months *vs.* 6-months, $p=0.006$, 16-months *vs.* 12-months, $p=0.006$]. In addition, differences with age were detected in the kyphosis score. The older the

age, the greater the degree of postural alteration in both groups [Age effect, A, ANOVA F (2,44) = 6.212, p=0.005].

Figure 1 illustrates the performance of the mice in the assessment of clasping and the ledge scores. Independently of the age, an increased incidence of the clasping score was observed in the 3xTg-AD group, reaching statistical significance in the second and third trials (T2, T3) [Genotype effect, Fisher exact test, T2 df (2) =7.159 p=0.019; T3 df (2) =8.555 p=0.009]. Also, the performances at 16-months of age were sensitive to detect G differences, in the second trial, with 80% of 3xTg-AD mice exhibiting clasping reflex compared to only 20% in the NTg group [16-months, Fisher exact test df (2) = 6.895, p=0.002] (See figure 1A). No differences in age were detected in the clasping score.

The ledge score showed differences in G and A in all groups. Deterioration of coordination was observed in the 3xTg-AD animals that worsened with age and the repetition of the trials. Thus, the results in T3 at 16 months were the ones that allowed to record the most significant impairment, and T2 was also quite sensitive [Age effect, A, 3xTg-AD, Fisher exact test, T3 df (2) =18.652, p<0.001 and T2 df (2) =8.842 p=0.010, respectively]. On the other hand, genotype differences are also observed in T3, confirming the deterioration of 3xTg-AD animals [Genotype effect, G, Fisher exact test T3 df (10) =24.155, p=0.001] (See figure 1C).

Furthermore, distinct severity of coordination impairment in the ledge and clasping scores was found (see figures 1B and 1D). In the clasping score was recorded as follows: 6-months, NTg-mild 11% (2/18), 3xTg-AD-mild 33% (6/18), 3xTg-AD-moderate 6% (1/18); 12-months, NTg-mild 11% (2/18), 3xTg-AD-mild 24% (5/21), 3xTg-AD-moderate 14% (3/21); 16-months, NTg-mild 22% (6/27), 3xTg-AD-mild 55% (18/33), 3xTg-AD-moderate 9% (3/33). In the ledge score, the severity was as follows: 6 -months, NTg-mild 17% (3/18), 3xTg-AD-mild 22% (4/18); 12-months, NTg-mild 22% (4/18), 3xTg-AD-mild 10% (2/21), NTg-moderate 6% (1/18); 16-months, NTg-mild 44% (12/27), 3xTg-AD-mild 70% (23/33), 3xTg-AD-moderate 3% (1/33)].

4. Discussion

In the present work, we demonstrate the impairment in the physical phenotype score in the males 3xTg-AD mice described its features according to the incidence and severity at three stages of the disease. The scores showed the early appearance of impairments worsening with age in the 3xTg-AD mice. Previous studies of ataxia models [10,11,16] have demonstrated that transgenic males with harbouring P301S AD-mutation show changes in the four score items, while these changes were not evidenced in females [8]. Also, the TRIM32 model shows differences in both sexes, with impaired coordination and hindlimb clap [17]. Our results may have similarities with ataxia models. We found differences in the clasping, gait, and total scores, with age differences indicating significant deterioration in advanced stages of the disease, particularly in the ledge test, kyphosis, and total score. For the first time, the physical phenotype score system detected impaired motor coordination through two scores, the ledge score and gait analysis. Here, it is important to note that the ledge score is demanding since it also requests balance strongly affected by ageing and where weight differences can become a confounding factor. However, in the present study, weight interference can be ruled out since the factor effect on the ledge test was found on age but not genotype.

In normal conditions, adult rodents picked up by the tail and slowly descending towards a horizontal surface extend all four limbs in anticipation of contact [10,18]. This extension reflex response may be triggered by either visual or tactile stimuli [19]. However, in the case of mutant mice with CNS pathologies, a flexion response is displayed instead, often characterized by paw-clasping and a bat-like posture [8,18,20,21]. This response has been recently observed in Aβ plaque-bearing APP23 mice (genetic background C57BL/6J) and Tg2576 (C57B6/SJL) mutants. This pathological reflex has been also observed in APP751SWE/LD + PS1/M233T + L235P mutant mice on a B6/CBA/129SV background [18,21–23]. This report confirms that clasping is also present in 3xTg-AD mice. The description of its features indicates increased incidence and severity with the progress of the disease compared to control animals. In addition, clasping worsened with the repeated test, with 3xTg-AD males showing the most significant impairments in the third trial, a question that is methodologically relevant and also points at worse muscular endurance in this model, in agreement with previous reports in the rotarod [3].

The assessment of the ledge score allowed to identify deterioration in coordination in the 3xTg-AD mice, with genotype differences at 16 months of age compared to controls and the increase in the incidence of deterioration as age increased. Coordination and balance in mice are based on the location of the paws on the testing surface [17,24]. Thus, the ledge score

can indicate alterations related to coordination and may also show balance alteration.
Concerning the findings in gait score, we can highlight that the deterioration was present in 3xTg-AD animals already at 6 months of age and that genotypic differences with their NTg counterparts were sustained at all ages. Previously, we have described that gait motor performance in 3xTg-AD is accompanied by a series of bizarre behaviours that interfere with the trajectory and movement, even limiting the possibilities of exploration, which is evidenced from six months [4]. Also, these alterations were also identifiable in a scenario of social isolation in 13-month-old animals [3]. Moreover, genotype differences may arise if different types of kyphosis (postural and structural) are identified, as kyphosis may interfere with the gait and exploration of mice [25]. In this report, 3xTg-AD and NTg mice show an increased incidence of structural kyphosis from 12 months, while postural kyphosis is noted from 6 months of age in 3xTg-AD animals. In addition, kyphosis may be associated with animals' functional and frailty status [25].
On the other hand, a consistent correlation of the motor phenotype and cerebellar histopathological changes or cognitive deficits has not yet been reported [7]. In the 3xTg-AD mice model, the loss of cerebellar nuclei was previously described in the molecular layer at 6 months of age, when neuropsychiatric and cognitive symptoms were already apparent, mimicking the early stages of the disease [12]. The present findings may explain the impairments related to coordination, as pointed out by studies on cerebellar atrophy and its relation to the appearance of the clasping [7,20].

5. Conclusion

In summary, the 3xTg-AD mice show deficiencies in the elements of the physical phenotype score at different stages of Alzheimer's disease progression. In particular, clasping was increased independently of age, and its severity worsened with repeated testing. In contrast, the impairment of coordination is further exacerbated with the progress of the disease. The gait score was sensitive to genotype and could be recorded already at the early stages of the disease (6 months of age). Kyphosis and ledge score were sensitive to an age effect, with a worse ledge score in 3xTg-AD mice evident at 16 months of age.

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Table 1. Phenotype scoring system

Phenotype scoring system		6-months	12-months	16-months	Factors' effects
Clasping score	**NTg**	0.17 ± 0.11	0.22 ± 0.22	0.22 ± 0.09	**G***
	3xTg-AD	0.44 ± 0.14	0.52 ± 0.21	0.73 ± 0.14	
Ledge score	**NTg**	0.17 ± 0.16	0.33 ± 0.17	0.44 ± 0.11	**A$^{##}$**
	3xTg-AD	0.22 ± 0.16	0.10 ± 0.06	**0.76 ± 0.10$^{\&\&}$**	
Gait score	**NTg**	-	0.06 ± 0.05	0.07 ± 0.04	**G***
	3xTg-AD	0.11 ± 0.07	0.19 ± 0.06	0.15 ± 0.052	
Kyphosis score	**NTg**	-	0.50 ± 0.22	0.56 ± 0.17	**A$^{##}$**
	3xTg-AD	0.11 ± 0.11	0.43 ± 0.20	0.73 ± 0.14	
Total score	**NTg**	0.33 ± 0.27	1.11 ± 0.18	1.30 ± 0.21	**G*; A$^{###}$**
	3xTg-AD	0.89 ± 0.23	1.24 ± 0.34	**2.36 x 0.27 $^{\$\$\&}$**	

Phenotype scoring system. The results are expressed as mean ± SEM. Statistics: one-way ANOVA, Genotype effect (G), * $p < 0.05$; Age effect (A), ##$p < 0.01$, ###$p < 0.001$, followed by *post-hoc* Bonferroni test. Differences between 16-months *vs.* 6-months, $^{\$\$}$ $p < 0.01$; 16-months *vs.*12-months, $^{\&}p < 0.05$; $^{\&\&}p < 0.001$.

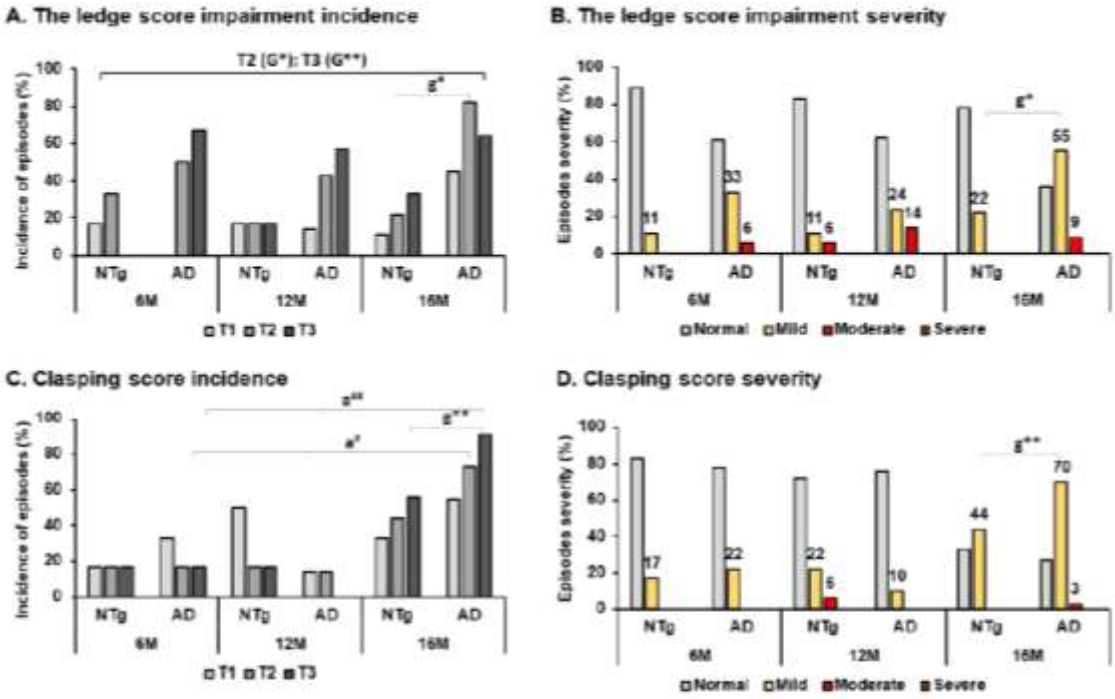

Figure 1. Clasping score and ledge score impairment. (A) Clasping score incidence; (B) Clasping score severity; (C) The ledge score impairment incidence, (D) The ledge score impairment severity. The results are expressed as incidence in percentage (%). Statistics: Fisher's exact test, Genotype effect (G), * $p < 0.05$, ** $p < 0.01$. Genotype differences in each age are expressed as (g) *$p < 0.05$. Age differences (A) in the 3xTg-AD group are expressed as (a); #$p < 0.05$, ##$p < 0.01$.

Conclusions

1) Male 3xTg-AD mice showed impairment in all physical phenotype score items.
2) Clasping increased independently of age and its severity worsened with repeated testing.
3) Coordination impairment worsened with disease progression.
4) Gait score was sensitive to genotype and the worst ledge score was evident at 16 months. Kyphosis and ledge scores were sensitive to the effect of age.

FRAILTY, FROM HUMANS TO MOUSE MODELS

Most authors agree that the clinical manifestations of frailty syndrome in humans include: involuntary decreases in body weight, muscle strength and strength, impaired balance and gait, and decreased physical mobility. This report aims to highlight the most useful mouse models used in research based on the biological hypothesis of human frailty syndrome. Animal studies provide opportunities to understand the mechanisms that trigger frailty. They also provide empirical evidence on pathophysiological pathways and mechanisms and identify potential biomarkers to generate interventions and treatments to modulate or counteract the syndrome.

Specific objective:

4. To identify the most useful mouse models used in research based on the biological hypothesis of human frailty syndrome.

Gerontology And Geriatric Research

SCIENTIFIC LITERATURE

Special Issue Article "Frailty"

Research Article

Frailty, from Humans to Mouse Models

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ABSTRACT

The frailty corresponds to a syndrome of well-defined biological and clinical characteristics within its physical phenotype, is multidimensional, dynamic and non-linear. It has a high prevalence in the elderly population and increases after 65 years of age. The syndrome of frailty can be considered as a state of predis-capacity or risk of developing a disability and dependence from a situation of incipient functional limitation. It is identified by a decrease in the resistance and the physiological reserves that lead to a deterioration of the physiological systems, causing adverse effects on health. This report aims to highlight the most useful mouse models used on the research based on the biological hypothesis of human frailty syndrome. Animal studies provide opportunities that can help us understand the mechanisms that trigger frailty. In addition, they provide empirical evidence on their pathways and physio pathological mechanisms, as well as the identification of potential biomarkers to generate interventions and treatments that modulate or counteract the syndrome.

INTRODUCTION: FRAILTY AS CLINICAL SYNDROME

Frailty is a concept that has been increasing substantially since the 1980s [1]. Different authors emphasize diverse aspects of frailty incorporating physical function, cognitive function and psychological and psychosocial factors, making it possible to differentiate characteristics of a phenotype which evolves towards a state of dependence, loss of the physiological reserves, uncoupling from the environment, chronic illnesses and their complications [2-4]. Most definitions includes an excessive reduction of lean body mass (sarcopenia), a reduced ability to ambulate and move, and less physical activity with an added sense of weakness [5].

From the clinical point of view, frailty is considered a syndrome whose phenotypic expression is the result of a progressive decline of physiological functions in multiple body systems. In addition, it is accompanied by a state of greater vulnerability to stress that leads to an increased risk of dependence, functional deterioration, hospitalization and mortality in elderly people [1-3,6]. In 1988 Woodhouse defined frail elderly people as those more than 65 years of age who depended on others for the activities of daily living and were often under institutional care [7]. Later Gillick complements this concept emphasizing the social consequences of frailty [8].

Among geriatrics, the concept of frailty include the presence of chronic diseases, alteration of gait, sensory deficits, poor self-perception of health, repeated falls, polypharmacy, frequent hospitalizations [1,9]. Also, it includes functional criteria established in terms of dependence on basic activities of daily life and dependence

on instrumental activities. Among the cognitive and affective criteria, the concept of frailty includes depression and cognitive impairment. With respect to socioeconomic criteria it can be identified: living alone, recent widowhood, age over 80 and low income [3,6,7]. It is widely accepted that the prevalence of frailty increases dramatically with age, and appears to be a result of a vicious cycle influenced by endogenous and exogenous factors [4,9].

Now, the recognition of frailty supposes the recognition of frailty is an important challenge for clinicians and health agencies, since their presence suggests a greater risk of adverse effects on health, increased needs for long-term care, greater dependency and disability, as well as an increase in health spending, making necessary a timely intervention.

FRAILTY CLINICAL PHENOTYPE

Strategies to differentiate frailty phenotypes benefit from multifactorial approaches that allow us to differentiate genetic, cellular, psychological, physiological and environmental risk factors [2,5]. From this point of view, Brocklehurst's Dynamic model of frailty model allows us to differentiate a balance between assets that help a person maintain their independence in the community, and deficits that threaten this independence. Among the factors of advantage are: health, functional capacity, a positive attitude towards health and other resources (social, spiritual, financial and environmental). While in the deficits are: chronic diseases, disability, dependence on others for activities of daily living and the burden of caregivers [6]. Rockwood and collaborators add an interaction of assets and deficits, "medical" and "social", that maintain independence, reinforcing the model dynamically, whose changes in the state can be recognized by adjusting the weights of the various assets and deficits [1,6].

At the same time, Campbell and Buchner [10], considered that frailty arises from a decline in the reserve of multiple systems, which places the frail older person 'at risk' for disability or death with minor stresses, a notion they call 'unstable disability'. In more advanced ages, frailty is equated with an increased risk of death associated with age, being a complex factor present during aging [1]. For its part Aubertin-Leheudre et al. organize the risk factors into four categories: physiological, such as immune system dysfunction; doctors, such as diabetes or cognitive impairment; sociodemographic and psychological, such as depression [4].

On the other hand, many hypotheses have been proposed about the causes or origin of frailty, being the most consensus: genetic disorders, diseases and injuries, lifestyle and aging [9]. Essentially is the result of multiple alterations among which endocrine, immunological and musculoskeletal dysregulations have been reported. Among them, sarcopenia (loss of strength and muscle mass) represents a fundamental element [11]. As a result, this scenario predisposes the elderly to have a greater number of diseases and adverse effects, derived from a lack of compensatory mechanisms and loss of homeostasis, due to a decline in multiple bodily systems (muscular, immune, neuroendocrine, vascular) with decrease of their functional reservation [5,11,12].

ASSESSMENT CLINICAL SIGNS OF DETERIORATION AND DISABILITY IN THE FRAILTY SYNDROME

In recent decades, numerous attempts have been made to find which criteria best identify frail patients. Fried, in 2001, elaborated a definition of "frailty phenotype" that consisted of the presence of 3 of 5 elements to be evaluated: 1) unintentional loss of ≥ 10 pounds in the previous year, 2) feeling of "being exhausted" reported by the patient, 3) weakness (measured by the strength of the fist closure, 4) slow gait and 5) little physical activity [5]. The predictive value of this scale was determined based on the data obtained in a prospective cohort study on cardiovascular health in people over 65 years of age. This model has been validated later through the data of the Cardiovascular Health Study. Fried's study showed that patients who had three or more components of the phenotype had a higher risk of falls, loss of mobility, alteration in the ability to perform activities of daily living, hospitalization and death. The presence of up to two components would make up the risk group of preventive interventions. It was possible to demonstrate that the fragile group differed from the group with disability and from the group with comorbidity. In his work, Fried concludes that frailty is not synonymous with disability and that the terms are not exclusive [3,5]. Therefore, Fried's criteria have served as a model for the assessment of frailty in clinical scenarios where an accurate, easy and quick diagnosis is needed, including first

contact consultation for outpatients and frailty screening in different populations [4,11].

In the same way, Macknight and Rockwood have focused on investigating the presence of frailty as a predictor of morbidity and mortality in patients who live in nursing homes or patients in the perioperative period. They propose a multi-domain model that provides important ideas: (1) frailty represents greater vulnerability; (2) is heterogeneous; and (3) it is associated with chronological aging. In effect, it becomes biological, as opposed to chronological age [1]. Therefore, any definition of frailty must include the following: multisystem impairment, instability, change over time, an allowance for heterogeneity within a population, an association with aging, an association with an increased risk of adverse outcomes [1,13].

Therefore, physical frailty and cognitive frailty have been differentiated. Being physical frailty, a clinical condition characterized by an abnormal decrease in physiological reserves that increases stress and reduces the ability of an individual to maintain homeostasis and, therefore, leads to vulnerability [4,5]. There are different evaluation guidelines to measure physical frailty being very important to describe between frailty and normal aging since they seem to be indistinct because some factors, such as sarcopenia and strength (dynapenia), occur throughout the aging process. In turn, the term cognitive frailty has been used as a general descriptor for the cognitive impairment that occurs when people reach advanced age, or to refer to cognitive or pre-demential disorders that occur in association with other medical conditions. The term cognitive frailty implies a parallel with physical frailty. However, the definition of cognitive frailty depends on its diagnostic criteria [4].

To facilitate the ability to assess physical frailty, Studenski et al. [14] Report of the Global Clinic of Change in Physical Frailty of the Physical Environment of Frailty Includes: Medical understanding, Use of medical attention, Appearance, Perceived health, Activities of daily life, Emotional state and social status. His research looks at the geriatric clinical opinion about the change in physical frailty, agreeing on the evaluation and measurement criteria, so that his instrument discriminates the magnitude and direction of the change, capturing patterns of contributing impediments what makes it feasible to apply in clinical research [14,15].

There is also a consensus that frailty is a state of pre-disability, so that both its definition and measurement instruments should not appear determinants of disability. The overlap of frailty and disability is like the superposition of these with comorbidity. While many individuals who are fragile also have disabilities, frailty is not synonymous with disability, defined as the difficulty or dependency for some activities of daily life. In fact, frailty is a predictor of dependence as a physiological precursor of it [3-5].These hypotheses have been key the biological bases to develop the animal models.

BASIC CLINICAL MEASUREMENTS OF FRAILTY IN HUMANS AND MOUSE MODELS

Clinical studies on frailty have limitations inherent to the population under study, heterogeneity of frail elderly. Many of the advances made by studies, observations and clinical trials have been able to provide intervention strategies for this age group, but they have not completely resolved the problem [16,17]. Although clinical studies are beginning to understand frailty, there is still a real lack of evidence to guide clinicians to identify, assess and treat frailty. Hence, animal models can help the study of frailty, reducing genetic and lifestyle factors that contribute or confuse the observed phenotypes [18].

Mice are the models of mammals widely used in research due to their relative ease of genetic manipulation, low cost and short lifespan [19]. Through mouse reproduction technology, researchers have been able to reduce biological variation as a source of experimental noise and have thus achieved successful advances in different fields. On the other hand, both the frailty phenotype of Fried and the frailty index of Rockwood have been translated into mice, so its applicability and translation to humans is directly benefited [11,20].

The molecular basis of frailty, a syndrome rather than a disease is little known, mouse models of frailty would be of great value to determine which are the pathways that trigger frailty. That is why different preclinical models of frailty in animals have been developed to explore or mimic manifestations of frailty in humans, identifying keys that promote research in this field [16,21]. There are different models of study, from those that address biological protestors due to aging, others address the cumulative effects of deficits

and consequences of lifestyle, trying to quantify manifestations and deterioration. While others seek to recreate preclinical signs to improve rehabilitation strategies and timely treatments, recreating genetic models that recreate the etiology of frailty [16-19,21]. (Table 1) lists the most studied and applied models that simulate frailty in mouse models, in this table you can see the different measurements according to the clinical bases developed from the clinical studies conducted in different studies in humans.

Table 1: Mouse models for frailty: tools of comparative evaluation according to the parameters of the frailty index in humans.

Clinical basis	Animal model concept	Experimental subject	Study	Parameters of frailty assessment and their applications
Biological Age and frailty in aging mice	*Biologicals age*	C57BL/6J (male mice)	Ingram and Reynolds [22]	Evaluate biological age through a battery of psychomotor tests: rotarod (balancing on rotating rod), grip strength, exploratory behavior and wheel running tasks. This study is not specifically for frailty but, it is useful for measuring general health or biological age in animal experiments on aging.
Frailty in Genetically manipulated mice	*IL-10 knock-out mice*	Female IL-10^{tm1Cgn} mice on a C57BL/6J background	Walston et al. [24]	Based on the characterization of IL-10^{tm1Cgn} genetically modified model. To explore biological mechanisms of frailty. Model of inflammation and multisystemic decline.
Biological Age and frailty in aging mice	*Sarcopenia in frailty*	C57BL/6J (male breeder mice) and *Sprague Dawley male Rat*	Walter [9]	Characterization skeletal muscle aging in pre-clinical mammalian models. Measurement of muscular performance, size and architecture through micro X-ray computed tomography (micro-CT) imaging and muscle histology.
Based on Rockwood's Frailty Index	*Mouse frailty index*	C57BL/6J (male and female mice)	Parks et al. [19]	Evaluate different health parameters: activity levels, hemodynamics measures, body composition and basic metabolic status. The Mouse *frailty index* can be used to quantify frailty in aging mice.
Biological Age and frailty in aging mice	*C57BL/6J neuromuscular healthspan-scoring*	C57BL/6J (male mice)	Graber et al. [26]	The Neuromuscular healthspan scoring system provide a score each animal from three individual scores obtained from the functional assessment: rotarod, grip strength and the maximal isometric force. Also provide information the in vitro muscle contractility.
Based on Fried's Frailty Phenotype	*Frailty phenotype index*	C57BL/6J (male mice)	Liu and Graber (2014)	Assess levels of physical performance: grip strength, walking speed (rotarod), physical activity (voluntary wheel running), endurance (average of grip strength and walking speed test)
Based on Rockwood's Frailty Index	*Mouse clinical frailty index*	C57BL/6J (male and female mice)	Whitehead et al. [20]	Evaluate the parameters of possible deficits related to aging principally through visual inspection of the evaluator: Integument, physical/musculoskeletal, vestibulocochlear/auditory, ocular/nasal, digestive/urogenital, discomfort and body weight and temperature. This model is based on deficit accumulation throughout life and exhibits features observed in clinical studies in human.
Frailty in Genetically manipulated mice	*Cu/Zn superoxide dismutase knockout mouse*	Sod1KO mice	Deepa et al. [15]	The model shows alterations similar that characteristics to define human frailty: weight loss, weakness, low physical activity and exhaustion. Sod1KO mice show increased inflammation and sarcopenia. Useful to study the etiology of frailty.
Based on Fried's Frailty Phenotype	*Inactivity as a model of frailty (Valencia Score)*	C57BL/6J (male mice)	Gomez-Cabrera et al. [27]	Score for frailty based on five Fried's criteria for frailty in human: they propose a Valencia score (frailty in rodents): weight loss unintentional, weakness, grip strength, poor endurance and energy, slowness and low physical activity level (light-rope test). The study speared in two groups the animals: sedentary mice and spontaneous wheel-runners.

From the biological point of view, models such as the one developed by Ingram and Reynolds in male C57BL76J mice have been described. They observed at the same chronological age different biological ages as a manifestation of biological processes related to the passage of time, among the individual variables survival has a positive relationship, the lower the rate of decline in performance, the longer the life of the individuals [22]. The study by Ingram and Reynolds does not have a direct relationship with frailty, but it makes an approach to the individual differences between individuals from the biological point of view and the process linked to changes during aging [21,23].

On the other hand, Walston et al. [24] explores biological mechanisms of frailty based on an inflammatory and immunological cellular model, which points towards the multisystemic decline that surrounds this syndrome [17,21]. The IL-10 model does not express the anti-inflammatory cytokine interleukin 10 (IL-10) and, like frail human beings, is more susceptible to the activation of the inflammatory pathway [24]. Walston suggests that increasing the age of IL-10 mice would develop physical and biological characteristics like those of humans, since it develops an inflammation and a decrease in strength that is compatible with human frailty at a younger age compared to the control type mice C57BL/6J [16,17,21].

In addition to the IL-10 model recognized as a genetic model, Deepa et al. [15], they developed a new genetic model Cu/Zn superoxide dismutase, which exhibits four characteristics that define frailty in humans: weight loss, weakness, low activity and exhaustion. The Sod1 KO animals of this model show increased inflammation and sarcopenia, playing a role in the etiology of frailty at the level of oxidative stress, mitochondrial dysfunction and cellular senescence. Although both genetic models are the

best available models of their type, there is no evidence of the role played by the expression of their genes in human frailty [15].

With a physiological approach based on the Rockwood Frailty Index, Whitehead et al., [20], proposes a series of parameters related to possible aging deficits [18]. Establishes a clinical index of frailty based on the concept of accumulated deficits in people providing information on activity monitoring, hemodynamic status, body composition, basic metabolism and organ function. In their study, 31 variables measured in male and female C57BL/6J mice were incorporated [15,25]. Their results demonstrated that a clinical index of non-invasive frailty can be used to quantify frailty in mice. In addition, their clinical frailty index showed a progressive increase with the age of the subjects [20].

In consideration with the impact that age has on the biological and physiological alterations of the frailty syndrome Parks et al., [19] developed a frailty detection and quantification tool in a mouse model associated with aging based on the frailty model of Rockwood [16]. Parks et al. [19] Developed an approach to quantify frailty with a Frailty Index (FI). To quantify frailty, they measured many health-related variables linked to the function of different systems that are known to change with age in both human and animal models. They selected 31 specific variables chosen to provide information about activity levels, hemodynamic status, body composition, basic metabolism and organ function. They measured all these variables in a small group of adult and aged mice to generate a unique FI score for each animal and we compared these scores between different groups (age, sex). Their results showed that the levels of frailty were similar in aged males and females. Furthermore, they found that there were no differences between the sexes in the parameters used to construct the FI in the aged group, although the middle-aged females had lower systolic blood pressure, lower lean tissue mass, and more body fat than the males as reported previously in mouse models [19]. The frailty index developed by Parks et al. [19]. Is one of the most used indices in current investigations of frailty in mouse models. It has also allowed us to understand the relationship between frailty and cardiac changes that occur with aging.

Alternatively, Whitehead et al. [20] continued the research based on the Frailty index proposed by Parks et al. Whitehead et al, used a physiological approach that included the systems: musculoskeletal, ocular and digestive, by measuring 31 criteria that allowed quantification non-invasively in animals [16]. Frailty was studied in male and female C57BL/6J mice through a longitudinal study. As a result, they obtained that the frailty index score increases gradually in adult (5 months) to old (19 months) and very old (28 months) animals in males and females. In addition, the range of deficits accumulated in mice was like those observed in the clinic of human frailty. For the validation of the scale created by Whitehead et al., it was submitted to a correlation with scales applied in humans in its 31 criteria, as well as with the frailty index of Parks [20]. The limitation of the current indices applied in mouse models by both authors Parks and Whitehead, is that they do not include cognitive criteria or a social and hierarchical relationship between animals. Undoubtedly both indexes are very useful for the development of research in this field.

Also based on the frailty model of Rockwood, Graber et al. [26] developed a system of evaluation of frailty based on physiological and functional measurements to test the efficacy of possible interventions for sarcopenia and frailty in animal models of aging [16]. They developed a neuromuscular scoring system of the healthspan, evaluating male C57BL/6J mice of three ages: adults (6-7 months of age, 100% survival), old (24-26 months of age, 75% survival), and group of elderly people (> 28 months of age, ≤50% survival). The functional performance was obtained from the rotarod tests and the inverted grip test. In addition, muscular contractility in vitro was determined. Among their results, they found that both functional capacity and strength deteriorate with age in the C57BL / 6J mouse as evidenced by decreases in the grip test, rotarod and muscle contractility [25]. This model can be used as a tool for researchers to evaluate interventions from the point of view of motor performance related to frailty syndrome.

Finally, among the most recent models of frailty study in mouse models is the one developed by Gomez-Cabrera et al. [27]. This model is based on the human frailty phenotype of Fried and aimed to create a score for frailty in experimental animals called "Criteria de Valencia". They also sought to determine

the effect of physical inactivity on the development of frailty. They included male C57Bl / 6J mice and compared the sedentary lifestyle versus the active lifestyle in terms of frailty by evaluating the clinical criteria used in humans: involuntary weight loss; bad resistance (execution time); slowness (running speed); weakness (grip strength) and low level of activity (motor coordination) in five different ages: 17, 20, 23, 26 and 28 months of age. Each criterion had a designated cut-off point to identify the mice with the lowest performance. Among its results it can be uncovered that spontaneous life-long exercise significantly delays frailty contrary to what happened in sedentary animals that become fragile as they get older. Gomez-Cabrera et. al, propose physical inactivity as an experimental model for the study of frailty [27].

CONCLUSIONS

The concept of frailty is key in the context of geriatric care. It has evolved from Linda P. Fried's phenotypic frailty model and Kenneth Rockwood's cumulative deficit model, creating the theoretical construct that has allowed the understanding of the processes that frailty involves. Most authors agree that the most common clinical manifestations are an involuntary decrease in body weight, strength and muscle strength, balance and gait disturbances and a decline in physical mobility. The study of these clinical signs has allowed the understanding of the processes that condition the loss of the capacity of adaptation that elderly people present with frailty. The studies carried out in humans have limitations due to the heterogeneity of the syndrome, its manifestations involve affectation of several organs and bodily systems making it multidimensional. Faced with limitations in human studies, preclinical studies in animals provide opportunities to provide this evidence empirically, helping us to understand the mechanisms of frailty, identify potential biomarkers and explore interventions to modulate and generate treatments for frailty syndrome.

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PHASE 2: MODULATION AND INTEGRATION OF PRIMARY AND SECONDARY PSYCHOMOTOR SIGNATURES OF ALZHEIMER'S DISEASE DYSFUNCTION AND EXTRINSIC FACTORS

Research articles:

A. Translational Modelling of Psychomotor Function in normal and AD-Pathological aging with special concerns on the effects of social Isolation
B. Kyphosis and bizarre patterns impair spontaneous gait performance in end-of-life mice with Alzheimer's disease pathology while gait is preserved in normal ageing
C. Impact of behavioural assessment and re-test as functional trainings that modify survival, anxiety and functional profile (physical endurance and motor learning) of old male and female 3xTg-AD mice and NTg mice with normal aging

Research article submitted:

Annex 7: Phenotypical, behavioural and systemic hallmarks in end-point-mice scenarios.

Poster presentation:

Annex 8: Translational modelling of psycho-motor function in normal and pathological aging with special concerns on the effects of isolation. International Psychogeriatrics (2020), doi:10.1017/S1041610220002732.

TRANSLATIONAL MODELLING OF PSYCHOMOTOR FUNCTION IN NORMAL AND AD-PATHOLOGICAL AGING WITH SPECIAL CONCERNS ON THE EFFECTS OF SOCIAL ISOLATION

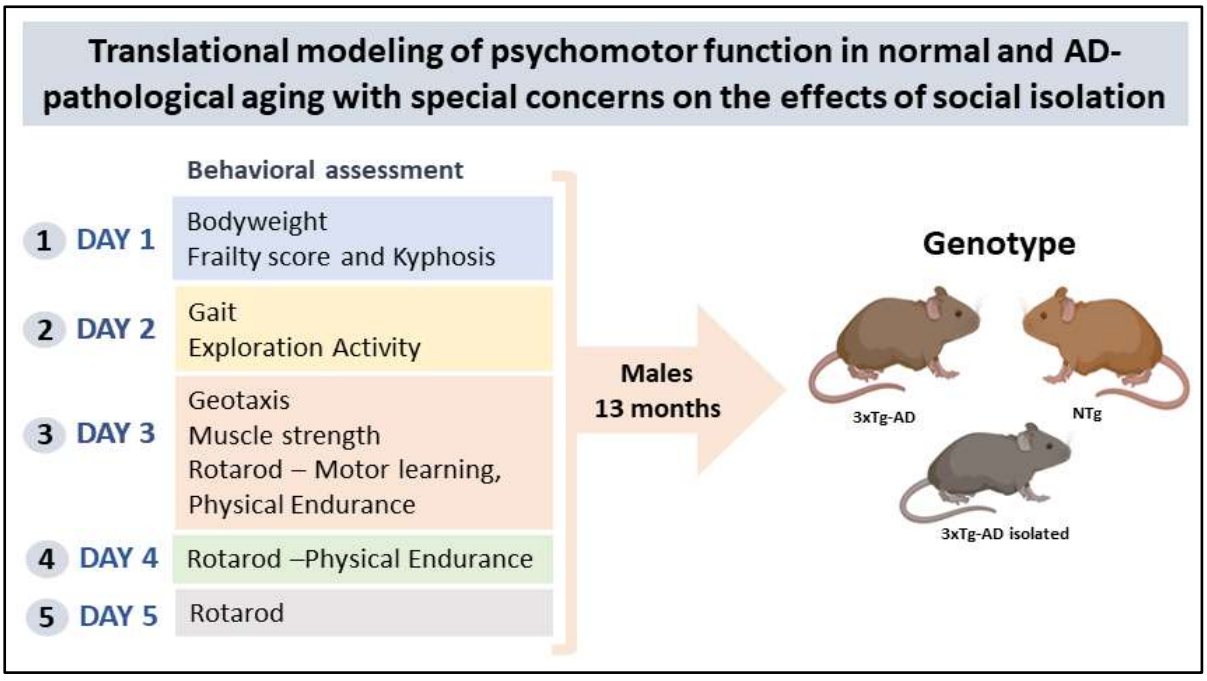
This work evaluated psychomotor functions in normal ageing and pathological Alzheimer's disease male mice and the impact of “naturalistic isolation” in a subgroup of 3xTg-AD mice. Thirteen-month-old male 3xTg-AD and C57BL/6 mice were included. A battery of tests was used to assess four psychomotor functions: spontaneous gait analysis, muscle strength, motor performance, and the physical frailty phenotype.

Specific objectives:

5.1. To explore the psychomotor performance of 13-month-old male NTg and 3xTg-AD mice, corresponding to normal ageing and advanced stages of Alzheimer's disease.

5.2. To assess the impact of isolation in a subgroup of male 3xTg-AD mice that lost their partners and lived alone for the last 2-3 months after ten months of social life.

Experimental design



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Translational Modeling of Psychomotor Function in Normal and AD-Pathological Aging With Special Concerns on the Effects of Social Isolation

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One year after the start of the COVID-19 pandemic, its secondary impacts can be globally observed. Some of them result from physical distancing and severe social contact restrictions by policies still imposed to stop the fast spread of new variants of this infectious disease. People with Alzheimer's disease (AD) and other dementias can also be significantly affected by the reduction of their activity programs, the loss of partners, and social isolation. Searching for the closest translational scenario, the increased mortality rates in male 3xTg-AD mice modeling advanced stages of the disease can provide a scenario of "naturalistic isolation." Our most recent work has shown its impact worsening AD-cognitive and emotional profiles, AD-brain asymmetry, and eliciting hyperactivity and bizarre behaviors. Here, we further investigated the psychomotor function through six different psychomotor analysis in a set of 13-month-old 3xTg-AD mice and their non-transgenic counterparts with normal aging. The subgroup of male 3xTg-AD mice that lost their partners lived alone for the last 2–3 months after 10 months of social life. AD's functional limitations were shown as increased physical frailty phenotype, poor or deficient psychomotor performance, including bizarre behavior, in variables involving information processing and decision-making (exploratory activity and spontaneous gait), that worsened with isolation. Paradoxical muscular strength and better motor performance (endurance and learning) was shown in variables related to physical work and found enhanced by isolation, in agreement with the hyperactivity and the appearance of bizarre behaviors previously reported. Despite the isolation, a delayed appearance of motor deficits related to physical resistance and tolerance to exercise was found in the 3xTg-AD mice, probably because of the interplay of hyperactivity and mortality/survivor bias. The translation of these results to the clinical setting offers a guide to generate flexible and personalized rehabilitation strategies adaptable to the restrictions of the COVID-19 pandemic.

Keywords: traslacional neuroscience, Alzheimer disease, psychomotor function, motor performance, gait, frailty, isolated, COVID-19

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INTRODUCTION

The COVID-19 pandemic is causing high morbidity and dramatic mortality worldwide. Unfortunately, it has also put pressure on healthcare systems and altered our lifestyles, leading to many worrisome secondary impacts (Brown et al., 2020). Severe measures to curb its spread have been adopted and are still implemented in the new waves, restricting physical and social contact between people, with the elderly population being among the most affected (El Haj et al., 2020). Consequently, preventive strategies and therapeutical interventions for older people, such as promoting social activities, physical and environmental stimulation critical for those with dementia, have been kept to a minimum (Canevelli et al., 2020).

Physical activity is essential to control symptoms and risk factors for many diseases (Warburton and Bredin, 2017). The closures of gyms, swimming pools, and exercise clubs, in addition to laws limiting access to outdoor space and free movement, have inevitably reduced opportunities to exercise or play sports. Decreased physical activity levels in many people may increase other unhealthy lifestyles, but it is also triggering a worsening of the clinical symptoms of diseases, such as Alzheimer's disease (Lautenschlager et al., 2008; Abate et al., 2020; Lara et al., 2020). Exercise is essential to reduce sarcopenia, falls, and fall-related injuries in healthy older adults. Also, the cognitive, cardiorespiratory, and musculoskeletal benefits will be directly affected by the cessation of its performance (Palmer et al., 2020). The closure of day centers has left those whose fragility requires permanent rehabilitation programs at home. Therefore, unprofessional home care may not be enough to meet complex diseases' needs and demands (Wang et al., 2020).

Alzheimer's disease is a complex neurodegenerative disease that leads not only to hallmark cognitive impairment but also to psychomotor dysfunction (O'Leary et al., 2020). Thus, it is one of the leading causes of disability and dependency among older people worldwide. Due to their cognitive and functional deficits, AD patients are vulnerable during crises, especially during the COVID-19 pandemic, and confinement seems to affect neuropsychiatric symptoms in AD patients with low baseline cognitive function (Boutoleau-Bretonnière et al., 2020). It can be overwhelming for those affected and for their caregivers and families, with very high pressure on the direct and indirect healthcare costs (Wang et al., 2020). In the current scenario, people with AD are a particularly vulnerable population due to their complex cognitive and psychomotor dysfunction (Verlinden et al., 2014). Memory problems enhance their difficulties in understanding what is happening (Lara et al., 2020; Wang et al., 2020). This pandemic further exacerbates their vulnerability due to morbidity and mortality from the virus and the pandemic's indirect effects on the health system and support networks on which they depend (Brown et al., 2020). Some studies have reported alterations and exacerbation of cognitive and behavioral symptoms related to confinement and its effects on AD. Worsening of cognitive symptoms, particularly of memory and orientation abilities, the appearance of alterations, such as agitation-aggression, apathy, and depression, the most practical manifestations, have been detected (Boutoleau-Bretonnière et al., 2020; El Haj et al., 2020; Palmer et al., 2020).

Therefore, despite the main clinical characteristic of Alzheimer's disease is cognitive decline and impairment, motor disorders, such as bradykinesia, extrapyramidal stiffness, and gait disturbances are also significant. They will also be affected by the limitations and restrictions dictated to contain and prevent the COVID-19 pandemic (Abate et al., 2020). More excellent knowledge of these psychomotor dysfunctions will contribute to improving the actions to intervene on these deficiencies and impediments that restrict the independence and autonomy of people and their environment.

Like what happens in patients with AD, different mouse models mimic psychomotor deficiencies on a translational level. These deficiencies indicate disease progression when they increase in severity (Buchman and Bennett, 2011; Wagner et al., 2019), making them an essential phenotype for the study of AD progression (O'Leary et al., 2018). The 3xTg-AD model (Oddo et al., 2003) has been widely studied for the impact of Aβ and tau at different study levels, from synaptic plasticity to behavior (España et al., 2010). It mimics various AD symptoms in a temporal and neuroanatomical pattern similar to that observed in humans (Belfiore et al., 2019). After 12 months, a neuropathological profile corresponding to the disease's advanced stages can be observed (Oddo et al., 2003; Belfiore et al., 2019). Thus, this model has made it possible to carry out numerous basic research studies to know the factors related to the progression of the disease as well as preclinical investigations that seek to verify the effect of preventive and therapeutic therapies (Martini et al., 2018).

Therefore, the current study aimed to explore the psychomotor performance of 13-month-old male N1g and 3xTg-AD mice corresponding to normal aging and advanced stages of Alzheimer's disease. We have used a battery to evaluate six different psychomotor functions: spontaneous gait analysis, muscle strength, motor performance, the physical phenotype of frailty. Also, we assessed the impact of isolation in a subgroup of male 3x1g-AD mice that lost their partners and, after 10 months of social life, lived alone for the last 2–3 months.

MATERIALS AND METHODS

Animals

A total of forty-six homozygous 3xTg-AD ($n = 31$) and non-transgenic (NTg, $n = 15$) male mice of 13 months of age in a C57BL/6J background (after embryo transfer and backcrossing of at least 10 generations) established at the Universitat Autònoma de Barcelona (Baeta-Corral and Giménez-Llort, 2014) were used in this study. The 3xTg-AD mice harboring transgenes were genetically modified at the University of California at Irvine, as previously described (Oddo et al., 2003). Animals were kept in groups of 3–4 mice per cage (Macrolon, 35 × 35 × 25 cm) filled with 5 cm of clean wood cuttings (Ecopure, Chips6, Date Sand, UK; uniform cross-sectional wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 × 20 cm, White). In the current work, 7 of the 31 3xTg-AD mice had lost their cage mates and lived alone in their

cage for 2–3 months. In all cases, standard home cages covered with a metal grid allow the perception of olfactory and auditory stimuli from the rest of the colony. All animals were kept under standard laboratory conditions of food and water ad lib, 20 ± 2°C, 12 h light cycle: dark with lights on at 8:00 a.m. and 50–60% relative humidity.

Behavioral Assessment

Psychomotor behavior was measured in a behavioral battery consisting of six consecutive steps: (1) Physical Frailty Phenotype, (2) Spontaneous Gait Phenotype: Exploratory activity and (3) Quantitative parameters of gait), (4) Muscular Strength: Forelimb Grip Strength and muscular endurance—Hanger test, (5) Motor performance: Learning, Physical Endurance, and Coordination—Rotarod, and (6) Hindlimb clasping and Geotaxis. Assessments were performed under dim white light (20 lx) during the light cycle of the light cycle: dark (10 a.m. to 1 p.m.). Behavioral evaluations were carried out in 3 days and a counterbalanced manner by observing two independent observers blind to the genotype. The tests were carried out during the morning; 30 min were assigned to habituate the animals in the test room before starting the measurements. We made the following distribution: Day 1—Physical frailty phenotype (body weight, kyphosis, alopecia, etc.) and 2 h later, it was done spontaneous gait analysis; Day 2—Muscle strength and Motor performance; Day 3—Hindlimb clasping and geotaxis. All procedures followed the Spanish legislation on "Protection of animals used for experimental and other scientific purposes" and the EU Directive (2010/63/EU) on this issue. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used (Kilkenny et al., 2010).

Physical Frailty Phenotype

Physical signs of frailty were identified through a physical phenotype that includes the following measurements: body weight, body position, palpebral closure, piloerection, alopecia, tail position, tremor, and kyphosis. These measurements were made before the different tests that are described later. A score of 0 was assigned for normal aspects or 1 for abnormal aspects. Besides, a photographic record was taken of each animal to demonstrate these physical aspects. Also, were measured geotaxis and Hindlimb clasping. Geotaxis was measured using a 10 × 12 cm grid; the time it took for the animal to reach the vertical position from an inverted position at a 90° angle on the grid was recorded in a single trial. Hindlimb clasping closure is a marker of disease progression and severity in several neurodegeneration models in mice. We have included the test described by Chou et al. (2008) and illustrated by Guyenet et al. (2010), which consists of holding the mouse by the tail near its base, observing the hindlegs' position for 10 s in three trials. If the hindlegs are extended continuously outward, away from the abdomen, it is scored with a 0, indicating normality. If one or both hindlegs are retracted toward the abdomen for more than 5 s, a score of 1 and 2 are assigned, respectively. If its hind legs are fully retracted and touching the abdomen for more than half the time, a score of 3 is assigned, indicating greater severity. After each test, the animal is given 30 s of rest.

Spontaneous Gait Phenotype

To assess spontaneous gait, the mice were placed in a 27.5 × 9.5 cm transparent test box and observed during a total period of 2 min.

Exploratory Activity

In trial 1, the latency to start the movement (taking as reference the movement of the hind legs), the number of explorations (visited corners), the latency and the number of rearing were recorded. Bizarre behaviors were identified during the execution of the walk and classified according to our previous work (Baeta-Corral and Giménez-Llort, 2014). **Figure 2C** shows the path of the circling trajectory of a representative animal. During the tests, defecation and urination were also recorded.

Quantitative Parameters of Gait

Two 1-min trials were performed, and gait was recorded by video recording from the undersurface (Cheng et al., 1997). The KINOVEA 8.26 free software was used to identify the metacarpal and metatarsal fore and hind legs and perform the analysis. The quantitative parameters were those described by Wang et al. (2017). A representative animal is illustrated in **Figures 2A,B**.

Muscular Strength: Forelimb Grip Strength and Muscular Endurance—Hanger Test

The forelimbs' muscular strength was measured using the hanger test, which is based on a mouse's tendency to grasp a grid or bar instinctively when suspended by the tail. The three trials of the test (1 min ITT) allow discriminating grip strength and muscular endurance, according to the suspension times used (Giménez-Llort et al., 2002). In the first and second trials, grip strength is assessed holding on the animal with its front legs for 5 s at the height of 40 centimeters. In the third trial, the animal is suspended for 60 s in a single attempt to assess muscular endurance. A box with sawdust is placed under the animal to protect it from a possible fall in both cases. The bar used is graduated in 5-cm blocks to obtain the distance covered when the animal moves through the bar; the latency and movement distance are recorded.

Motor Performance—Rotarod

To assess motor learning, coordination, and endurance training, mice were evaluated on the constant, accelerated, and rocking Rotarod mode (Ugo basile®, Mouse RotaRod NG). The apparatus consists of five 3 cm diameter cylinders, which are suitably machined to provide grip. Six 25 cm diameter dividers make for five lanes, each 5.7 cm wide, enable five mice to be assessed on the rotor simultaneously. The height to fall is 16 cm. The mice were placed on the rod with their back to the experimenter to measure motor learning, and the rod began to accelerate until it reached 10 rpm. The necessary tests were carried out so that each animal was kept at least 60s on the rod with 1 min of rest between each learning trial. To measure the resistance of the animals, we used the protocol described by Brown and Wong (2007) in which the mice are placed on the rotating rod facing in the opposite direction to the movement of the rod, with an acceleration of 0–48 rpm during a test of 6 min

maximum. The test includes six trials with a 1-min rest to start each. A single test measured coordination in the device's rocking mode until reaching 20 rpm with 10 revs, and this mode allows rotations in both directions of the rod. In all tasks, the latency achieved by each animal was recorded.

Statistics

Statistical analyses were performed using SPSS 23.0 software. Results were expressed as the mean ± standard error of the mean (SEM) for each task and trial. The factors were analyzed with ANOVA, MRA, Student's *t*-test, and Chi-square or Fisher's exact test. The magnitude of the association was measured with Bonferroni. Variables that did not have a normal distribution were transformed using a square root to apply the parametric statistical tests. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

In the first place, we characterized the genotypic differences in psychomotor performance of 13-month-old male 3xTg-AD mice, an age mimicking advanced stages of the disease, compared to age matched NTg mice with normal aging. **Table 1** summarizes the main results obtained, where a clear difference between NTg ($n = 15$) and 3xTg-AD ($n = 31$) mice stands out. To verify our hypothesis that the 3xTg-AD mice that recently lost their home-cage partners exhibited different psychomotor functions, the data of 3xTg-AD mice was depicted in two subgroups, according to their most recent housing conditions. **Figures 1**–**6** also show the impact of social isolation in the behavior and psychomotor. We analyzed the genotype differences and the effect of isolation according to frailty parameters (see **Table 2** and **Figure 1**). Our results showed that the 3xTg-AD/ISO mice subgroup ($n = 7$) had a high motor performance in physical endurance and muscular strength tests but low performance in exploratory activity and spontaneous gait, considered basic daily life activities.

Physical Frailty Phenotype

In all groups, the animals had a low prevalence of signs of frailty (**Table 2**). It must be pointed out that, at this middle age, the NTg group presented overweight [$F_{(2, 43)} = 25.925$, $p = 0.000$ (43 ± 1.8 g)], 33% (5/15) exhibited tremor in the anterior or posterior limbs [X^2 (df 2), $p = 0.003$] and the sign of hindlimb clasping with a scale of mild and moderate severity [$F_{(2, 43)} = 31.355$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$] (**Figure 1A**). No statistically significant differences were found in the geotaxis, but a trend of transgenic animals performing the test more quickly was noted [NTg = 16.5 ± 5.9; 3xTg-AD = 9.5 ± 2.5; 3xTg-AD/ISO = 7.0 ± 1.3] (**Figure 1B**). Also, 57% (4/7) of 3xTg-AD/ISO animals presented alopecia in some areas of their body [X^2 (df 1), $p = 0.042$].

Spontaneous Gait Phenotype

The sequence of behavioral events developed in the gait test is detailed in **Figure 2**; the gait analysis of representative animals with normal and bizarre gaits are illustrated in **Figure 3**, whereas quantitative gait indicators are depicted in **Figure 4**.

TABLE 1 | Genotype differences between 13-month-old male 3xTg-AD mice and NTg mice in the assessment of psychomotor functions.

Genotype differences	NTg mice $n = 15$ (Mean ± SEM)	3xTg-AD mice $n = 31$ (Mean ± SEM)	Statistics
Physical frailty phenotype	(See **Table 2** and **Figure 1**)		
Spontaneous gait	(See **Figures 3**, **4**)		
Phenotype: exploratory activity			
Freezing (latency of movement, s)	5.53 ± 2.38	23.23 ± 2.51	**
Rearing (latency, s)	25.61 ± 3.49	52.64 ± 2.69	***
Vertical activity (n of counts)	3.80 ± 0.36	0.51 ± 0.16	***
Horizontal activity (n of counts)	10.26 ± 0.77	2.03 ± 0.56	***
Quantitative parameters of gait			
Stride length (cm)	4.88 ± 0.22	2.03 ± 0.34	***
Variability of stride length (%)	20.10 ± 2.83	9.60 ± 2.26	**
Support base of forelimbs (cm)	2.51 ± 0.12	2.64 ± 0.10	n.s.
Support base of hindlimbs (cm)	3.96 ± 0.11	3.48 ± 0.19	n.s.
Speed (cm/s)	6.66 ± 0.75	2.13 ± 0.46	***
Cadence (steps/s)	2.67 ± 0.24	0.96 ± 0.20	***
Muscular strength: Hanger test	(See **Figure 5**)		
Grip strength (latency, s)	0.88 ± 0.14	2.55 ± 0.24	***
Grip distance (cm)	0.0 ± 0.0	2.17 ± 0.73	*
Muscular endurance (latency, s)	0.92 ± 0.15	17.80 ± 3.87	**
Muscular endurance (distance, cm)	0 ± 0.0	9.35 ± 2.24	**
Motor performance: Rotarod	(See **Figure 6**)		
Motor learning (latency, s)	6.33 ± 1.67	19.51 ± 8.07	*
Trials learning (n of trials needed)	9.66 ± 0.61	3.38 ± 0.37	***
Physical endurance (latency, s)	32.55 ± 3.83	157.02 ± 9.56	***
Coordination (latency, s)	10.93 ± 2.09	74.64 ± 11.80	**
Spin (n)	1 ± 0.0	1.70 ± 0.19	*
Geotaxis (latency, s)	16.46 ± 5.01	9.89 ± 1.00	n.s.

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

Since the beginning of the test, genotype-dependent differences were found in freezing behavior since it was only present in both groups of transgenic mice [Trial 1, Fisher (df 2), $p = 0.004$; Trial 2, Fisher (df 2), $p = 0.006$]. Backward movement and stretching were also observed. These bizarre behaviors were elicited in greater frequency in transgenic animals, with the most frequent stretching recorded in the 3xTg-AD/ISO group [Trial 1, X^2 (df 2); $p = 0.042$]. Both types of movements indicate the intention to explore before traveling through a given space (**Figure 3D**).

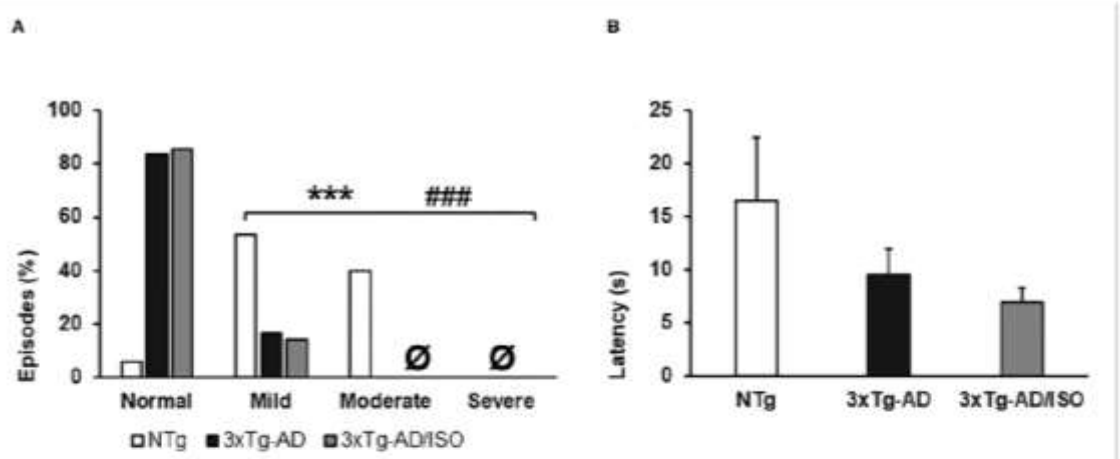

FIGURE 1 | Physical frailty phenotype. **(A)** Hindlimb clasping, the results are expressed as episodes of hindlimb clasping (%). Statistics: $X2$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in 3xTg-AD vs. the NTg group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in 3xTg-AD/ISO vs. the NTg group. **(B)** Geotaxis, the results are expressed as mean ± SEM. Statistics: One-way ANOVA followed by *post-hoc* Bonferroni test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in 3xTg-AD vs. the NTg group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in 3x1g-AD/ISO vs. the N ig group. **Ø** indicates 0 data in this group.

On the other hand, the latency to initiate horizontal (freezing latency of movement) in transgenic animals was increased [Freezing (latency of movement), $F_{(2, 45)} = 7.429$, $p = 0.002$; *post-hoc*: 3xTg-AD *vs.* NTg $p = 0.027$, 3xTg-AD/ISO vs. NTg $p = 0.002$]. In this variable, an effect of isolation was shown as a higher motion latency than its group-housed transgenic counterpart [Students' *t*-test, $p = 0.015$] (**Figure 3A**). In the vertical activity, higher latency was shown in the 3xTg-AD groups compared with other NTg mice [Rearing: $F_{(2, 45)} = 18.860$ $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.012$] (**Figure 3B**). Despite this could be due to the presence of freezing, the total vertical (rearings episodes) and horizontal (crossings episodes) exploratory activity was also lower in both groups of transgenic animals compared to the NTg group [N rearing, $F_{(2, 45)} = 50.400$, $p = 0.000$; *post-hoc*: 3x1g-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$], [N visited corners, $F_{(2, 45)} = 36.322$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$] (**Figure 3C**).

In addition, as illustrated in **Figure 4**, all the quantitative gait indicators (stride length, variability of stride, speed, and cadence) showed alterations and deficits in displacement and trajectory [Stride length, $F_{(2, 45)} = 11.552$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.015$], [Variability of stride, $F_{(2, 45)} = 4.714$, $p = 0.014$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.011$], [Speed, $F_{(2, 45)} = 14.393$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.002$], [Cadence, $F_{(2, 45)} = 15,341$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.001$] (**Figures 4A–E**), but not in the anterior or posterior base of support, which remained preserved [Forelimbs: $F_{(2, 45)} = 0.61$, $p = 0.772$; Hindlimbs: $F_{(2, 45)} = 1.651$, $p = 0.204$] (**Figure 4C**). Increased defecation was recorded in the 3xTg-AD group, reaching 41% of the episodes [X^2 (df 2); $p = 0.000$].

Muscular Strength

The assessment of muscle strength of the forelimbs, illustrated in **Figure 5**, showed a deficit in the NTg control group, while the highest grip strength in the 3xTg-AD/ISO group and the muscular endurance in the 3xTg-AD group. [Grip strength-latency, $F_{(2, 45)} = 12.958$, $p = 0.000$; *post-hoc*: 3xTg-AD *vs.* NTg $p = 0.001$, 3xTg-AD/ISO vs. NTg $p = 0.000$], [Muscular endurance-latency, $F_{(2, 45)} = 8.622$, $p = 0.001$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.001$, 3xTg-AD/ISO vs. NTg $p = 0.016$] (**Figures 5A,C**). In the same way, the distance covered by the animal while it was suspended was greater in transgenic animals in the two strength tasks [Grip-distance, $F_{(2, 45)} = 5.303$, $p = 0.009$; *post-hoc*: 3xTg-AD/ISO vs. NTg $p = 0.008$], [Endurance-distance, $F_{(2, 45)} = 6.113$, $p = 0.005$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.004$] (**Figures 5B,D**).

Additionally, we have detected that the isolated animals have a higher grip and displacement force on this test than their transgenic group-housed counterparts [Grip strength: Students' *t*-test, $p = 0.007$; Grip-distance. Students' *t*-test, $p = 0.018$].

Motor Performance

In learning and physical endurance tests, transgenic animals' motor performance was higher than that of NTg animals. Transgenic animals needed an average of three trials to learn the test, unlike N1g animals that required an average of nine trials, so the latencies obtained were consequently higher in the transgenic group, albeit they did not reach statistical significance. [Trials learning, $F_{(2, 45)} = 41.824$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$] (**Figures 6A,B**).

Physical endurance, as well as learning, was higher in transgenic animals, with the 3x1g-AD/ISO group being the one who achieved the best physical performance in the six trials.

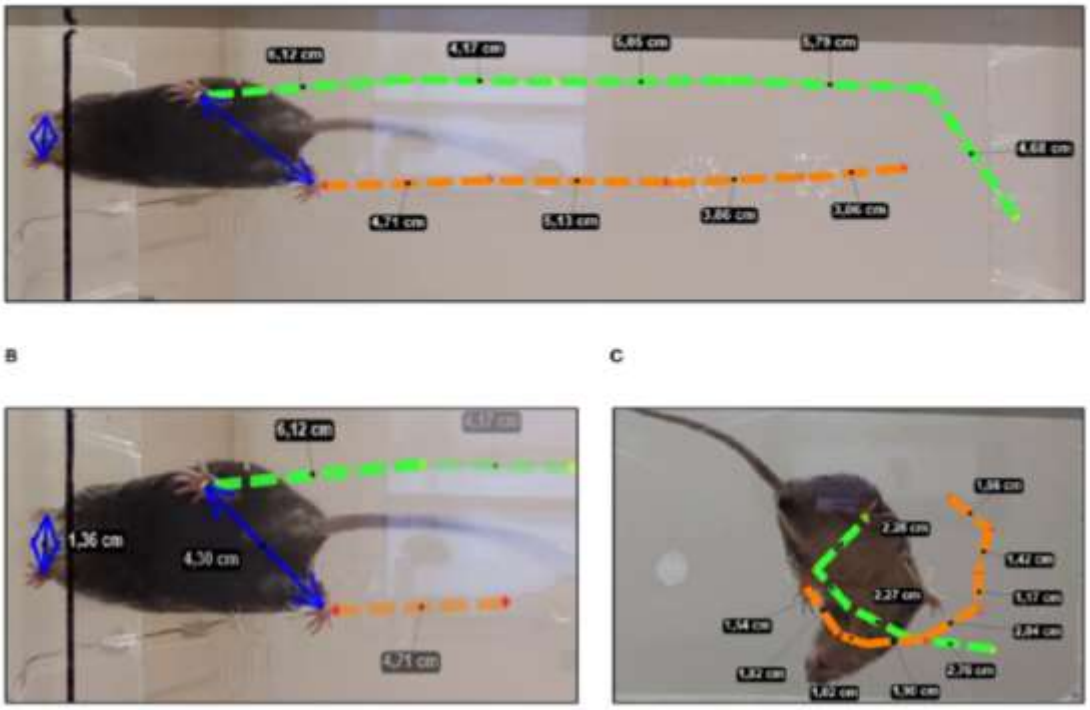

FIGURE 2 | Spontaneous gait analysis. **(A)** Normal gait stride length and **(B)** base of support measurement using hindlimbs and forelimbs paw prints in mice with a normal gait. **(C)** Bizarre circling in a representative 3x Tg-AD mice showing a general directional movement, with traced route being short and wide when making normal circles or having many loops.

[Physical endurance-latency. $F_{(2, 43)} = 45.507$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$] (**Figure 6C**). [Physical endurance trial by trial. MRA, $F = 45.515$, $p = 0.000$, [ANOVA T1-T6; T1: $F_{(2, 43)} = 11.304$, $p = 0.001$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.001$, 3xTg-AD/ISO vs. NTg $p = 0.000$; T2: $F_{(2, 43)} = 15.791$, $p = 0.000$; *post-hoc*: 3xTg-AD *vs.* NTg $p = 0.000$, 3xTg-AD/ISO *vs.* NTg $p = 0.000$; T3: $F_{(2, 43)} = 35.788$, $p = 0.000$ *post hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO *vs.* NTg $p = 0.000$; *post-hoc*: 3xTg-AD/ISO *vs.* 3xTg-AD $p = 0.033$; T4: $F_{(2, 43)} = 54.453$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$; *post hoc*: 3xTg-AD/ISO vs. 3xTg-AD $p = 0.024$; T5: $F_{(2, 43)} = 37.370$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$; T6: $F_{(2, 43)} = 30.044$, $p = 0.000$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$, 3xTg-AD/ISO vs. NTg $p = 0.000$] (**Figure 6D**).

Regarding the coordination measured in the rotarod, the 3xTg-AD animals exhibited higher latency than the NTg animals, exceeding 60 s in this test. In addition, there was a greater use of spin or postural strategies to stay on the bar while turning in both directions. [Coordination-latency: $F_{(2, 44)} = 8.786$, $p = 0.001$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.000$]. [Spin: $F_{(2, 43)} = 5.461$, $p = 0.008$; *post-hoc*: 3xTg-AD vs. NTg $p = 0.010$] (**Figures 6E,F**).

DISCUSSION

The present work assessed the psychomotor functions in male mice with normal and AD-pathological aging and the impact of "naturalistic isolation" in a subgroup of 3xTg-AD mice. Male sex was chosen to explore further the stronger sex-dependent motor effects of aging reported in male C57BL/6 mice than in females (Baeta-Corral and Giménez-Llort, 2015) and because at this age, the singularity of the natural isolation scenario only occurs in male 3xTg-AD mice as a result of their neuroimmunoendocrine derangement and increased mortality rates (Giménez-Llort et al., 2008). The results indicated genotype differences with paradox better performance in motor variables involving more significant physical work in 3xTg-AD animals independently of social isolation, and a delayed appearance of motor deficits related to physical resistance and tolerance to exercise in 3xTg-AD mice survivors that remained isolated during 2–3 months. However, in

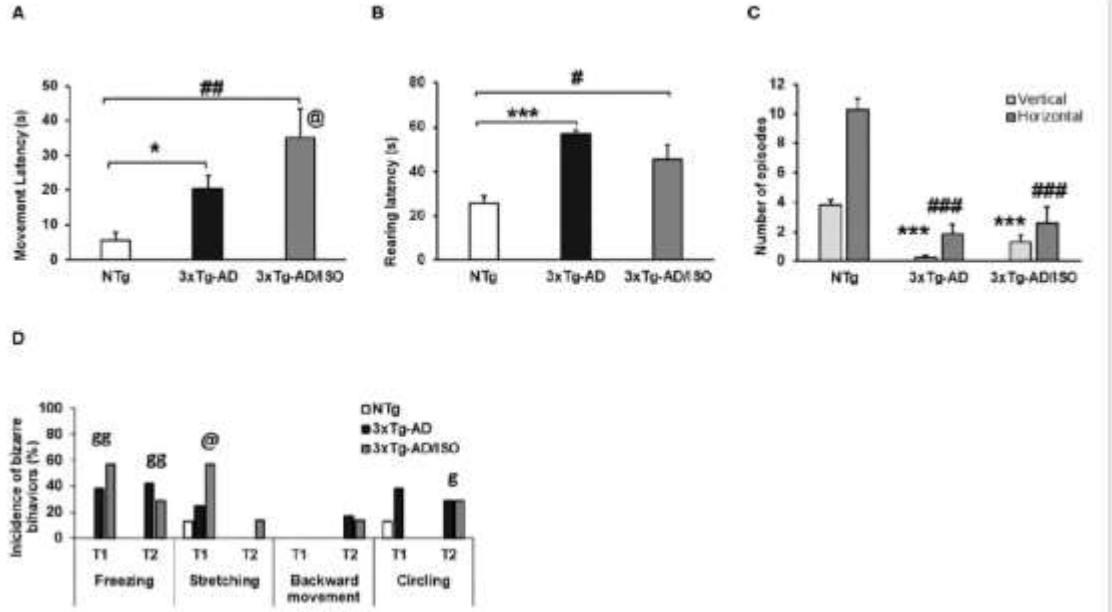

FIGURE 3 | Spontaneous gait phenotype: exploratory activity. Results are expressed as mean ± SEM. **(A)** Freezing (latency of movement); **(B)** Rearing; **(C)** Vertical and horizontal activities. Statistics: One-way ANOVA followed by post-hoc Bonferroni test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in 3xTg-AD vs. the NTg group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in 3xTg-AD/ISO vs. the NTg group. Student's *t*-test, 3xTg-AD vs. NTg, in Freezing (Latency of movement), @ indicates isolation. **(D)** Behaviors associated with exploration and circling bizarre behaviors, results are expressed as incidences of bizarre behaviors (%). Statistics: X^2, $^{**}p < 0.01$, $^{**}p < 0.05$, $^{*}p < 0.05$, $^{n.s}p > 0.05$, g indicates genotype, and @ indicates isolation.

the variables that involve information processing and decision-making to perform a task (exploration and gait), these animals exhibited poor or deficient performance that includes circling as bizarre behavior.

Regarding the physical frailty phenotype, middle-aged NTg animals were obese and presented tremors in the extremities accompanied by partial alopecia in some animals. In the 3xTg-AD mice, the frequency of alopecia was similar to that of NTg animals, accompanied by a high, stiff tail that is associated with alert and arousal in animals. Most 3xTg-AD/ISO animals presented some degree of alopecia in their body, but normal parameters were found in the rest of the variables. Unlike the results obtained by Kane et al. (2018), 3xTg-AD mice were more fragile than NTg males, accompanied by higher mortality. This was in agreement with our recent work in end of life scenarios (Muntsant et al., 2021).

On the other hand, geotaxis and buckling may indicate NTg animals' alterations due to body weight, like that observed in rotarod, and lighter mice perform better than heavier mice (Stover et al., 2015). In particular, the grip has been described as an alteration of the extremities' reflexes due to motor coordination deficits, neurological signs that resemble myoclonic movements, epileptic seizures, or pathological reflexes that alter gait (Lalonde et al., 2012). However, we have already shown in male C57BL/6 mice that grip strength and prehensility are also sensitive to body weight and fat composition associated with the aging process (Baeta-Corral et al., 2018).

Frailty and dementia are closely related and share similar common risk factors, such as sociodemographic factors, comorbidities, and lifestyle factors (Buchman et al., 2008; Wallace et al., 2018; Petermann-Rocha et al., 2020). According to the results obtained in human beings in several studies, the decrease in grip strength and a slow gait speed or the deterioration of balance have been attributed to a worse cognitive condition among people with frailty, which contributes to the incidence of dementia, including AD (Li, 2002; Hanlon et al., 2018; Lim et al., 2018; Petermann-Rocha et al., 2020).

In this respect, it is noteworthy that the gait of the 3xTg-AD mice showed deficits similar to gait with an aging pattern, accompanied by a series of bizarre behaviors that can interfere with the trajectory and movement similar to that reported by Muntsant and Giménez-Llort (2020a) in long-term isolation in male mice. At the beginning of the task, a long freezing period is accompanied by a high latency in the exploratory activity that interferes with the horizontal and vertical activity counts compared to the NTg group. This neophobic response corresponds to one of the most sensitive ethological behaviors of the 3xTg-AD phenotype detected in previous studies (Giménez-Llort et al., 2007; Giménez-Llort, 2010; Muntsant and Giménez-Llort, 2020a). Likewise, we can

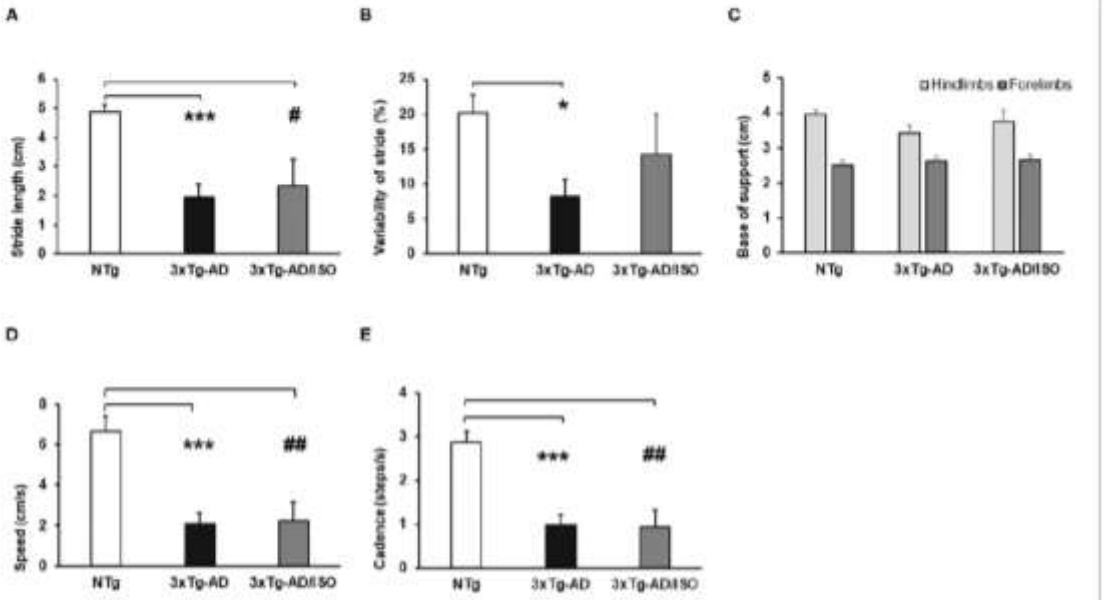

FIGURE 4 | Spontaneous gait phenotypic quantitative parameters of gait. Results are expressed as mean + SEM. **(A)** Stride Length; **(B)** Variability of Stride Length; **(C)** Base of Support; **(D)** Speed; **(E)** Cadence. Statistics: One-way ANOVA followed by *post-hoc* Bonferroni test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in 3xTg-AD vs. the NTg group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in 3xTg-AD/ISO vs. the NTg group.

distinguish that the 3xTg-AD group-housed animals presented several episodes in the horizontal and vertical components slightly lower than the 3xTg-AD isolated animals, contrary to what occurs in the movement latency at the beginning of the test, where the isolated animals 3xTg-AD took longer to perform movements, thus influencing the results of all quantitative gait parameters.

Bizarre behaviors are mainly related to psychiatric and neurological disorders (Giménez-Llort et al., 2002; Baeta-Corral and Giménez-Llort, 2014; Cordón-Barris et al., 2016). In previous studies, it has been reported that these behaviors can also be provoked when animals are subjected to unfamiliar environments, mainly those used to evaluate anxiety behavior as a manifestation or response of stress (Willner, 1991; Giménez-Llort et al., 2002, 2007). We described that is at the age of 6 months when the initial freezing response observed in 3xTg-AD and NTg animals of both sexes when assessed under anxiogenic conditions, such as the open field test or the corner test is more likely to be followed by the elicitation of bizarre behaviors (Baeta-Corral and Giménez-Llort, 2014). During the tests, the animals exhibited behaviors considered bizarre that were classified as stereotyped stretching, stereotyped rearing, backward movement, and jumping, apparently without a purpose but considered coping-with-stress strategies. In the Morris water maze, a stressful scenario for mice, we have already reported circling swimming behavior in 6-month-old 3xTg-AD mice (Castillo-Mariqueo and Giménez-Llort, 2019). The presence of this bizarre behavior worse with the progress of the disease as it is a distinctive swimming pattern in male 3xTg-AD mice at 13 months of age, modeling advanced disease stages (Baeta-Corral and Giménez-Llort, 2015). In the present work, the results corroborate that when faced with novelty and recognition of places, 3xTg-AD mice exhibit these behaviors, which delays the appearance of horizontal and vertical exploration. Stretching and circling were behaviors exhibited by isolated animals, suggesting that stretching behavior or risk assessment was sensitive to social conditions.

In the quantitative gait parameters, 3xTg-AD mice showed deficits like gait with an aging pattern, accompanied by a series of bizarre behaviors that can interfere with trajectory and movement, as mentioned above. The gait analysis reported by Brown's laboratory in 16-month-old animals did not show significant differences in the length or width of the stride between genotypes or sexes (Garvock-de Montbrun et al., 2019), whereas at 6 months of age, the animals exhibited a longer stride than NTg mice (Stover et al., 2015). In the present work, we have detected a decrease in all variables related to stride length in the transgenic group (cadence, speed, and stride variability) without altering the base of support of the front and rear extremities are similar to those detected in the control group.

Gait disorders in patients with AD have been described within the group of disorders known as "frontal gait," and in particular, gait in AD has been defined as "cautious gait" (Pirker and Katzenschlager, 2017; Baker, 2018). At the same time, cautious

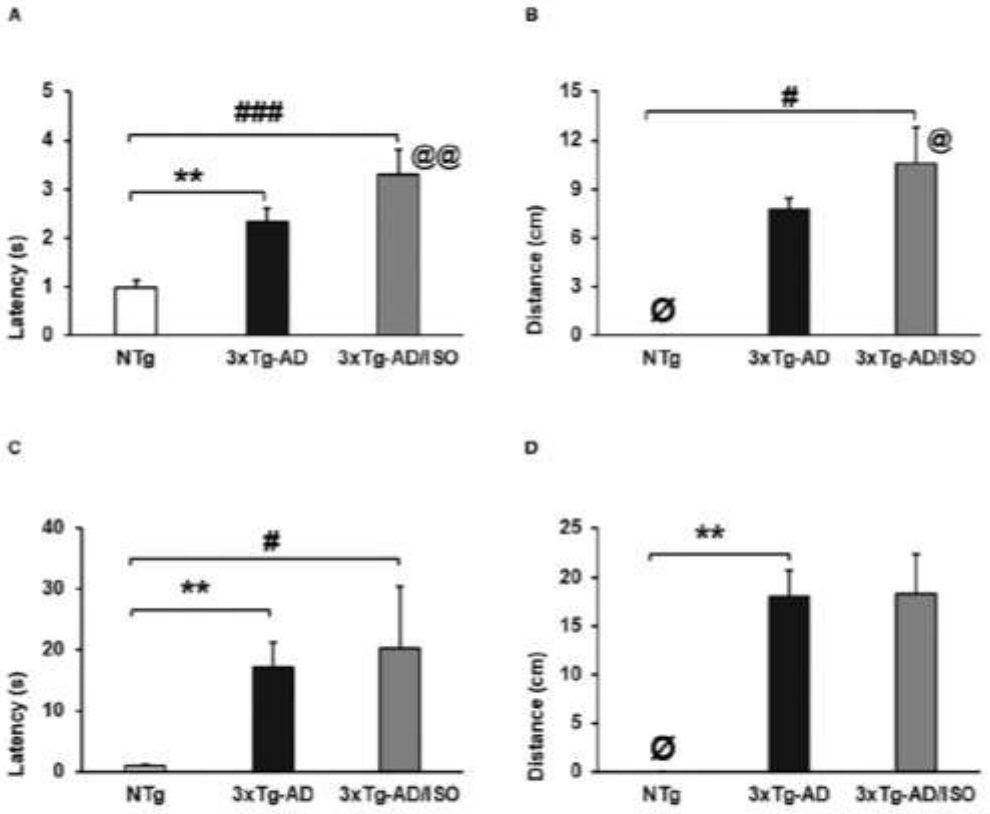

FIGURE 5 | Muscular strength—hanger test. Results are expressed as mean ± SEM. **(A)** Grip Strength-latency, **(B)** Grip-distance, **(C)** Muscular Endurance-latency, **(D)** Endurance-distance. Statistics: One-way ANOVA followed by post-hoc Bonferroni test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in 3xTg-AD vs. the NTg group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in 3xTg-AD/ISO vs. the NTg group. Students' *t*-test, 3xTg-AD vs. NTg in Grip Strength–stency and Grip-distance, @ indicates isolation., **Ø** indicates 0 data in this group.

gait occurs more frequently in patients with mild dementia (Clinical Dementia Rating Scale: Hughes CDR, stage 1). This gait pattern is like the one observed in aging, and it may present a decrease in speed, stride length, and gait postural stability, which is manifested more specifically in static and dynamic balance, with a widened support base (Scherder et al., 2007). Dynamic instability has also been observed in mild and moderate AD (Mesbah et al., 2017). In advanced AD stages, the disorder becomes more prominent, and the gait has been described as "frontal gait." At this stage, the person has difficulties standing up and postural maladjustments that prevent the change to different positions in coordination with the segments, such as arms and legs, causing difficulties to achieve a stable position (Munoz et al., 2010; Pirker and Katzenschlager, 2017). It is complex to mimic human motor disorders in mice, but we have detected some similarities in gait execution. The onset of gait in humans is affected as occurs in Parkinson's disease, with bradykinesia, some patients try to start gait by swinging the trunk laterally or by exaggerated movements of the arms, there is dragging of the feet, but it disappears after walking a few steps with what the gait usually improves (Beauchet et al., 2016; Montero-Odasso and Perry, 2019). Also, freezing or freezing episodes can occur, especially when turning and when facing obstacles (Muir et al., 2012). Although AD's clinical feature is declining cognition, the motor signs that frequently accompany AD often precede and predict AD's clinical diagnosis (Munoz et al., 2010). In 3xTg-AD animals, the bizarre behaviors described above appear to be a translational approach to detecting the severity of the psychomotor disorders presented in Alzheimer's disease, and they differentiated the effects of social isolation.

We have found significant differences in muscular strength that indicate that 3xTg-AD animals have a conserved strength in isolation, and this is the first time this finding has been reported. Already at 6 months of age, Stover et al. (2015) reported that the 3xTg-AD mice had a lower strength than the NTg mice at 16 months, Garvock-de Montbrun et al. (2019)

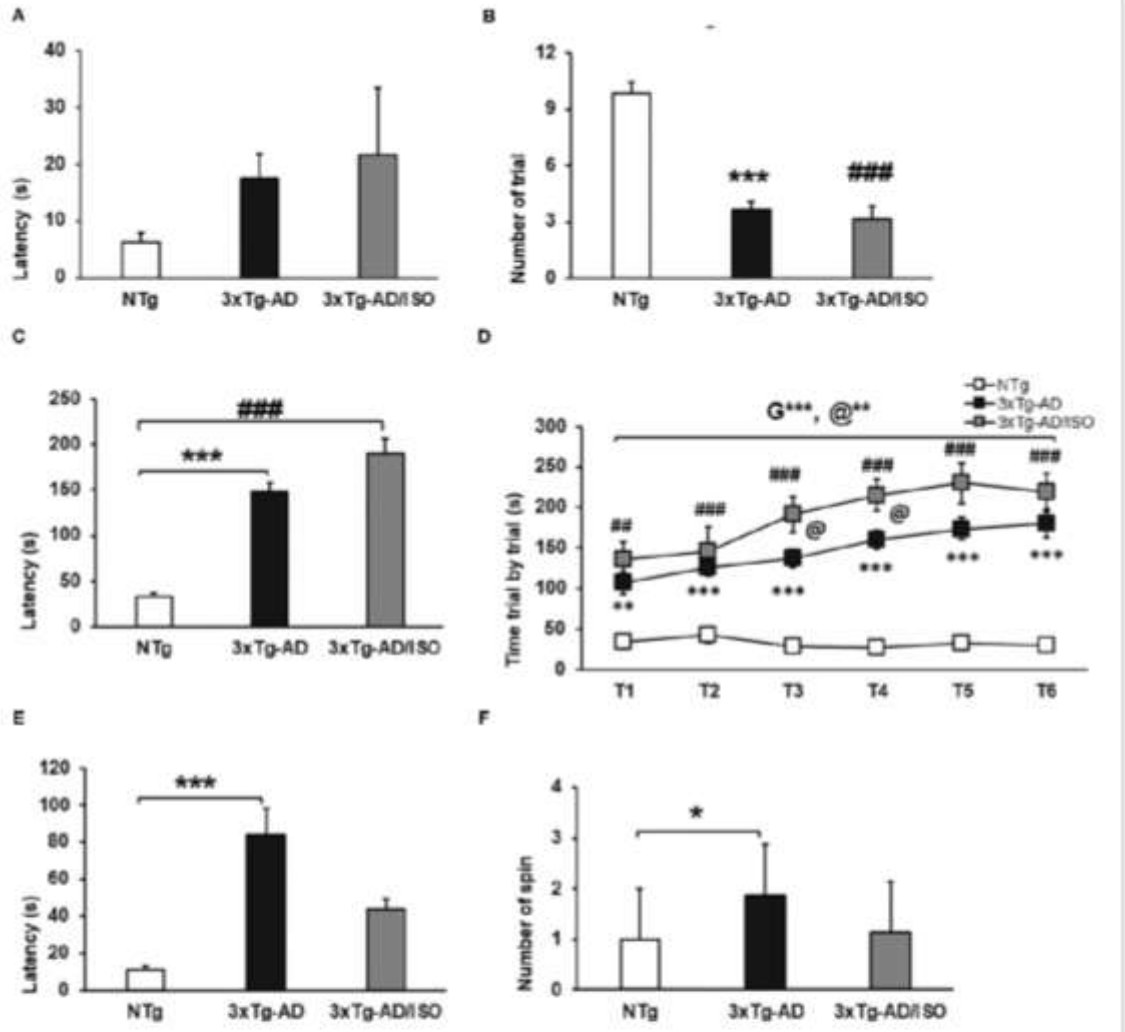

FIGURE 6 | Motor performance—Rotarod. Results are expressed as mean ± SEM. **(A)** Motor learning—Latency; **(B)** Trials learning; **(C)** Physical Endurance-latency; **(E)** Coordination-Latency; **(F)** Spins. Statistics: One-way ANOVA followed by *post-hoc* Bonferroni test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in 3xTg-AD vs. the NTg group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in 3xTg-AD/ISO vs. the NTg group. **(D)** Physical Endurance trial by trial. Statistics: MRA T1-T6, followed by *post-hoc* Bonferroni test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, G: indicates differences in genotype, @: indicates differences in isolation conditions. One-way ANOVA T1, T2, T3, T4, T5, T6, followed by *post-hoc* Bonferroni test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in 3xTg-AD vs. the NTg group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in 3xTg-AD/ISO vs. the NTg group.

found no significant differences, although the 16-month-old mice were heavier than those of 6 months, his grip strength did not decrease.

It has been previously reported that in humans, there is an association between the pathology of AD in the cognitive regions and the grip strength or grip strength (Buchman et al., 2008; Boyle et al., 2009). Also, the loss of strength and muscle mass is frequent in the aging; even BMI and frailty are associated with AD's risk (Boyle et al., 2009; Moon et al., 2019). Thus, muscle mass and strength are not related to each other in the male and female groups. In AD patients, large muscle mass does not mean more significant power. A simple assessment of lower extremity muscle strength is effectively predicted cognition than mass muscle measurement in male patients (Moon et al., 2018, 2019). Stover et al. (2015) reported that 6-month-old 3xTg-AD mice had a higher motor performance in both strength and rotarod tests

TABLE 2 | Physical frailty phenotype.

Physical frailty phenotype	NTg $n = 15$	3xTg-AD $n = 31$	3xTg-AD $n = 24$	3xTg-AD/ISO $n = 7$	Statistics
Body weight	43 ± 1.8 g	32 ± 0.1 g	33 ± 0.6 g	32 ± 0.9 g	***, ggg
Kyphosis	–	–	–	–	–
Alopecia	3/15 (20%)	9/31 (29%)	5/24 (21%)	4/7 (57%)	@
Body position	–	–	–	–	–
Palpebral closure	–	–	–	–	–
Piloerection	–	–	–	–	–
Tail position	–	4/31 (13%)	4/24 (17%)	–	n.s.
Tremor	5/15 (33%)	–	–	–	***, gg

*ANOVA: X_2., ***$p < 0.01$, **$p < 0.05$, *$p < 0.05$, n.s $p > 0.05$, g, genotype, @, isolation.*

than their NTg counterparts. In the current 13-month-old NTg animals studied, we cannot rule out that body weight may be the variable that modifies muscle strength as this group is made up of obese animals, so it is necessary to contrast these results with normal-weight animals.

The motor performance performed by the isolated 3xTg-AD animals is even higher than the 3xTg-AD grouped animals, which has allowed us to discriminate its effect in this group. At the clinical level, exercise is a widely used therapeutic resource to contribute to the treatment of the diseases symptoms and improve patients' quality of life with AD (Dao et al., 2013; Meng et al., 2020). Physical exercise is related to maintaining an optimal cognitive state and adequate maintenance of the mainly musculoskeletal and cardiovascular systems, making it a protective factor of health (Fielding, 1995; Taylor, 2014; Langhammer et al., 2018). In these animals, the practice of exercise during the six trials studied shows an increase in physical resistance as the test develops. This variable may indicate that the basal physical state of these animals is optimal and that the effect of physical exercise enhances their performance. We can highlight that although the rotarod results indicate that mice improved motor performance, they performed worse in other tasks that relate to cognitive and affective variables, in agreement with the hallmarks of the disease.

On the other hand, the 3xTg-AD animals in the coordination test carried out in the rotarod reached a higher performance without isolation effects. This group managed to stay on the rotating bar for more than 1 min, performing an average of two turns, reflecting the postural adjustment necessary to avoid falling from the bar. In contrast, in humans, it has been shown that alterations in balance and coordination are clinically demonstrable in people with mild cognitive impairment and AD (Franssen et al., 1999). These findings indicate that balance control aspects deteriorate with increasing severity of cognitive impairment and that executive function plays an essential role in controlling balance and coordination (Franssen et al., 1999; Eggermont et al., 2010; Tangen et al., 2014).

One of the main differences concerning the genotype NTg group may be due to changes typical of aging. We know that there is a functional decline as age increases. Also, there is a slow and gradual sensory deterioration (Cavazzana et al., 2018). Studies of auditory, visual, and vestibular sensory deficits and alterations in the C57BL/6 strain suggest a deterioration in these systems, leading to functional and cognitive deterioration in this group of animals. As a result, the impaired sensory system could induce poor performance in some of the animals' tests (Shiga et al., 2005; Vijayakumar et al., 2015). Besides, the obesity present in animals can be interference to achieve optimal performance in some tests, for example, those related to more excellent work of physical resistance and muscular strength and deficits and alterations, such as tremor together with hindlimb clasping. Some studies indicate that this strain tends to develop severe obesity if put on a high-fat diet (Brownlow et al., 1996; Williams et al., 2003). Other studies point to obesity in C57BL/6J animals as one of the changes associated with aging, in which the increase in adipose tissue alters energy metabolism and cardiovascular function (Krishna et al., 2016; Chu et al., 2017). Here, it is interesting to note that in previous work, we have shown that 6-month-old C57BL/6 with the same bodyweight but higher fat composition due to d-galactose-induced accelerated-aging exhibited reduced equilibrium, muscular strength, coordination, and prehensility, and these effects were only found in male sex (Baeta-Corral et al., 2018). In that work, the effects of accelerated aging on balance, motor coordination, and learning were also tested on an accelerating rotarod showing differences in the number of training trials needed to learn to walk on the lane, and the distance traveled once the task was learned.

Social isolation, from this perspective of social deprivation, would increase the vulnerability to stress episodes (Bartolomucci et al., 2003). Studies carried out in different mouse models (Swiss CD-1, Tg2576, 3xTg-AD) point out that individually housed mice show a reduced neophobic reaction and decreased anxiety compared to group-housed mice (Dong et al., 2004, 2008; Rothman et al., 2012). Furthermore, an anxious animal shows a more significant latency to explore the novel environment (Palanza et al., 2001). Bartolomucci et al. (2003) pointed out that when Swiss CD-1 mice are challenged with a new stimulus, individually housed mice respond with less fear and more extraordinary exploration and locomotion than group-housed mice. Similarly, we have recently shown that naturalistic isolation in 3xTg-AD elicited hyperactive patterns, as measured in both gross and

fine-motor functions (Muntsant and Giménez-Llort, 2020a). Pathophysiology is critical to differentiate the underlying mechanisms that trigger these responses. In the present study, animals were left alive for monitoring until the more advanced ages of life, and therefore, the impact on the HPA axis and neuropathology could not be determined. However, in our precedent work using the same "naturalistic isolation" approach, isolated 3xTg-AD mice showed increased AD-brain asymmetry in the hippocampus and cortical areas, and the above mentioned behavioral alterations were correlated to increased hippocampal tau pathology (Muntsant and Giménez-Llort, 2020a,b). Previous work in these findings in the literature suggests that 3xTgAD mice are more vulnerable than control mice to chronic psychosocial stress, resulting in an exacerbation of Aβ accumulation and impairs neurotrophic signaling (Rothman et al., 2012). On the other hand, Tg2576 mice exhibit increases in plasma corticosterone and increases in the expression of GR and CRFR1 in the cortex and hippocampus, in association with increases in the level of Aβ in brain tissue, plaque deposition of Aβ, and atrophy of the hippocampus (Dong et al., 2008).

In summary, we found standard and distinctive psychomotor features between the normal and pathological aging AD samples and the impact of the social isolation scenario. We can highlight the genotype factor and physical activity level as a protective mechanism, although physical frailty phenotype indicators are present. While the 3xTg-AD mice showed more significant deterioration in the physical aspects, their motor learning capacity remained preserved. Additionally, these animals exhibited higher performance in exercise tolerance and muscle strength tests, where the genotype seems to be a determining factor in general performance. On the other hand, the "naturalistic isolation" studied here seemed to interfere with motor performance. The presence of freezing at the beginning of the exploratory activity and spontaneous gait test was associated with increased functional limitation in this group. On the contrary, the physical parameters: strength, and physical performance in rotarod, apparently are not altered, showed a coincidence with hyperactivity or anxiety, one of the manifestations of the advanced stages of AD.

These findings generate new hypotheses to study the underlying biological mechanisms and have been useful to be applied in translational scenarios of geriatric rehabilitation (Castillo-Mariqueo et al., 2020), where timely geriatric interventions (Giménez-Llort, 2010) should be one of the priorities to counteract the second impact of the current pandemic in the older adults with dementia.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All procedures are approved by CEEAH and are in accordance with Spanish legislation on Protection of Animals Used for Experimental and Other Scientific Purposes and the EU Council 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.

AUTHOR CONTRIBUTIONS

LG-L: conceptualization. LC-M: performance, analysis of behavior, and illustrations. Both authors equally contributed to the scientific discussions, the writing, and approval of the 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.

Conclusion

1) 3xTg-AD mice showed more significant impairment in physical aspects, their motor learning ability remained preserved.
2) 3xTg-AD mice showed higher performance in exercise tolerance and muscle strength tests, where genotype seems to be a determining factor in overall performance.
3) “Naturalistic isolation” seemed to interfere with motor performance.
4) The presence of freezing at the start of the exploratory activity and the spontaneous gait test was associated with greater functional limitation in the isolation group.
5) The physical parameters: strength, and physical performance in rotarod, are apparently not altered in the isolated group.

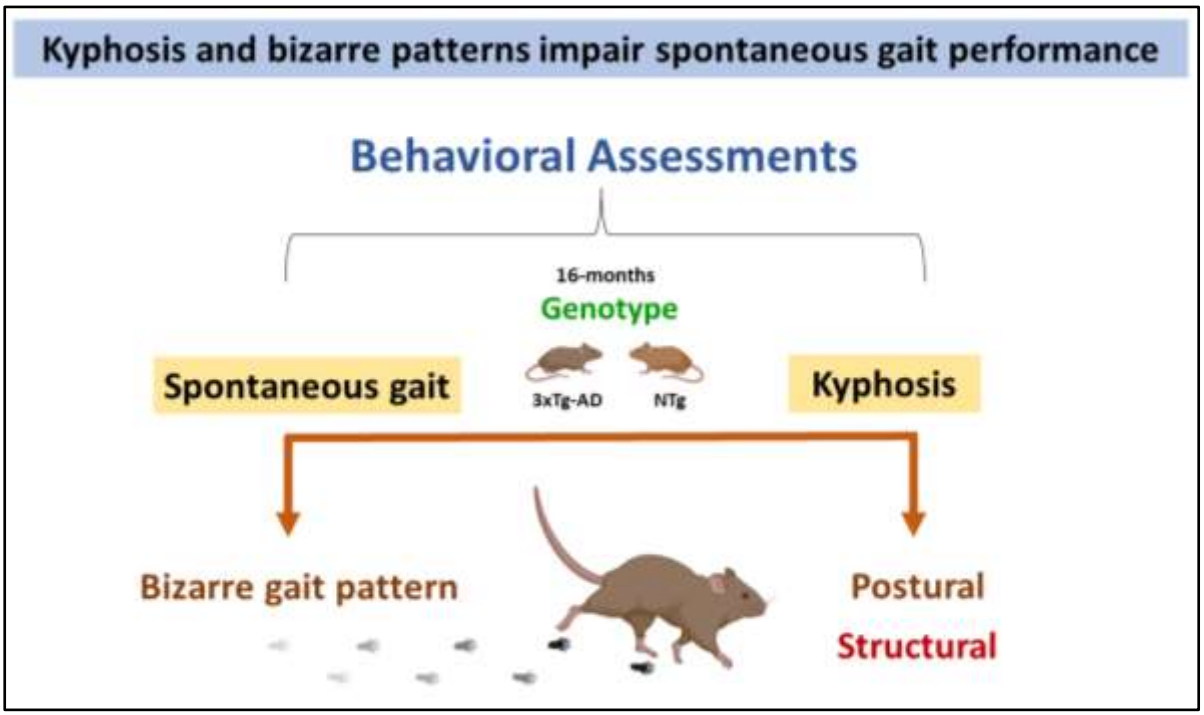
KYPHOSIS AND BIZARRE PATTERNS IMPAIR SPONTANEOUS GAIT PERFORMANCE IN END-OF-LIFE MICE WITH ALZHEIMER'S DISEASE PATHOLOGY WHILE GAIT IS PRESERVED IN NORMAL AGING

In this work, kyphosis and bizarre gait patterns associated with functional gait limitations and exploratory activity were studied in male 3xTg-AD and NTg mice at 16 months of age. The male sex was chosen as sex-dependent psychomotor effects of ageing are stronger in NTg males than in females, and, at this age, male 3xTg-AD mice are near the end of life due to higher mortality rates.

Specific objective

6.1. To identify kyphosis and bizarre gait patterns associated with functional gait limitations and exploratory activity in male 3xTg-AD and NTg mice at the age of 16 months.

Experimental design



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Kyphosis and bizarre patterns impair spontaneous gait performance in end-of-life mice with Alzheimer's disease pathology while gait is preserved in normal aging

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Abstract:
The shorter life spans of mice provide an exceptional experimental gerontology scenario. We previously described increased bizarre (disruptive) behaviors in the 6-month-old 3xTg-AD mice model for Alzheimer's disease (AD), compared to C57BL/6J wildtype (NTg), when confronting new environments. In the present work, we evaluated spontaneous gait and exploratory activity at old age, using 16-month-old mice. Male sex was chosen since sex-dependent psychomotor effects of aging are stronger in NTg males than females and, at this age, male 3xTg-AD mice are close to an end-of-life status due to increased mortality rates. Mice's behavior was evaluated in a transparent test box during the neophobia response. Stretching, jumping, backward movements and bizarre circling were identified during the gait and exploratory activity. The results corroborate that in the face of novelty and recognition of places, old 3xTg-AD mice exhibit increased bizarre behaviors than mice with normal aging. Furthermore, bizarre circling and backward movements delayed the elicitation of locomotion and exploration, in an already frail scenario, as shown by highly prevalent kyphosis in both groups. Thus, the translational study of co-occurrence of psychomotor impairments and anxiety-like behaviors can be helpful for understanding and managing the progressive functional deterioration shown in aging, especially in AD.

Keywords: Alzheimer's disease; bizarre; exploratory activity; gait; 3xTg-AD mice; kyphosis.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of cognitive, language, and behavioral functions [1]. In addition, dementia can have multifaceted clinical presentations [2]. Thus, a wide range of behavioral and psychological symptoms of dementia (BPSD) can manifest, reaching more than 90% in most patients [3]. Therefore, BPSD requires great efforts for caregivers and society in general [4]. The gap between the clinical characteristics of this disease and its elicitation in animal models entails great efforts by researchers to achieve a replicable approach. However, they have begun to be addressed in some of these models in the last decade [5]. In this way, we described early symptoms similar to BPSD in the 3xTg-AD mouse model for the first time at 2.5 months of age [5], and bizarre behaviors at 6 months of age [6]. Subsequently, we described bizarre behaviors in swimming performance in the Morris water maze [7]. Thus, at 13 months of age, it was possible to identify non seeking, floating, and circling behaviors among genotypes more precisely, the latter group being the characteristic behavior of 3xTg-AD animals [7]. These findings were later confirmed in a study conducted on 6-month-old male animals [8]. Furthermore, these behaviors are sensitive to environmental factors. Thus, we recently reported that naturally isolated 13-month-old 3xTg-AD male animals, as a social isolation model, exhibit bizarre behaviors that interfere with exploratory activity and locomotion in gait, with stretching and circling behaviors being the most sensitive behaviors exhibited by isolated animals [9].

In the clinical setting, the recognition of neurological disorders of gait, balance, and posture are important to offer clues about the underlying pathology, especially in those patients with a clinical diagnosis who are in the early stages of their evolution and who will progress to more complex and disabling disorders [10,11]. However, many clinicians find these functional gait disorders difficult to diagnose, as they are often seen in conjunction with other functional movement disorders or other neurological signs [10]. Specifically, in

dementia, gait disorders have been described as an alteration called 'cautious gait,' and it occurs more frequently in patients with mild dementia, while in advanced stages, the gait disorder is called 'frontal gait' [12–14]. This gait pattern is similar to that observed in aging. They may present: decrease in speed, stride length, and postural stability of gait, which is manifested more specifically in static and dynamic balance, with a widened base of support [15]. Dynamic instability has also been observed in both mild and moderate AD [12].

On the other hand, kyphosis is one of the characteristic signs of animal models for Marfan or Scheuermann disease [16,17]. It has been described as a sign characterized by anterior wedging of the vertebral bodies accompanied by a variation in their height and its consequent asymmetry at the thoracic level, increasing with age [16]. Increased anterior curvature of the thoracic spine in humans is typical in older people and may result from changes at the musculoskeletal and biomechanical level of the whole body accompanied by geriatric syndromes such as frailty [18]. Although it has been reported that thoracic kyphosis is not directly associated with physical function in people over 65 years of age [19], its severity can alter lung function [20]. However, there are few reports of kyphosis in mouse models asociated with gait or exploratory activity. In female C57BL/6 mice, the loss of body mass in senescence is associated with the appearance of other characteristics of the aging phenotype, such as kyphosis, baldness and loss of coat color [21].

The bizarre behaviors previously described as manifestations of BPSD, we have considered 'bizarre gait patterns,' and in this way, we have transferred them to a functional analysis that allows detecting abnormalities in gait and exploratory activity. We have also selected kyphosis as an indicator of severity (postural and structural) and physical frailty that limits the functional performance of 16-month-old male 3xTg-AD mice in an advanced AD stage compared to non-transgenic (NTg) mice with normal aging.

1. Materials and Methods

2.1. Animals

A total of twenty-one homozygous 3xTg-AD (n = 11) and non-transgenic (NTg, n = 10) male mice of 16 months of age in a C57BL/6J background (after embryo transfer and backcrossing of at least ten generations) established at the Universitat Autònoma de Barcelona were used in this study. The 3xTg-AD mice harboring transgenes were genetically modified at the University of California at Irvine, as previously described [22]. Animals were kept in groups of 3-4 mice per cage (Macrolon, 35 × 35 × 25 cm3) filled with 5 cm of clean wood cuttings (Ecopure, Chips6, Date Sand, UK; uniform cross-sectional wood granules with 2.8-1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm x 20 cm, White). All animals were kept under standard laboratory conditions of food and water ad lib, 20 ± 2◦C, 12 h light cycle: dark with lights on at 8:00 a.m. and 50-60% relative humidity. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used [23].

1.2. Experimental desing

A cross-sectional study was carried out to evaluate the interaction between kyphosis, bizarre gait patterns with gait variables and exploratory activity using a brief evaluation battery applied prior to the euthanasia of the animals. Measurements were applied to old animals that met the end point criteria of Reynolds et al., 1985 and Talan and Engel, 1986 [24,25].

2.3. Behavioral assessment

Behavioral evaluations were carried out in a single day and balanced by observing two independent observers blind to the genotype. During the morning, the tests were carried out; 30 minutes were allowed to habituate the animals in the test room before starting the measurements. The evaluation protocol, bizarre gait patterns, and physical phenotype of frailty used here are recently reported in Castillo-Mariqueo and Gimenez-Llort's 2021 study [9]. In addition, videos of gait were taken for posterior analysis with KINOVEA 0.8.15 free software.

2.3.1. Survival, Kyphosis and physical phenotype of frailty

Survival and mortality ratios were analyzed retrospectively, considering the cohort of siblings from the same litter of mice included in the study. A total of 32 male NTg and 3xTg-AD mice Thus, a total of 32 homozygous 3xTg-AD (n = 16) and non-transgenic (NTg, n = 16) male mice were included for these analysis.

Kyphosis was differentiated into two types: postural and structural. These were measured during spontaneous locomotion in the housing box, and later it was confirmed in the postural inspection where the animal is placed on a

fence where it can be held from the front legs, then small traction is made from the tail to lengthen the body of the animal, and check the degree of deformation at the thoracic level. A score of 0 to 2 is assigned, where 0 indicates the absence of kyphosis; 1 indicates postural kyphosis, which disappears during the traction of the tail, or during the march; 2 indicates structural kyphosis, the one that is maintained during the inspection and is confirmed during the necropsy.
The physical phenotype of frailty includes body conditions, body weight, alopecia, whisker loss, piloerection, tremor, and others, and then a score of 0 was assigned for normal appearances or 1 for abnormal appearances [26,27]. In addition, a photographic record was taken of each animal to demonstrate these physical aspects.

2.3.2. Bizarre gait patterns and quantitative parameters of gait

The gait of the animals on a transparent housing box measuring 27.5 x 9.5 cm was recorded by video and direct observation for one minute. For the discrimination of bizarre gait patterns, the trajectory in the path of each animal was differentiated according to the form of displacement, being possible straight, scanning, or circling during more than half the observation time or repeated over three episodes in the case of circling. Then the postural pattern of movement was differentiated: normal, shrink, or stretching. And finally, the rhythmicity or type of step in the displacement was differentiated: rhythmic and symmetric, skipping or backward movement. These movements were visually analyzed through videos and direct observation in the path of forwarding displacement in the transverse and sagittal planes. Their presence or absence was recorded for each one.
The KINOVEA 8.15 software was used for the quantification and analysis of the gait trajectory. The quantitative parameters were measured according to Castillo-Mariqueo and Gimenez-Llort's 2021 protocol [9] and described previously by Wang in 2017 [28].

2.3.3 Exploratory activity, geotaxis, quadriceps and triceps surae muscles related to sarcopenia

The exploratory activity was recorded during the spontaneous gait test according to the Castillo-Mariqueo and Gimenez-Llort's 2021 protocol [9] as well as 90° geotaxis. In addition, the 45° geotaxis test was included to reduce the degree of difficulty of the test and grant more possibilities of registration in those animals whose fragility is greater.
One hour after the evaluation of the performance, the animals were sacrificed and necropsied to extract the quadriceps and triceps sural muscles, which were weighed individually. The 'sarcopenia index' [29] was applied to obtain an indirect measure of sarcopenia as a biological marker of frailty.

2.4. Statistics

Statistical analyses were performed using SPSS 15.0 software. Results were expressed as the mean ± standard error of the mean (SEM) for each task and trial or incidence in percentage. The variables that did not present a normal distribution were normalized with a square root or were fractionated with Fractional rank [30]. The variables were analyzed using the Student's t-test and Chi-square or Fisher's exact test. Correlations were analyzed with Point-Biserial Correlation. The survival curve was analyzed with the Kaplan-Meier test (LonRank). Statistical significance was considered at $p < 0.05$.

3. Results

The animal cohort was analyzed from birth to 16 months of age of the animals (16 NTg and 16 3xTg-AD). Only male siblings from the same litter belonging to mice meeting the endpoints were considered. Log-rank analysis does not show a significant genotype-dependent difference [$\chi2$ (1) = 3.669, p = 0.055], although it is possible to note a shorter lifespan in NTg mice [mean survival days: NTg, 459.28 $\pm$ 15.57, CI: 428.75 - 489.79; 3xTg-AD 502.75 $\pm$ 3.52, CI: 495.84 - 509.65]. Additionally, we have expanded the cohort by incorporating the animals born in the same time interval into the analysis, completing one n = 81 mice. The results show a difference in the mortality of the cohort, the 3xTg-AD animals reach 45% (26/58) and the NTg 22% (5/23), despite not being significant [$\chi2$ (1) = 0.968, p = 0.325], it shows a longer lifespan cuts in NTg mice [Mean survival days: NTg, 452.57 $\pm$ 14.21, CI: 424.72 - 480.43; 3xTg-AD, 488.91 $\pm$ 7.61, CI: 473.99 - 503.83.
The animals present similar indicators of frailty in all variables without significant statistics. The variable of interest of the physical frailty phenotype, kyphosis, was 90% (10/11) of the transgenic animals and 80% (8/10) in the NTg group. Also, it is possible to differentiate a higher incidence in structural kyphosis, which has been higher in 3xTg-AD animals with 81% (9/11). The animals presented a similar body

weight in both groups, reaching 27.8 + 1.0 g in the NTg animals and 27.1 + 1.1 g in the 3xTg-AD mice.
There is also a higher incidence of the wound, dermatitis, or prolapse in NTg animals that (eye discharge 30%, dermatitis 20%, wound 50%, and rectal prolapse 20%), unlike 3xTg-AD, present a higher incidence of postural alterations such as tail position (18%), body position (9%) and kyphosis previously mentioned. However, the high incidence in both groups of piloerection stands out (NTg 80%, 3xTg-AD 54%), see Table 1.
On the other hand, as illustrated in figure 1, the incidence of bizarre gait patterns exhibited in the 3xTg-AD group reached 82% (9/10), and those in the NTg group 40% (4/10) with a high incidence in circling that reached 36% (4/11) followed by backward movement with 18% (2/11) in transgenic animals. In the case of NTg, the behavior with the highest incidence was stretching at 20% (2/10), see Figure 1A. In addition, a decrease in stride length, cadence, and speed was observed in quantitative gait parameters in 3xTg-AD mice [stride length: Student's t-test p = 0.040; speed: Student's t-test p = 0.041; Mann Whitney U cadence p = 0.036], see Figure 1B, 1C, 1D, 1E. Also, the correlation analysis between kyphosis and quantitative gait parameters shows us a statistically significant correlation in stride length and speed [stride length: r2=-0.202*, p=0.040; speed: r2=-0.260*, p=0.018], see figure 1F, 1G.
In the exploratory activity, genotype-dependent differences were found where 3xTg-AD animals take longer to start locomotion [freezing: Student's t-test p = 0.015]. See figure 2A. In the same way, horizontal (corners) and vertical (rearings) activity is decreased compared to the non-transgenic group, which is significant in horizontal activity [horizontal activity: Student's t-test p = 0.006; ratio: Student's t-test p = 0.048], see figure 2B. Also, the rearing latency was significant, being higher in 3xTg-AD mice [rearing latency: Student's t-test p = 0.040]. There were no significant differences in defecation and urine between the groups [defecation: NTg, 0.6 $\pm$ 0.3 bolis; 3xTg-AD, 0.5 $\pm$ 0.2; urination: NTg, frequency of 70% (7/10); 3xTg-AD frequency of 55% (6/11]. For his part, 45º geotaxis was statistically significant in 3xTg-AD mice, taking longer to complete the test [geotaxis 45º: Student's t-test p = 0.036]. No differences were detected at 90º of the test. See figure 2C.
Furthermore, bizarres gait patterns correlated with horizontal activity [r2=-0.261*, p=0.018], and vertical activity [r2=-0.191*, p=0.047]. Also, episodes de bizarres gait patterns correlated with rearing latency [r2=0.338**, p=0.006], see figure 2D, 2E, 2F.
As illustrated in figure 2G, differences in the weight of the quadriceps muscle were detected, being higher in the 3xTg-AD mice [quadriceps: Student's t-test p = 0.029]. This significance was also obtained by applying sarcopenia index [sarcopenia index-quadriceps: Student's t-test p = 0.016]. The tripes surae muscle did not show statistically significant differences.

4. Discussion

In this research, kyphosis and bizarre gait patterns associated with functional limitations of gait and exploratory activity were studied in male 3xTg-AD and NTg mice at the age of 16 months. Although these behaviors have been previously reported in the open field, gait tests, and the Morris water maze [6–9], this is the first time confirmed in such an old age, using 16-month-old animals. In this way, the results corroborate that in the face of a novelty situation and the recognition of place, old 3xTg-AD mice exhibit bizarre behaviors, and most importantly, they interfere with their locomotion and spontaneous exploration, in an aging scenario that already includes kyphosis, an indicator of frailty with high incidence in both groups.
The manifestation of bizarre behaviors in anxiety tests suggests that these behaviors could be related to coping with stress [6]. The bizarre behavior patterns in 3xTg-AD mice from 6 to 13 months of age differ from NTg animals and correlate with other anxiety behaviors, locomotion, and emotionality [6,7]. However, bizarre behaviors can be heterogeneous and with low incidence, which is a limitation to detect genotype differences. Also, most of them depend on sex, with females being the ones that exhibited these behaviors to a greater extent [6]. Therefore, it is relevant that in the present study using the male sex, we detected circling and backward movements as the behaviors with the highest incidence in old transgenic animals mimicking very advanced stages of the disease. Specifically, horizontal and vertical exploratory activity were found affected. Observing a correlation between the presence of these behaviors called 'bizarre gait patterns' and a decrease in exploration with a delay in the first episode of vertical exploration, of which the 3xTg-AD mice are the most affected in the horizontal exploratory activity and the latency of movement, suggesting that once these behavioral patterns appear, they persist as the disease progresses.
On the other hand, previous studies of gait in 13-month-old male 3xTg-AD mice indicate deficits that coincide with an aging pattern,

accompanied by a series of bizarre behaviors that can interfere with trajectory and movement [9,31,32]. Alteration in stride length decreased speed and cadence in 3xTg-AD animals seem to be the most sensitive variable, being even modified by postural alterations such as structural kyphosis in stride length and gait speed. In the Tg2576 mouse model, ten-month-old mice in the pre-plaque stage exhibited significantly altered duty cycle and step patterns with decreased stride length and stride time. However, base-of-support, stride time variability, stride length variability, cadence, phase dispersions, and gait symmetry indices remain were unaltered [33].

Alternatively, it has been described that spontaneous microhemorrhages appear in rodent models of AD associated with a vascular phenotype that could partly explain the deficiencies observed in gait. Recent studies have identified these age-related vascular lesions as a factor that exacerbates gait dysfunctions [34,35].

Additionally, the animals show similar frailty phenotype alterations with some distinctions regarding a higher incidence of posture alterations in transgenic mice and injuries or wounds in non-transgenic mice, both coinciding with a high incidence of piloerection. It has previously been reported that old mice will have a subset of injuries as part of the natural progressive deterioration of organ function that defines normal aging [36–39], some of which are not lethal, even meeting criteria for euthanasia [36].

Due to the higher heterogeneity of the aging process under normal and pathological conditions, the survival curves are a must to understand in which scenario the results are referred to. Previously, an increase in the vulnerability of 3xTg-AD mice associated with the sex [40,41] has been reported, but more recently, it has been reported that different cohorts differ in their survival curves, with females independent of genotype being the ones that have a life shorter than males [42]. In our report, we have detected the opposite scenario, where it seems that factors such as frailty or kyphosis could play a different role in normal aging. In the case of 3xTg-AD animals, it could be due to 'survivors,' who, despite their functional deterioration, reach more years of life but with considerable functional limitations. Also, we have determined the compromise of functional performance when there is the presence of kyphosis and bizarre gait patterns, which point to a more significant restriction of daily life functions and that in a translational way could be a cause of dependency in humans. In addition, we can highlight that body size and weight seem to be critical factors that play a different role in aging, and their interaction with frailty is fundamental for the description of the scenario we are studying.

The results obtained in the gait of the 3xTg-AD mice coincide with the results reported in studies carried out in humans. On the one hand, gait deficiencies are associated with factors related to the severity of the disease [43], and specifically in stride length and speed, patients with early AD walk more slowly and with shorter steps than healthy older adults [44–46]. Also, kyphosis harms the elderly population [47]. It has been associated with adverse health effects and increased mortality [48], it has even been associated with an increase in the risk of falls [49], so it seems interesting to us to be able to investigate this variable and establish the added limitations in the progression of AD.

Finally, we have observed a decrease in the volume of the quadriceps muscle of non-transgenic animals, without alteration in the triceps surae muscle, which suggests that despite greater fragility at the aged animals' muscle level it does not affect exploratory activity or performance of the march that remains preserved. These results are currently under further investigation.

5. Conclusions

It is known that the causes of gait disorders are diverse and multifactorial, which makes their intervention complex. That is why any gait disorder must be thoroughly evaluated to determine the triggers and thus improve the mobility and independence of patients in order to prevent complications and at the same time improve non-pharmacological rehabilitation therapies.At the translational level, the present study of psychomotor disturbances and anxious behaviors provides evidence that besides classical cognitive analysis, the assessment of anxiety-like patterns and psychomotor disturbances can be helpful to understand and manage the progressive functional deterioration related to aging and the nuances in the AD scenario, with a translational value for the older people, especially those with AD.

Supplementary Materials: Not aplicable.

Author Contributions: Conceptualization, L.G.L.; methodology, L.G.L and L.C.M; resources, L.G.L.; data curation, L.C.M; Statistical analysis: L.C.M.; writing-L.C.M.; writing-revision and editing- L.M.C. and L.G.L.: funding acquisition: L.G.L. Both authors have read and agreed to the published version of the manuscript.

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) the 8th of March 2019

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Informed Consent Statement: Not applicable.

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Tables

Conditions	Normal aging (n=10)	AD-pathological aging (n=11)	p value
1. Survival *(mean ± SEM days, 95% Confidence Interval)*			
Mortality ratio (*Sibilings, n=32*)	459.28 ± 15.57 CI: 428.75 - 489.79	502.75 ± 3.52 CI: 495.84 - 509.65	*0.055*
Mortality ratio (*Cohorts, n=81*)	452.57 ± 14.21 CI: 424.72 – 480.43	488.91 ± 7.61 CI: 473.99 – 503.83	*n.s.*
2. Kyphosis *(animals, %)*	8 (80%)	10 (90%)	*n.s.*
Postural	2 (20%)	1 (9%)	*n.s.*
Structural	6 (60%)	9 (81%)	*n.s.*
3. Physical conditions *(animals, %)*			
Body weight (*g*)	27.8 ± 1.0	27.1 ± 1.1	*n.s.*
Alopecia	3 (30%)	2 (18%)	*n.s.*
Loss of whiskers	1 (10%)	5 (45%)	*n.s.*
Palpebral closure	-	-	*n.s.*
Tail position	-	2 (18%)	*n.s.*
Postural body	-	1 (9%)	*n.s.*
Piloerection	8 (80%)	6 (54%)	*n.s.*
Tremor	2 (20%)	5 (45%)	*n.s.*
Eye discharge/swelling	3 (30%)	-	*n.s.*
Dermatitis/eczema	2 (20%)	-	*n.s.*
Wound (face, nose, or periorbital)	5 (50%)	5 (45%)	*n.s.*
Rectal prolapse	2 (20%)	-	*n.s.*

Kaplan Meier, *LongRank p<0.05**, n.s. p>0.005. X^2 n.s. p > 0.05.

Figure 1. Bizarre gait patterns and kyphosis

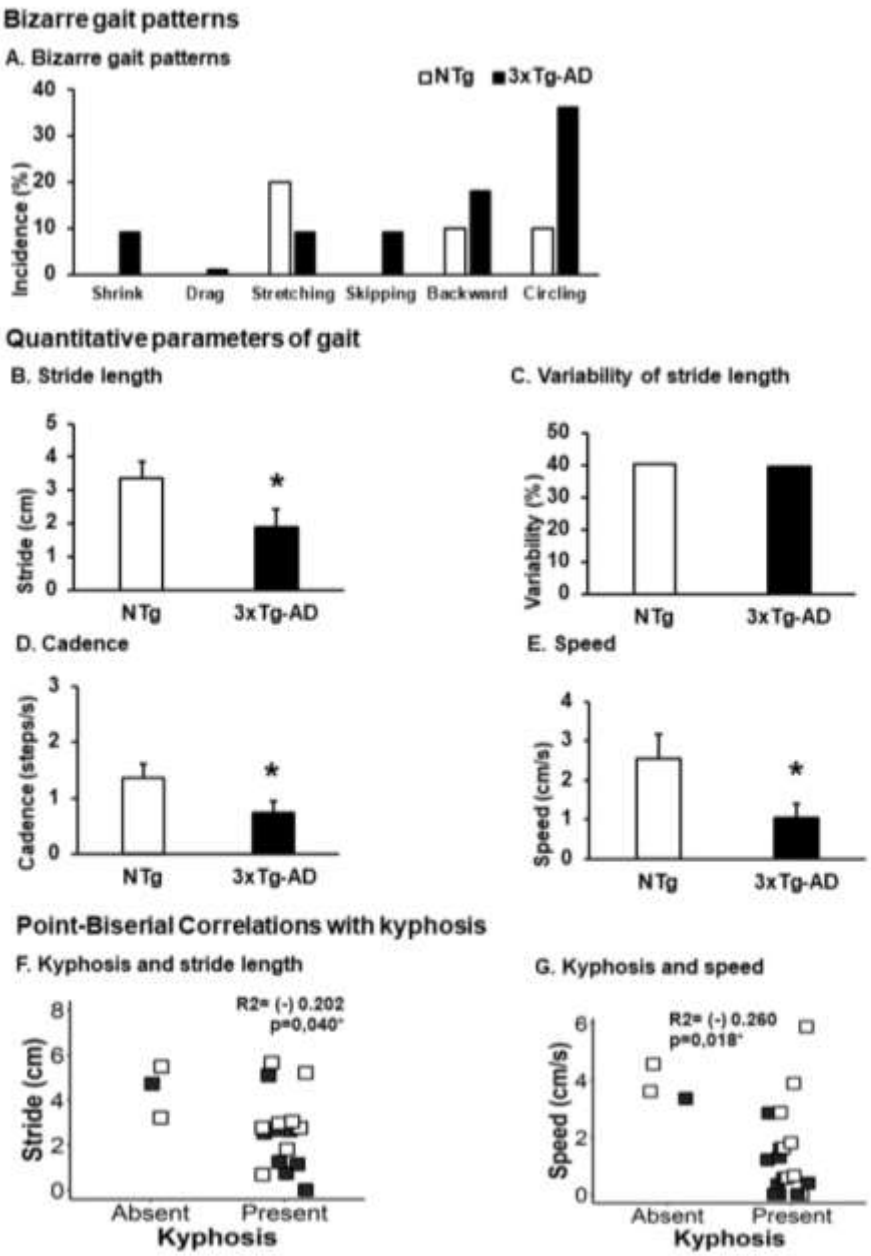


Figure 1. Bizarre gait patterns and kyphosis. (A) Bizarre gait patterns, the results are expressed as incidences of episodes of bizarres gait patterns (%), shrink, drag,stretching, skipping, backward movement, and circlin. Statistics: X^2, n.s. $p > 0.05$. Quantitative parameters of gait. Results are expressed as mean ± SEM. (B) Stride Length; (C) Variability of Stride Length; (D) Cadence; (E) Speed. Statistics: Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. $p > 0.05$. Point-Biserial Correlations analysis between kyphosis and bizarre gait patterns. Meaningful, Point-Biserial Correlation between Kyphosis and (F) Stride length, and (G). Statistics: Pearson r2, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 2. Exploratory activity, geotaxis and sarcopenia

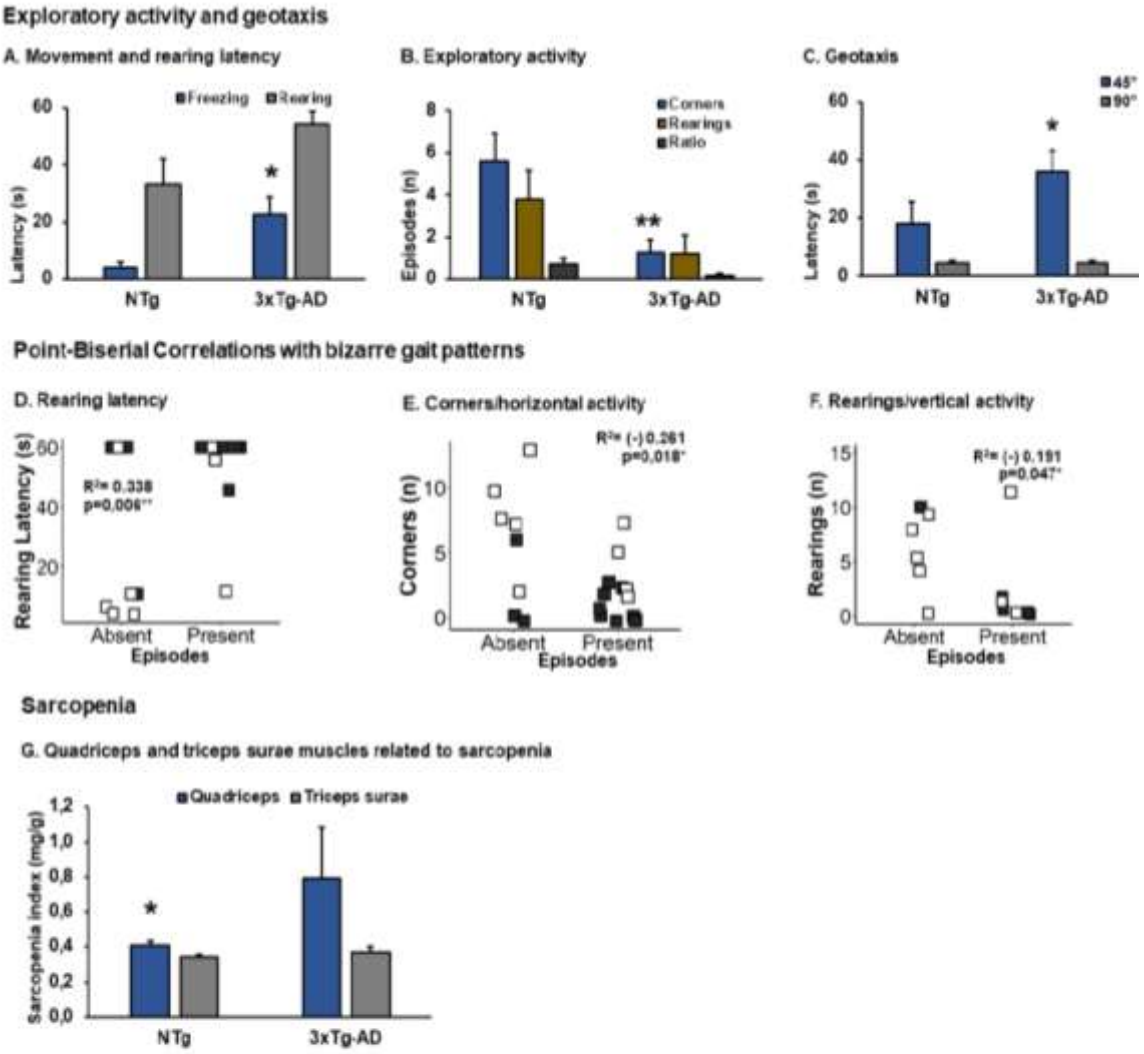


Figure 2. Exploratory activity, geotaxis and sarcopenia. Results are expressed as mean ± SEM. (A) Movement and rearing latency; (B) Exploratory activity; (C) Geotaxis; (G) Sarcopenia index- Quadriceps; (D) Sarcopenia index- Triceps sura Quadriceps and triceps surae muscles related to sarcopenia. Statistics: Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. $p > 0.05$. Meaningful, significant Point-Biserial Correlation between bizarres gait patterns and (D) Rearing latency, (E) Corners (horizontal activity), (F) Rearings (vertical activity) Statistics: Point-Biserial Correlation R^2, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Conclusions

1) Kyphosis altered stride length and gait speed in 3xTg-AD mice.
2) Bizarre gait patterns limited exploratory activity in transgenic mice.
3) Structural and postural kyphosis as a primary gait disturbance in 3xTg-AD mice.
4) Bizarre gait patterns as a secondary impairment to exploratory activity in 3xTg-AD mice.
5) Despite frailty, gait function is not impaired in non-transgenic mice.
6) Piloerection is the primary marker of macroscopic examination indicating severity in the in-of-life setting at 16 months in 3xTg-AD and NTg mice.

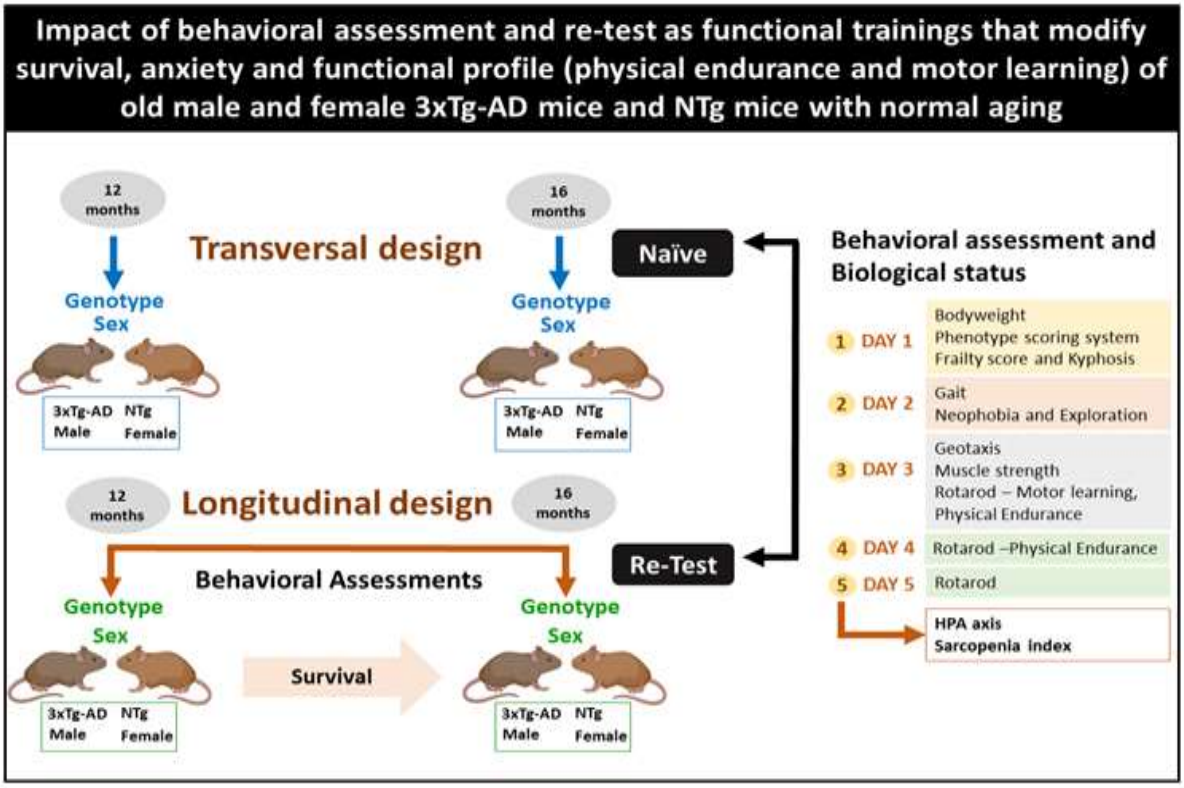
IMPACT OF BEHAVIOURAL ASSESSMENT AND RE-TEST AS FUNCTIONAL TRAININGS THAT MODIFY SURVIVAL, ANXIETY AND FUNCTIONAL PROFILE (PHYSICAL ENDURANCE AND MOTOR LEARNING) OF OLD MALE AND FEMALE 3XTG-AD MICE AND NTG MICE WITH NORMAL AGING

This study investigated the impact of two factors, sex and retesting, by evaluating the behavioural outcomes of 12- and 16-month-old male and female 3xTg-AD and non-transgenic mice in longitudinal and cross-sectional experimental designs. Fifty-six behavioural variables, functional profile and biological status (HPA axis and sarcopenia index) were included. Firstly, the sex factor was studied, characterising the psychomotor phenotype of middle-aged (12 months) and older (16 months) females in comparison with males of the same age. In addition, in the long term, the administration of behavioural batteries and their dragging effect was studied in contrast to naïve mice in these tests at 16 months of age.

Specific objectives:

7. To determine the effects of retesting on the behavioural performance of animals tested in two scenarios:
   7.1. In a longitudinal design, with within-subjects analysis of a set of 12-month-old animals retested four months later, at 16 months of age;
   7.2. In a cross-sectional design, by comparing 16-month-old animals that had (retested) or had not (naïve) experienced the test battery.

Experimental design:



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Impact of Behavioral Assessment and Re-Test as Functional Trainings That Modify Survival, Anxiety and Functional Profile (Physical Endurance and Motor Learning) of Old Male and Female 3xTg-AD Mice and NTg Mice with Normal Aging

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Abstract: Longitudinal approaches for disease-monitoring in old animals face survival and frailty limitations, but also assessment and re-test bias on genotype and sex effects. The present work investigated these effects on 56 variables for behavior, functional profile, and biological status of male and female 3xTg-AD mice and NTg counterparts using two designs: (1) a longitudinal design: naïve 12-month-old mice re-tested four months later; and (2) a cross-sectional design: naïve 16-month-old mice compared to those re-tested. The results confirmed the impact as (1) improvement of survival (NTg rested females), variability of gait (3xTg-AD 16-month-old re-tested and naïve females), physical endurance (3xTg-AD re-tested females), motor learning (3xTg-AD and NTg 16-month-old re-tested females), and geotaxis (3xTg-AD naïve 16-month-old males); but (2) worse anxiety (3xTg-AD 16-month-old re-tested males), HPA axis (3xTg-AD 16-month-old re-tested and naïve females) and sarcopenia (3xTg-AD 16-month-old naïve females). Males showed more functional correlations than females. The functional profile, biological status, and their correlation are discussed as relevant elements for AD-pathology. Therefore, repetition of behavioral batteries could be considered training by itself, with some variables sensitive to genotype, sex, and re-test. In the AD-genotype, females achieved the best performance in physical endurance and motor learning, while males showed a deterioration in most studied variables.

Keywords: Alzheimer's disease; aging; survival; anxious profile; functional profile; motor performance; frailty; training; gait; kyphosis

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1. Introduction

Specific motor skills impaired in old age include a broad and varied spectrum that involves a reduction in gait speed, loss of strength and muscle mass, and decline of balance [1–3]. However, aging has become increasingly recognized as a potentially modifiable risk factor for chronic disease and frailty [4,5]. The deterioration of motor performance related to cognitive dysfunction in Alzheimer's disease (AD) has recently gained importance in clinical research [6–9]. Particularly, gait impairment and its association with cognitive impairment [10] could shed light on potentialities to distinguish AD [1]. Inclusive, higher levels of Aβ and tau are associated with more significant memory decline, but not with changes in executive function [11]. The study by Sperling points out that these results could explain why some clinically active patients presented elevated tau and Aβ levels [11]. Thus, Aβ and tau proteins can serve as markers of cognitive impairment; however, they are insufficient and cannot detect all cases of dementia, especially in the early stages [11,12]. For this part, gait speed, for example, is longitudinally associated with cognitive decline,

dementia, and falls in older adults [13,14], with slower gait associated with increased fall risk and poor baseline cognition [6]. However, motor dysfunctions and deterioration remain poorly explored. Consequently, functional and cognitive decline comorbidity is a warning sign for increased disability [8], a growing public health problem [15], and it is already present at preclinical stages of Alzheimer's disease [5].

On the other hand, aging is a frequent risk factor for different diseases, including dementia [16]. Recently, in a review of the literature that examined the pathophysiological basis and biomarkers of AD and other neurodegenerative diseases, it was pointed out that the predisposing factors for neuroinflammation are aging, metabolic diseases, hypertension, cerebrovascular accidents, depression and depression, dementia, among others [17]. In addition, healthy aging would be associated with chronic inflammation, contributing to a greater vulnerability to anxiety and depression [17]. Thus, cause–effect relationships can become bidirectional in the pathogenesis of multifactorial diseases, leading to a disease-prone state [18]. Age-related deficits in the ability to process contextual information and regulate responses to threat, addressing that structural and physiological alterations in the prefrontal cortex and medial temporal lobe determine cognitive changes in advanced aging, which may eventually cause patterns of cognitive dysfunction seen in patients with AD and mild cognitive impairment (MCI) [19].

Furthermore, it is known that AD is characterized by high heterogeneity in the disease's manifestation, progression, and risk factors [20]. Such a high phenotypic variability is considered one of the most significant obstacles in early diagnosis and clinical trial design [20]. Therefore, there is great interest in identifying factors driving variability used for patient stratification [20,21]. Additionally, the impact of sex on the disease varies throughout its progression [22,23]. It is important to identify the role of sex differences in the cognitive dimension if potentially more precise diagnoses and treatments should emerge [24,25], but few studies have reported differences in the psychomotor functional dimension of the disease.

In the last decade, at the translational level, the impact of interventions on age-related disability, frailty, and the onset of AD has been investigated in animal models to develop clinically relevant measures that provide indications for the approach and management of disability, frailty, and illness [26,27]. In addition, genotype and sex differences in cognitive, emotional, and locomotor performance have been studied at the preclinical level to assess the effects of promising interventions before their application in clinical settings [28–30].

Recently, our laboratory has developed a study method to identify psychomotor impairments and deficits at different stages of Alzheimer's disease [31]. Previously, we reported a functional impairment phenotype in male mice's gait and physical performance of the 3xTg-AD transgenic model in the initial, intermediate, and advanced stages of the disease. The results showed that 3xTg-AD mice show a significant functional impairment in the quantitative variables of gait and exploratory activity, movement limitations, and muscle weakness related to functional decline in the different stages of severity of the disease intensify with increasing age. In addition, signs of frailty accompany functional deterioration, and sarcopenia is evident in an advanced stage of AD, with differences in the morphological characteristics of muscle fibers and the number of fat cells [31].

Furthermore, we differentiate the disorders and postural patterns into two types of kyphosis (postural and structural) that differ in severity and limit the exploratory activity. In addition, the results indicated that the presence of bizarre gait patterns accounts for behavior similar to anxiety when 3xTg-AD mice face novelty situations and recognition of places, with circling and backward movements being the most frequent, in an already frailty setting [32,33].

The present study was designed to investigate the impact of two factors, sex, and repeated test, assessing the behavioral outputs of 3xTg-AD mice and mice with normal aging in longitudinal and transversal experimental designs. According to our previous work, a battery of psychomotor tests: gait, exploration, muscle strength, motor learning, physical endurance, and frailty status, was used [31]. In addition, phenotype of frailty

and biological status (HPA axis and sarcopenia index) was also included [32,33]. Thus, in the first place, we studied the sex factor by characterizing the psychomotor phenotype of middle—(12 months) and old—(16 months) age females, that in the 3xTg-AD mice corresponds to two neuropathological stages of the disease [31], as compared to that of aged-matched males. On the other hand, long-term studies provide better insights for assessing interventions with preclinical validity, but the administration of behavioral batteries is not exempt from carryover effects. In addition, behavioral batteries and repeated tests can be considered behavioral stimulation [34]. Therefore, we aimed to investigate the effects of repeated tests on the behavioral performance of animals assessed in two scenarios: 1) in a longitudinal design, with within-subjects analysis of a set of 12-month-old animals re-tested four months later, at 16 months of age; and 2) in a transversal design, when comparing 16-month-old animals that had (re-tested) or not (naïve) that experienced the battery of tests.

2. Materials and Methods

2.1. Animals

A total of 191 male and female mice were included for the survival analysis, and ninety-six of them, homozygous 3xTg-AD (n = 54) and non-transgenic (NTg, n = 42) male and female mice of 12 to 16 months of age in a C57BL/6J background (after embryo transfer and backcrossing of at least ten generations), established at the Universitat Autònoma de Barcelona, were included in the experimental study. As previously described, the 3xTg-AD mice harboring transgenes were genetically created at the University of California at Irvine [35]. Animals were kept in groups of 3–4 mice per cage (Macrolon, 35 cm × 35 cm × 25 cm) filled with 5 cm of clean wood cuttings (Ecopure, Chips, Date Sand, UK; uniform cross-sectional wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm × 20 cm, White). In all cases, standard home cages covered with a metal grid to allow the perception of olfactory and auditory stimuli from the rest of the colony. All animals were kept under standard laboratory conditions of food and water ad libitum, 20 ± 2 °C, 12 h light cycle:dark with lights turned on at 8:00 a.m. and 50–60% relative humidity. The study complied with the ARRIVE guidelines developed by the NC3Rs and aimed to reduce the number of animals used [36].

2.2. Experimental Design

A longitudinal and a transversal study were carried out to evaluate the anxious-like and functional profiles of male and female 3xTg-AD and NTg mice. Biological variables (corticosterone and sarcopenia) of animals at the end point (16 months of age) were also included. For this purpose, animals were randomly assigned into two experimental batches (see Figure 1, Experimental design)

2.3. Behavioral Assessment and Biological Status

The assessment consisted of four consecutive evaluation steps conducted during 5 days, as follows: Day 1, bodyweight, phenotype scoring system, and frailty; Day 2, gait and exploration; Day 3, geotaxis, muscle strength, and rotarod; Days 4–5, rotarod. The procedures and protocol were based on the protocol used by Castillo-Mariqueo and Giménez-Llort [31]. Assessments were performed under dim white light (20 lx) in the light cycle (10:00 a.m. to 1:00 p.m.). Behavioral evaluations were carried out in a counterbalanced way by two independent observers, blind to the genotype. Animals were habituated to the test room 30 min before the start of the tests.

2.3.1. Survival, Bodyweight, Phenotype Scoring System, Frailty Score, and Kyphosis

Survival curves were analyzed considering the cohort of siblings from the same litter of mice included in the study, from birth to 16 months of age. A total of 191 male and female mice, NTg and 3xTg-AD were included in this analysis (NTg males = 49; NTg females = 58; 3xTg-AD males = 50; 3xTg-AD females = 34). All animals were weighed and

evaluated with the phenotype scoring system that includes four subtests and scores: ledge, grip, gait, and kyphosis [37,38]. Individual measures were scored from 0 (the absence of the relevant phenotype) to 3 (the most severe manifestation) [37]. The measures can be analyzed individually or combined into a composite phenotype score [39,40]. On the other hand, frailty was assessed using an adaptation of the MCFI by Whitehead [41], which includes 30 assessment items from the clinical setting. The 12 elements with the highest incidence previously reported by our laboratory were selected [42]. Their incidence was reported through an absence (0), presence (1) score. The clinical evaluation included physical aspects, injuries and wounds, alopecia, piloerection, body and tail position, tremor, and urogenital alterations.

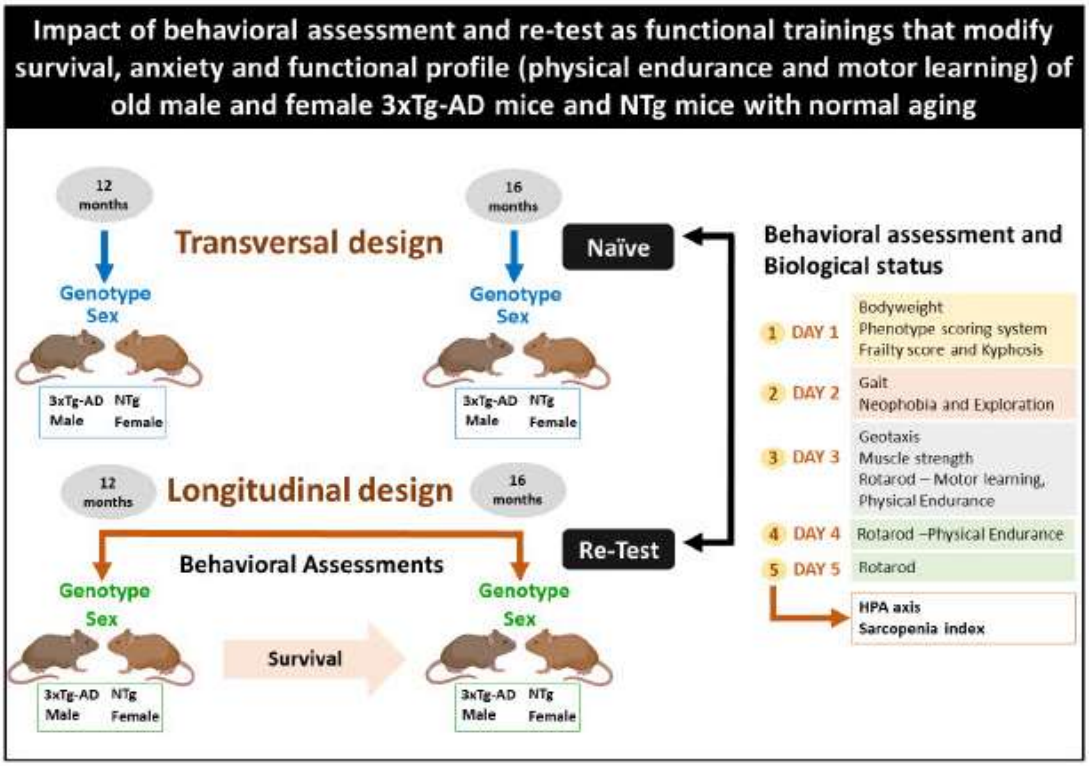

Figure 1. Experimental design. Longitudinal design: the first group was assessed in the behavioral battery at the age of 12 months and again when the animals reached 16 months of age. Transversal design: the second group was housed in standard conditions without manipulation until they were tested at 16 months of age, so they could be compared to re-tested 16 months old animals.

2.3.2. Quantitative Parameters of Gait, Neophobia, and Exploration

The quantitative parameters of the gait and exploration were recorded by filming the spontaneous gait of the mice for 1 min. Later the videos were analyzed using KINOVEA 0.8.15 free software according to the Castillo-Mariqueo and Gimenez-Llort protocol [31]. Stride length, stride length variability, speed, and cadence were included according to the methodology used by Wang et al. [43]. The examination included observation of body position, limb support, and movement. In addition, neophobia (immediate fear of a new place) was assessed by means of the corner test [44] and the recording of freezing (latency of movement), the number of explorations on the horizontal axis (visited corners), the latency and number of explorations on the vertical axis (rearings).

2.3.3. Muscular Strength—Hanger Test and Geotaxis

The muscle strength was measured in the forelimbs using the hanger test. Three trials were performed to observe the tendency of a mouse to instinctively grasp a rack or bar when suspended by the tail. In the first and second trials, grip strength was assessed by holding the animal with its front legs for 5 s at the height of 40 cm. In the third trial, the animal is suspended for 60 s in a single attempt to assess muscular endurance. This test allows discriminating grip strength and muscular endurance according to the suspension times used by mice [45]. A box with sawdust is placed under the animal to prevent a possible fall in each trial. The bar used is graduated in 5-cm blocks to obtain the distance covered when the animal moves through the bar. The latency and movement distance are recorded. Geotaxis was measured using a 10 cm×12cm grid. A single trial registered the time it took for the animal to reach the vertical position from an inverted position at a 90° angle on the grid.

2.3.4. Motor Performance: Learning and Physical Endurance—Rotarod

Six micro training cycles were carried out during three consecutive days with a previous learning session and psychomotor coordination. The animals were trained in the Rotarod apparatus (Ugo basile®, Mouse RotaRod NG) according to a training volume established in our previous research laboratory investigations [31]. An incremental intensity of 5 to 48 rpm was applied according to individual tolerance with a maximum duration of 360 s in each microcycle with a 1-min recovery between trial.

2.3.5. Biological Status: HPA Axis and Sarcopenia Index

The animals were euthanized and the muscle tissues were necropsied. Plasma from a blood sample was obtained by centrifugation and stored and −80 °C until corticosterone analysis. Corticosterone content (ng/mL) was analyzed using a commercial kit (Corticosterone EIA Immunodiagnostic Systems Ltd., Boldon, UK). Absorbance was read at 450 nm with Varioskan LUX ESW 1.00.38 (Thermo Fisher Scientific, Massachusetts, MA, USA) [42]. The weights of the quadriceps and triceps surae muscles of the right lower extremity of each animal were recorded and kept for future analysis. The sarcopenia index [46] was applied to obtain an indirect measure of sarcopenia as a biological marker of frailty.

2.4. Statistics

Statistical analyses were performed using SPSS 15.0 software. Results were expressed as the mean ± standard error of the mean (SEM) for each task and trial. The variables recorded were analyzed with Student t-test, Chi-squared or Fisher's exact test, one-way ANOVA, and multiple regression analysis (MRA). The split-plot ANOVA design with factors genotype (G), sex (S), previous experience either as a re-test (R) in the longitudinal approach or as naïve (N) in the transversal approach, were included. Their G×S, G×R, and S×N factor interactions were also studied. *Post hoc* comparisons were run with Bonferroni corrections. Pearson's correlations were made to analyze the functional correlations with (1) corticosterone, (2) sarcopenia index, and (3) phenotype score system. The survival curve was analyzed with the Kaplan–Meier test (Log rank). In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Survival, Bodyweight, Phenotype Scoring System, Frailty Score, and Kyphosis

Figure 1 shows the data obtained for survival, frailty score and postural and structural kyphosis. Thus, the animal cohort was analyzed from birth to 16 months (16 months NTg and 16 months 3xTg-AD). Only siblings from the same litter belonging to mice meeting the end-points were considered. Log rank analysis showed statistically significant differences dependent on genotype and sex (G, χ^2 (1) = 20.044, $p < 0.001$; S, χ^2 (1) = 33.531, $p < 0.001$), see Figure 2A. In this way, it was possible to observe that females of both genotypes have higher mortality than males, and that of them the NTg is even higher (days of average

survival: Males, NTg = 445.05 ± 15.75, CI: 414.16–475.94; 3xTg-AD = 505.57 ± 13.04, CI: 480.01–531.13. Females, NTg = 343.60 ± 15.39, CI: 313.42–373.78; 3xTg-AD = 442.76 ± 18.40, CI: 406.69–478.83). In addition, the female NTg cohort reached 79% (46/58) of mortality and 3xTg-AD the 50% (17/34) with ages 11 to 13 months having the greatest death. For their part, NTg males reached 37% (18/49) and 3xTg-AD 24% (12/50), with 15 to 16 months being the age of greatest death. During the follow-up of the animals that started the battery at 12 months, 20 deaths were detected, the NTg males had 29% (4/14), the 3xTg-AD males a 20% (4/20), the NTg females a 35% (7/20), and 3xTg-AD females 31% (4/13), see Figure 2A.

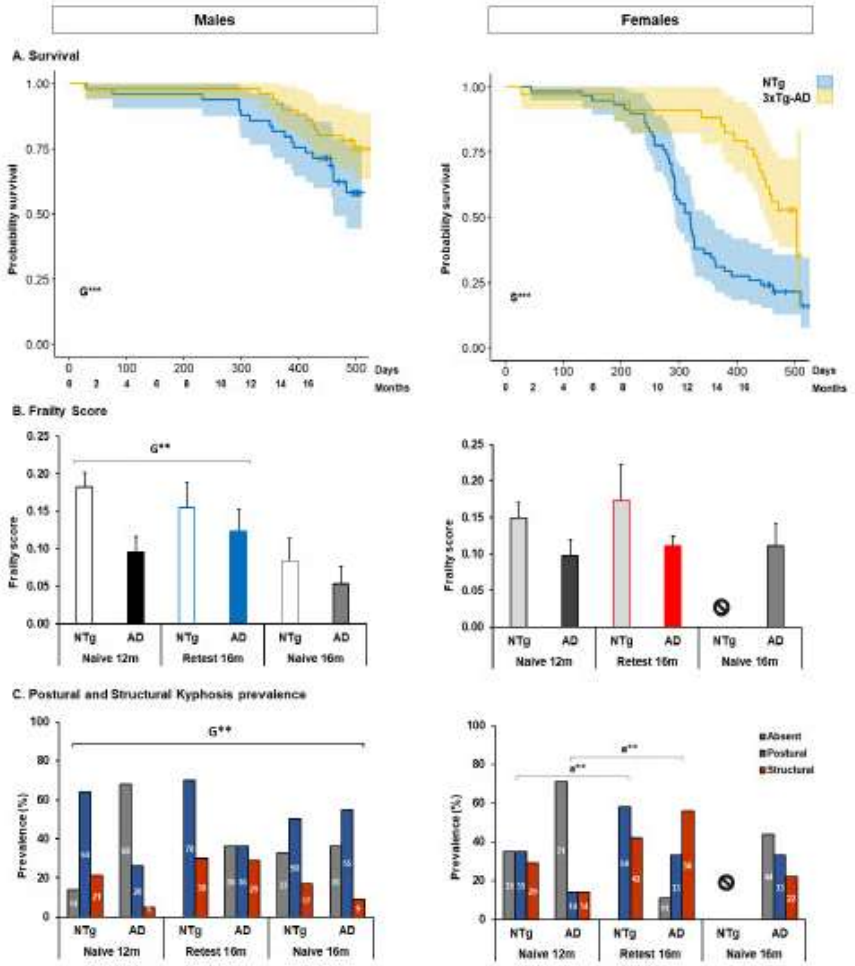

Figure 2. Survival, frailty score, and postural and structural kyphosis. (**A**) Survival. Statistics: Kaplan–Meier test—Log rank, G, genotype effect: χ^2, G*** $p < 0.001$***; S, sex effect: χ^2, S*** $p < 0.001$***. (**B**) Frailty score. Statistics: ANOVA, G, genotype effect: G** $p < 0.01$**. (**C**) Postural and structural kyphosis. Statistics: Fisher's exact test, G, genotype effect in males, G** $p < 0.01$**; a, aging effect in females: a** $p < 0.01$**. The symbol ⃠ indicates the absence of the group, and m, months.

In terms of frailty score, G effect were identified, the score is higher in NTg animals in naïve 12 months (frailty score, G, F (1, 62) = 11.159, $p = 0.001$), see Figure 2B. In addition, the severity of kyphosis has been identified, thus, in males, a higher prevalence of postural kyphosis has been observed, being higher in the case of NTg mice (severity, Fisher's exact

test df (15) = 24.403, $p = 0.023$. G effect, Fisher's exact test df (3) = 11.842, $p = 0.004$). In the case of females, the highest prevalence of cases was also postural kyphosis, and this increased in 3xTg-AD mice at 16 months, being the structural type disorder in this group the one with the highest prevalence attributable to age (severity, Fisher's exact test df (12) = 22.900, $p = 0.008$. 3xTg-AD naïve 16 months vs. re-test 16 months, Fisher's exact test df (3) = 16.137, $p = 0.001$), see Figure 2C.

Table 1 shows the phenotype scoring system obtained in males and females. Specifically, at naïve 12 months, differences were detected in the gait, kyphosis, and total score, with G effect in kyphosis and total score showing the high deterioration in the NTg group (kyphosis, G, F (1, 62) = 13.329, $p = 0.001$. Total score, G, F (1, 62) = 4.078, $p = 0.048$). In addition, there is an interaction of the G×S in gait, with 3xTg-AD females and NTg males presenting a lower (gait score, G×S, F (1, 62) = 7.776, $p = 0.007$). At the 16 months re-tests, the difference in gait score is maintained without significant differences in the other parameters (gait score, G×S, F (1, 44) = 10.709, $p = 0.002$). In contrast to naïve 16 months, differences were observed in genotype and sex in the clasping score and gait, being measured the genotype differences only in males and sex between the 3xTg-AD group (clasping score, F (2, 30) = 4.646, $p = 0.017$; male 3xTg-AD naïve 16 months vs. male NTg naïve 16 months, $p = 0.019$. G, in male group, T student t = −2.836, $p = 0.012$. S, in 3xTg-AD group T student t = −2.138, $p = 0.046$). If we consider the change between the groups after the re-test, we have detected differences in the different scores of the phenotype scoring system. The main differences were detected in the total score, kyphosis and ledge score (re-test, total score, F (1, 141) = 15,972, $p < 0.0001$. Kyphosis score, F (1, 141) = 14.596, $p < 0.000$ and G, F (1, 141) = 5.159, $p = 0.025$. Ledge score, F (1, 141) = 10.435, $p = 0.002$). In addition, differences in total score, gait, kyphosis, and ledge score were detected between males, with effect of previous experience and genotype (total score, F (5, 80) = 3.449, K= 0.007, 3xTg-AD naïve 12 months vs. 3xTg-AD re-test 16 months, k = 0.031; R, F (1, 80) = 13.002, $p = 0.001$. Ledge score, R, F (1, 80) = 7.447, $p = 0.008$. Gait, F (5, 80) = 4.303, $p = 0.002$, 3xTg-AD re-test 16 months vs. 3xTg-AD naïve 16 months, $p = 0.003$; 3xTg-AD re-test 16 m vs. NTg naïve 16 months, $p = 0.003$; R, F (1, 80) = 7.461, $p = 0.008$; and G, F (1, 80) = 5.560, $p = 0.021$. Kyphosis score, F (5, 80) = 3.269, $p = 0.010$; R, F (1, 80) = 6.310, $p = 0.014$; and G, F (1, 80) = 5.225, $p = 0.025$). In females, differences were found in total score, kyphosis and gait with effect of previous experience and genotype (total score, F (4, 60) = 2,800, $p = 0.034$; R, F (1, 60) = 4.517, $p = 0.038$; G, F (1, 60) = 4.767, $p = 0.033$. Kyphosis score, F (4, 60) = 3.375, $p = 0.015$, 3xTg-AD naïve 12 months vs. 3xTg-AD re-test 16 months, $p = 0.050$; R, F (1, 60) = 7.791, $p = 0.007$. Gait score, F (4, 60) = 2.909, $p = 0.029$; G, F (1, 60) = 5.632, $p = 0.021$).

Table 1. Phenotype scoring system.

Phenotype Scoring System		Naïve 12-Month-Old			Re-Test 16-Month-Old			Naïve 16-Month-Old			Statistics
		Males	Females	*p*-Value	Males	Females	*p*-Value	Males	Females	*p*-Value	
Clasping score	NTg	0.52 ± 0.16	0.43 ± 0.08	n.s.	0.67 ± 0.20	0.69 ± 0.17	n.s.	0.25 ± 0.06	NR	$s^{\$}$	n.s.
	AD	0.56 ± 0.17	0.62 ± 0.24		0.79 ± 0.19	0.30 ± 0.15		0.67 ± 0.18 $^{\$}$	0.26 ± 0.12		
Ledge score	NTg	0.40 ± 0.15	0.48 ± 0.14	n.s.	0.60 ± 0.09	0.68 ± 0.11	n.s.	0.31 ± 0.12	NR	n.s.	$R^{\&}$
	AD	0.25 ± 0.08	0.48 ± 0.13		0.58 ± 0.16	0.51 ± 0.07		0.22 ± 0.09	0.41 ± 0.14		
Gait score	NTg	0.21 ± 0.15	0.33 ± 0.12	G×S$^{\$\$}$	0.20 ± 0.13	0.38 ± 0.15	G×S$^{\$\$}$	-	NR	$s^{\$}$	G^{*}, $R^{\&\&}$, $r^{\&\&}$, $r^{\$\$}$
	AD	0.26 ± 0.10	-		0.64 ± 0.13$^{\&\&}$	-		0.03 ± 0.03	0.44 ± 0.24		
Kyphosis score	NTg	1.07 ± 0.16	1.0 ± 0.25	G^{**}	1.30 ± 0.15	1.38 ± 0.15	n.s.	0.72 ± 0.21, $s^{\$\$}$	NR	n.s.	G^{*}, $R^{\&\&\&}$, $r^{\&}$
	AD	0.30 ± 0.13	0.43 ± 0.20		0.95 ± 0.26	1.37 ± 0.27 $^{\&}$		0.47 ± 0.17	0.67 ± 0.28		
Total score	NTg	2.21 ± 0.37	2.24 ± 0.39	G^{*}	2.77 ± 0.37	3.14 ± 0.45	n.s.	1.28 ± 0.23	NR	n.s.	$R^{\&\&\&}$, $r^{\&}$ G^{*}
	AD	1.37 ± 0.30	1.52 ± 0.33		2.96 ± 0.58 $^{\&}$	2.18 ± 0.32		1.39 ± 0.28	1.78 ± 0.44		

Statistics: ANOVA, G, genotype effect, G^{**} $p < 0.01$, $p < 0.05$, n.s. $p > 0.05$. R, Re-test effect, R^{***} $p < 0.001^{***}$, R^{**} $p < 0.01^{**}$, $p < 0.05^{*}$, n.s. $p > 0.05$. G×S, genotype and sex interaction effect, G×S^{**} $p < 0.01^{**}$, $p < 0.05^{*}$, n.s. $p > 0.05$. Bonferroni post hoc test: g, genotype, g^{*} $p < 0.05^{*}$; s, sex; $ expressed genotype differences between sex, $s^{\$\$}$ $p < 0.01^{\$\$}$; & expressed differences between re-test groups, $r^{\&\&}$ $p < 0.01^{\&\&}$, $p < 0.05^{\&}$; $ expressed genotype differences between re-test group, $r^{\&}$ $p < 0.05^{\&}$, and $r^{\$\$}$, between sex differences. NR indicates the absence of the group.

In bodyweight at 12 months was high in 3xTg-AD, and at 16 months it decreased in females. In addition, males naïve 16 months weighed more than re-test males at the same, see Tables S1–S3.

3.2. Quantitative Parameters of Gait, and Neophobia and Exploration

Quantitative parameters of gait are shown in Figure 3. For naïve 12 months, statistically significant differences were observed in all quantitative gait variables. Stride length showed differences in G and S, with the longest stride length in NTg males and 3xTg-AD in females. This interaction was also observed in gait speed, high in NTg mice. At the same time, the variability of gait presented differences associated with S, with females showing less than males' variability and, therefore a gait with more homogeneous steps in its trajectory. Additionally, a genotype-dependent difference was observed in cadence, where NTg mice show better performance in this variable with marked differences between males. In the re-test at 16 months, this group registered a gait performance that shows the interaction between the G×S effect in stride length and speed, with the performance of 3xTg-AD females being the one with the best performance in both variables. The re-test of this group at 16 months showed differences in cadence, increasing its performance in the group of 3xTg-AD mice of both sexes and decreasing in the NTg group, see Figure 3A–D and Tables S1–S3.

On the other hand, mice at 16 months did not show significant differences in quantitative variables of gait. Differences could only be observed between the re-test and a naïve group of males at 16 months in stride length, cadence, and speed, where the re-test NTg mice presented a high performance in speed and cadence compared to the naïve NTg mice, 3xTg-AD re-test, and naïve, but lower performance in stride length than naïve mice. In addition, differences were detected between the 3xTg-AD males and females in the variable's variability and gait speed, with the 3xTg-AD naïve and re-test females showing less variability than the 3xTg-AD males. This difference was also present in gait speed, with a better performance of re-test females followed by naïve 16 months females over 3xTg-AD males in both conditions, see Figure 3A–D and Tables S1–S3.

For its part, the neophobia and exploratory activity presented sex differences in the ratio visited corners/rearings, being higher in females of both genotypes, see Figure 4. This difference was maintained at re-test 16 months, with the higher ratio in females. In addition, the ratio in MRA of the groups showed an interaction between G×S, indicating a lower performance in 3xTg-AD re-test males at 16 months and higher in re-test females, see Figure 4B and Tables S1–S3.

As in gait, no significant differences were detected in exploratory activity between the naïve 16 months group. However, in contrast to the re-test males at 16 months, the naïve males presented high vertical activity than the re-test, see Figure 4C,D. Between the group of 3xTg-AD mice, S effect was identified in vertical activity, where naïve males presented higher activity. Movement latency was also lower in naïve males, but the same was not observed in 3xTg-AD females, see Figure 4A and Tables S1–S3.

3.3. Muscular Strength: Forelimb Grip Strength and Muscular Endurance—Hanger Test and Response to Gravity: Geotaxis

Lower muscle strength can be observed in the resistance distance in the 3xTg-AD animals at the age of 12 months, which, despite not showing statistical differences, shows a trend with less strength in the 3xTg-AD males. No statistically significant differences were detected in the rest of the variables, although a worse performance of the animals was observed, see Table S1. At 16 months re-test and 16 months naïve, no significant differences were detected.

On the other hand, significant differences in geotaxis were detected. The group of 3xTg-AD male took longer to complete the test in the re-test 16 months group in contrast to the 12 months and naïve 16 months group, see Figure 5A. Notably, at 16 months in the re-test group, an interaction was observed between the G×S of the animals, showing

more significant latency in 3xTg-AD males and NTg females. G×S interaction was also observed between the group of naïve 16 months 3xTg-AD mice. In addition, there was an interaction between the G×R was detected among male mice at 16 months in contrast to naïve mice of the same age, with the test time being shorter in naïve mice, see Figure 5A and Tables S1–S3.

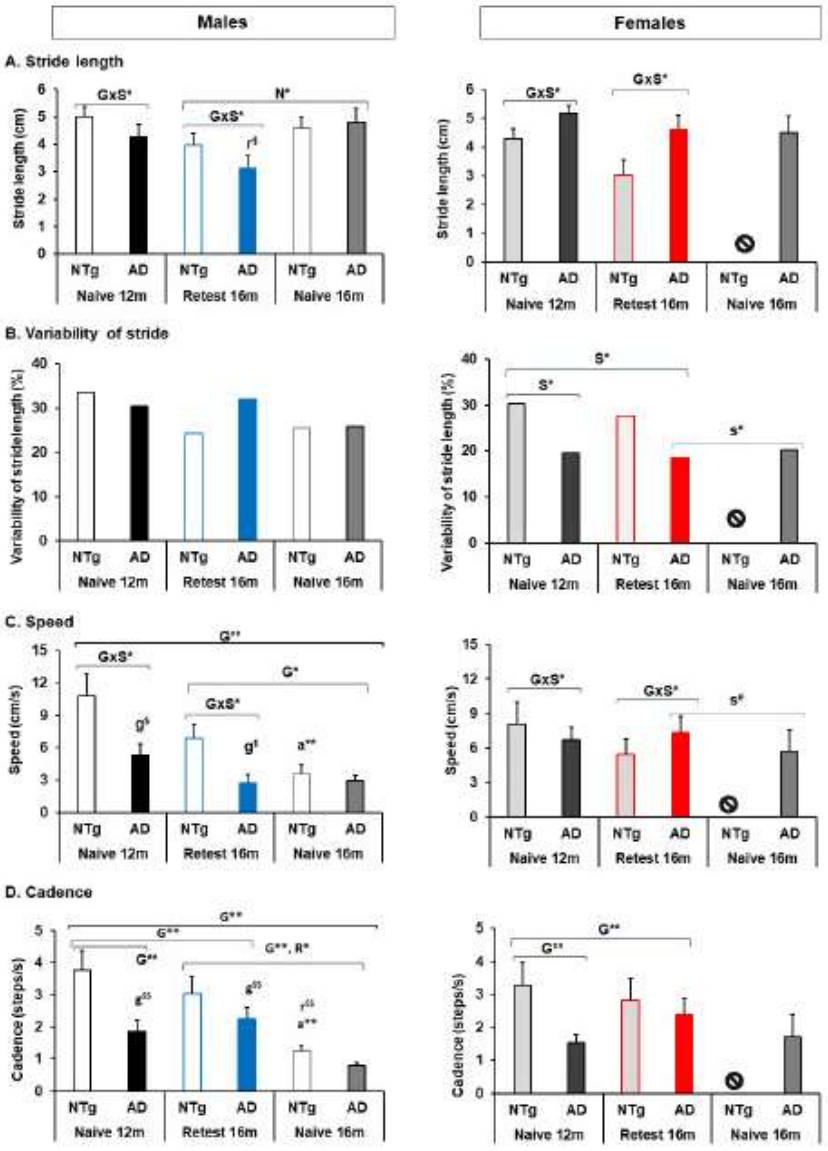

Figure 3. Quantitative parameters of gait. (**A**) Stride length, (**B**) variability of stride, (**C**) speed, (**D**) cadence. Statistics: ANOVA, G, genotype effect, G** $p < 0.01$**, G* $p < 0.05$*. S, sex effect, S* $p < 0.05$*. G×S, genotype and sex interaction effects, G×S* $p < 0.05$*. R, re-test effect, R* $p < 0.05$*. N, naïve effect, N* $p < 0.05$*. a, aging, a** $p < 0.01$**. Bonferroni *post hoc* test: g, genotype; s, sex; $ expressed genotype differences between sex, and # expressed sex differences between genotypes. The symbol ⦸ indicates the absence of the group, and m, month.

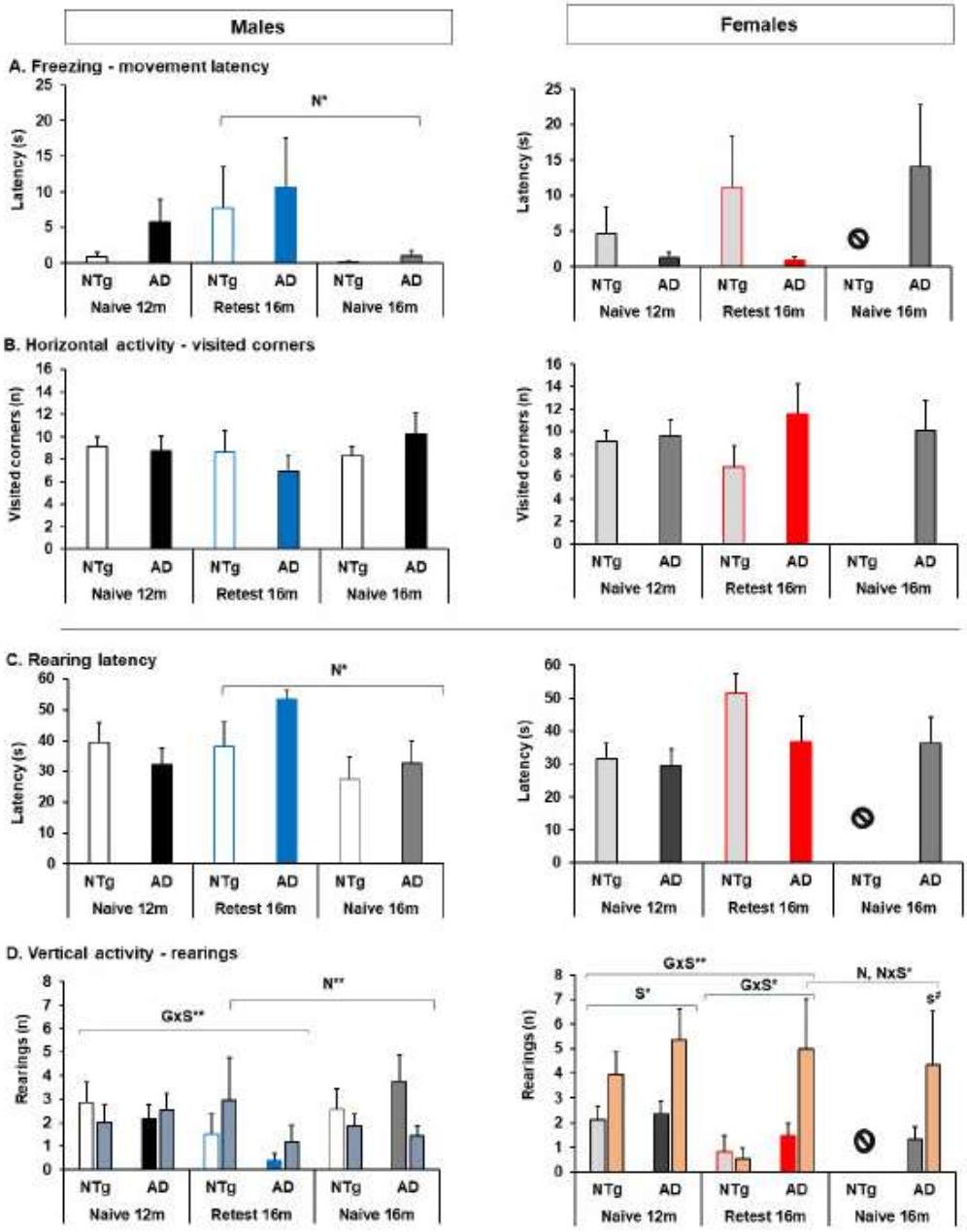

Figure 4. Ethogram of Neophobia and Exploratory activity. (**A**) Freezing, (**B**) horizontal activity, (**C**) rearing latency, (**D**) vertical activity. Statistics: ANOVA, S, sex effect, S* $p < 0.05$; G×S, genotype and sex interaction effects, G×S* $p < 0.05$*. N, naïve effects, naïve at 16 months *vs.* re-test 16 months, N** $p < 0.01$**, N* $p < 0.05$*. N×S, naïve and sex interaction effects, N×S* $p < 0.05$*. Bonferroni *post hoc* test: s, sex; and # expressed sex; differences between genotypes. The symbol ⊘ indicates the absence of the group, and m, month.

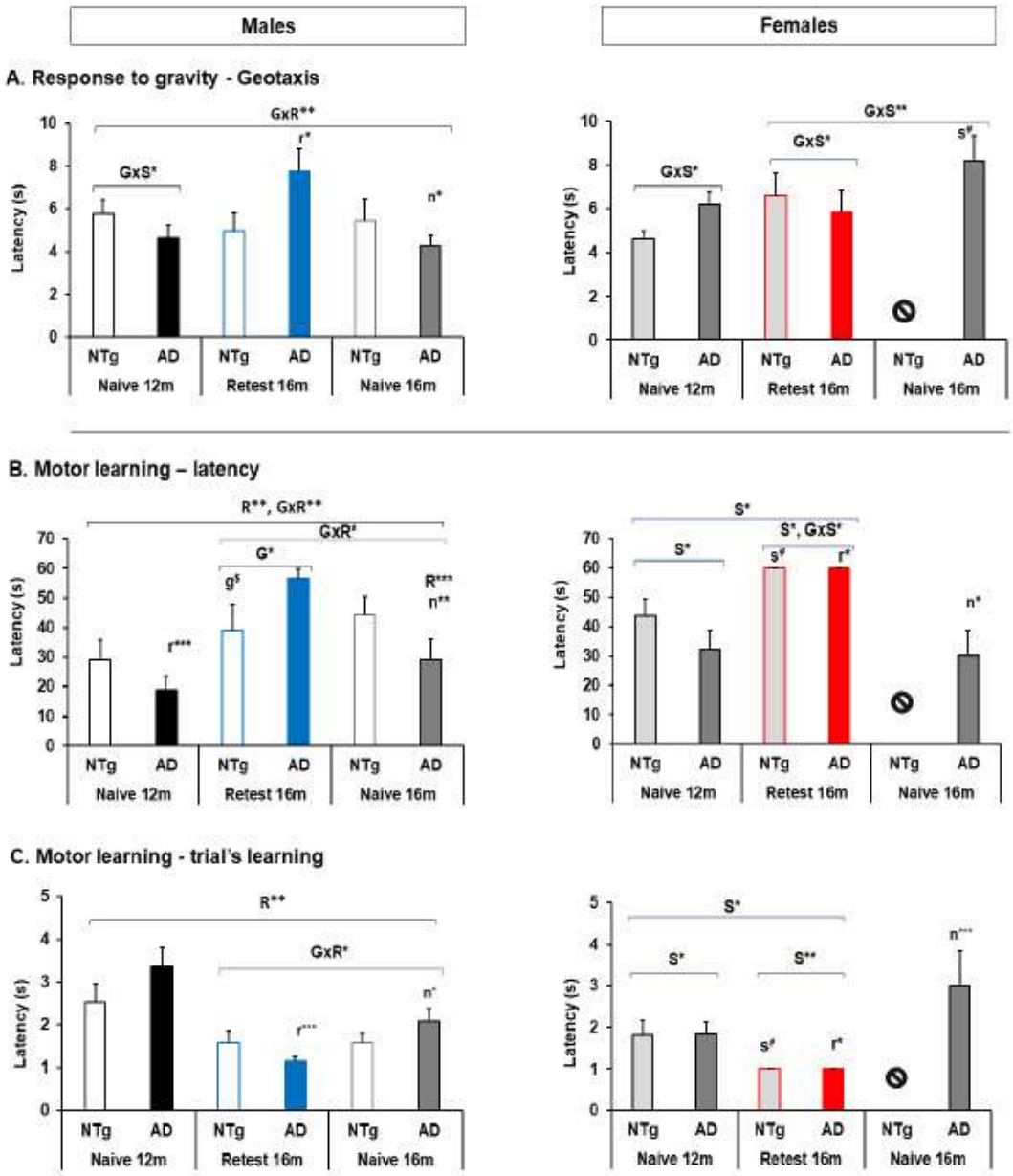

Figure 5. Geotaxis and motor learning—rotarod. (**A**) Geotaxis, (**B**) latency, (**C**) trial's learning. Statistics: ANOVA, G, genotype effect, G* $p < 0.05$. S, sex effect, S** $p < 0.01$, S* $p < 0.05$. R, Re-test effect, naïve 12 months and re-test 16 months, R** $p < 0.01$**, R* $p < 0.05$*. G×S, genotype and sex interaction effects, G×S** $p < 0.01$**, G×S* $p < 0.05$*. G×R, genotype and re-test effects, G×R** $p < 0.01$**, G×R* $p < 0.05$*. Bonferroni *post hoc* test: g, genotype, s, sex, r: re-test naïve 12 months vs.16 months, and n, naïve 16 months vs. re-test 16 months; $ expressed genotype, and # expressed sex differences between genotypes. The symbol ⊘ indicates the absence of the group, and m, month.

3.4. Motor Performance: Learning and Physical Endurance—Rotarod

The learning and motor performance tests in the Rotarod showed significant differences associated with different factors depending on the test or the group studied, see Figure 5. Among the males, significant differences were detected in learning and the number of trials between naïve and re-tests at 12 months and 16 months. In females, differences were detected in 3xTg-AD of 12 months and 16 months re-test and naïve, see Figure 5B,C and Tables S1–S3. In turn, for motor learning, the S effect plays an important role since females manage to learn earlier than males and spend more time on the wheel during the test at 12 months. At the re-test 16 months, the S effect was maintained in the number of trials, but in learning the G effect and G×S became important. In the same way, when performing MRA in the groups naïve at 12 months and re-test at 16 months, the S effect was the one that marked the statistical difference, see Figure 5B,C and Tables S1–S3. Nevertheless, there were no significant differences between the naïve 16 months group. However, between the re-test 16 months group vs. the naïve 16 months, differences were detected between males, where the R and G effects were significant. In addition, significant differences were also detected between the 3xTg-AD group, where the differences in S and R effects were the ones that obtained significance, see Figure 5B,C and Tables S1–S3.

At the same time, it is possible to differentiate physical endurance according to the interaction of G and S in the naïve 12 months, re-test 16 months, and naïve 16 months groups, see Figure 6. The NTg males have a physical endurance similar to that of 3xTg-AD females, followed by NTg females and finally 3xTg-AD males, whose performance is low and does not improve with training. This difference persisted in the re-test at 16 months. In males, differences were also detected in the physical endurance and each training days, with significance in the age of the NTg animals and the effect of Re-test in 3xTg-AD and the NTg (see Figure 6A and Tables S1–S3). In addition, on the first day of training, it was observed that 3xTg-AD males showed differences in R and aging effect among naïve mice (see Figure 6B, and Tables S1–S3). On the second day of training, the difference in G at 12 months and the effect of aging in the naïve 3xTg-AD group stand out. The changes observed on the third day of the test were recorded at 12 months, where the G has statistical significance and the R only in NTg group. For females, physical endurance was higher in the 3xTg-AD group. The re-test 16 months group had high latencies (see Figure 6A and Table S2). Differences were observed on Day 1 and Day 3, with differences was in the 16 months re-test NTg group and aging effect in the 3xTg-AD group (see Figure 6B and Table S3).

On the other hand, differences in genotype and sex were detected in the 12 months group (see Figure 6A and Table S1). In the 3 days of training, differences in effect were detected, with G distinction only on the second day (Figure 6B, and Table S1). Additionally, at 16 months in the re-test group, differences in G×S were recorded in physical endurance (see Figure 6A and Table S2). Days 2 and 3 showed differences in G×S, with no significant differences on the first day (see Figure 6B and Table S2). The re-test of this group corroborated the differences in G×S of the batch at 16 months (see Figure 6B and Table S2). However, at 16 months, groups of naïve mice did not show significant differences in this test. Yet, when comparing the re-test and naïve mice at 16 months, significant differences were detected between the group of males in physical endurance and the performance of Days 1 and 3 (see Figure 6A,B and Table S3). In addition, among the group of transgenic mice, differences in sex and re-test were detected between the groups, with the performance of the females being higher (see Figure 6A,B and Table S3).

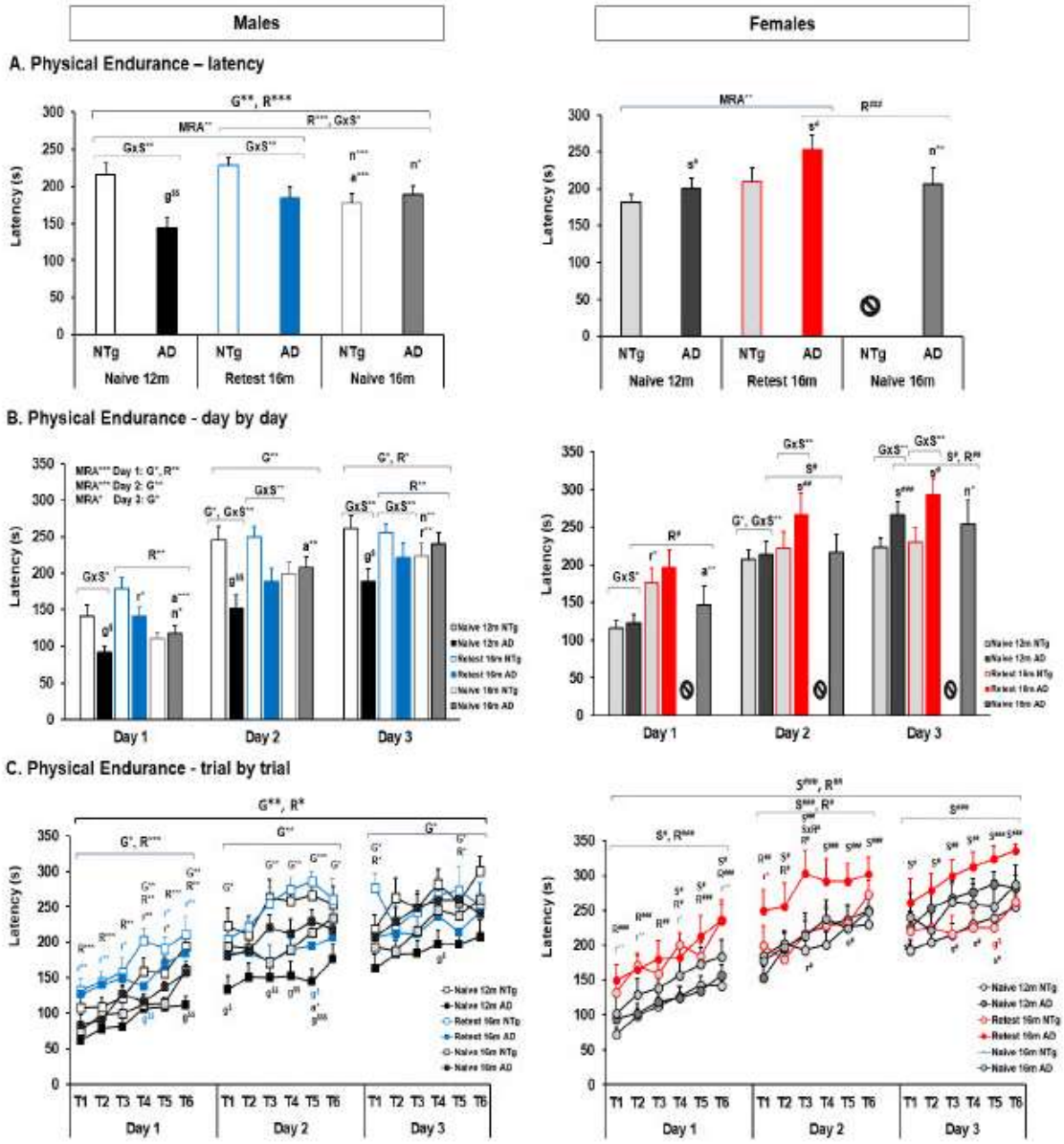

Figure 6. Physical endurance—rotarod. (**A**) Latency, (**B**) day by day, (**C**) trial by trial. Statistics: ANOVA, MRA-ANOVA, G, genotype effect, G** $p < 0.01$, G* $p < 0.05$. S, Sex effect, S*** $p < 0.001$***, S** $p < 0.01$**, S* $p < 0.05$. R, re-test effect, naïve 12 months vs. re-test 16 months, R*** $p < 0.001$***, R** $p < 0.01$**, R* $p < 0.05$*. G×S, genotype and sex effects, G×S** $p < 0.01$**, G×S* $p < 0.05$*. S×R, sex and re-test effects, S×R* $p < 0.05$*. Bonferroni *post hoc* test: g, genotype; s, sex; r. re-test, naïve 12 months vs. 16 months; n, naïve, naïve 16 months vs. re-test 16 months; $ expressed genotype, and # expressed sex differences between genotypes. The symbol ⊘ indicates the absence of the group, and m, month.

Furthermore, considering MRA between the groups, we can differentiate the effect of G, S, and R in the day-by-day and trial-by-trial tests, Table S4 shows the statistical differences from Figure 6C. The MRA analysis between males on Day 1 showed differences in G and S, see Figure 6C. It was observed that the NTg retest males improve with the repetition of the trials as well as the 3xTg-AD, but these do so to a lesser extent, and both the 12 months and 16 months naïve males have lower performance than retest. On Day 2, the genotype effect was observed here. The naïve 12 months NTg mice and the retest 16 months show a high latency in the test that increases with the execution of the trials. Naïve 16 months 3xTg-AD mice show the best performance within this group. In addition, the MRA trial-by-trial showed the differences in each trial and the animals' G and R differences. Here, it is highlighted that the first day plays an important role in the retest and then the differences of genotype. Also, among the females, significant differences were recorded in MRA trial by trial, with the 3xTg-AD retest of 16 months being the ones with the highest performance during all the test days. On the first day of training, differences in performance were obtained between the naïve 12 m NTg females and their retest 16 months, with a higher latency between the 16 months 3xTg-AD retest (Figure 6C). The second day of training did not record differences between the females, but on the third day, the highest performance of the 3xTg-AD retest 16 months was observed again.

Additionally, it is possible to differentiate females from males in the 3xTg-AD group, with females showing better performance in all tests. Thus, on Day 1 the mice differ in S and R. On the second day, the differences obtained on Day 1 are maintained, but the difference in the gender factor increases between the groups. On the third day, it is only possible to differentiate the gender factor between the groups. Specifically, the differences between the different factors have been identified in each trial. Thus, we can highlight specific differences be-tween the groups as detailed below on supplementary data for males and females. In addition, differences between 3xTg-AD males and females were detected in the following trials (see Figure 6C and Table S4).

3.5. *Biological Status: HPA Axis and Sarcopenia Index*

Higher differences were found in the corticosterone level in the re-test group compared to the naïve group (corticosterone, $F(6, 70) = 9.817$, $p < 0.001$ *post hoc:* male 3xTg-AD naïve vs. female 3xTg-AD naïve $p < 0.001$, male NTg naïve vs. male NTg re-test, $p = 0.001$). In addition, between group of males, the interaction of N had a lower level of corticosterone in the naïve group (N, $F(1, 47) = 25.163$, $p < 0.001$). In the 3xTg-AD group of animals, S effect and S×N interaction effects were differentiated (S, $F(1, 42) = 16.456$ $p < 0.001$. S×N, $F(1, 42) = 4.243$, $p = 0.046$), see Figure 7A.

The weight of the quadriceps and triceps sural muscles showed statistically significant differences (quadriceps, $F(6, 70) = 3.203$, $p = 0.008$. Triceps surae, $F(6, 70) = 7.126$, $p < 0.001$, *post hoc*: male naïve 3xTg-AD vs. female naïve 3xTg-AD, $p < 0.001$; female naïve 3xTg-AD vs. female re-test 3xTg-AD, $p = 0.022$). In addition, differences in the N effect were detected in male group, so, the muscle weight being greater in the naïve group in both muscles (quadriceps, N, $F(1, 46) = 8.965$, $p = 0.005$. Triceps surae, N, $F(1, 46) = 7.267$, $p = 0.008$). In the group of 3xTg-AD mice, differences in S and N were detected in the triceps surae muscle, the quadriceps muscle did not show significant differences in this analysis (triceps surae, S, $F(1, 44) = 14.955$, $p < 0.001$. S×N, $F(1, 44) = 6.998$, $p = 0.012$), see Figure 7B,C.

In the sarcopenia index, significant differences were observed in sarcopenia index-triceps surae (sarcopenia index, $F(6, 70) = 3.158$, $p = 0.008$, *post hoc*: male naïve 3xTg-AD vs. female naïve 3xTg-AD, $p < 0.001$; female naïve 3xTg-AD vs. female re-test 3xTg-AD, $p = 0.022$), see Figure 7E.

Furthermore, corticosterone levels were correlated with different variables, detecting a different correlation between males and females. In males, a negative correlation with the muscle weight of the quadriceps and triceps surae stands out, and a positive correlation with the variables, phenotype scoring system, frailty score, cadence and physical endurance on the first day (quadriceps, $r^2 = (-) 0.141$, $p = 0.008$; triceps surae, $r^2 = (-) 0.098$, $p = 0.03$).

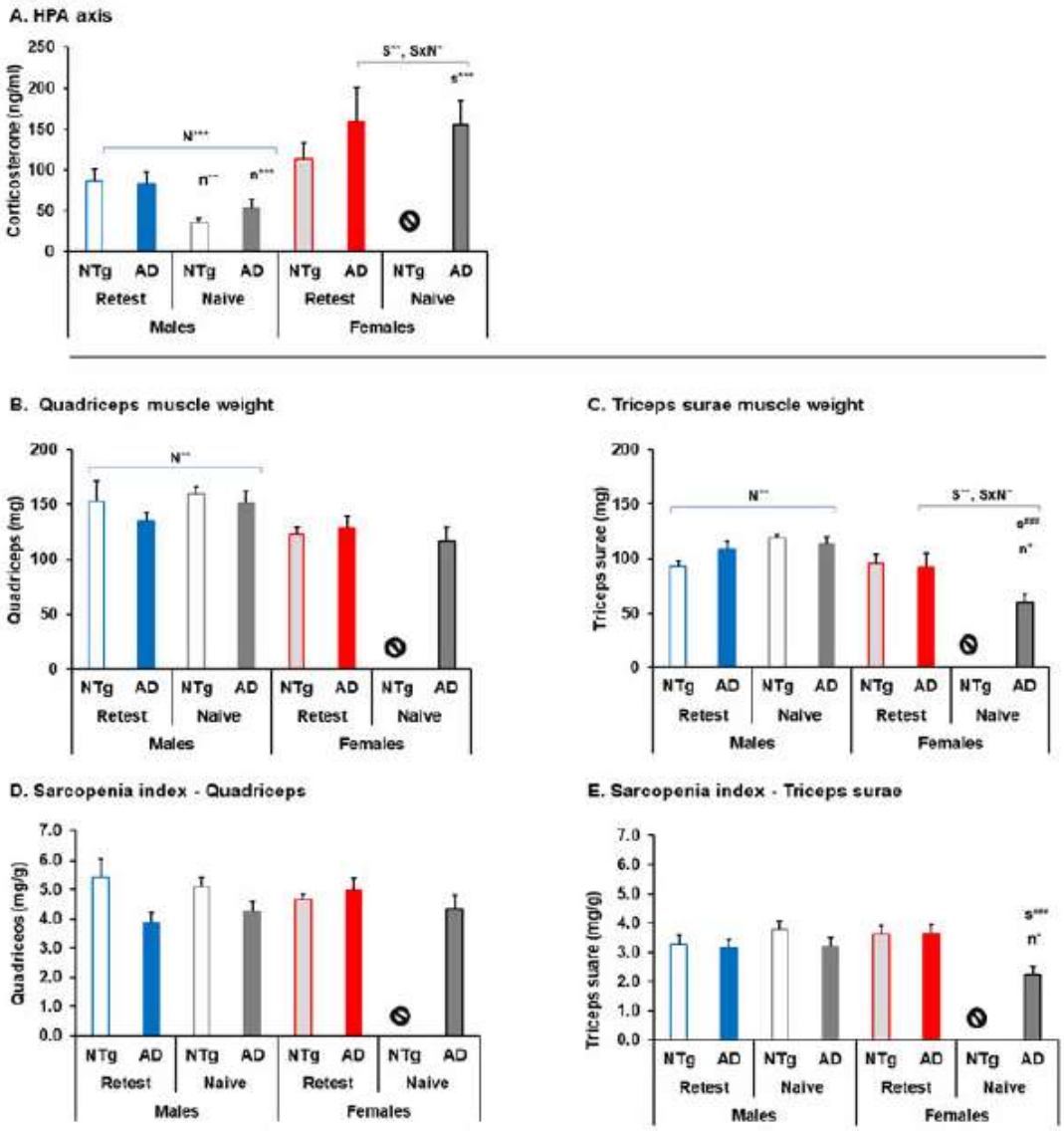

Figure 7. Biological status: HPA axis and sarcopenia index. (**A**) HPA axis, (**B**) quadriceps muscle weight, (**C**) triceps surae muscle, weight, (**D**) sarcopenia index—quadriceps, (**E**) sarcopenia index—triceps surae. Statistics: ANOVA, S, sex effect, S** $p < 0.01$**. N, naïve, naïve 16 months vs. re-test 16 months, N*** $p < 0.001$***, N** $p < 0.01$**. S×N, sex and naïve effects, S×N** $p < 0.01$**, S×N* $p < 0.05$*. Bonferroni *post hoc* test: s, sex; n: naïve, naïve 16 months vs. re-test 16 months; and # expressed sex differences between genotypes, $s^{###}$, $p < 0.001^{###}$. The symbol ⊘ indicates the absence of the group, and m, month.

Phenotype scoring system, $r^2 = 0.182$, $p = 0.002$; frailty score, $r^2 = 0.119$, $p = 0.016$; Cadence, $r^2 = 0.092$, $p = 0.036$; Physical endurance day 1, $r^2 = 0.190$, $p = 0.002$), see Figure 8A–F. In females, a positive correlation between corticosterone with performance in the rotarod on total, the second and third day were detected (physical endurance—total, $r^2 = 0.143$,

p = 0.039; physical endurance Day 2, r^2 = 0.157, p = 0.03, physical endurance Day 3, r^2 = 0.168, p = 0.024), see Figure 8G–I.

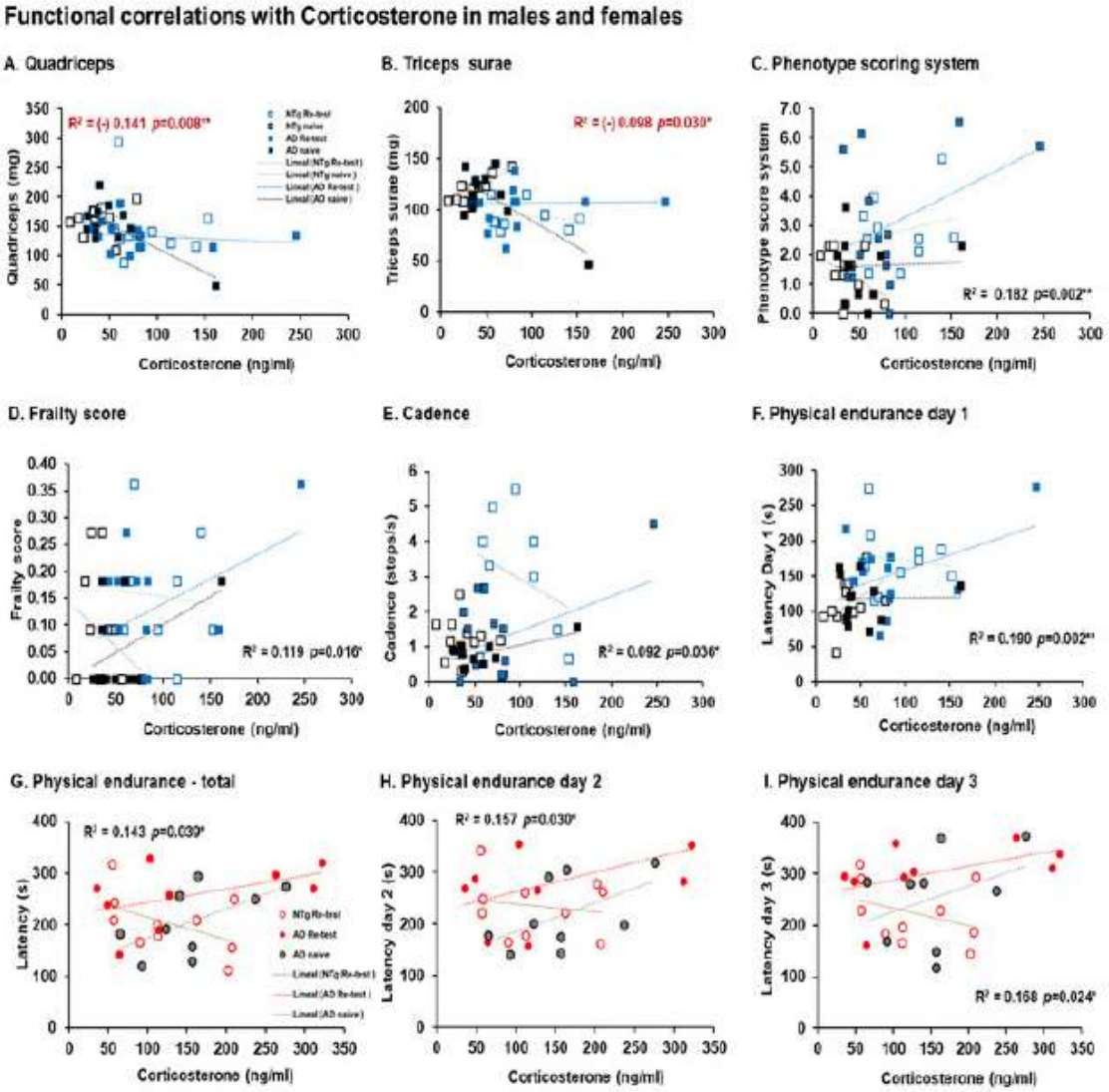

Figure 8. Functional corticosterone correlations in males and females. Pearson's Correlations analysis of corticosterone in males and females. Meaningful, Pearson's correlation in males between corticosterone and (**A**) quadriceps, (**B**) triceps surae, (**C**) phenotype scoring system, (**D**) frailty score, (**E**) cadence, and (**F**) physical endurance Day 1. Meaningful, Pearson's correlation in females between corticosterone and (**G**) physical endurance—total, (**H**) physical endurance Day 2, (**I**) physical endurance Day 3. Statistics: Pearson r^2, $^{**}p < 0.01$, $^{*}p < 0.05$.

In different way, only in male, functional correlations with sarcopenia index were detected. Thus, sarcopenia index-quadriceps correlations with physical endurance Day 1 and Day 2 (sarcopenia index-quadriceps—physical endurance Day 1, r^2 = 0.190, p = 0.002. sarcopenia index–quadriceps—physical endurance Day 2, r^2 = 0.084, p = 0.048). In ad-

dition, sarcopenia index–triceps surae correlation with the number of horizontal explorations (visited corners) (sarcopenia index–triceps surae—corners, $r^2 = (-)0.099$, $p = 0.029$), see Figure 9A–C.

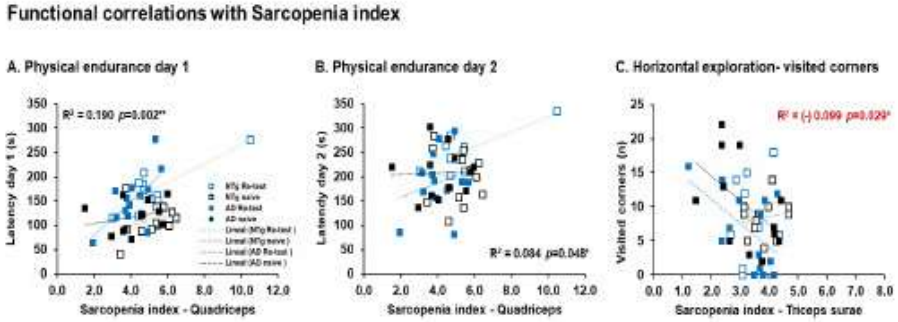

Figure 9. Functional correlations with sarcopenia index. Meaningful, Pearson's correlations analysis of sarcopenia index. Meaningful, Pearson's correlation between sarcopenia index quadriceps and (**A**) physical endurance Day 1, (**B**) physical endurance Day 2. Sarcopenia index, and triceps surae and (**C**) horizontal exploration—visited corners. Statistics: Pearson r^2, $^{**}p < 0.01$, $^{*}p < 0.05$.

On the other hand, males and females had a negative correlation between phenotype score system and functional variables. In the case of males, a negative correlation was detected between stride length and the phenotype scoring system (stride length—phenotype scoring system, $r^2 = (-)\ 0.178$, $p = 0.003$). In females, a negative correlation is observed with physical endurance—total (phenotype scoring system, $r^2 = (-)\ 0.208$, $p = 0.011$), see Figure 10A,B.

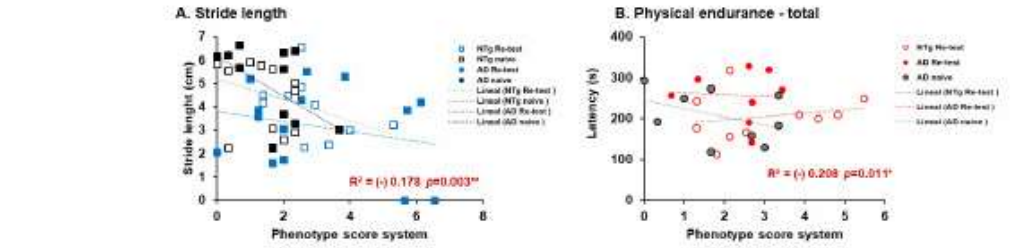

Figure 10. Functional correlations with phenotype score system. Pearson's correlations analysis of phenotype score system. Meaningful, Pearson's correlation between phenotype score system and (**A**) stride length in males, and (**B**) physical endurance—total in females. Statistics: Pearson r^2, $^{**}p < 0.01$, $^{*}p < 0.05$.

There is the summary of results in Table 2.

Table 2. Summary of results.

	Genotype Factor (G)	Sex Factor (S)	Re-Test Factor (R)	Naïve Factor (N)
Phenotype scoring system	⬆ deficits 3xTg-AD group ⬆ deterioration in 3xTg-AD males in the total score			
Frailty	⬆ 3xTg-AD males at 16 m in the re-test			
Kyphosis	⬆ 3xTg-AD males increased the severity in the re-test at 16 m			

Table 2. *Cont.*

	Genotype Factor (G)	Sex Factor (S)	Re-Test Factor (R)	Naïve Factor (N)
Quantitative parameters of gait	Speed: ⬆ 3xTg-AD males at 12 m, and ⬇ in the re-test at 16 m ⬇ 16 m naïve NTg and 3xTg-AD Cadence: ⬇ 3xTg-AD males at 12 m and 16 m Re-test ⬇ 3xTg-AD and NTg Re-test group at 12 and 16 m ⬇ Naïve 16 m NTg and 3xTg-AD males had a lower cadence than age-matched re-tests	Speed: ⬆ Re-test and naïve 16 m 3xTg-AD females Variability of stride length: ⬇ Re-test and naïve 16 m 3xTg-AD females	Stride length: ⬇ Re-test 3xTg-AD males at 12 m Cadence: ⬇ Naïve 16 m males 3xTg-AD and NTg ⬇ 3xTg-AD male in all groups	Stride length: ⬆ Naïve 3xTg-AD and NTg at 16 m
Exploration and neophobia		Exploratory activity (ratio): ⬆ NTg and 3xTg-AD females at 12 m		Freezing: ⬇ Naïve 3xTg-AD and NTg males at 16 m Vertical exploratory activity: ⬆ Naïve 3xTg-AD and NTg males at 16 m Exploratory activity (ratio): ⬆ Naïve 3xTg-AD and NTg males at 16 m ⬆ Naïve 3xTg-AD females at 16 m
Geotaxis		⬆ Naïve 3xTg-AD females at 16 m	⬆ Re-test 3xTg-AD in the re-test group compared to their performance at 12 m.	
Motor learning	⬆ Latency 3xTg-AD males Re-test at 16 m	⬆ Latency and trials females at 12 m in both genotypes. ⬆ Females at 16 m re-test group	⬆ Latency re-test 3xTg-AD males at 16 m ⬆ Latency re-test 3xTg-AD females at 16 m ⬆ N trials among males in re-test group ⬇ N trials among females in re-test group ⬆ NTg re-test males at 16 m	⬇ Latency naïve 3xTg-AD male and females at 16 m
Physical Endurance	⬇ 3xTg-AD males at 12 m and 16 m in the re-test group. ⬇ Day 2, 3xTg-AD males in all groups ⬇ Day 3, 3xTg-AD males at 12 m	⬆ 3xTg-AD females at 12 m and 16 m ⬆ Day 2–3, 3xTg-AD females at 12 m and 16 m	⬆ Re-test at 16 m in all group in 2nd and 3rd training days ⬆ Re-test at 16 m male groups in 1st training day ⬆ Re-test at 16 m female group in 1st and 2nd day	⬆ 16 m naïve 3xTg-AD males than 3xTg-AD Re-test at this age.
HPA axis		⬆ 3xTg-AD re-test at 16 m ⬆ Naïve females at 16 m		⬇ Naïve Re-test males at 16 m ⬆ Naïve females at 16 m
Sarcopenia index		⬇ Triceps surae and sarcopenia index naïve 3xTg-AD females		⬆ Quadriceps and triceps sura muscles naïve males Re-test at 16 m. ⬇ Triceps surae and sarcopenia index naïve 3xTg-AD females re-test females at 16 m
Survival	High mortality, mostly among NTg female mice, rescued in longitudinal designs			
Correlation's interactions	In males, negative correlations between corticosterone and quadriceps, triceps surae; and positive correlations between corticosterone and phenotype score system, frailty score, cadence, and physical endurance Day 1. Females, positives correlated between corticosterone and physical endurance–total, physical endurance Days 2 and 3. Positive correlations in males were detected between sarcopenia index-quadriceps and physical endurance on Days 1 and 2. In females, negative correlations were detected between sarcopenia index–triceps and horizontal activity. Negative correlations in males were identified between phenotype score system and stride length, and in females' phenotype score system and physical endurance—total.			

According to the factors, genotype (G), sex (S), re-test (R) and naïve (N), a summary of the main results of this study is presented. It also includes the correlation's interactions. The symbol ⬆ indicates increase, ⬇ indicates decreases, and m, month.

4. Discussion

Recently, we developed a battery of psychomotor tests that include gait, neophobia and exploration, muscle strength, motor learning, physical resistance, and frailty status [33]. The results, in males, indicated that 3xTg-AD mice exhibit a more significant functional impairment in the quantitative variables of gait and exploratory activity than age-matched NTg counterparts with normal aging. The presence of movement limitations and muscle weakness was determinant for the functional decline related to the stages of severity of the disease that worsened with age. In addition, we detected the presence of signs of physical frailty, which accompany the functional deterioration of these animals. The signs of sarcopenia were present in an advanced stage of AD [31,32]. Therefore, the present study was designed to investigate, for the first time, several aspects: (1) from a gender-medicine perspective, the impact of this functional impairment in 3xTg-AD females as compared to males; (2) the long-term effects of repeated test, either in longitudinal (the same set of animals at 12 and 16 months of age) or transversal (two different sets, pre-tested or naïve, at 16 months of age) designs, both in pathological and normal aging scenarios; (3) to include a phenotype of frailty and physical deterioration that may find a functional correlation with the biological status (HPA axis and sarcopenia), with nuances in male and female animals.

4.1. Survival, Bodyweight, Phenotype Scoring System, Frailty Score, and Kyphosis

4.1.1. Survival

The survival curves on the cohorts of 191 animals allowed us to record higher mortality in females, being the group of NTg females the one that presented the highest number of deaths between 8–12 months of age. Interestingly, only females under the longitudinal design survived and achieved 16 months of age, while the group of naïve NTg females perished before reaching that old age, suggesting that repeated testing might have some protective effects. These results agree with our previous reports in these colonies, where high mortality rates associated with increased frailty were reported in females, and NTg exhibited increased mortality from 12 months of age [42]. In the case of 3xTg-AD mice, females that reached old age were survivors who overcame the disease's advanced neuropathological stages and exhibited lower behavioural differences with their NTg counterparts except for cognitive AD-hallmarks [47]. We have also described that, in male 3xTg-AD mice, an increase of mortality rates is associated with impairment in the neuro-immune-endocrine system compared to their females counterparts or the NTg genotype [48–50]. Noteworthy, we have recently reported survival bias and crosstalk between chronological and behavioral age in an APPswe model, where age- and genotype-sensitivity tests defined behavioral signatures in middle-aged, old, and long-lived mice with normal and AD-associated aging [51]. Therefore, the present work provides further evidence on sex and genotype-dependent differences in life expectancy and supports the key role of frailty and compensatory mechanisms as previously reported by our and other laboratories using different models of AD [29,49–52].

4.1.2. Frailty

In the present work, the frailty results showed genotype differences between males, with NTg being the ones with the highest score. Only 12 of the 30 MCFI parameters were included as the incidence of the other indicators was very low or null. Kane and Brown [29] reported that 3xTg-AD male mice have a higher frailty index (FI) than NTg mice and 3xTg-AD females, and it was associated with their higher mortality ratios. Their study also indicated an increase in the frailty associated with age. On the other hand, in the present work, functional correlations in males found that their corticosterone levels correlated with frailty score and phenotype scoring system, both measures of functional decline. These results could indicate less deficit accumulation or functional capacity at the time of measurement in 3xTg-AD mice [53]. Therefore, it is plausible that other factors contribute to the survival/mortality of animals, and a complex multifactorial scenario be specific for each sex and biological age/stage of disease. In addition, in female C57BL/6 mice, greater frailty

from 17 months of age with higher mortality at 26 months has been recently described in contrast to the non-fragile mice that reached 29 months of life [54]. These data have made it possible to identify that the prevalence of frailty in female mice increases throughout life and accurately predicts mortality [54]. Additionally, the animals' bodyweight presented genotype differences that coincide with previous data [33] but the re-test decreased the weight in males, probably due to the training carried out at 12 months of age.

4.1.3. Kyphosis

On the other hand, the severity of kyphosis was differentiated into postural and structural [31,32]. Here, genotype differences between males have been detected that corroborate previous reports, with greater severity in 3xTg-AD mice [31]. In females, here described for the first time, the severity of kyphosis increased with age and was more significant in the 3xTg-AD mice at 16 months in the re-test group, where the structural type predominates. The differences detected in males corroborate our other recent reports [32].

4.1.4. Phenotype Scoring System

Kyphosis is also one of the scores included in the phenotype scoring system [39,40], which has recently been functionally differentiated by a severity classification that allows more information to be collected in contrast to other variables, such as those associated with gait and exploratory activity [32]. Thus, in the phenotype scoring system, we detected that kyphosis at 12 months of age was more significant in NTg of both sexes, a significance that was not reproduced at 16 months in these animals, which corroborates our differentiation of severity in the presence of kyphosis since the postural condition can be positionally modified. In addition, in the gait score, an increase in functional impairment was detected in 3xTg-AD males and females, which appears in the re-test group at 16 months. This variable makes it possible to discriminate a significant impairment of movement and exploratory activity since bizarre behaviours may occur that interfere with movement [31]. The deficits detected in the quantitative parameters of gait will be discussed in the following section.

4.1.5. Clasping

Finally, the presence of increased clasping in naïve 3xTg-AD mice at 16 months can also be highlighted. It was related to a more significant involved or progression of the disease [55,56]. The present results also suggest that repeated tests exerted protective effects in this respect. Lalonde [55] described brain regions and genes affecting limb-clasping responses. In the C57BL/6 strain, age-dependent locomotor deficits, including hindlimb clasping, are associated with a decreased number of dopaminergic neurons in aged mice, with reduced dopamine levels in the striatum [57]. Interestingly, alterations in the dopaminergic system described in 3xTg-AD mice and other AD models may also explain the presence of increased clasping.

4.2. Quantitative Parameters of Gait, and Neophobia and Exploration

4.2.1. Stride Length

Quantitative parameters in the gait analysis indicated that stride length was shorter in re-tested (16-month-old) male mice compared to age-matched naïve animals, and that this variable correlated with the gait phenotype score system. Interestingly, re-tested 3xTg-AD mice had the shortest stride length among the males compared to the naïve. In addition, differences in genotype and sex were observed at 12 and 16 months in the re-test group with greater stride length at 12 months in 3xTg-AD females and re-test in NTg males. In addition, the stride variability in females was lower than that of males, and the 3xTg-AD in all groups had the best performance, so their movement had more homogeneous steps throughout the trajectory. Previously, in our study in male 3xTg-AD mice of 6, 12, and 16 months of age, no differences in stride length or variability were detected, although a trend to increase stride length with age was observed in the case of 3xTg-AD mice while remained stable in the NTg genotype [31]. However, in another study at 6 months of

age, increased stride length was reported in 3xTg-AD mice with no sex difference [58]. In addition, at 16 months of age, the gait of 3xTg-AD has been described as normal, without differences in genotype and sex [59,60]. According to the results, we propose that using the variability of the stride can help discriminate the trajectory of the movement during the gait analysis similar to humans where recently the variability was identified as a marker of cortical-cognitive dysfunction in AD patients [61,62].

4.2.2. Speed

A significant decrease in speed in the male 3xTg-AD mice in all groups was observed. This decrease may be associated with a progressive functional decline in the 3xTg-AD male mice and coincides with the findings at 13 months of age we have previously reported [33]. Cadence had a lower performance in the 3xTg-AD males at 12 months of age. However, it increased at 16 months in the re-test group, differing from naïve at this age. Thus, cadence and speed are the variables with the highest sensitivity to discriminate genotypic differences in male mice and differentiate changes in gait attributable to pathological aging in the 3xTg-AD genotype. In the case of 3xTg-AD females, speed in-creases slightly in the 16-months re-test group and was higher than in males in all groups. At the clinical level, the identification of early changes in gait is of great relevance for identifying psychomotor disorders that in the case of AD may be related to the timing of steps and gait speed [63]. Additionally, corticosterone levels were positively correlated with a cadence in males.

4.2.3. Neophobia and Exploration

The neophobia response, expressed as freezing, of 12 and 16-month-old naïve male mice was lower than in re-test mice in both genotypes, and statistically significant when contrasted with 16-month-old naïve mice. In females at 16 months of age, re-tested and naïve, a higher freezing was observed than in 16-month-old naïve females, albeit did not reach the statistical significance.

This neophobia emotional response is a characteristic of the 3xTg-AD model that is ac-companied by reduced immediate exploratory behaviour in a novel environment, as we first described in these animals in the open field test and the corner test already at the early 'premorbid' age of 2.5 months and worsened with the progress of the disease [64]. In addition, it corresponds to more sensitive ethological behaviours of the 3xTg-AD phenotype that has been reported in several other studies [31,33,42]. In addition, the horizontal exploratory activity did not report statistically significant differences.

However, in the vertical exploratory activity (number and latency of rearings), differences between the re-test male mice at 16 months and the naïve of the same age were more statistically significant than the activity in naïve mice. In addition, the ratio (visited corners/rearings) in the re-test male mice of 12 and 16 months increased in the re-test but differed from the females at both ages, being lower in males at 12 months in both genotypes. At 16 months in re-test, NTg male's ratio was high than NTg females, and in 3xTg-AD case, the ratio was increased in females 3xTg-AD. This decrease in activity over time, which is also observed in NTg mice, has been previously described as due to normal aging [64], with 3xTg-AD mice exhibiting less activity in most cases, which is attributed as a pathological trigger similar to BPSD that appear later in NTg mice due to normal aging [64]. In addition, in males was observed that correlated horizontal activity with triceps sural weight.

4.3. Muscular Strength: Forelimb Grip Strength and Muscular Endurance—Hanger Test and Response to Gravity—Geotaxis

4.3.1. Muscular Strength

Muscular strength is associated with global cognitive function in older people [65]. In addition, skeletal muscle mass index and physical performance (timed up and go test and grip strength) have decreased in older adults with AD [66]. Our results have not detected significant differences, although, at 12 months, it seems that females have a superior performance in grip strength and muscular endurance. Previously, we have reported that

13-month-old 3xTg-AD mice in natural isolation have preserved muscular strength [33] and that muscle strength and endurance would be associated with aging [31]. The laboratory of Brown also reported that at 6 months, 3xTg-AD mice have a deficit in grip strength [58], but at 16 months these results are not reproduced [59]. Additionally, the reduction in muscle weight and the appearance of sarcopenia may not yet be evident in the loss of muscle strength and resistance, or aging in this variable has greater importance than the distinction of the effects of the pathology in humans [67–69].

4.3.2. Geotaxis

On the other hand, geotaxis showed differences between the males, with the 3xTg-AD re-test at 16 months being the ones that obtained a worse performance and the 3xTg-AD naïve females at 16 months. In addition, females take longer to pass the test, which is reflected in the differences in GxS in the 16-month-old re-test and naïve group. The usefulness of this test has been previously described [70]. Specifically, the geotaxis has allowed us to differentiate the animals' postural positioning and balance strategies to pass the test and thus detect a possible functional deficit [31,33]. Therefore, 3xTg-AD re-test males and naïve females at 16 months show the most significant deterioration in this task.

4.4. Motor Performance: Learning and Physical Endurance—Rotarod

The motor performance showed superior performance in females of both genotypes. The motor learning tests and the number of trials reached the maximum values of the test in the re-test at 16 months. The increased performance may be due to pretraining done at 12 months, which can produce cognitive improvements with a long-term wheel of activity. In 16-month-old naïve 3xTg-AD males and females, lower latency and high number of trials were observed to achieve motor learning. Male 3xTg-AD mice have the most inferior performance in all tests.

As in motor learning, females have a high physical endurance. The 3xTg-AD females in the re-test group at 16 months achieved the highest performance over the male 3xTg-AD naïve and re-test, and female 3xTg-AD naïve 16-month-old females, and with similar performance to the NTg males of the same age. In addition, all groups increased their performance with training from Day 1 to Day 3, which is evident to a greater extent on the third day of training, and the effect of the re-test is observed at 16 months with an effect on different days for males and females, being in males on the first day of training and in females on the first and second day of training. Additionally, it was possible to distinguish the effect of aging in the naïve male NTg in contrast to the naïve at 12 months and re-test at 16 months. In addition, among the 3xTg-AD group, the sex differences between the 16-month-old re-test mice are distinguished from Day 1 to Day 3 of training. The 3xTg-AD males present the lowest performance among all groups, although with the training in the first day increased de physical endurance at 16 months in re-test group, on the following days, their performance is below 3xTg-AD naïve for 16 months.

The motor performance of 3xTg-AD mice has been reported in different studies. The performance in coordination and motor learning of 3xTg-AD mice has been highlighted over the performance of NTg mice, and these results are observable from 6 months and are reproduced at 16 months [58–60]. It has even been mentioned that 3xTg-AD females perform better than males at these ages [58,59]. In our laboratory, only reproduced the results of Stover et al. and Garvock-de Montbrun et. al. at 13 months, where the 3xTg-AD male mice presented a higher performance than the NTg, but in the latter, the weight factor interfered in the results [33]. Decreased motor function is also associated with aging, as reported in C57BL/6 mice of different ages [71–73]. In addition, we have differentiated the conceptualization of motor performance into motor learning—latency and motor learning—trials learning, since after physical exercise, the animals must manage to stay on a moving wheel in a coordinated manner. Consequently, in the first trials, physical endurance has a workload associated with an anaerobic exercise that progresses to aerobic exercise as the trials and their respective recovery times are replicated. In humans, the decrease in

endurance exercise performance and its physiological determinants with aging appear to be mediated mainly by a reduction in the intensity (speed) and volume of exercise performed during training sessions [74]. Under this hypothesis, in their study, Pena et al. reported that 3xTg-AD mice improve their maximum latency in rotarod when subjected to aerobic exercise [75].

These results are accompanied by correlations with corticosterone levels and behave differently between males and females. In the case of males, corticosterone correlates positively with physical endurance on the first day of training, and in the case of females, it correlates positively with total physical endurance and physical endurance on the second and third days. On the other hand, a positive correlation was also detected in males between index-quadriceps sarcopenia and rotarod performance on the first and second days. A negative correlation was also detected between total physical endurance and the phenotype score system in females. Therefore, these interactions could explain the differences in performance between the groups studied.

4.5. Biological Status: HPA Axis and Sarcopenia Index

4.5.1. Corticosterone

Corticosterone levels differed between groups due to sex and re-test factors, but not genotype. Males exhibited lower corticosterone levels in naïve mice of both genotypes, with similar levels between 3xTg-AD and NTg in the re-test group. On the contrary, in females, higher plasma corticosterone levels were observed in the 3xTg-AD re-test, and naïve females had similar levels that exceed the NTg re-test. It is also possible to distinguish that naïve 3xTg-AD females had higher levels than their male counterparts. The results agree with the sexual dimorphism reported by Muntsant et al., with higher plasma corticosterone levels in females [42], and also with plasma levels similar to the intervals described by Giménez-Llort et al. [76]. Additionally, corticosterone levels showed functional correlations with different variables depending on sex. In males, the correlation was inversely associated with the muscle mass of the quadriceps and triceps surae, and positively with frailty and gait cadence indicators. On the other hand, higher corticosterone levels correlated with higher performance on the first day of training in physical endurance. In females, the correlation with corticosterone was related to physical endurance performance with greater significance on the second and third training days. These results could indicate chronic stress if there is a long-term activation of the HPA axis in the case of females [77]. A report suggested that the combination of emotional and physical stress in a period of 5 h of exposure severely affected memory in NTg mice and increased the alterations in 3xTg-AD mice as a consequence of the reduction in the number dendritic spines and increase in the Aβ levels [50]. Additionally, the elevated corticosterone may precede cognitive impairments in genetically vulnerable 3xTg-AD females [78,79] and may, in turn, be related to frailty [80].

4.5.2. Sarcopenia

Furthermore, we have observed that the quadriceps and triceps surae muscles have a greater weight in naïve male mice, whereas in 3xTg-AD females, a lower weight is observed in the triceps surae muscle with significant differences with the group of 3xTg-AD females and re-test and males of this genotype. These differences in naïve 3xTg-AD females are also observed in the sarcopenia index of the triceps surae muscle. In humans, sarcopenia is closely related to dementia, particularly AD, and may be involved in the pathophysiological process of AD [68,81]. On the other hand, poor muscle function but not reduced lean muscle mass drives the association of sarcopenia with cognitive decline in old age [67,82]. Sarcopenia, low grip strength, and slow walking speed were significantly associated with mild cognitive impairment in the community-dwelling elderly [80,83]. Therefore, our results can be helpful to study what occurs in human pathology through a translational approach to motor dysfunction at different levels of disability [31].

Moreover, in the case of males, the weight of the quadriceps and triceps surae muscles negatively correlated with plasma corticosterone levels. A positive correlation of the quadriceps sarcopenia index with physical endurance Days 1 and 2 was also found. In the case of the sural triceps sarcopenia index, it correlated negatively with the number of corners visited in the exploratory activity. These correlations were not found in females.

Finally, the study's limitations were given by the high mortality rate of NTg females that resulted in the lack of 16-month-old naïve group. Therefore, the genotype differences between 3xTg-AD and NTg females could not be contrasted. However, the analyses were carried out to detect the sex differences between the 3xTg-AD group. In future research, it would be interesting to compare the results of this study with NTg females since their functional profile may differ from males in physical or biological variables, such as in 3xTg-AD females.

5. Conclusions

From the results, it is possible to highlight that the high mortality rate in females, and among them that in the NTg group, was prevented in the group of females behaviorally assessed at 12 months of age, and these females were able to reach the age of 16 months completing the longitudinal design. In addition, higher corticosterone levels were detected in females and lower muscle weight of the triceps surae, which could indicate sarcopenia and alteration of the HPAaxis, which was more significant in the naive group at 16 months. Additionally, there were genotype-sensitive variables such as the phenotype scoring system, frailty and kyphosis in which the group of 3xTg-AD males showed physical deterioration. In turn, the motor learning and physical endurance variables were sensitive to re-testing, with 3xTg-AD females achieving the best performance when repeating the behavioral battery at 16 months. In addition, the females exhibited a better performance in gait, where their stride was homogeneous and straight. Additionally, females exhibited less severe scores in physical variables, such as kyphosis, which could explain males' more significant deterioration in some motor tests. On the other hand, males showed deterioration in most of the variables studied. For their part, the correlations could explain the differences obtained between males and females, being positive in females between corticosterone and physical endurance, and the case of males between sarcopenia index and physical endurance as well as corticosterone with physical variables. The present results highlight the complexity of experimental scenarios in neurodegenerative diseases, such as Alzheimer's disease, confirming not only the different impact of factors depending on genotype, sex, and age but their interplay with the methodological approach. They provide evidence that genotype, sex and age-dependent impact of behavioral assessment, as well as the repetition of behavioral tests, should not be underestimated. Conversely, and most importantly, the ability of behavioral assessment and repeated tests to modify the behavioral outputs indicates that they could be considered functional trainings that modify survival, anxiety, and functional profile (physical endurance and motor learning) of old male and female 3xTg-AD mice and also NTg mice counterparts with normal aging.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10050973/s1, Table S1: Physical performance in males and females Naïve 12-month-old 3xTg-AD and NTg mice; Table S2: Physical performance in males and female 3xTg-AD and NTg after Re-test to 16m; Table S3: Physical performance in males and females Naïve 16-month-old 3xTg-AD and NTg mice; Table S4: Statistics Figure 5C.

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Conclusion

1) High mortality, mainly among female NTg mice, rescued in longitudinal designs.

The genotype factor was sensitive in:

1) Phenotype scoring system detected kyphosis, gait and clasping deficits, which were more significant in the 3xTg-AD group. 3xTg-AD males showed significant deterioration in the total score on phenotype scoring system.
2) Frailty score increased in 3xTg-AD males at 16m at retest.
3) Kyphosis − 3xTg-AD males increased severity of kyphosis at retest at 16m.
4) Gait speed − 3xTg-AD males at 12m had lower speed than NTg and decreased at the 16m retest. In addition, 16m naïve NTg and 3xTg-AD performed worse than at the 16m retests.
5) Cadence − 3xTg-AD males had a lower cadence than NTg males at 12m and 16m retests. In the retest group, lower cadence was also detected in females at 12m and 16m. In addition, naïve NTg and 3xTg-AD males at 16m had lower cadence than age-matched retests.
6) Motor learning (latency) at 16m in the male retest group − 3xTg-AD males achieved high performance compared to the age-matched NTg.
7) Physical endurance − 3xTg-AD males had the lowest performance at 12m and 16m in the retest group. On the second training day, the differences between males increased, with the 3xTg-AD males underperforming. In addition, the 3xTg-AD males performed the poorest in each test during the 3 days of training, and among them, the 12m males performed the worst.

The sex factor was sensitive in:

1) Gait variability − 16m 3xTg-AD females in the retest and the naïve groups had the lowest gait variability. In addition, in all groups, 3xTg-AD females had the best performance.
2) Gait speed − 16m 3xTg-AD females in the retest and the naïve groups had a higher gait speed than 3xTg-AD males.
3) Exploratory activity (ratio) − NTg and 3xTg-AD females at 12m had more significant exploratory activity.
4) Geotaxis - Naïve 3xTg-AD females at 16m took longer to complete the test than naïve 3xTg-AD males at 16m.
5) Motor learning (latency) and motor learning tests learning −12m females learned earlier and achieved higher latency than males of both genotypes. In the 16m retest group, females achieved the highest test performance in both genotypes.
6) Physical endurance − 3xTg-AD females at 12m and 16m in the retest group had higher latencies than 3xTg-AD males at the same ages. Physical endurance differed between 3xTg-ADs on the second and third day of training, where retest females at 12m and 16m outperformed 3xTg-AD males of the same age. In most tests, it was possible to differentiate 16m 3xTg-AD retest females from age-matched 3xTg-AD males.

7) Corticosterone levels – They were higher between 3xTg-AD retest and naïve females at 16m.
8) Naïve 3xTg-AD females have a lower triceps surae muscle weight than males, which corresponds with the findings on index-triceps surae sarcopenia.

The retesting factor (16m vs. 12m) was sensitive in:

1) Stride length – The 3xTg-AD males at 16m in the retest group had a shorter stride length compared to their stride length at 12m.
2) Cadence among males − 16m naïve males had a slower cadence than those retested of the same age in both genotypes.
3) Geotaxis − Time to complete the test increased at 16m in 3xTg-AD males in the retest group compared to their performance at 12m.
4) Motor learning (latency) − 3xTg-AD males increased their latency at 16m in the retest group compared to their performance at 12m. In addition, 3xTg-AD males at 16m in the retest group had a higher latency than naïve 3xTg-ADs of the same age. Furthermore, 3xTg-AD females increased their performance at 16m in the retest group compared to 12m.
5) Motor learning test learning among males − Retesting decreased the number of trials to pass the test in both genotypes. In females, 3xTg-AD at 16m in the retest group reduced the number of trials to pass the test compared to their performance at 12m.
6) Physical endurance − NTg males retested at 16m had a greater latency than naïve males of the same age, and the greater latency was reproduced in 3xTg-AD females at the same age. In all groups, the first and third day of training differentiated retests from naïve at 16m. On the first-day testing of males, it was possible to distinguish the retested from the naïve at 12m and 16m. In females, it was possible to distinguish the trained from the naïve at 12m and 16m on the first and second day.

The naïve factor (16m naïve vs. 16m retested) was sensitive in:

1) Stride length − Among males, the 16m naïve group had a longer stride length than the retest group in both genotypes.
2) Freezing − 16m naïve males had lower freezing in both genotypes.
3) Vertical exploratory activity − Naïve 16m males had higher vertical activity than retested males at the same age in both genotypes.
4) Exploratory activity (ratio) − Naïve 16m males had more significant exploratory activity than retested males of the same age in both genotypes. Furthermore, this was replicated among naïve 3xTg-AD females.
5) Motor learning − 16m naïve 3xTg-AD males and females underperformed the retest group in both sexes.
6) Physical endurance − 16m naïve 3xTg-AD males had greater physical endurance than 3xTg-AD retested males at this age. The opposite is true for 3xTg-AD females, with 16m retest group performing better.
7) Corticosterone levels − Among naïve males at 16m were lower than retested males.

Quadriceps and triceps surae muscle weight – They were higher among naïve males compared to retested males. In addition, naïve 3xTg-AD females had lower muscle weights than 3xTg-AD retested females, consistent with findings on sarcopenia of the index-triceps surae.

CHAPTER 5. DISCUSSION

PHASE 1: CHARACTERISATION OF PRIMARY AND SECONDARY MOTOR SIGNATURES OF PSYCHOMOTOR DYSFUNCTION IN NORMAL AGEING AND ALZHEIMER'S DISEASE

The 3xTg-AD mouse model shows early cognitive impairment (Oddo, et al. 2003a; Oddo, et al. 2003b). Specifically, associative learning deficits are evident between 3 and 5 months of age and problems in spatial working memory are recognisable from 6 months onwards in the Morris water maze paradigm (Webster et al., 2014). Furthermore, in this paradigm, between 9 and 11 months, deficits in recognition memory are evident (Roda et al., 2020; Webster et al., 2014).

On the other hand, alterations in exploratory activity are first detected in the open field test (OF) at 2.5 months of age, and at later ages in the corner test (CT), with less habituation to novelty and disinhibition at 6 months, and neophobia at 12 months (Roda et al. 2020). While most behavioural tests show an increase in emotionality in adulthood and old age (Giménez-Llort et al. 2007). Likewise, BPSDs are evident from 5 months of age, manifesting as neophobia and anxious behaviour (Roda et al. 2020; Baeta-Corral and Giménez-Llort 2018).

Therefore, in phase 1, results have been reported for male 3xTg-AD mice of different disease stages compared to non-transgenic mice at similar ages that account for primary and secondary signatures of motor dysfunction in both normal ageing and AD. The first study demonstrated that BPSD behaviour in the early stages of the disease could be evidenced through the study of floating behaviour and bizarre circling that would accompany cognitive impairment traditionally reported through MWM. In addition, the second study modulated and integrated of primary and secondary motor signatures of AD in interaction with extrinsic factors was developed in the 3xTg-AD model. Males aged 6, 12, 13 and 16 months and females aged 12 and 16 months were included. Also, the third study found that motor dysfunction in 3xTg-AD mice is associated with poorer performance at older ages and is accompanied by coordination deficits as measured by the Phenotype Scoring System.

Finally, the fourth study identified frailty and physical and behavioural characteristics of 16-month-old male mice of the standard golden strain C57BL/6J at the end-point status. It hypothesised that, despite presenting positive criteria for euthanasia, aged animals could maintain their functional performance and body weight regardless of organometric system impairment or loss of muscle mass. In addition, a review article on the different models of frailty with translation to human study models was included.

Indices for flotation and circling, two non-search behaviours in the water maze, sensitive to d-galactose–induced accelerated ageing and Alzheimer's disease

The study of BPSD has been neglected in most experimental research on AD, classically focused on cognitive symptoms. The aquatic environment of MWM involves a stressful condition for mice that leads to cognitive performances with the presence of other emotionally related behaviours.

The aim of this work was to provide a quantitative (number of episodes and duration) and qualitative (prevalence) analysis of floating and circling; the most common “non-search behaviours” elicited in MWM. The expression of these behaviours was studied in 6-month-old wild-type gold-standard C57BL/6 mice and 3xTg-AD mice and when both genotypes were subjected to D-galactose-induced chronic accelerated ageing. The age of 6 months was chosen because, in the 3xTg-AD model, it corresponds to the onset of the disease, detecting only intraneuronal βA oligomers in the hippocampus and basolateral amygdala underlying their cognitive deficits and anxious profile (España et al., 2010). Furthermore, this adult age would help us to analyse these behaviours without age-related confounding factors, while providing a stable adult scenario to observe the impact of accelerated induced ageing.

Elicitation of floating and circling was recorded during three standard MWM paradigms: visual perceptual learning, place task for spatial reference memory and a final probe trial for short-term memory. The results show that the rate of “flotation”, characteristic of non-transgenic performance, is sensitive (reduced) to accelerated ageing and AD. Floating may be due to lack of motivation or behavioural despair, but the results indicate that young non-transgenic mice show the most positive behaviour, and that accelerated ageing reduces floating. In addition, some mice are able to float because they can orient themselves effortlessly. It is not yet clear whether a high rate of floating is beneficial or detrimental in the context of cognitive performance. In contrast, circling behaviour, characteristic of 3xTg-AD mice, may be an additional tool to assess BPSD-like symptoms in AD models, while its rate unveils bizarre behaviour induced by D-galactose-induced ageing.

These results may be useful for preventive and therapeutic interventions targeting AD, but may also be suitable for assessing possible risk factors in normal ageing animals.

Modelling Functional Limitations, Gait Impairments, and Muscle Pathology in Alzheimer’s Disease: Studies in 3xTg-AD Mice

This work translated the components of the conceptual framework of the International Classification of Functioning, Disability and Health (ICF) used in human rehabilitation and their functional equivalence in 3xTg-AD mice. Thus, this study presented the gait impairments and functional limitations in the exploratory activity of the 3xTg-AD mouse model and compared it to C57BL/6 with normal ageing.

Specifically, in walking, as in humans (Coelho et al. 2012; Chiaramonte and Cioni 2021), speed was the variable with the highest sensitivity for detecting deficiencies in movement and locomotion. In addition, bizarre gait patterns also alter speed, accompanied by stride length and cadence. Likewise, ageing reported in C57BL/6 mice modifies foot strike patterns with a decrease in cadence and stride time (Tarantini et al., 2019). On the other hand, in novelty situations, 3xTg-AD mice respond with neophobia and anxiety behaviours (España et al., 2010; Giménez-Llort et al., 2007). Neophobia modifies exploratory activity with increasing age, accentuating symptoms (Muntsant and Giménez-Llort, 2020). However, we have described a relationship between unusual gait patterns and the horizontal and vertical components of exploratory activity. Thus, the bizarre gait patterns that limit locomotion in

3xTg-AD mice do the same in NTg mice, increasing with age. In 3xTg-AD mice, these behaviours are mainly related to psychiatric and neurological disorders (Baeta-Corral and Giménez-Llort, 2014; Cordón-Barris et al., 2016; Giménez-Llort et al., 2002).

In addition, overall mobility was interfered with by periods of freezing. The 12-month-old mice can be distinguished as they present several freezing episodes in both genotypes, in contrast to the 6-month-old mice, where the 3xTg-AD group presented prolonged freezing behaviour, taking longer to perform the first movement before starting to walk, which may also influence the decrease in exploration and quantitative gait parameters, similar to what occurs in social isolation scenarios (Castillo-Mariqueo and Giménez-Llort, 2021a).

At the same time, the results showed that both 3xTg-AD and C57BL/6 mice show a higher incidence of structural kyphosis from 12 months of age onwards, which could explain, from a postural point of view, the decrease in exploration in both groups with increasing age. Furthermore, by indirect measurement of sarcopenia, the quadriceps muscle was found to be associated with sarcopenia in the normal-aged mice, whereas in the transgenic group, sarcopenia appears at 16 months of age. Interestingly, the triceps surae muscle also indicated sarcopenia in the 16-month-old 3xTg-AD mice. Morphological differences were found between the groups, where the fibres are homogeneously distributed, with differences between them, but maintain a similar distribution. However, a difference in the number of nuclei was observed in the NTg control animals, which seems to be higher than in the 3xTg-AD, especially at 12 months. The presence of adipose cells was also detected. Thus, adipose cells were present to a lesser extent in the quadriceps, irrespective of genotype and age, with intramuscular predominance. In contrast, adipose cells showed a peripheral or intramuscular location depending on genotype and age in the triceps. Thus, in the NTg control group, adipose cells were found in more peripheral areas, with a more significant proportion at 16 months. On the other hand, in the 3xTg-AD group, adipose cells were more intramuscular, with a higher proportion at 12 months. Interestingly, NTg control mice had similar weights at each age, while the weight of 3xTg-AD mice increased with age.

In this way, the functional limitations detected are equivalent to the difficulties that older adults usually face in carrying out activities of daily living and that we can consider as markers of deterioration of functional health. Furthermore, the use of the ICF as a conceptual framework allows functional status to be described, facilitating its interpretation and application in the rehabilitation of people with AD.

Clasping and ledge-score coordination impairment as primary behavioural markers of functional impairment in Alzheimer's disease (status: accepted)

This work has characterised the motor dysfunction of the 3xTg-AD mouse model in different motor tasks, focusing on the abnormal clasping reflex and coordination impairments measured through the Phenotype Scoring System, which includes four screening items originally developed for ataxia models (Ditzler et al., 2003; Guyenet et al., 2010). Male 3xTgAD mice (n=24) at 6, 12, and 16 months of age (mimicking early, advanced, and late disease stages, respectively) and their age-matched non-

transgenic counterparts (NTg, n=21) with normal ageing were included. Differences in this score were found with the incidence or severity of the four items and the total score. The 3xTg-AD mice showed impairments in all items of the score. Clasping increased independently of age, and its severity worsened with repeated testing.

In contrast, coordination impairment worsened with disease progression. The gait score was sensitive to genotype, and the worse ledge score was evident at 16 months. The kyphosis and ledge scores were age-sensitive. The impairments and functional limitations of male 3xTg-AD mice related to Alzheimer's disease stages provide a scenario to understand the heterogeneity of non-cognitive symptoms.

Currently, our research group has developed a new study method that allows us to observe the psychomotor performance of the 3xTg-AD experimental model at different stages of Alzheimer's disease progression (Castillo-Mariqueo and Giménez-Llort, 2021). We have also reported in this model an increase in bizarre (disruptive) behaviours that appear from 6 months of age compared to wild-type C57BL/6J (NTg) when confronted with new environments (Baeta-Corral and Giménez-Llort, 2015; Castillo-Mariqueo and Giménez-Llort, 2019; Giménez-Llort et al., 2007).

Frailty, from Humans to Mouse Models

Currently, the most widely used mouse models in research are based on the biological hypothesis of frailty syndrome in humans. Much of this research addresses limitations due to the heterogeneity of the syndrome and its manifestations, as they involve several organs and body systems, making it multidimensional and challenging to study in humans (Kane et al., 2016; Zglinicki et al., 2016). There are different models of study, ranging from those that address biological protestors due to ageing, others address the cumulative effects of lifestyle deficits and consequences, seeking to quantify manifestations and deterioration. Still others seek to recreate preclinical signs to improve rehabilitation strategies and timely treatments by recreating genetic models that recreate the aetiology of frailty (Howlett, 2015; Kane et al., 2016; Seldeen et al., 2015; Zglinicki et al., 2016). Thus, animal studies provide opportunities to help us understand the mechanisms that trigger frailty. They also provide empirical evidence on pathophysiological pathways and mechanisms and identify potential biomarkers to generate interventions and treatments to modulate or counteract the syndrome.

PHASE 2: MODULATION AND INTEGRATION OF PRIMARY AND SECONDARY PSYCHOMOTOR SIGNATURES OF ALZHEIMER'S DISEASE DYSFUNCTION AND EXTRINSIC FACTORS

There are now several studies that report on the main cognitive and emotional deficits in the different stages of AD, including findings in the motor and psychomotor domains in humans. Stiffness, slowness, gait impairment, and other movement disorders accompany AD at different stages of the disease (Kurlan, 2000). There is an important need to accurately characterise movement disorders in AD studies to clarify the clinical phenomenology and neurobiology of the disease and to accurately distinguish AD from other degenerative dementias (Scarmeas et al. 2004; Albers et al. 2015; Kurlan 2000).

In this phase, a naturalistic model of isolation, normal and pathological ageing and behavioural battery learning and retesting were included in a longitudinal design with within-subjects analysis and a cross-sectional design contrasting the between-subjects retest. Thus, dysfunctions associated with gait, body posture, frailty, neophobia, and exploratory activity present in normal and pathological ageing related to the stages of AD progression were detected. In addition, we demonstrated that postural alterations, such as kyphosis and bizarre gait patterns, are associated with functional limitations in gait and exploratory activity in male 3xTg-AD mice at 16 months of age, preventing delays in the performance of both activities.

The results obtained in these studies highlight the complexity of experimental scenarios in neurodegenerative diseases, confirming the impact of genotypic factors, sex and age, as well as the repetition of behavioural tests on the results of psychomotor variables, currently little explored, which should be considered in future research.

Translational Modelling of Psychomotor Function in normal and AD-Pathological ageing with special concern on the effects of social Isolation

The aim of this work was to explore the psychomotor performance of 13-month-old male 3xTg-AD mice, corresponding to an advanced stage of AD. The impact of isolation was assessed in a naturalistic model of mice that lost their siblings and were left alone in their housing boxes after ten months of social life. AD survivors who remained isolated for 2-3 months. A psychomotor battery was applied including: spontaneous gait, muscle strength, rotarod motor performance, and physical phenotype of frailty.

The results indicated paradoxical genotypic differences in terms of better performance on motor variables, with more significant physical performance in 3xTg-AD animals, regardless of social isolation and late onset of motor impairment related to physical endurance and exercise tolerance of the 3xTg-AD mice. However, in variables involving information processing and decision-making to perform a task (exploration and gait), these animals showed poor performance, including circling as a bizarre behaviour. Thus, the motor performance achieved by the isolated 3xTg-AD animals is even superior to

that of the grouped 3xTg-AD animals. In these animals, exercise during the 6 rotarod tests studied showed an increase in physical endurance as the test progressed. This variable may indicate that the basal physical condition of these animals is optimal and that the effect of physical exercise improves their performance. We can highlight that although the results of the rotarod indicate that the mice improved their motor performance, they performed worse in other tasks related to cognitive and affective variables, in accordance with the characteristics of the disease.

On the other hand, spontaneous gait performance and exploratory activity reported freezing episodes at the beginning of the tests, which may be associated with more significant functional limitations in the 3xTg-AD mice group and in the isolated mice. In contrast, the physical parameters: strength and physical endurance in rotarod, are not altered, showing coincidence with hyperactivity or anxiety, one of the manifestations of the advanced stages of AD. In addition, these animals showed higher performance in muscle strength, where genotype seems to be a determining factor in overall performance. Furthermore, it is possible to highlight the factor of genotype and physical activity level as a protective mechanism, although phenotypic indicators of physical frailty are present. While 3xTg-AD mice showed a more significant impairment in physical aspects, their motor learning ability remained preserved.

These findings generate new hypotheses for studying the underlying biological mechanisms useful in translational geriatric rehabilitation scenarios.

Kyphosis and bizarre patterns impair spontaneous gait performance in end-of-life mice with Alzheimer's disease pathology while gait is preserved in normal ageing

In this report, kyphosis and bizarre gait patterns associated with functional limitations of gait and exploratory activity in male 3xTg-AD and NTg mice at 16 months were studied; the presence of bizarre behaviours in dry first-time trials in old age has been confirmed. It is also corroborated that in novelty and place recognition situations, old 3xTg-AD mice exhibit bizarre behaviours. Most importantly, they interfere with their locomotion and spontaneous exploration and include kyphosis, an indicator of frailty with a high incidence in 3xTg-AD mice and non-transgenic mice. In addition, piloerection is the primary marker of macroscopic examination indicating severity in the in-of-life setting at 16 months in 3xTg-AD and NTg mice.

Previously, it has been reported that females have a higher incidence of these behaviours with sex differences (Giménez-Llort et al. 2007; Baeta-Corral and Giménez-Llort 2014). Also, at 13 months in males, gait impairments have been found to coincide with an ageing pattern, accompanied by a series of bizarre behaviours that may interfere with trajectory and movement (Castillo-Mariqueo et al., 2021; Lang et al., 2003). Furthermore, a correlation was observed between the presences of these so-called “bizarre gait patterns” and a decrease in exploration with a delay in the first episode of vertical exploration, with 3xTg-AD mice being the most affected by inactivity. Horizontal exploration and movement latency suggest that they persist as the disease progresses once these behavioural patterns

appear. We have also identified compromised functional performance when kyphosis and bizarre gait patterns are present, pointing to a more significant restriction of daily living functions, which translationally could be a cause of dependence in humans.

Impact of behavioural assessment and re-test as functional trainings that modifying survival, anxiety and functional profile (physical endurance and motor learning) of old male and female 3xTg-AD mice and NTg mice with normal ageing

This study was designed to investigate the effects of retesting on the behavioural performance of non-transgenic and 3xTg-AD male and female animals tested in two scenarios: 1) in a longitudinal design, with within-subject analysis of a set of 12-month-old animals tested repeatedly four months later, at 16 months of age; and 2) in a cross-sectional design, comparing 16-month-old animals that had undergone (retesting) or not (naïve) the test battery. The impact of two factors, sex and retesting, was contrasted. In line with our previous work, a psychomotor test battery was used: gait, exploration, muscle strength, motor learning, physical endurance and frailty status. In addition, frailty phenotype and biological status (HPA axis and sarcopenia index) were included.

The results confirmed the impact of the longitudinal study with a training intervention through a behavioural battery at 12 months and its repetition, increasing the survival of 3xTg-AD and non-transgenic females at 16 months. In addition, gait variability improved in 3xTg-AD females at 16 months, and physical endurance in 3xTg-AD females exceeded that of the other groups, also increasing their motor learning with the non-transgenic male group, as well as geotaxis in 3xTg-AD males. In contrast, anxiety increased in 3xTg-AD males retested at 16 months.

On the other hand, a cross-sectional analysis contrasting the trained groups with a 16-month-naïve group showed higher corticosterone levels in the 3xTg-AD females in both groups, accompanied by sarcopenia in the 16-month-naïve group.

In addition, there were genotype-sensitive variables, such as phenotype scoring system, frailty and kyphosis, in which the 3xTg-AD male group showed physical impairment. Motor learning and physical endurance variables were sensitive to test repetition, and 3xTg-AD females performed best when repeating the behavioural battery at 16 months. On the other hand, the females performed better in gait, where their gait was homogeneous and straight. They also showed less severe scores in physical variables, such as kyphosis, which could explain the more significant impairment of males in some motor tests. On the other hand, males showed dysfunction in most of the variables studied. Thus, correlations could explain the differences obtained between males and females, being positive in females between corticosterone and physical endurance, and in the case of males between sarcopenia index and physical endurance and corticosterone with physical variables.

Functional profile, biological status and their correlation are discussed as relevant to AD pathology. Therefore, the repetition of behavioural batteries could be considered as training, with some variables

sensitive to genotype, sex and test repetition. In the AD genotype, females performed best in physical endurance and motor learning, while males performed worse in most of the variables studied. These results highlight the complexity of experimental scenarios in neurodegenerative diseases such as Alzheimer's disease, confirming the different impact of factors according to genotype, sex and age and their interaction with the methodological approach.

SUMMARY

The 3xTg-AD mice are a model that expresses the functional and motor impairment of primary and secondary psychomotor dysfunction that limits the activities and performance of mice, similar to what occurs in humans and according to the temporality of AD impairment.

In gait, the decrease in stride length, speed, and cadence seems to be the most sensitive variable of psychomotor dysfunction, being even modified by postural alterations such as structural kyphosis, which increases the deterioration of stride length and gait speed in male 3xTg-AD mice. In addition, kyphosis is sensitive to age, showing a progression from postural to structural kyphosis in 3xTg-AD and non-transgenic animals. Similarly, the clasping reflex indicates the severity of AD, a primary signature like kyphosis. On the other hand, the frailty phenotype accompanies general psychomotor impairment in mice and increases with age.

Similarly, physical endurance is sensitive to sex, with 3xTg-AD and non-transgenic females showing higher performance that increases with training; however, they show the highest indicators related to frailty, sarcopenia and HPA axis alteration with worse indicators at 16 months in the 3xTg-AD when no behavioural battery or repetition training is applied at later ages. Table 4 summarizes the psychomotor dysfunctions in the 3xTg-AD model presented in this thesis.

Finally, functional impairments and alterations in 3xTg-AD mice are related to Alzheimer's disease stages, providing a scenario to understand the heterogeneity of non-cognitive symptoms of motor performance.

Table 4. Summary psychomotor dysfunction and frailty related to sarcopenia in 3xTg-AD mice model

Age tested	Sex tested	Motor dysfunction and frailty related to sarcopenia	References
6 months	Males	(1) Bizarre behaviour "Circling" was characteristic of 3xTg-AD mice also in the aquatic Morris water maze at onset of disease	(Castillo-Mariqueo and Giménez-Llort, 2019)
6, 12, and 16 months	Males	(1) Functional impairment in gait (speed, cadence and stride length) and exploratory activity (horizontal and vertical explorations); (2) Muscle weakness marks the functional decline related to disease severity stages that intensify with increasing age; (3) Motor performance in 3xTg-AD is accompanied by bizarre behaviours that interfere with gait trajectory; (4) Incidence of kyphosis with postural predominance at 12 months, increasing in severity at 16 months (structural); (5) Clasping reflex present in all groups, irrespective of age; (6) Motor deficits increase with age in the rotarod; (7) Motor learning and physical endurance severely impaired at 16 months in the rotarod; (8) Signs of physical frailty accompany functional decline; (9) Signs of sarcopenia are present at an advanced stage of AD, with differences in fibre distribution, the number of cell nuclei and the presence of adipose tissue in the triceps surae and quadriceps muscles.	(Castillo-Mariqueo et al., 2021)
13 months	Males	(1) The isolated 3xTg-AD group showed higher performance in exercise tolerance and muscle strength tests; (2) The presence of freezing at the start of the exploratory activity and spontaneous gait test was associated with greater functional limitation in this group; (3) Hindlimb clasping is present during the tail suspension test; (4) Motor learning ability was preserved in 3xTg-AD mice in the rotarod; (5) Increased endurance performance in isolated mice in the rotarod; (6) The physical frailty phenotype is present in 3xTg-AD mice.	(Castillo-Mariqueo and Giménez-Llort, 2021a)
16 months	Males	(1) Kyphosis modified stride length and gait speed in end-of-life 3xTg-AD mice; (2) Bizarre gait patterns limit exploratory activity in transgenic end-of-life mice; (3) Structural and postural kyphosis as a primary impairment that modifies gait in 3xTg-AD end-of-life mice; (4) Bizarre gait patterns as a secondary impairment to exploratory activity in 3xTg-AD mice; (5) The animals show similar frailty phenotype alterations with some distinctions regarding a higher incidence of postural alterations in transgenic mice and injuries or wounds in non-transgenic mice, both coinciding with a high incidence of piloerection; (6) Despite frailty, gait function is not impaired in non-transgenic mice.	(Castillo-Mariqueo and Giménez-Llort, 2021b)
12, 16 months	Males Females	(1) 3xTg-AD females, irrespective of age, showed improved gait performance, where their stride was homogeneous and straight; (2) Decreased speed in male 3xTg-AD mice in all groups; (3) Increased freezing at 16 months in males' vertical exploration in the retest group; (4) Kyphosis modifies quantitative gait parameters in males. The 3xTg-AD males at 16 months had structural kyphosis, reflecting greater severity and functional limitations with predominance in the retest group;	(Castillo-Mariqueo and Giménez-Llort, 2022)

	(5) Presence of increased clasping in naïve 3xTg-AD mice at 16 months; (6) Motor learning and physical endurance variables were sensitive to retesting, with 3xTg-AD females performing best when the behavioural battery was repeated at 16 months; (7) 3xTg-AD males had greater physical frailty at 16 months, indicating more significant associated functional impairment; (8) Quadriceps and triceps surae muscles are heavier in naïve male mice. Lower triceps surae and quadriceps muscle weights are observed in 3xTg-AD females.

CONTRIBUTIONS AND LIMITATIONS

This thesis contributes to advances in research on the non-cognitive symptomatology present in AD, which has gained greater importance among the scientific community as it generates a more significant impact on the dependence and disability of people with AD and their caregivers.

On the other hand, due to the intrinsic difficulty of breeding programs with transgenic animals, in the more complex experimental designs, several experimental series are needed to complete the sample size. In these cases, to overcome this experimental limitation, every experimental set is established with a representation of at least half of the experimental groups and performed in a counterbalanced manner.

To overcome mortality bias, groups of females of both genotypes could have been included for comparison at ages below 12 months. Likewise, 16-month-old naïve NTg females that could be compared with 3xTg-AD females of the same age were not included, as mortality in the non-transgenic group was higher than expected, not reaching the formation of this group.

Finally, as a future projection, we will seek to investigate biomarkers that will allow us to correlate primary and secondary markers of psychomotor dysfunction that complement these findings and those detected in muscle and fat tissue.

CHAPTER 6. CONCLUSIONS

CONCLUSIONS

The main conclusions of this thesis are:

1. The hindlimb clasping reflex is also a primary impairment indicating worsening AD symptomatology, present in 3xTg-AD mice regardless of age.
2. Structural and postural kyphosis constitutes the main impairment modifying stride length and gait speed in 3xTg-AD mice, and its severity increases with age.
3. Structural kyphosis characterizes end-point mice, while postural kyphosis is for normal aging.
4. Piloerection is the primary marker of macroscopic examination indicating severity in the in-of-life setting at 16 months in 3xTg-AD and NTg mice.
5. Bizarre behaviours constitute the secondary dysfunction that limits the exploratory activity of 3xTg-AD mice in dry tests and alters swimming patterns and searching behaviour in the Morris water maze test.
6. Bizarre circling is a feature of 3xTg-AD mice and manifests early in the disease in MWM. In addition, bizarre gait patterns are a distinctive marker in 3xTg-AD mice.
7. The frailty that accompanies functional impairment in 3xTg-AD mice is associated with mortality in females, even in non-transgenic mice.
8. Signs of sarcopenia associated with frailty are observed in advanced stages of AD, with 3xTg-AD females showing the greatest impairment.
9. Secondary dysfunctions can be modulated by external factors such as isolation and behavioural battery retest, where 3xTg-AD mice improve motor performance in females and isolation in males.
10. 3xTg-AD females show more significant impairment associated with frailty and sarcopenia, but perform better in physical endurance tests, with high corticosterone levels.

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ANNEX

ANNEX 1: BIZARRE BEHAVIOURS LIMIT EXPLORATORY ACTIVITY AND IMPAIR SPONTANEOUS GAIT PERFORMANCE IN AGED MICE WITH AD PATHOLOGY. 2ND INTERNATIONAL ELECTRONIC CONFERENCE ON BRAIN SCIENCES (2021) MPDI, DOI:10.3390/IECBS2021-10671.

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Bizarre behaviors limit exploratory activity and impair spontaneous gait performance in aged mice with Alzheimer's disease

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Abstract: The shorter life spans of mice provide an exceptional experimental gerontology scenario. We previously described increased bizarre (disruptive) behaviors in the 6-month-old 3xTg-AD mice model for Alzheimer's disease (AD), compared to C57BL/6J wildtype, when confronting new environments. In the present work, we evaluated spontaneous gait and exploratory activity at old age, using 16-month-old mice. Male sex was chosen since sex-dependent psychomotor effects of aging are stronger in C57BL/6J males than females and, at this age, male 3xTg-AD mice are close to an end-of-life status due to increased mortality rates. Mice's behavior was evaluated in a transparent test box during the neophobia response. Stretching, jumping, backward movements and bizarre circling were identified during the gait and exploratory activity. The results corroborate that in the face of novelty and recognition of places, old 3xTg-AD mice exhibit increased bizarre behaviors than mice with normal aging. Furthermore, bizarre circling and backward movements delayed the elicitation of locomotion and exploration, in an already frail scenario, as shown by highly prevalent kyphosis in both groups. Thus, the translational study of co-occurrence of psychomotor impairments and anxiety-like behaviors can be helpful for understanding and managing the progressive functional deterioration shown in older people, especially those with AD.

Keywords: Alzheimer's disease; bizarre; exploratory activity; gait; 3xTg-AD mice; kyphosis; circling

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1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of cognitive, language, and behavioral functions [1]. In addition, dementia can have multifaceted clinical presentations [2]. Thus, a wide range of behavioral and psychological symptoms of dementia (BPSD) can manifest, reaching more than 90% in most patients [3]. Therefore, BPSD requires great efforts for caregivers and society in general [4].

The gap between the clinical characteristics of this disease and its elicitation in animal models entails great efforts by researchers to achieve a replicable approach. However, they have begun to be addressed in some of these models in the last decade [5]. In this way, we described early symptoms similar to BPSD in the 3xTg-AD mouse model for the first time at 2.5 months of age [5], and bizarre behaviors at 6 months of age [6]. Subsequently, we described bizarre behaviors in swimming performance in the Morris water maze [7]. Thus, at 13 months of age, it was possible to identify non-seeking, floating, and circling behaviors among genotypes more precisely, the latter group being the character-

istic behavior of 3xTg-AD animals [7]. These findings were later confirmed in a study conducted on 6-month-old male animals [8]. Furthermore, these behaviors are sensitive to environmental factors. Thus, we recently reported that naturally isolated 13-month-old 3xTg-AD male animals, as a social isolation model, exhibit bizarre behaviors that interfere with exploratory activity and locomotion in gait, with stretching and circling behaviors being the most sensitive behaviors exhibited by isolated animals [9].

The present work aimed to identify distinctive patterns of bizarre behaviors related to deficiencies and functional limitations of spontaneous gait and exploratory activity in 16-month-old male 3xTg-AD mice in an advanced AD stage compared to non-transgenic (NTg) mice with normal aging.

2. Materials and Methods

2.1. Animals

A total of twenty-one homozygous 3xTg-AD (n = 11) and non-transgenic (NTg, n = 10) male mice of 16 months of age in a C57BL/6J background (after embryo transfer and backcrossing of at least ten generations) established at the Universitat Autònoma de Barcelona were used in this study. The 3xTg-AD mice harboring transgenes were genetically modified at the University of California at Irvine, as previously described [10]. Animals were kept in groups of 3-4 mice per cage (Macrolon, 35 × 35 × 25 cm3) filled with 5 cm of clean wood cuttings (Ecopure, Chips6, Date Sand, UK; uniform cross-sectional wood granules with 2.8-1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm x 20 cm, White). All animals were kept under standard laboratory conditions of food and water ad lib, 20 ± 2◦C, 12 h light cycle: dark with lights on at 8:00 a.m. and 50-60% relative humidity. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used [11].

2.2. Behavioral assessment

Behavioral evaluations were carried out in a single day and balanced by observing two independent observers blind to the genotype. During the morning, the tests were carried out; 30 minutes were allowed to habituate the animals in the test room before starting the measurements. The evaluation protocol, bizarre behaviors registered, and physical phenotype of frailty used here are recently reported in Castillo-Mariqueo and Gimenez-Llort's 2021 study [9]. In addition, videos of gait were taken for posterior analysis with KINOVEA 0.8.15 free software.

2.3. Statistics

Statistical analyses were performed using SPSS 23.0 software. Results were expressed as the mean ± standard error of the mean (SEM) for each task and trial or incidence in percentage. The factors were analyzed using the Student's t-test or U-Mann Whitney test and Chi-square or Fisher's exact test. In all cases, $p<0.05$ was considered statistically significant.

3. Results

The animals presented a similar body weight in both groups, reaching 27.8 ± 1.0 g in the NTg animals and 27.1 ± 1.1 g in the 3xTg-AD mice. The variable of interest of the physical frailty phenotype, kyphosis, was present in 91% (10/11) of the transgenic animals and 80% (8/10) in the NTg group.

In the exploratory activity, genotype-dependent differences were found where 3xTg-AD animals take longer to start locomotion [Mann-Whitney U p = 0.016]. In the same way, horizontal and vertical activity is decreased compared to the non-transgenic group, which is significant in horizontal activity [Mann-Whitney U p = 0.008].

On the other hand, the incidence of bizarre behaviors exhibited in the 3xTg-AD group reached 82% (9/10), and those in the NTg group 40% (4/10) with a high incidence in circling that reached 36% (4/11) followed by backward movement with 18% (2/11) in transgenic animals. In the case of NTg, the behavior with the highest incidence was stretching at 20% (2/10).

Similar to what occurs in exploratory activity, a decrease in stride length was observed during gait with a low speed of the steps in 3xTg-AD mice [stride length: Mann-Whitney U $p = 0.024$; speed: Student's *t*-test $p = 0.041$].

4. Discussion and conclusions

In this research, bizarre behaviors and the functional limitations of the exploratory activity and gait performance of male 3xTg-AD and NTg mice at the age of 16 were studied. Although these behaviors have been previously reported in the open field, gait tests, and the Morris water maze [6–9], this is the first time confirmed in 16-month-old animals. In this way, the results corroborate that in the face of a novelty situation and the recognition of place, old 3xTg-AD mice exhibit bizarre behaviors, and most importantly, they interfere with their locomotion and spontaneous exploration, in an aging scenario that already includes kyphosis, an indicator of frailty with high incidence in both groups.

The manifestation of bizarre behaviors in anxiety tests suggests that these behaviors could be related to coping with stress [6]. The bizarre behavior patterns in 3xTg-AD mice from 6 to 13 months of age differ from NTg animals and correlate with other anxiety behaviors, locomotion, and emotionality [6,7]. However, bizarre behaviors can be very varied and heterogeneous, which is a limitation to detect genotype differences. Also, most of them depend on sex, with females being the ones that exhibited these behaviors to a greater extent [6]. Therefore, it is relevant that in the present study using the male sex, we detected circling and backward movements as the behaviors with the highest incidence in old transgenic animals mimicking very advanced stages of the disease.

On the other hand, previous gait studies of 13-month-old male 3xTg-AD mice indicate deficits that coincide with an aging pattern, accompanied by a series of bizarre behaviors that can interfere with trajectory and movement [9]. A high period of freezing at the beginning of the test accompanied by a high latency in the exploratory activity that interferes in the horizontal and vertical activity is consistent with our findings at 16 months, making us note that once these behavioral patterns appear, they persist as the disease progresses.

The study of co-occurrence of psychomotor impairments and anxiety-like behaviors can be helpful for understanding and managing the progressive functional deterioration related to aging and the nuances in AD-scenario, with a translational value for older people, especially those with AD.

Supplementary Materials: Not aplicable.

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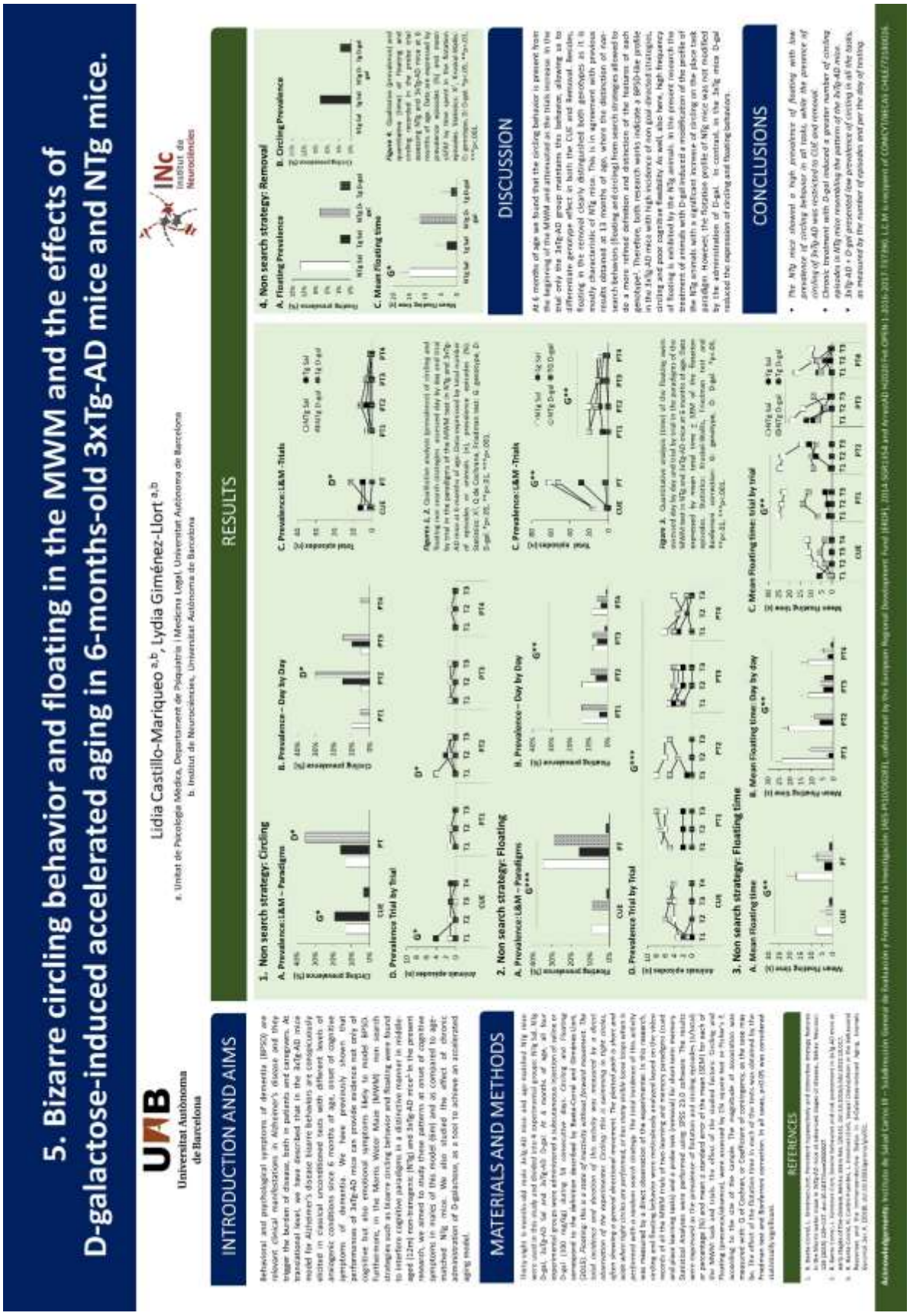
5. Bizarre circling behavior and floating in the MWM and the effects of D-galactose-induced accelerated aging in 6-months-old 3xTg-AD mice and NTg mice.
UAB Universitat Autònoma de Barcelona
Lidia Castillo-Mariqueo a,b, Lydia Giménez-Llort a,b
a. Unitat de Psicologia Mèdica, Departament de Psiquiatria i Medicina Legal, Universitat Autònoma de Barcelona
b. Institut de Neurociències, Universitat Autònoma de Barcelona
INc Institut de Neurociències
INTRODUCTION AND AIMS
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DISCUSSION
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REFERENCES

UAB INc Institut de Neurociències

Departament de Psiquiatria i Medicina Legal UAB

Folio:90

Fragilidad física y sistémica, situaciones de novedad y supervivencia en el envejecimiento y la enfermedad de Alzheimer: Una aproximación traslacional a las residencias de larga estadía

Lidia Castillo-Mariqueo[1,2] , Lydia Giménez-Llort[1,2]

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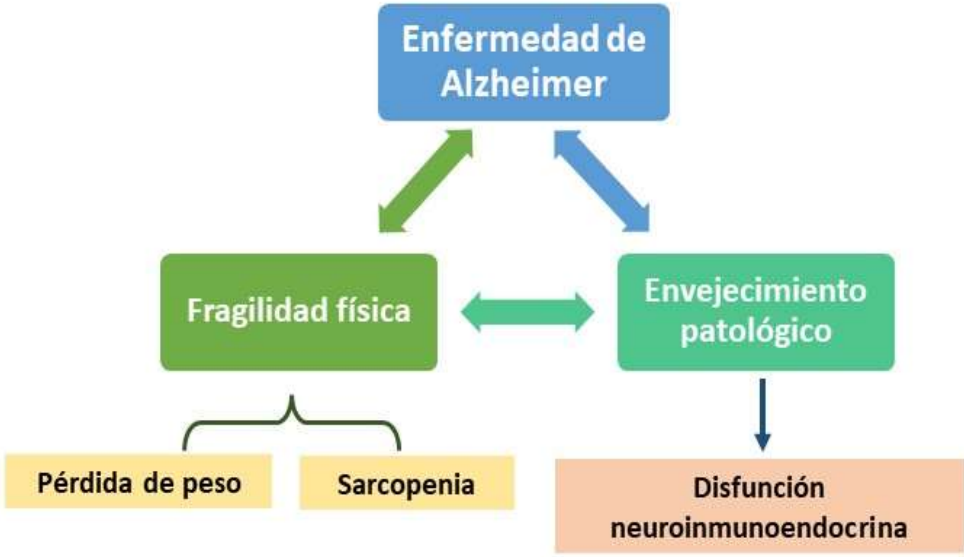
Introducción

Modelo animal de ratones 3xTg-AD

$PS1_{M146V}$, $APPS_{we}$, y tau $_{P301L}$

Especie *Mus musculus*
Cepa: C57BL/6

Mimetiza varios síntomas de la EA en un patrón temporal y neuroanatómico similar al observado en los humanos.
Frank M LaFerla, UCI, USA (Oddo et al., 2003)



Objetivo

Escenario de novedad

- Actividad exploratoria → Actividad horizontal y vertical
- Estado emocional → Neofobia

Material y método

Estudio longitudinal

Muestra: Machos y hembras 3xTg-AD/NTg (*n=67*)

→ **Traslación a Humanos**

Sexo	Genotipo	n
Macho	NTg	14
	AD	20
Hembra	NTg	20
	AD	13

meses: 12m, 13m, 14m, 15m, 16m

años: 50a, 55a, 60a, 65a, 70a

Test de esquina/Corner Test

Marcadores funcionales

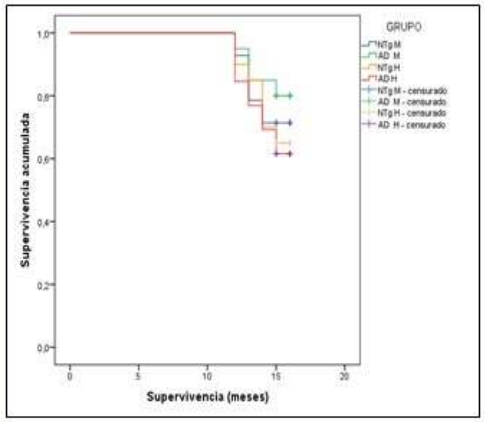
1. Sobrevivencia
2. Peso corporal
3. Actividad horizontal y vertical
4. Peso de tejidos y órganos

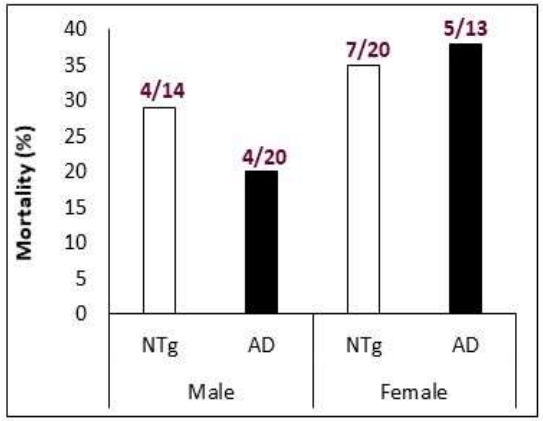
Disección de tejidos y órganos — Músculos | Bazo | Hígado

Statistics: ANOVA, MRA, X^2. Kruskal Wallis, U-Mann Whitney *p<.05, **p<.01, ***p<.001.

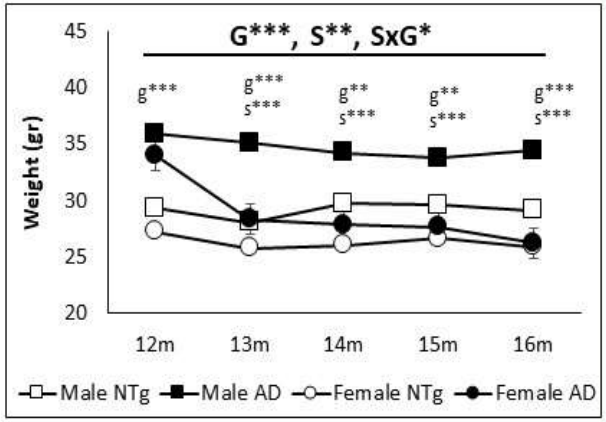
Resultados

1. Supervivencia

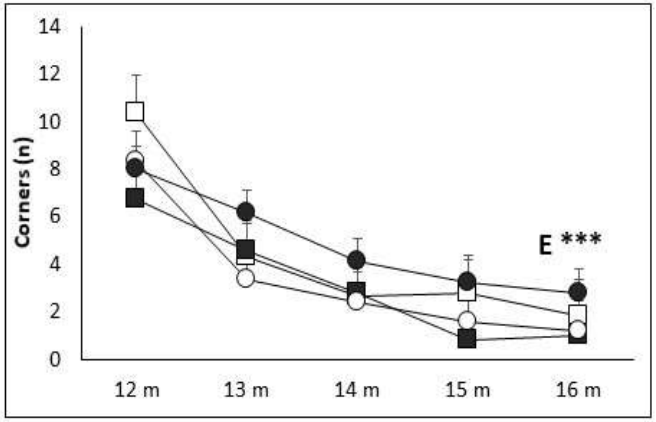




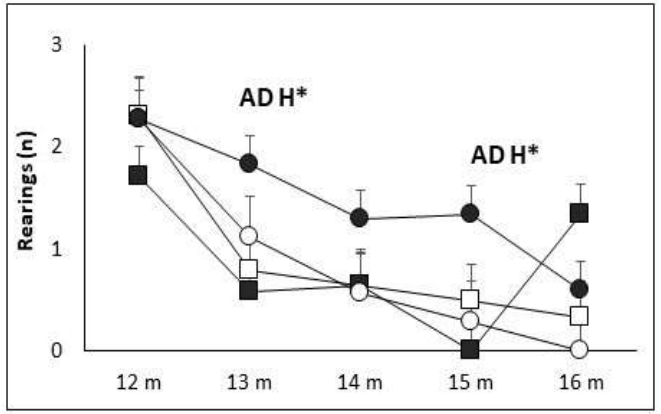
2. Peso corporal



3. Actividad horizontal



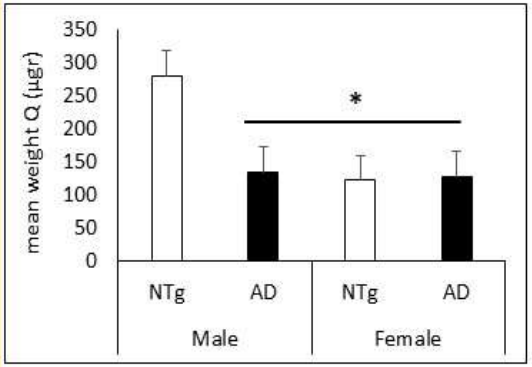
Actividad vertical



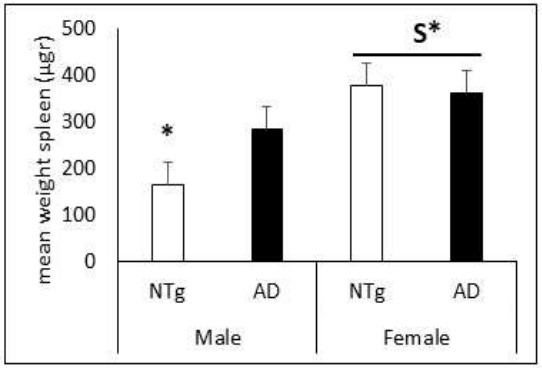
1.Supervivencia: Kaplan-Meier, grupo, p=0,668 - G, p=0,669- S, p=0,273.
Mortalidad (%) X_2, p=0,866.
2.Peso corporal: MRA, (S, G) $F_{(1,67)}$= 3839,820, p=0,000. MRA (SxG), $F_{(1,67)}$=2,729, p=0,030
3.Actividad horizontal (n de esquinas) MRA, grupo $F_{(1,67)}$= 69,236, p=0,000, efecto de la edad.
Actividad vertical (n verticalizaciones). T-student, 13m AD H: p=0,013 ; 15m AD H: p=0,046

Resultados

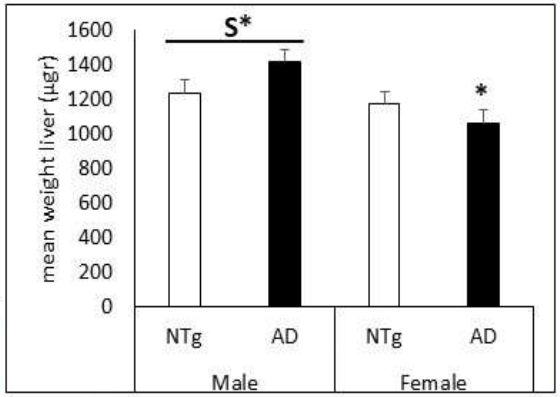
4. Peso de Cuádriceps



Bazo



Hígado



4. Cuádriceps p=0,010 – Bazo p=0,044 – Hígado p=0,032 (Kruskal Wallis).
Genotipo: Cuádriceps, Bazo, Hígado n.s.. Sexo: Cuádriceps n.s. – Bazo p=0,015 – Hígado: 0,004

Conclusión

1 — Mortalidad

Mayor mortalidad en **hembras** en ambos genotipos

13m corresponde a la **edad de mayor decesos** en todos los grupos (55años)

2 — Fragilidad física

Existe **pérdida progresiva de peso** en todos los grupos

Las **hembras** pierden más peso comparado con machos **en todas las edades**

3 — Envejecimiento Neofobia

La **actividad motriz horizontal disminuye** en todos los grupos **a medida que aumenta la edad.**

La **actividad vertical** es **mayor** en **hembras** AD a los **13m y 15m**

4 — Envejecimiento patológico Alteración sistémica

La **masa muscular** en animales **AD machos y hembras** es menor, junto con las **hembras NTg** **Sarcopenia**

A nivel **inmuno-endocrino**, existe una tendencia al **aumento de peso** del **bazo** en las **hembras** independiente del genotipo

De manera contraria, a nivel **metabólico,** el **hígado** de los **machos** posee un mayor peso

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Acknowledgements: UAB GE-260408 - CONICYT/BECAS CHILE/72180026

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EPO-006

Gait impairments and functional limitations in the exploratory activity in an animal model of Alzheimer's disease

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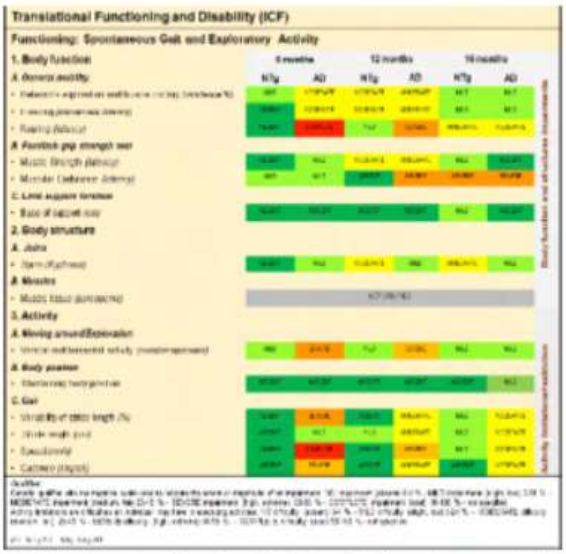
Background and aims: Gait impairments in Alzheimer's disease (AD) result from structural and functional deficiencies that generate limitations in the performance of activities and restrictions in an individual's biopsychosocial participation. In a translational way, we have used the conceptual framework proposed by the International Classification of Disability and Health Functioning (ICF) to classify and describe the functioning and disability on gait and exploratory activity in the 3xTg-AD animal model.

Methods: We developed a behavioral observation method that allows us to differentiate qualitative parameters of psychomotor performance in animals' gait, similar to the behavioral patterns observed in humans. The functional psychomotor evaluation allows measuring various dimensions of gait and exploratory activity at different disease progression stages in dichotomy with aging. We included male 3xTg-AD mice and their non-transgenic counterpart (NTg) of 6, 12, and 16 months (n=45).

Results: Here we present the preliminary results. The 3xTg-AD mice show more significant functional impairment in gait and exploratory activity quantitative variables. The presence of movement limitations and muscle weakness mark the functional decline related to the disease severity stages that intensify with increasing age. Motor performance in 3xTg-AD is accompanied by a series of bizarre behaviors that interfere with the trajectory, which allows us to infer poor neurological control. Besides, signs of physical frailty accompany the functional deterioration of these animals.



Gait impairments and functional limitations in the exploratory activity: Translational approach of Functioning and Disability

Conclusion: The use of the ICF as a conceptual framework allows the functional status to be described, facilitating its interpretation and application in the rehabilitation of people with AD.

Disclosure: Nothing to disclose.

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A-21-02225 Gait impairments and functional limitations in the exploratory activity in an animal model of Alzheimer's disease

INc Institut de Neurociències

Lidia Castillo-Mariqueo[1,2] and Lydia Giménez-Llort[1,2]

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

UAB Universitat Autònoma de Barcelona

INTRODUCTION

Gait impairments in Alzheimer's disease (AD) result from structural and functional deficiencies that generate limitations in the performance of activities and restrictions in an individual's biopsychosocial participation. In a translational way, we have used the conceptual framework proposed by the International Classification of Disability and Health Functioning (ICF) to classify and describe the functioning and disability on gait and exploratory activity in the 3xTg-AD animal model.

AIMS

In this research, we developed a method for the characterization of qualitative parameters of psychomotor performance in the gait of 3xTg-AD animals at different ages, corresponding to an initial, intermediate, and advanced stages of AD in contrast to aging similar to the observed behavior patterns in humans.

METHODS

For this purpose, the functional psychomotor evaluation allows measuring various dimensions of gait and exploratory activity at different stages of disease progression in dichotomy with aging. We included male 3xTg-AD mice and their non-transgenic counterpart (NTg) of 6, 12, and 16 months (n = 45).

RESULTS

Translational Functioning and Disability (ICF)

Functioning: Spontaneous Gait and Exploratory Activity

1. Body function	6 months		12 months		16 months		
A. General mobility:	NTg=6	AD=6	NTg=6	AD=7	NTg=9	AD=11	
• Behavior's exploration and bizarre circling *(incidence %)*	MILD	MODERATE	MODERATE	MODERATE	MILD	MILD	Body function and structures impairments
• Freezing *(movement latency)*	ABSENT	MODERATE	MODERATE	MODERATE	MILD	MILD	
• Rearing *(latency)*	ABSENT	COMPLETE	MILD	SEVERE	MODERATE	MODERATE	
B. Forelimb grip strength test							
• Muscle Strength *(latency)*	ABSENT	MILD	MODERATE	MODERATE	MILD	ABSENT	
• Muscular Endurance *(latency)*	MILD	MILD	ABSENT	SEVERE	SEVERE	SEVERE	
C. Limb support function							
• Base of support *(cm)*	ABSENT	ABSENT	ABSENT	ABSENT	MILD	ABSENT	
2. Body structure							
A. Joins (Kyphosis)	ABSENT	MILD	MODERATE	MILD	MODERATE	MILD	
B. Muscles (sarcopenia)	NOT SPECIFIED						
3. Activity:							
A. Moving around/Exploration							
• Vertical and horizontal activity *(number episodes)*	MILD	SEVERE	MILD	SEVERE	MILD	MILD	Activity limitations/restriction
B. Body position							
• Maintaining body position	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	MILD	
C. Gait							
• Variability of stride length *(%)*	ABSENT	SEVERE	ABSENT	MODERATE	MILD	MODERATE	
• Stride length *(cm)*	ABSENT	MILD	MILD	MODERATE	MILD	MODERATE	
• Speed *(cm/s)*	ABSENT	COMPLETE	ABSENT		MILD	MODERATE	
• Cadence *(steps/s)*	ABSENT	SEVERE	ABSENT	MODERATE	ABSENT	MODERATE	

Qualifier:
Generic qualifier with the negative scale used to indicate the extent or magnitude of an impairment: NO impairment (absent) 0-4 % - MILD impairment (slight, low) 5-24 % - MODERATE impairment (medium, fair) 25-49 % - SEVERE impairment (high, extreme) 50-95 % - COMPLETE impairment (total) 96-100 % - not specified.
Activity limitations are difficulties an individual may have in executing activities: NO difficulty (absent) 0-4 % - MILD difficulty (slight, low) 5-24 % - MODERATE difficulty (medium, fair) 25-49 % - SEVERE difficulty (high, extreme) 50-95 % - COMPLETE difficulty (total) 96-100 % - not specified.

DISCUSSION / CONCLUSIONS

In our preliminary results, we have found common and distinctive characteristics between the AD pathological aging sample and the normal aging sample. We can stand out: (1) The 3xTg-AD mice show more significant functional impairment in gait and exploratory activity quantitative variables. (2) The presence of movement limitations and muscle weakness mark the functional decline related to the disease severity stages that intensify with increasing age. (3) Motor performance in 3xTg-AD is accompanied by a series of bizarre behaviors that interfere with the trajectory, which allows us to infer poor neurological control. (4) Signs of physical frailty accompany the functional deterioration of these animals. According to the literature, this is the first report that comprehensively presents the gait impairments and functional limitations in the exploratory activity of the 3xTg-AD mouse model.

Finally, the use of the ICF as a conceptual framework allows the functional status to be described, facilitating its interpretation and application in the rehabilitation of people with AD.

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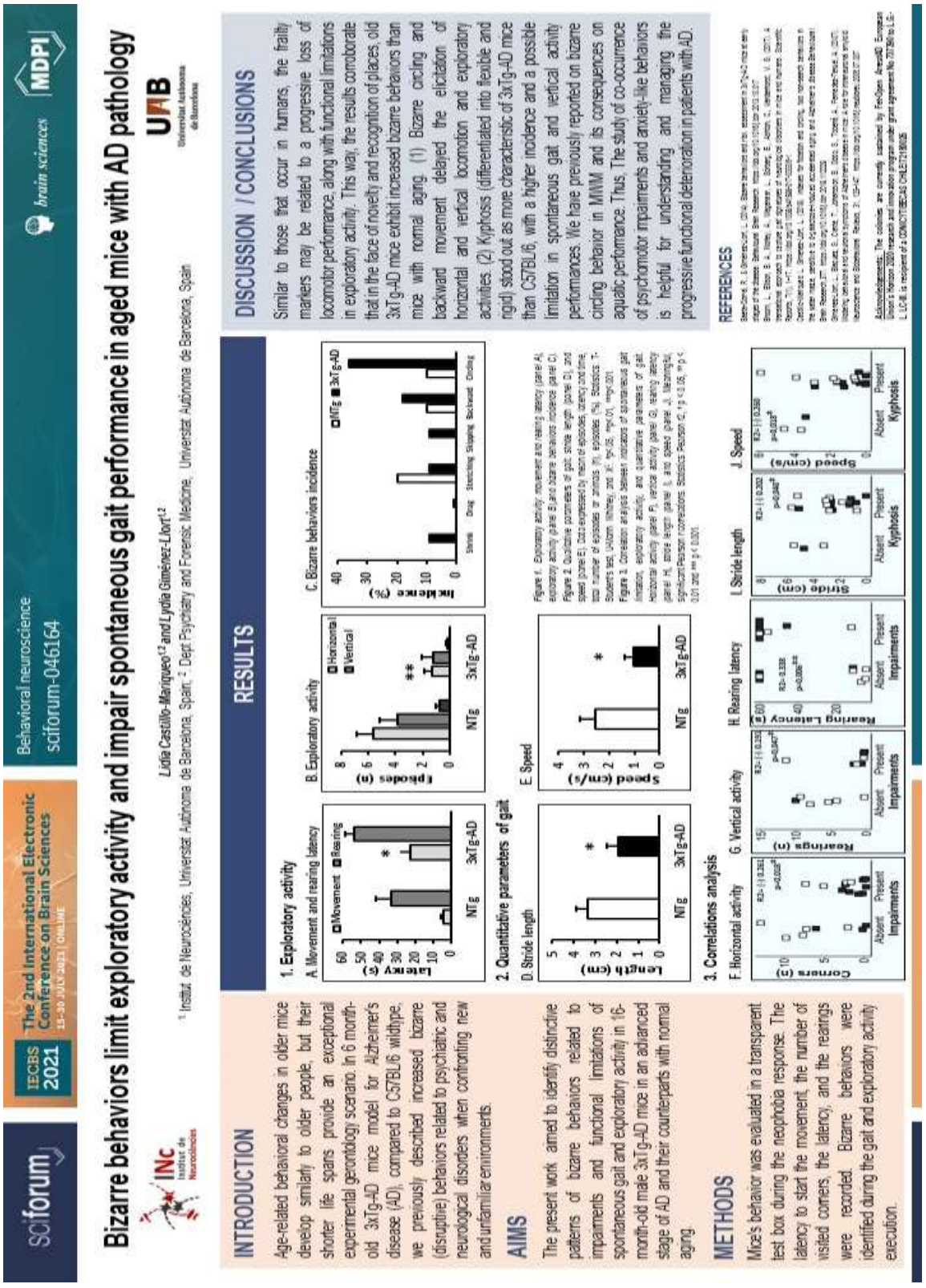
ANNEX 5: BIZARRE BEHAVIOURS LIMIT EXPLORATORY ACTIVITY AND IMPAIR SPONTANEOUS GAIT PERFORMANCE IN AGED MICE WITH AD PATHOLOGY. 2ND INTERNATIONAL ELECTRONIC CONFERENCE ON BRAIN SCIENCES (2021)



EPO-007

Hindlimb clasping, kyphosis and piloerection: Frailty markers from middle to very old ages in mice

L. Castillo Mariqueo [1], D. Alveal-Mellado [2], L. Gimenez-LLort [1]

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

Background and aims: The state of frailty is a clinical-biological syndrome that affects the elderly population with a higher risk of functional dependence. It is produced by the dysfunction of multiple organs and systems, causing a significant hospitalization, disability, and death rate. However, individual variability increases with the aging process, and divergence between chronological and biological age becomes more prominent. This research aims to identify distinctive aspects of physical frailty, from a behavioral neurology translational approach.

Methods: The animal model 3xTg-AD for Alzheimer's disease (n=37) and its non-transgenic (NTg) counterpart with normal aging (n=14), from 12 to 21 months modeling middle-age to very-old scenarios, were used. The animals' functional limitations and impairments were assessed to define their Physical Frailty Phenotype. The classical open-field anxiety test was included to control for general horizontal and vertical activities.

Results: We have detected common elements of physical frailty and functional performance in all the animals, independently of their genotypes and age. Signs such as piloerection, kyphosis, and hindlimb clasping seemed to be the ones that better defined the level of severity and deterioration, as confirmed by end-of-life scenarios. They are important to note since they may influence the other physical and behavioral results.

Physical Frailty Phenotype	NTg N=14	3xTg-AD N=37	Statistics
Body position	-	6/37 (16%)	n.s.
Kyphosis	14/14 (100%)	35/37 (95%)	n.s.
Alopecia	4/14 (29%)	6/37 (16%)	n.s.
Palpebral closure	2/14 (14%)	4/37 (11%)	n.s.
Piloerection	11/14 (79%)	28/37 (76%)	n.s.
Tail position	-	4/37 (11%)	n.s.
Tremor	2/14 (14%)	12/37 (32%)	n.s.
Hindlimb clasping:			
• *Normal*	5/14 (36%)	10/37 (27%)	n.s.
• *Mild*	7/14 (50%)	20/37 (54%)	n.s.
• *Moderate*	2/14 (14%)	6/37 (16%)	n.s.
• *Severe*	-	1/37 (3%)	n.s.

X_2 *** p < 0.01, ** p < 0.05 * p < 0.05, n.s. p > 0.05

Table 1. Physical Frailty Phenotype

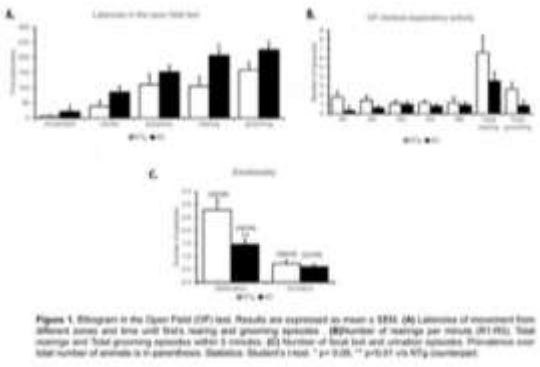


Figure 1. Ethogram in the Open Field test (OF)

Conclusion: Inter-individual heterogeneity from the middle to ancient age can disrupt the relationship between chronological age and animals' physical status/frailty. Identifying markers of frailty independent of chronological age may help us translate them into clinical settings and better design interventions in the frailest population with normal and/or neuropathological aging.

Disclosure: Nothing to disclose.

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Hindlimb clasping, kyphosis and piloerection: Frailty markers from middle to very old ages in mice with normal and AD-pathological aging

INc Institut de Neurociències

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

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INTRODUCTION

The state of frailty is a clinical-biological syndrome that affects the elderly population with a higher risk of functional dependence. It is produced by the dysfunction of multiple organs and systems, causing a significant hospitalization, disability, and death rate. However, individual variability increases with the aging process and divergence between chronological and biological age becomes more prominent.

AIMS

This research aims to identify distinctive hallmarks of physical frailty in middle to very old mice with normal aging and pathological AD from a translational approach to behavioral neurology.

METHODS

We looked for the physical and behavioral characteristics of a set of mice from 16 to 20 months of age from 3xTg-AD mice compared to NTg of the same age with normal aging. A single-day behavioral battery was applied: Corner test, Open Field, and Physical frailty phenotype.

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RESULTS

Table 1. Physical Frailty Phenotype

Physical Frailty Phenotype	NTg N=14	3xTg-AD N=37	Statistics
Body position	-	6/37 (16%)	n.s.
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Tail position	-	4/37 (11%)	n.s.
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Hindlimb clasping:			
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• *Severe*	-	1/37 (3%)	n.s.

Figure 1. Ethogram in the Corner Test (CT)

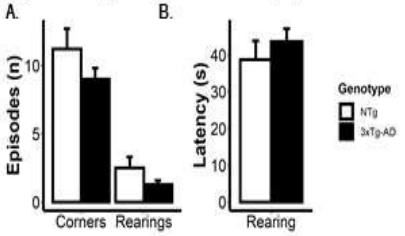

Figure 2. Ethogram in the Open Field (OF) test.

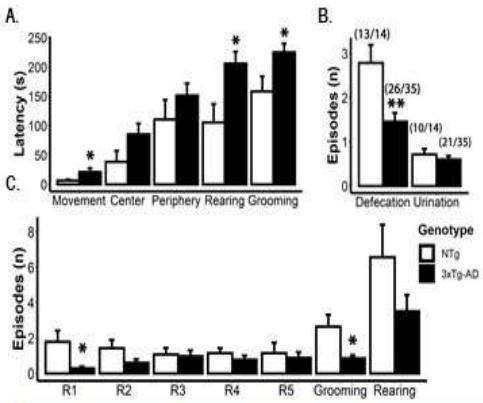

Table 1. Physical Frailty Phenotype is an adaptation based on the conceptual description of Frailty. We have included a physical domain that shows the animals' general impairments without subjecting them to an evaluation that involves handling and stress for the animals. X2, *** p < 0.01, ** p < 0.05 * p < 0.05, n.s. p > 0.05.

Figure 1. Ethogram in the Corner Test (CT). Results are expressed as mean ± SEM. Number of corners and rearings carried out within the 60s of the test (A) and latency until the first rearing (B). Statistics. Student's t-test (NTg v/s AD): p>0.005

Figure 2. Ethogram in the Open Field (OF) test. Results are expressed as mean ± SEM. (A) Latencies of movement from different zones and time until first rearing and grooming episodes. (B) Number of fecal boli and urination episodes. Prevalence over total number of animals in the parenthesis. (C) Number of rearings per minute (R1-R5). Total rearings and Total grooming episodes within 5 minutes. Statistics : Student' s t-test. *p>0.005 , **p>0,001 (AD v/s NTg)

DISCUSSION / CONCLUSIONS

We have detected common elements of physical frailty and functional performance in all the animals, independently of their genotypes and age. (1) Signs such as hindlimb clasping, kyphosis and piloerection seemed to be the ones that better defined the level of severity and deterioration, as confirmed by end-of-life scenarios. They are important to note since they may influence the other physical and behavioral results. (2) Inter-individual heterogeneity from middle to very old age can disrupt the relationship between chronological age and the physical status/frailty of animals. Identification of markers of frailty independent of chronological age may help us to translate them into clinical settings and better design interventions in the frailest population with normal and/or neuropathological aging. and application in the rehabilitation of people with AD.

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ANNEX 7: PHENOTYPICAL, BEHAVIOURAL AND SYSTEMIC HALLMARKS IN END-POINT MICE SCENARIOS (CURRENT STATUS: SUBMITTED AT JOURNAL OF APPLIED ANIMAL WELFARE SCIENCE, TAILOR & FRANCIS GROUP)

Phenotypical, behavioural and systemic hallmarks in end-point mice scenarios

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Abstract

The state of frailty is a clinical-biological syndrome that affects the elderly population with a higher risk of functional dependence. Animal models can provide a tool to study this complex scenario. In the present work, we analysed the physical and behavioural hallmarks of a set of 16-month-old mice (C57BL/6J) at their end-point compared to age-matched counterparts with normal aging. A group of 6-month-old mice was added for control for age bias. First, we identified 'structural kyphosis' (visible and unmodifiable deformation in locomotion) correlated with piloerection as the hallmarks of physical frailty phenotype compared to 'postural kyphosis' (adjustment to counteract increased visceral volume but attenuated during locomotion) of old mice with normal aging. Alopecia (barbering) was presented in both old groups. Normal levels of exploratory activity in the corner test for neophobia and triceps surae muscle weight but increased latency of rearing indicated the poorest emotional phenotype with the possible contribution of structural kyphosis. The presence of hepatomegaly and splenomegaly counteracted the significant WAT loss commonly associated with end-of-life traits, which should have on body weight but preserved muscle mass.

Keywords: end-point, euthanasia, welfare, bodyweight, sarcopenia, C57BL/6J mice.

Introduction

Animal models of aging research have critical implications for human aging and age-related diseases (Mitchell et al., 2015). Their physical and mental health effects are measurable by readily gross examination and behavioural assessment (Turner et al., 2012). However, exist heterogeneity in the aging and complexity of the age-related scenario in old animals with the reduced survival of animals and the concomitant increase of laboratory costs (Baeta-Corral et al., 2018; Giménez-Llort et al., 2019; Torres-Lista et al., 2019). Old mice have a subset of injuries due to the progressive deterioration of the organs' function and systems expressing frailty and age-related diseases (Pettan-Brewer and Treuting, 2011; Zglinicki et al., 2016).

On the other hand, in many studies that use young and adult mice, the scientific end-point usually is related to time, a defined event, or a condition in the animal that occurs or does not occur after a particular intervention (Toth, 2018; Trammell et al., 2012). When the possibility of animal pain, distress, or suffering exists, researchers should delineate the research objectives and procedures for assessing animal health and ensuring the animal's well-being (Fries, 1980; Suckow & Gimpel, 2020; Trammell et al., 2012). The end-point at which an animal is sacrificed must be established according to clinical or experimental criteria (Burkholder et al., 2012; Morton & Griffiths, 1985; Suckow & Gimpel, 2020). Currently, the methods used to assess the condition of a mouse and establish these criteria may include observation of behaviour, assessment of physical appearance, and measurement of body weight (Beynen et al., 1987; Mei et al., 2019; Morton & Griffiths, 1985; Ullman-Culleré, 1999). Behavioural parameters include observing unprovoked behaviour and responses to external stimuli (Burkholder et al., 2012; Mei et al., 2019). Frequently, physical appearance includes exophthalmia or enophthalmia, runny nose or eye, rough coat, and kyphosis (Burkholder et al., 2012). These findings have been described as standard indicators of ill health, allowing an animal to be monitored over time as health declines (Mei et al., 2019; Ullman-Culleré, 1999). Additionality, decreased consumption of food and water is an important sign of declining health (Olfert, 1995) as it generally results in weight loss (Redgate & Boggs, 1991). Being a weight loss of 20%, a criterion for euthanasia alone is already a reliable predictor of clinical deterioration (Talbot et al., 2020). Likewise, it has been described that there is an age-related decrease in body temperature (Reynolds et al., 1985), animals that exhibit higher body temperatures and more excellent temporal stability tend to live longer, particularly in the C57BL/6 mice strain (Reynolds et al., 1985; Talan & Engel, 1986). Although there is consensus that frailty implies multi-organ dysfunction and greater vulnerability to chronic diseases and mortality (Zglinicki et al., 2016), the systemic effects on the aging of the mouse and its functional implications have not yet been well defined (Palliyaguru et al., 2019; Pettan-Brewer & Treuting, 2011).

Therefore, the present work aims to determine if the deterioration of an animal with an end-point indication

could be previously inferred not only by hallmark parameters of its physical frailty phenotype (including bodyweight) but also through ordinary and easy-to-take homecage housekeeping tasks that allow measurement of exploration and neophobia. Since we are interested in targeting body weight loss and the physical frailty phenotype, the liver and spleen were chosen to include the effects of aging at the systemic level. Organometric analysis of post-mortem systemic conditions could indicate that weight can be an indirect measurement of sarcopenia. In addition, we have selected the *triceps surae* muscle as an indicator of sarcopenia and white adipose tissue as an underlying frailty criterion. On the other hand, we also hypothesized that despite presenting positive criteria for the indication of euthanasia in the end-point state, animals with advanced age might maintain their functional performance and body weight regardless of the affectation in the weight of organs or loss of muscular mass. We have studied 16-month-old C57BL/6J mice in two clinical aging scenarios: the end-point and normal aging. In addition, a set of 6-month-old mice were added to control for the age factor.

Materials and methods

Animals

A total of twenty-two male C57BL/6J mice were used. A group of 16-month-old mice at end-point status (16M end-point, *n*=9) was compared with one of the same age but with normal aging (16M Aging, *n*=7). The third group of 6-month-old mice (6M Adult, *n*=6) was included to monitor the age factor. Animals were housed three or four per cage and maintained in Macrolon cages (35 × 35 × 25 cm) under standard laboratory conditions of food and water ad libitum, 22 ± 2 °C, a 12 h light: dark cycle starting at 8.00 a.m. and relative humidity 50–60%. All procedures were by Spanish legislation on '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).

Experimental design

A cross-sectional study was conducted to assess the appearance of sarcopenia through an indirect study method. The measurements were applied to adult and old animals' *triceps surae* muscle compared with old animals that met end-point criteria (Reynolds et al., 1985) and Talan and Engel (Talan & Engel, 1986). Before euthanasia, the animals were evaluated with a brief behavioural assessment to verify their physical frailty phenotype, geotaxis, and exploratory activity.

Physical frailty phenotype, geotaxis and exploratory activity

- Physical frailty phenotype - Includes the body conditions, body weight, alopecia, loss of whiskers, kyphosis, piloerection, tremor, eyes discharge, dermatitis, wounds, rectal prolapse, and others. These measurements were made before the exploratory activity and geotaxis. A score of 0 was assigned for normal aspects or 1 for abnormal aspects. Besides, a photographic record was taken of each animal to demonstrate these physical aspects.

  Specifically in the kyphosis variable It was differentiated two types: 'postural kyphosis' and 'structural kyphosis' (Castillo-Mariqueo & Giménez-Llort, 2021b). These were measured during the locomotion and exploratory activity of the animals and later confirmed with the anatomical deformation observed in the postural evaluation.
- Geotaxis- It was measured using a 10 ×12cm grid, at an angle of 90 °. The animal was placed in the grid, in an inverted position. The time spent to reach the vertical position in one trial was measured.
- Exploratory activity - It was assessed through spontaneous exploration in the corner test. The mice were placed in a 27.5 x 9.5 cm transparent test box and observed for 1 minute. The latency to start the movement (taking as reference the hind legs' movement), the number of explorations (visited corners), the latency, and the rearing number were recorded. Defecation and urination were also considered.

Hepatic, splenic, and WAT indexes related to Sarcopenia

One hour after the behavioural evaluation, the animals were euthanized, and the organs and tissues (liver, spleen, WAT, and triceps surae muscle) were necropsied, weighed and preserved for future analysis. According to the 'sarcopenia index' (Edström & Ulfhake, 2005), it was recorded each animal's body weight together with the triceps surae muscle's weight without the calcaneal tendon to calculate sarcopenia in the animals. Second, the 'sarcopenia index' was adjusted by subtracting the larger volume organs' weight carcass without a liver, spleen, and WAT to control their weight as a confounding bias for 'bodyweight loss'. Therefore, the differences between the 'sarcopenia index' and our new measure, the 'carcass index', were verified. Besides, we have individually recorded the weight of organs and tissues to demonstrate differences between the groups.

Statistics

SPSS 15.0 software and the open-source programming language R software, version 4.0.3 was used for statistical analysis. The results are expressed as mean ± SEM. ANOVA and Bonferroni post hoc test evaluated differences among three independent groups. Finally, correlations were analysed with Pearson's correlation. Statistical significance was considered at $p < 0.05$.

Results

The physical frailty phenotype checklist included alopecia, loss of whiskers, kyphosis, piloerection, tremor, eye discharge and swelling, dermatitis and eczemas, wound and rectal prolapse. The incidence of these variables was indicative of the animals' end-point status (see table 1A). Incidence of kyphosis, characteristic of old ages [X^2, p=0.046 vs. adult mice], was found the most sensitive variable to show the difference between groups [Fisher exact test, per group, p=0.001], with the highest incidence in mice at the end-point [89%, 8/9 mice; Fisher's exact test (df 2), p = 0.002]. Besides, it was differentiated two levels of severity in kyphosis, 'postural' and 'structural'. In end-point animals, a higher prevalence

of structural kyphosis was observed, indicating greater severity in this variable [89%, 8/9 mice; Fisher's exact test (df 2), p = 0.001].
Incidence of piloerection was also specific of end-point [78%, 7/9 mice; Fisher's exact test (df 2), p = 0.004]. Despite being rare, wounds in the body may be present in adult [17%, 1/6 mice] and aging animals [14%, 1/7 mice] but were more frequent in end-point mice [56%, 5/9 mice]. Eye discharge and swelling [22%, 2/9 mice] and dermatitis/eczema [22%, 2/9 mice] had a low incidence and presented in the end-point group.
As illustrated in figure 1A, no statistically significant differences were found in the geotaxis, but a tendency to increase the speed to achieve the vertical geotaxis position was observed in old animals [adult, 3.3 ± 0.37 s; aging, 4.1 ± 0.44 s; end-point, 4.7 ± 0.86 s]. Similarly, despite kyphosis and piloerection distinctive frailty scores in old animals at end-point, the exploratory test (see figure 1B) indicated no differences between groups, except those age-dependent. An increased fearful response measured as a delay in the latency of the first rearing was shown in old groups compared with adult animals [p=0.042]. The exploratory activity, as measured by the horizontal and vertical ratios, was also age-dependent [p=0.07] but no differences were observed between both aged groups (see figure 1C). Age-dependent trend of changes in emotionality were shown as decreased defecation boli [adult, 1.3 ± 0.61 bolis; aging, 0.4 ± 0.20 bolis; end-point = 0.3 ± 0.17 bolis] and increased presence of urination [adult, 33.3% (2/6 mice); aging, 14.3% (1/7 mice); end-point, 66.7% (6/9) mice].
Figure 2 illustrates the bodyweight and different organ and tissues indexes. The *triceps surae* did not show differences in the groups [ANOVA, F (2,19) = 1.475, p = 0.25, *n.s.*] but a lower weight in the end-point animals [adult 30.4 ± 1.21g, aging 28.8 ± 0.6g end-point 27.8 ± 1.01g]. Significant WAT loss was found associated to old age [ANOVA, F (2,19) = 8.558, p=0.0022; *post-hoc* 16M end-point *vs.* 6M adult, p=0.002; 16M aging *vs.* 6M adult, p=0.026; both old groups *vs.* adult, p=0.001], [aging vs end-point p=0.232] but was not enough to differentiate 16M end-point animals from those with normal aging.
Also, this drop in visceral adipose tissue did not translate into body weight loss because the other visceral organs increased with old age, and more specifically with the end-point status. Thus, hepatomegaly [ANOVA, F (2,19) = 9.556, p=0,0013; *post-hoc* 16M end-point *vs.* 6M adult, p=0,0011] and splenomegaly [ANOVA, F (2,19) = 11.96, p=0,00043, *post-hoc* 16M end-point *vs.* 6M adult, p=0,0001; both 16M old groups *vs.* 6M adult, p=0.001] were found. Sarcopenia index [ANOVA, F (2,19) = 1.382, p=0.28, *n.s.*] and its corrected value excluding visceral organs [ANOVA, F (2,19) =1.247, p=0.28, *n.s.*] did not show group effects.
Table 1B depicts the correlation analysis between piloerection and structural kyphosis and as the physical frailty makers and the other weight and behavioural variables. Piloerection correlated with splenomegaly [r^2=0.390**, p=0.002], WAT loss [piloerection, r^2= -0.279**, p=0.004] and hepatomegaly [r^2=0.234*, p=0.023]. Structural-Kyphosis and piloerection were positively correlated [r^2=0.274*, p=0.012]. Structural-Kyphosis was related to hepatomegaly [r^2=0.245*, p=0.019], splenomegaly [r^2=0.184*, p=0.046] but not with WAT [r^2= -0.106, p=0.138]. Only kyphosis seemed to correlate with latency of rearing, albeit did not reach statistical significance [r^2= -0.176, p=0.052].
Figure 3 illustrates the correlation analysis to assess the contribution of the weight of organs (liver and spleen) and tissues (*triceps surae* and WAT) to the body weight. At a general level, a negative correlation with the spleen [r^2=-0.23*, p=0.024] and a positive correlation in WAT [r^2=0.449**, p=0.002] was found. If the body weight adjustment to carcass was carried out (Body weight - Weight of liver, spleen and WAT) correlations evidenced, per order of magnitude, WAT [r^2=0.43***, p=0.0008], spleen [r^2=0.27*, p=0.012], liver [r^2=0.22*, p=0.028] and *triceps surae* [r^2=-0.19*, p=0.045]. Regarding the correlation between weight and functional performance, a positive correlation in the latency of the first rearing correlated with the absolute [r^2=0.45*, p=0.048] and adjusted body weight [r^2=0.42=, p=0.05] in the 16M end-point animals.
Table 1C shows the correlations between behavioural performance, systemic phenotype, and carcass index. Thus, 'Carcass index' correlated with rearing latency in the end-point group [r^2=0.42*, p=0.05], and correlates positively with each of the variables of the systemic phenotype, and negative correlation with liver and spleen [liver r^2=-0.22*, p=0.028; spleen r^2=-0.27*, p=0.012], and positive with WAT and triceps surae [WAT r^2=0.43***, p=0.0008; spleen r^2=0.19*, p=0.045]. A positive correlation with WAT is observed in the adult group [r^2= 0.74*, p=0.028].

Discussion

The present work studied 16-month-old C57BL/6J mice who met the euthanisation criteria compared to age-matched mice with normal aging and 6-month-old adults. The results showed piloerection and structural kyphosis as their end-point physical frailty hallmarks. In contrast, increased latency of rearing indicated the poorest functional phenotype not justified by muscular loss since the weight of triceps surae was normal but could be suggestive as derived of their structural kyphosis. In addition, hepatomegaly and splenomegaly counteracted the impact that their significant WAT loss, commonly associated with age (Pappas & Nagy, 2019), should have on body weight. Therefore, organ indexes were calculated and body weight-adjusted to the 'carcass index' to find better indicators of sarcopenia for their end-point status. All of them correlated with piloerection and structural kyphosis.
Aging is characterized by a progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death (López-Otín et al., 2013). In humans, these mechanisms and their triggers have been studied in mice, with a special focus on frailty (Castillo-Mariqueo & Giménez-Llort, 2019; Schorr et al., 2018). Thus, in recent research, frailty has been highlighted as a simple and potentially useful indicator to predict animals' health status and mortality associated with biomarkers of aging (Zglinicki et al., 2016). The end-point status of old animals is defined by a clinical scenario where the severity of their pathologies generates significant functional limitations or compromises the animal's well-being. Causes of death are often not reported in studies in aged mice (Snyder et al., 2016), and the number of reports is lower when it comes to old animals with naturally occurring pathologies or injuries. In

survival studies of this colony, a sex factor has been evidenced with a worse survival rate in females but higher frailty in males (Muntsant et al., 2021).

Using our colony of C57BL/6J strain, the gold standard in experimental research and (Mekada et al., 2009), the physical frailty phenotype for end-point criteria was similar to that described in previous studies, with differences in the clinical incidence of some physical conditions. In this mice strain, Pettan-Brewer and Treuting (Pettan-Brewer and Treuting, 2011) showed that aged mice belonging to the University of Washington colony had four common clinical presentations: rectal prolapse, alopecia and dermatitis, eye lesions, and palpable masses. Besides, dying animals can be euthanized due to relatively nonspecific signs such as hunched over, cold to the touch with loss of body condition, and increased respiratory effort (Pettan-Brewer & Treuting, 2011). The presence of kyphosis and piloerection stood out as the frequent ones, mentioned as nonspecific signs in that study. Background C57BL/6J mice usually present dermatitis, one of the most commonly observed clinical problems in these mice (Burkholder et al., 2012). Generally, lesions that occur in dermatitis are due to pruritus-induced self-trauma, which progresses from superficial abrasions to deep ulcerations (Williams et al., 2012). This situation can explain wounds in the sample studied in the present work, where they were already present at six months and reached 56% in end-point animals. These ulcerative-like lesions of strain C57BL/6J may be a secondary result of strain-related behavioural characteristics (Williams et al., 2012).

Piloerection has been described as a sign of dehydration in animals when evaluating their health status (Burkholder et al., 2012). It is also described as an involuntary bristling of the coat as a reflex due to the activation of the sympathetic nervous system (Van Meer & Raber, 2005; Whitehead et al., 2014) as part of the evaluation criteria for frailty (Whitehead et al., 2014). At 17 months of age, males showed more deficits than females in piloerection, indicating more significant signs of frailty in C57BL/6J mice (Kane et al., 2019). In addition, these authors found frailty being linked to pro-inflammatory cytokines in a sex-specific manner.

Furthermore, the high incidence of kyphosis and alopecia exhibited by male mice at 16 months is similar to what was previously reported in this colony (Castillo-Mariqueo et al., 2021a; Castillo-Mariqueo & Giménez-Llort, 2021a; Castillo-Mariqueo & Giménez-Llort, 2021b; Castillo-Mariqueo & Giménez-Llort 2021c; Castillo-Mariqueo et al., 2021b). Also, in old female mice of this strain but at a higher age, the loss of body mass in senescence has been described and associated with the appearance of other characteristics of the aging phenotype, such as kyphosis, baldness, and loss of coat colour (Fahlström et al., 2011). Additionally, the Physical Frailty Phenotype distinguished the type of kyphosis that the animals presented and thus assigned a severity scale according to their anatomical and functional characteristics (Castillo-Mariqueo & Giménez-Llort, 2021b). Postural kyphosis refers to compensatory postural adjustments in response to increased visceral volume and disappears or attenuates during locomotion. In the case of 'structural kyphosis,' an anatomical change was found as an evolution of postural kyphosis with visible and unmodifiable deformation in locomotion. Interestingly, the end-point group showed greater severity in this variable, exhibiting a high prevalence of structural kyphosis. This result could indicate functional alterations that affect the gait and locomotion of the animals, as demonstrated in a previous study of male 3xTg-AD mice, where structural and postural kyphosis constitute a primary impairment that modifies stride and gait speed (Castillo-Mariqueo & Giménez-Llort, 2021b).

Interestingly, exploratory activity and geotaxis showed values that indicated preserved functional levels regardless of age and end-point status. Exploratory activity is associated with behavioural deficiencies in advanced ages, including motor skills (Shoji et al., 2016). However, certain behavioural domains of the mouse are preserved, as is the case with exploratory activity similar to adult mice in Fahlström's study in females (Fahlström et al., 2011). . Also, neophobia in the corner test showed a low latency of movement and shows the fear to the novelty, expressed of freezing, was almost non-existent in adult animals and slightly increased at 16 months in the aging and end-point groups, previously reported (Baeta-Corral & Giménez-Llort, 2014; Castillo-Mariqueo & Giménez-Llort, 2021a; Gimenez-Llort & Alveal-Mellado, 2021; Torres-Lista et al., 2019; Castillo-Marique et al., 2021b). The progressive reduction of skeletal muscle can result from normal aging without an underlying pathological process, although many chronic diseases can accelerate muscle loss (Roubenoff, 2000a; Roubenoff, 2000b). In the present work, the body weight and the weight of the triceps surae muscle did not show differences between the groups. However, it was possible to observe a trend towards decreasing in these values in old animals, with less than 15% body mass. An element of complexity in this scenario is the heterogeneity of aging. Thus, studies with samples of older animals (males 21 - 25 months) have shown that the muscle mass of the hind limb is lower compared to 10-month-old mice, with a significant decrease in daily physical activity and the strength of muscle grip (van Dijk et al., 2017). Therefore, since the 16-month-old animals presented alterations in functional performance related to increased latency of rearing, it was explored if the loss of muscle mass or kyphosis could explain the poor functional performance. The correlation analysis suggested that structural kyphosis could be related to the reduced latency to perform the first rearing in end-point mice. Their total vertical and horizontal exploration in the corner test did not differ from that exhibited by age-matched counterparts with normal aging.

Geotaxis is a widely used test to measure sensorimotor milestones at the postnatal level in mice (Abramov et al., 2012; Arakawa & Erzurumlu, 2015), also included in the primary screening of adult animals (Rogers et al., 1997). It corresponds to an innate response to gravitational signals that require vestibular and motor coordination to orient the body uphill in an angled plane (Thiessen & Lindzey, 1967). We have also proposed geotaxis as a functional test to recognize deterioration at this level in old animals (Castillo-Mariqueo & Giménez-Llort, 2021a). The test requires the constant support of the body by the extremities and a coordinated body balance so that the mouse can rotate its entire body on the declined surface (Thiessen & Lindzey, 1967). Although an increase in turning time in the old animals was detected, the differences did not reach statistical significance. Moreover, halftime did not exceed 5 seconds in all groups and was not dependent on body weight, indicating

optimal functionality at this sensorimotor level. For comparison, a recent study showed the influence of body weight on the performance of this proof, where at the age of 13 months, overweight animals took about 15 seconds to complete the test (Castillo-Mariqueo & Giménez-Llort, 2021a).

Liver and spleen organs presented statistically significant differences in the old group with end-point status, showing an increase in the weight of these organs. Previously in old C57BL/6J mice, an increase in the weight of several systemic organs, namely, the liver, heart, kidney, and spleen, have been shown (Lessard-Beaudoin et al., 2015). These findings also coincide with other strains of mice, such as B6C3F1, where the same phenomenon is observed (Marino, 2012). The liver has reported its weight gain up to 23-28 months, and a decrease is only observed in ancient mice (Lessard-Beaudoin et al., 2015). These organs are relevant in metabolic, inflammatory, and degenerative processes (Jonker et al., 2013). Thus, spleen weight can indicate alterations in cell number and distribution (Turner & Mabbott, 2017), causing the immune system to be seriously compromised as age increases (Jonker et al., 2013). Older female C57BL/6J mice have been reported to have an increased spleen precisely (Menees et al., 2021), and we have also reported splenomegaly mainly associated with female sex and exacerbated in the 3xTg-AD mice, an animal model of Alzheimer's disease (Giménez-Llort et al., 2008).

In the case of WAT, both the aging and end-point status groups showed a decrease in the weight of this enteric fatty tissue, being significant in both cases. The body fat percentage is constant in C57BL/6J mice until six months of age, then increases between 6 and 12 months (Glatt et al., 2007). Its decrease in older ages is associated with cachexia, accompanied by decreased body weight (Morton & Griffiths, 1985). Besides, we have detected a negative correlation in the spleen and a positive correlation in WAT concerning body weight. If the bodyweight adjustment to estimate the weight of carcass is made (bodyweight without liver, spleen, and WAT), these correlations are also evidenced in the liver and *triceps surae* with a negative and positive relationship. These findings coincide with Lessard-Beaudoin's (Lessard-Beaudoin et al., 2015) in the liver but differ from the spleen.

The clinical parameters and end-point criteria are essential to determine the point at which the animals will be sacrificed, making their use relevant based on the body condition and behaviour of the animals (Toth, 2018). In this sense, we have not detected functional impediments or sarcopenia through indirect measures in animal end-points. In turn, to diagnose underlying diseases such as vascular, inflammatory, and degenerative, gross *in vivo* determination of underlying pathologies of these animals is required, but their confirmation through histological studies carried out after necropsy is also necessary (Pettan-Brewer & Treuting, 2011).

This study provides a physical phenotype of findings in male C57BL/6J mice that match end-point criteria within the animal welfare regulations. Thus, it was detected that despite presenting physical alterations at an age that are considered aged animals, an optimal geotaxis and exploratory activity is maintained in the animals, similar to its counterpart that is considered with normal aging. Moreover, the necropsy of organs and tissues had been carried out, it was verified alterations in the weight of the liver and spleen organs, with hepatic and splenomegaly, and significant loss of the white adipose tissue. However, no alterations at the muscular level in the case of the triceps surae muscle were found, which maintained its weight concerning adult animals of the same strain. These data expand our understanding of the anatomical changes that occur with aging and provide reference values for further studies in C57BL/6J mice, complementary to those few reported in the literature. These observations also offer the potential to explore the effects of interventions targeting sarcopenia in older mice. Thus, the combination of studies in pathology with in vivo data will fully characterize the effect of proven interventions in multiple chronic diseases and the health of aged mice with a better translation to human aging and age-associated injuries.

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Table 1. Physical frailty phenotype and Correlation analysis

A. Physical frailty phenotype

Physical conditions	6M Adult (n=6)	16M Aging (n=7)	16M End-point (n=9)	p value
Alopecia	2 (33%)	5 (71%)	4 (44%)	n.s.
Loss of whiskers	-	1 (14%)	1 (11%)	n.s.
Piloerection	-	1 (14%)	7 (78%)	**, #
Kyphosis	-	3 (43%)	8 (89%)	**, #
Postural	-	2 (29%)	-	n.s.
Structural	-	1 (14%)	8 (89%)	**, #
Tremor	-	-	1 (11%)	n.s.
Eye discharge/swelling	-	-	2 (22%)	n.s.
Dermatitis/eczema	-	-	2 (22%)	n.s.
Wound (face, nose, or periorbital)	1 (17%)	1 (14%)	5 (56%)	n.s.
Rectal prolapse	-	-	1 (11%)	n.s.

B. Correlation analysis between the hallmarks of the physical frailty phenotype at end-point and the behavioural and the systemic phenotypes

Correlations	Kyphosis	Structural-Kyphosis	Piloerection
Behaviours			
90° geotaxis (s)	R^2 = 0,004 p= 0,773	R^2 = 0,0003 p= 0,936	R^2 = 0,166 p= 0,060
Corners (n)	R^2 = (-) 0,089 p= 0,177	R^2 = (-) 0,013 p= 0,601	R^2 = (-) 0,002 p= 0,812
Rearing (n)	R^2 = (-) 0,008 p= 0,682	R^2 = 0,002 p=0,811	R^2 = 0,004 p= 0,767
Rearing latency (s)	R^2 = 0,055 p= 0,291	R^2 = 0,002 p= 0,818	R^2 = 0,131 p= 0,098
Systemic Phenotype			
Liver (g)	R^2 = 0,261 p= 0,015 *	R^2 =0,245 p= 0,019 *	R^2 = 0,234 p= 0,023 *
Spleen (g)	R^2 = 0,137 p= 0,089	R^2 = 0,184 p= 0,046 *	R^2 = 0,380 p= 0,002 **
WAT (g)	R^2 = (-) 0,176 p= 0,052	R^2 = (-) 0,106 p= 0,138	R^2 = (-) 0,279 p= 0,004 **
Triceps surae (g)	R^2 = (-)0,003 p= 0,780	R^2 = (-) 0,132 p= 0,096	R^2 = (-)0,145 p= 0,080
Physical Frailty Genotype			
Structural-Kyphosis	-	-	R^2 = 0,274 P= 0,012*

C. Correlation analysis between carcass index and behavioural and the systemic phenotype

Correlations	Young (n=6) 6 months	Aging (n=7) 16 months	End-point (n=9) 16 months	General p value
Behavioural Phenotype				
90° geotaxis (s)	R^2 = 0.4 p=0.18	R^2 = 0.13 p=0.43	R^2 = 0.3 p=0.13	R^2 = 0.13 p=0.090
Corners (n)	R^2 = 0.26 p=0.26	R^2 = 0.29 p=0.21	R^2 = 0.046 p=0.58	R^2 = 0.0025 p=0.82
Rearing (n)	R^2 = 0.011 p=0.84	R^2 = 0.026 p=0.73	R^2 = 0.2 p=0.23	R^2 = 0.009 p=0.24
Rearing latency (s)	R^2 = 0.11 p=0.52	R^2 = 0.027 p=0.72	R^2 = 0.42 p=0.05*	R^2 = 0.16 p=0.060
Systemic Phenotype				
Liver	R^2 = 0.084 p=0.56	R^2 = 0.27 p=0.23	R^2 = (-)0.44 p=0.051	R^2 = (-) 0.22 p=0.028*
Spleen	R^2 = 0.51 p=0.11	R^2 = (-)0.22 p=0.29	R^2 = (-)0.17 p=0.27	R^2 = (-) 0.27 p=0.012*
WAT	R^2 = 0.74 p=0.028*	R^2 = 0.053 p=0.62	R^2 = 0.38 p=0.078	R^2 = 0.43 p=0.00087***
Triceps surae	R^2 = 0.091 p=0.64	R^2 = 0.22 p=0.29	R^2 = 0.4 p=0.067	R^2 = 0.19 p=0.045*

Statistics: Table 1A, Fisher's exact test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ used for group differences (adult, aging and end-point). X^2, # $p < 0.05$ and ## $p < 0.01$, ### $p < 0.001$ used for age differences (adult *vs.* old). Table 1B-1C, Pearson r correlations test, * $p \leq 0.05$, ** $p < 0.01$ and *** $p < 0.001$ used for group differences.

Figure 1. Geotaxis and exploratory activity

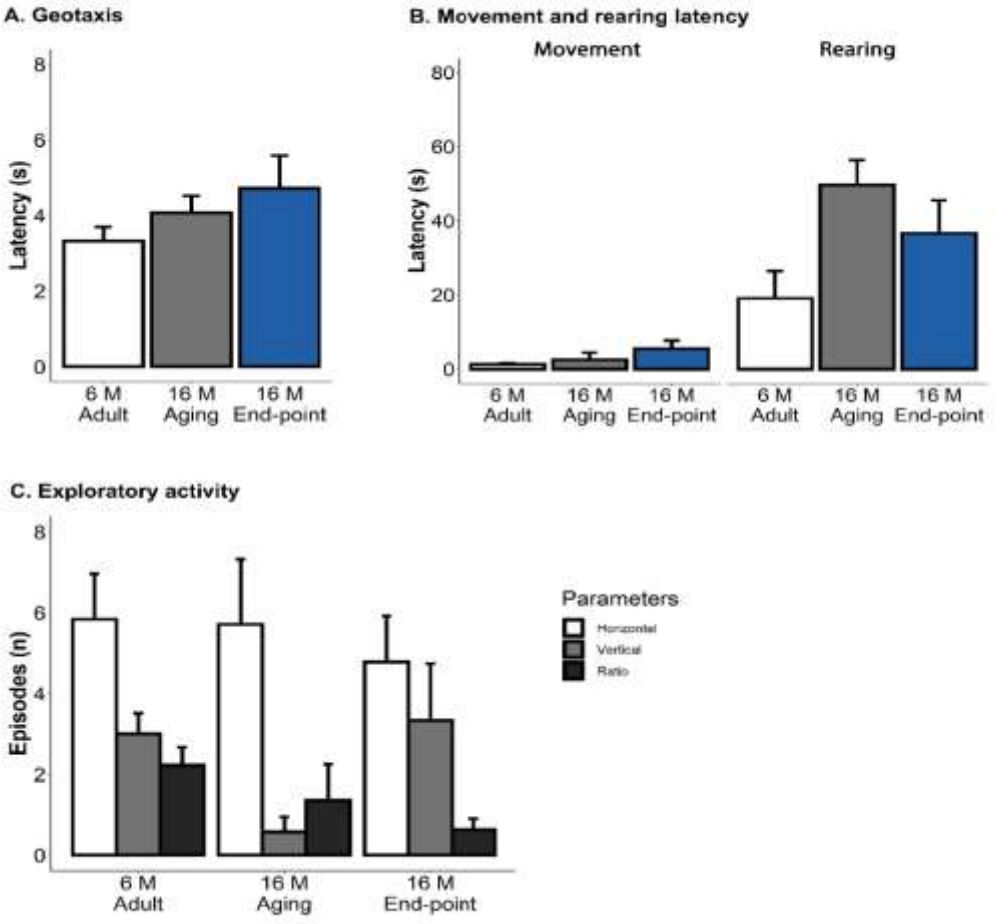

Figure 1. Geotaxis, latency of movement and rearing, and exploratory activity. The results are expressed as mean ± SEM. (A) Geotaxis. (B) Movement and rearing latency. C) Exploratory activity. Statistics: Student's t-test * $p<0.05$ and ** $p<0.01$, **** $p < 0.001$ adult 6M *vs*. both old aged 16M.

Figure 2. Hepatic, splenic, and WAT indexes related to Sarcopenia

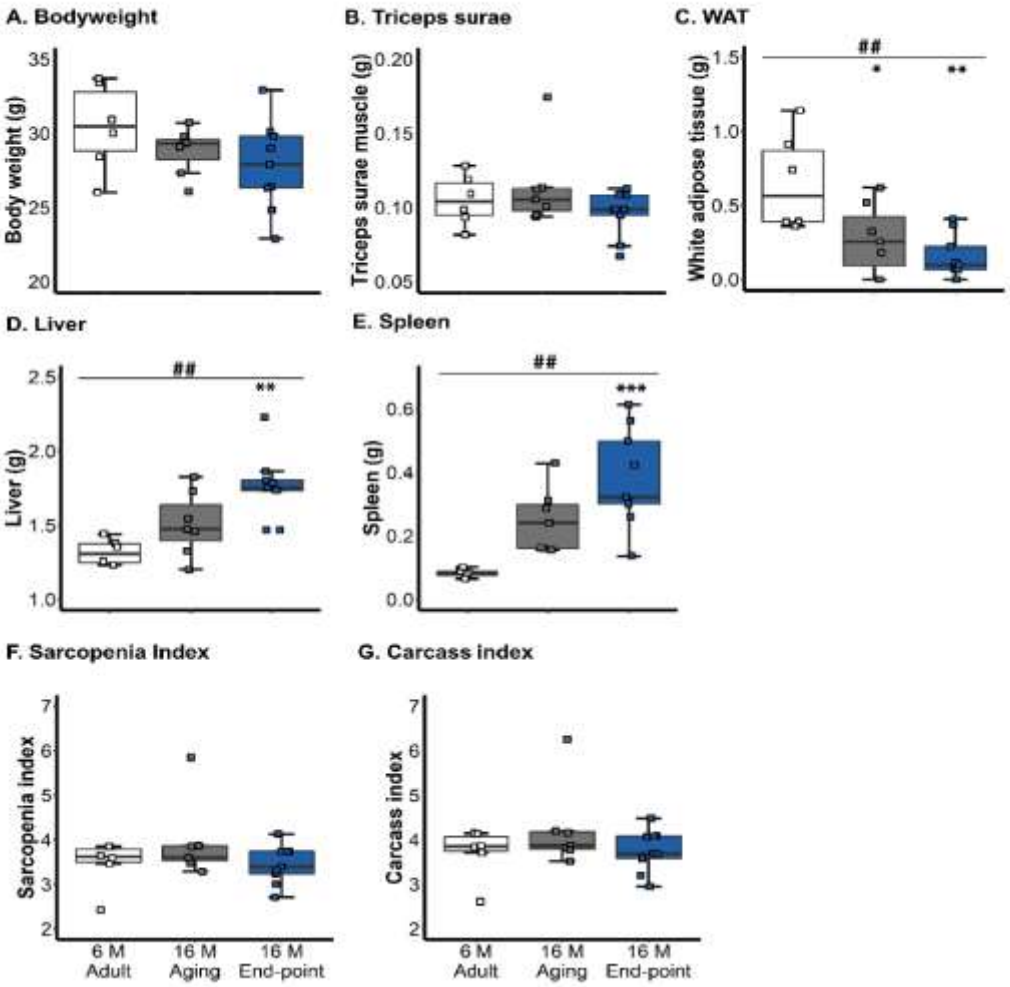

Figure 2. Hepatic, splenic, and WAT indexes related to Sarcopenia. Results are expressed as mean ± SEM. (A) Body weight; (B) *Triceps surae*; (C) WAT (D) Liver; (E) Spleen; (F) Sarcopenia index; (G) Carcass index. Statistics: One-way ANOVA followed by *post-hoc* Bonferroni test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Student's *t*-test $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ adult 6M vs both old aged 16M.

Figure 3. Correlation analysis between body weight and organ or triceps sural muscle in C57BL/6 male mice

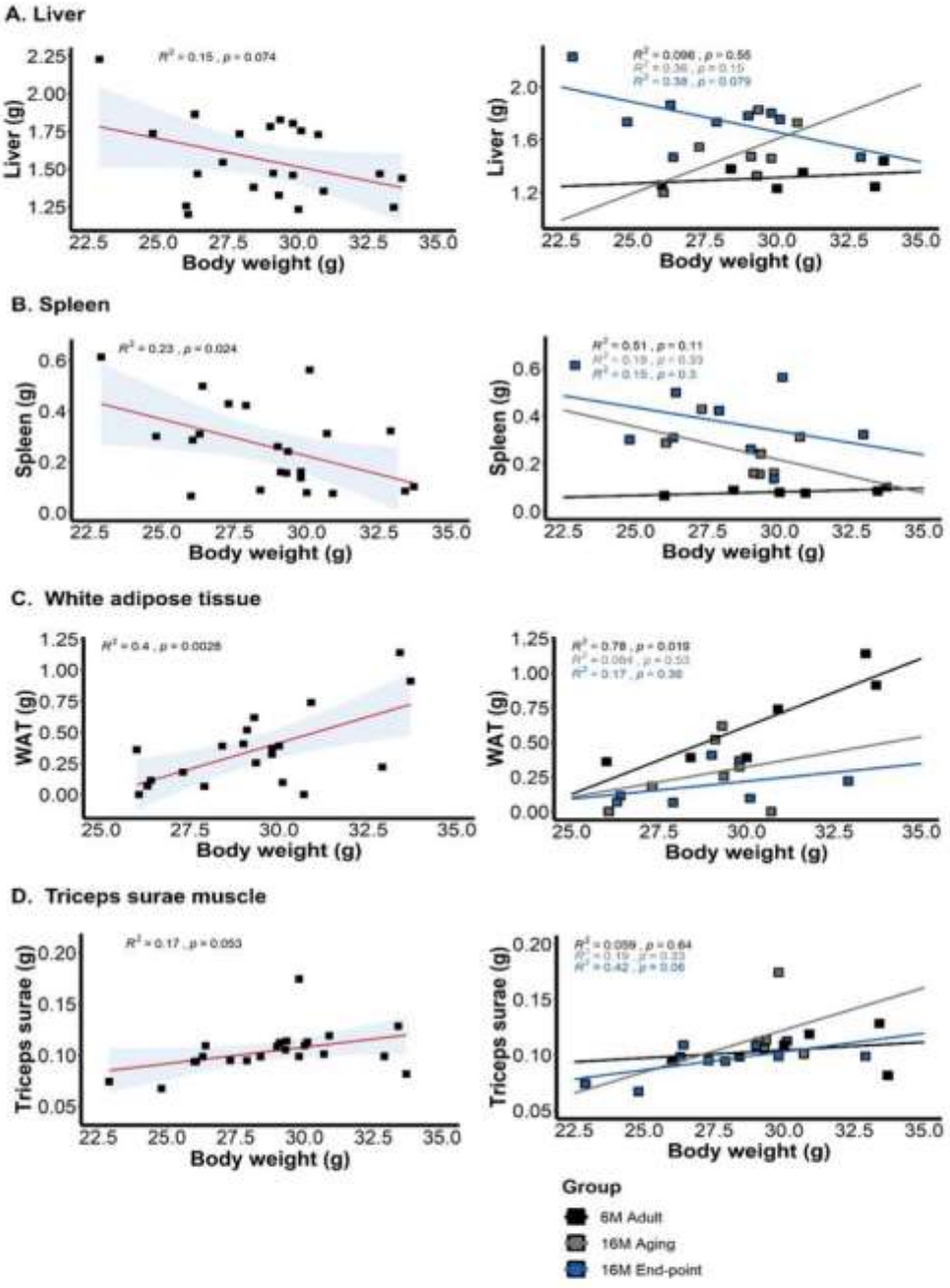

Figure 3. Correlation analysis between body weight and weight of organs (liver and spleen) and tissues (*triceps surae* and WAT) in C57BL/6J male mice. Meaningful, significant Pearson r correlations between body weight and (A) Liver, (B) Spleen, (C) White adipose tissue, (D) Triceps surae muscle. Statistics: Pearson r^2, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

420 - Translational modeling of psycho-motor function in normal and pathological aging with special concerns on the effects of isolation

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Time factor and human support are major constrains in the management of the COVID-19 pandemic and they enhance the challenges to adapt the lifestyles and intervention programs, with greater impact on the elderly people, especially those who are the most physically or cognitively frail. The restrictive confinement and the closing of the day centers has left those whose frailty requires permanent rehabilitation programs at home. In the case of Alzheimer's disease (AD) and other dementias, non-professional home care may not be enough to cover the needs and demands of these complex disorders. On the other hand, as elder people, these patients can be particularly affected by social isolation, which can cause changes in behavior and decrease functional performance in the basic activities of daily life, worsening their BPSD and cognitive impairment. In this context, and under the gaze of normal and pathological aging, we are developing a functional model of psycho-motor evaluation that allows us to study psycho-motor function, including motor learning and memory. Its translational value relays in the modeling of tests used in clinical settings. Here we present the very first results. We have selected the gold standard C57BL/6 mice together with the triple transgenic model of AD (3xTg-AD) to apply our psycho-motor protocol. We have included a series of measurements that make possible to differentiate several dimensions of basal motor learning, and the learning associated with fragile situations. We have found common as well as distinctive features between the sample of normal and AD-pathological aging, and under the isolation scenario. Among all, we can highlight the gender factor and the level of physical activity as a protective mechanism when indicators of frailty are present. Particularly, the 3xTg-AD mice show greater deterioration in physical aspects, but they retain their motor learning capacity comparable to the controls. On the other hand, higher performance in tests of exercise tolerance and muscle strength stand out in these mice, where genotype and gender appear to be determinant factors in overall physical performance: This generates new hypotheses of underlying biological protection mechanisms in translational scenarios relevant for the rehabilitation of geriatric and AD-patients.

Translational modeling of psycho-motor function in normal and pathological aging with special concerns on the effects of isolation

2020 IPA VIRTUAL CONGRESS

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INTRODUCTION

Time factor and human support are major constrains in the management of the COVID-19 pandemic and they enhance the challenges to adapt the lifestyles and intervention programs, with greater impact on the elderly people, especially those who are the most physically or cognitively frail. The restrictive confinement and the closing of the day centers has left those whose frailty requires permanent rehabilitation programs at home. In the case of Alzheimer's disease (AD) and other dementias, non-professional home care may not be enough to cover the needs and demands of these complex disorders. On the other hand, as elder people, these patients can be particularly affected by social isolation, which can cause changes in behavior and decrease functional performance in the basic activities of daily life, worsening their BPSD and cognitive impairment.

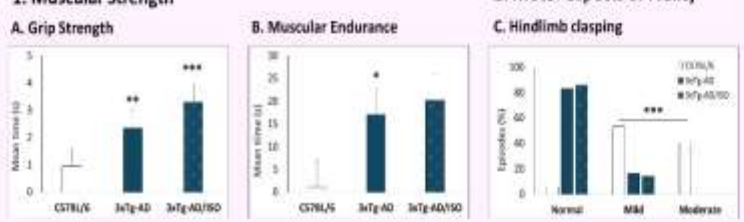
METHODS

Forty-six, 15-month-old male 3xTg-AD mice with their respective controls (C57BL/6) were used and 3 experimental groups were formed: C57BL/6 (control), 3xTg-AD (Alzheimer's disease) and 3xTg-AD/ISO (isolated). A psycho-motor protocol was applied that including motor learning and memory as well as a series of measurements that make possible to differentiate several dimensions of basal motor learning, and the learning associated with fragile situations. Statistical Analyses were performed using SPSS 23.0 software. Results were expressed as the mean ± standard error of the mean (SEM) for each task and trial. The effect of the factors was studied with the statistics: ANOVA, MRA and Chi square. The magnitude of the association was measured with Bonferroni, Duncan and Q contingency coefficient. In all cases, p 0.05 was considered statistically significant.

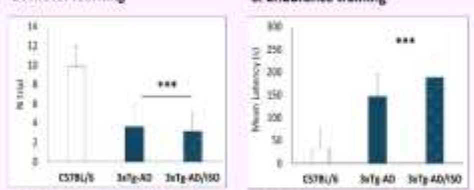
RESULTS

1. Muscular Strength

2. Motor aspects of Frailty



3. Motor performance



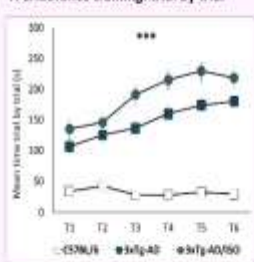


Figure 1, 3. Quantitative analysis muscular strength (Grid suspension, panel A, B) and motor performance (panel D, E,F - Rotarod: learning trials, 10RPM for 1 minute; 6 training trials, 5 to 40RPM for 6 minutes). **Figure 2.** Qualitative analysis of motor aspects of frailty(panel C). Data expressed by mean of episodes, latency and time, total number of episodes or animals (n), episodes (%). Statistics: ANOVA, MRA, X², *p<.05, **p<.01, ***p<.001.

4. Gait analysis

Figure 4. Gait analysis in progress. Quantitative and qualitative aspects of the steps, stride, base of support will be differentiated as well as the speed and trajectory that the mouse performs within its box without sawdust. (1) C57BL/6, (2) 3xTg-AD, (3) 3xTg-AD/ISO. KINOVEA Software.

CONCLUSIONS

We have found common as well as distinctive features between the sample of normal and AD-pathological aging, and under the isolation scenario. Among all, we can highlight the gender factor and the level of physical activity as a protective mechanism when indicators of frailty are present. Particularly, the 3xTg-AD mice show greater deterioration in physical aspects, but they retain their motor learning capacity comparable to the controls. On the other hand, higher performance in tests of exercise tolerance and muscle strength stand out in these mice, where genotype appear to be determinant factors in overall physical performance: This generates new hypotheses of underlying biological protection mechanisms in translational scenarios relevant for the rehabilitation of geriatric and AD-patients.

The present study is in the preliminary data analysis phase, so no differences have yet been registered with respect to gender and the isolation in the control group, in the coming months it is expected to complete the experimental protocol.

REFERENCES

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