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Survival, Behavioral And Food Find Test's Olfactory Signatures In Male and Female Mice with Alzheimer's disease and Normal Aging Under Naturalistic and Forced Social Isolation

Daniela Marín Pardo

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Ph.D. Dissertation presented by

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"El cuerpo hace lo que quiere. Yo soy el cuerpo, pero soy también la mente. Me he permitido ser siempre la que soy.

Rita Levi-Montalcini

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Abbreviations

AD Alzheimer's disease.

APP Amyloid precursor protein.

BPSD Behavioral and psychological symptoms associated to dementia.

DNC Neurocognitive disorders.

PS1 Presenilin-1.

ON Neurofibrillar tangles.

ROS Reactive oxygen species.

DNF Neurofibrillar degeneration.

Aβ Peptide β – amyloid.

Corner test

CT Corner Test.

CTc Total corners visited.

CTLatR Latency of the first vertical exploration (Rearing).

CTR Number of vertical explorations.

Open field

OF Open field.

OFLatm First Move Latency.

OFLatc Time to leave the center point.

OFLatp Time to enter the periphery.

OFLatR Latency of the first exploratory ertical survey (rearings).

OFLatG Self-care latency, Grooming.

R Number of vertical exploratory surveys.

OFTC Total crosses in the open field.

Cc1 Crossing in the center during the first minute.

Cc 2 Crossing in the center during the second minute.

Cc 3 Crossing in the center during the third minute.

Cc 4 Crossing in the center during the fourth minute.

Cc 5 Crosses in the center during the fifth and final minute.

Cp1 Crosses on the periphery during the first minute.

Cp2 Crosses on the periphery during the second minute.

Cp3 Crosses on the periphery during the third minute.

Cp4 Crosses on the periphery during the fourth minute.

Cp5 Crosses on the periphery during the fifth and last minute.

Cc2'1 Crosses in the center during the first minute in the second triial.

Cp2'1 Crosses in the periphery during the first minute in the second trial.

Object Recognition

N New.

F Familiar.

T-maze

TM T-Maze.

TMA1A2 Time to explore both arms.

TMLatT Time required to start exploring the arms.

TMLatEnd Time required to finish the test.

TMRTArmsErrors Number of errors made when visiting arms that have already been visited previously.

R Number of vertical exploratory surveys.

Dark and light box

DLB Dark Light Box.

D1_latGir Time to turn at the starting of the test, on day 1.

D2_latGir Time to turnat the starting of the test, on day 2.

D1_latR Latency of the first exploratory vertical survey (rearings) day 1.

D2_latR Latency of the first exploratory vertical survey (rearings) day 2.

D1latX Latency of body entry to the light compartment, on day 1.

D2latX Latency of body entry to the light compartment, on day 2.

D1latCX Latency of head entry to the light compartment, on day 1.

D2latCx Latency of head entry to the light compartment, on day 2.

D1R Number of vertical exploratory surveys in dark compartment, on day 1.

D2R Number of vertical exploratory surveys in dark compartment, on day 2.

D1RL Number of vertical exploratory surveys in light compartment, on day 1.

D2RL Number of vertical exploratory surveys in light compartment, on day 2.

D1TCC Total crosses in the dark compartment, on day 1.

D2TCC Total crosses in the dark compartment, on day 2.

TCL1 Total crosses in the light compartment, on day 1.

D2TCCL2 Total crosses in the light compartment, on day 2.

D1TOT_R Total vertical exploratory surveys in DLB, on day 1.

D2TOT_R Total vertical exploratory surveys in DLB, on day 2.

D1TTCC Total crosses in DLB on day 1.

D1TTCC Total crosses in DLB on day 2.

D1TX1D Tolat number of body entries in DLB on day 1.

D2TX Tolat number of body entries in DLB on day 2.

D1TCX1D Tolat number of head entries in DLB on day 1.

D2TCX Tolat number of head entries in DLB on day 2.

TX(light)D1 Total time spent in the light compartment on day 1.

TX(light) D2 Total time spent in the light compartment on day 1.

Food finding test

FFT Food Finding Test.

LatSniffing when the nose makes a direct contact with a surface and the vibrissae are in maximum extension and are in contact with the surface in the same way.

LatFinding “finding a food pellet” was defined as digging, touching, and holding the pellet in the front paws for more than 3 s.

LatEating Eating the hidden food holding the pellet in the front paws for more than 3 s of continuous eating.

Abstract

The latest evidence highlights the significant inter-individual variability observed in both normal aging and Alzheimer's disease (AD), along with elevated mortality rates and the urgent need for specific end-of-life prognosticators. Nevertheless, the investigation of such heterogeneity remains constrained in very old male AD mouse models due to their diminished survival rates. In this comprehensive investigation, using 915 (432 APP23 and 483 C57BL/6 littermates) mice, we confirmed the better survival curves in male than female APP23 mice and respective wildtypes, providing the chance to characterize behavioral signatures in middle-aged, old, and long-lived male animals. The sensitivity of a battery of seven paradigms for comprehensive screening of motor (activity and gait analysis), neuropsychiatric and cognitive symptoms was analyzed using a cohort of 56 animals spanning 12-, 18-, and 24-month-old male APP23 mice and wildtype littermates, revealed age-related differences in several variables. It is noteworthy that variables associated with stress coping, thigmotaxis, frailty, gait abnormalities, and cognitive impairment effectively distinguished the behavioral phenotype of male APP23 mice across the three age groups when compared to controls. Our findings revealed non-linear age- and genotype-dependent behavioral patterns in long-lived animals, indicating the presence of intricate interactions between chronological and biological/behavioral aging processes. Moreover, the temporal trajectory and severity of sensory system involution during aging are of critical importance in perceiving and recognizing the world. Sensory impairments have been demonstrated to significantly increase the risk of biological, psychological, and social decline in older individuals. Olfactory loss is regarded as an early biomarker in Alzheimer's disease neurodegeneration. To examine olfactory function in both typical and AD-related aging, we analyzed olfactory ethograms in middle-aged male and female C57BL/6 and 3xTg-AD mice, a genetic model of AD with cognitive impairment and a notable neuropsychiatric-like phenotype. A one-day food deprivation paradigm was employed to analyze ethological patterns in olfactory inspection and food-seeking behaviors. The results revealed that AD exhibited distinct olfactory signatures that differed from those observed in normal aging. Notably, male 3xTg-AD mice displayed an early olfactory signature compared to females, which differed from the patterns observed in control mice. Furthermore, social isolation accentuated olfactory

patterns and disrupted behavioral correlates, particularly in male 3xTg-AD mice. Lastly, the Food Finding Test (FFT) olfactory paradigm was employed to examine olfaction in aged animals, without overnight food deprivation. An ethogram of three goal-directed behaviors directed towards hidden food was meticulously analyzed in male and female 3xTg-AD mice and age-matched C57BL/6 counterparts. This new protocol demonstrated sensitivity to sex, genotype, and social context, particularly in males and AD mice, with isolation-inducing modifications in the ethogram and functional correlations. In light of these considerations, a comprehensive evaluation of the behavioral phenotypes was conducted in 3xTg-AD mice, a widely utilized transgenic model for Alzheimer's disease (AD). A range of behavioral assessments were employed, including the Corner Test, Open-Field Test, T-maze, Dark-Light Box Test, and Food Finding Test, in order to investigate the cognitive and emotional deficits characteristic of these mice. The results indicate significant age-dependent cognitive impairments and anxiety-like behaviors, including increased neophobia, reduced locomotor activity, and delayed food-seeking responses. It is noteworthy that the study also identified sex differences, with female 3xTg-AD mice displaying more pronounced deficits compared to males. These findings align with human epidemiological data and highlight the need for sex-specific therapeutic approaches. The observed correlations between various behavioral measures suggest that there are interconnected cognitive and emotional dysfunctions, which provide a deeper understanding of AD pathology. In sum, Recent research underscores the substantial inter-individual variability observed in both normal aging and Alzheimer's disease (AD), underscoring the necessity for the development of specific end-of-life prognosticators. The objective of this study was to investigate behavioral differences across age and genotype in APP23 transgenic and C57BL/6 littermates. The male APP23 mice exhibited superior survival rates compared to females and non-transgenic mice, thereby revealing disparate age-related behavioral patterns, particularly in the domains of stress coping, frailty, and cognitive impairment. The olfactory function of the 3xTg-AD and NTg mice was evaluated using the Food Finding Test (FFT), which revealed the presence of early olfactory alterations in the male AD mice, which were further exacerbated by social isolation. A comprehensive behavioral assessment revealed significant cognitive and emotional deficits in 3xTg-AD mice, with females exhibiting more pronounced symptoms than males. These findings are consistent with data from human subjects, thereby underscoring the necessity for sex-specific therapeutic interventions. These

findings contribute to our understanding of Alzheimer's disease pathology and suggest the potential value of olfactory testing as an early diagnostic tool.

Resumen

Las pruebas más recientes ponen de relieve la importante variabilidad interindividual observada tanto en el envejecimiento normal como en la enfermedad de Alzheimer (EA), junto con las elevadas tasas de mortalidad y la urgente necesidad de contar con pronosticadores específicos del final de la vida. Sin embargo, la investigación de esta heterogeneidad sigue estando limitada en modelos de ratones macho muy viejos con EA debido a sus reducidas tasas de supervivencia. En esta investigación exhaustiva, examinamos 915 ratones (432 APP23 transgénicos y 483 C57BL/6 compañeros de camada) para confirmar las tasas de supervivencia superiores observadas en los ratones APP23 macho en comparación con las hembras y sus respectivas contrapartes de tipo salvaje. Esto permitió la caracterización de firmas conductuales en animales machos de mediana edad, viejos y longevos. El análisis de una batería que comprendía siete paradigmas, que abarcaban la función motora (análisis de la actividad y la marcha), síntomas neuropsiquiátricos y deterioro cognitivo en un subconjunto de 56 animales que abarcaban ratones APP23 macho de 12, 18 y 24 meses de edad y compañeros de camada de tipo salvaje, reveló diferencias relacionadas con la edad en varias variables. Cabe destacar que las variables asociadas con el afrontamiento del estrés, la trigmotaxis, la fragilidad, las anomalías de la marcha y el deterioro cognitivo distinguieron eficazmente el fenotipo conductual de los ratones APP23 macho en los tres grupos de edad en comparación con los controles. Nuestros hallazgos revelaron patrones conductuales no lineales dependientes de la edad y el genotipo en animales longevos, lo que indica la presencia de intrincadas interacciones entre los procesos de envejecimiento cronológico y biológico/conductual. Además, la trayectoria temporal y la gravedad de la involución del sistema sensorial durante el envejecimiento son de vital importancia para percibir y reconocer el mundo. Se ha demostrado que las deficiencias sensoriales aumentan significativamente el riesgo de deterioro biológico, psicológico y social en las personas mayores. La pérdida olfativa se considera un biomarcador temprano en la neurodegeneración de la enfermedad de Alzheimer. Para examinar la función olfativa tanto en el envejecimiento típico como en el relacionado con la EA, analizamos etogramas olfativos en ratones C57BL/6 y 3xTg-AD machos y hembras de mediana edad, un modelo

genético de EA con deterioro cognitivo y un notable fenotipo de tipo neuropsiquiátrico. Se empleó un paradigma de privación de alimentos durante un día para analizar patrones etológicos en la inspección olfativa y los comportamientos de búsqueda de alimentos. Los resultados revelaron que la EA presentaba firmas olfativas distintas de las observadas en el envejecimiento normal. En particular, los ratones macho 3xTg-AD mostraban una firma olfativa más precoz que las hembras, que difería de los patrones observados en los ratones control. Además, el aislamiento social acentuó los patrones olfativos y alteró los correlatos conductuales, particularmente en los ratones machos 3xTg-AD. Por último, se empleó el paradigma olfativo Food Finding Test (FFT) para examinar la olfacción en animales envejecidos, sin privación de alimento durante la noche. Se analizó meticulosamente un etograma de tres comportamientos dirigidos hacia alimentos ocultos en ratones 3xTg-AD machos y hembras y en ratones C57BL/6 de la misma edad. Este nuevo protocolo demostró sensibilidad al sexo, genotipo y contexto social, particularmente en machos y ratones AD, con modificaciones inducidas por el aislamiento en el etograma y correlaciones funcionales. A la luz de estas consideraciones, se llevó a cabo una evaluación exhaustiva de los fenotipos conductuales en ratones 3xTg-AD, un modelo transgénico ampliamente utilizado para la enfermedad de Alzheimer (EA). Se emplearon diversas evaluaciones conductuales, como la prueba de la esquina, la prueba del campo abierto, el laberinto en T, la prueba de la caja oscura y luminosa y la prueba para encontrar comida, con el fin de investigar los déficits cognitivos y emocionales característicos de estos ratones. Los resultados indican deficiencias cognitivas significativas dependientes de la edad y comportamientos similares a la ansiedad, incluyendo un aumento de la neofobia, una reducción de la actividad locomotora y un retraso en las respuestas de búsqueda de comida. Cabe destacar que el estudio también identificó diferencias de sexo, ya que las hembras de ratones 3xTg-AD mostraban déficits más pronunciados que los machos. Estos resultados coinciden con los datos epidemiológicos humanos y ponen de relieve la necesidad de enfoques terapéuticos específicos para cada sexo. Las correlaciones observadas entre diversas medidas conductuales sugieren que existen disfunciones cognitivas y emocionales interconectadas, lo que aporta una comprensión más profunda de la patología de la EA. En resumen, investigaciones recientes subrayan la sustancial variabilidad interindividual observada tanto en el envejecimiento normal como en la enfermedad de Alzheimer (EA), lo que subraya la necesidad de desarrollar pronosticadores específicos del final de la vida. El objetivo de este estudio era investigar las diferencias conductuales en función de la

edad y el genotipo en ratones transgénicos APP23 y en compañeros de camada C57BL/6. Los ratones machos APP23 mostraron un comportamiento similar al de los ratones transgénicos. Los ratones machos APP23 mostraron tasas de supervivencia superiores en comparación con las hembras y los ratones no transgénicos, revelando así patrones de comportamiento dispares relacionados con la edad, particularmente en los dominios de afrontamiento del estrés, fragilidad y deterioro cognitivo. La función olfativa de los ratones 3xTg-AD y NTg se evaluó mediante el Test de Búsqueda de Alimentos (Food Finding Test, FFT), que reveló la presencia de alteraciones olfativas tempranas en los ratones machos AD, que se agravaron aún más con el aislamiento social. Una evaluación exhaustiva del comportamiento reveló importantes déficits cognitivos y emocionales en los ratones 3xTg-AD, y las hembras presentaban síntomas más pronunciados que los machos. Estos resultados coinciden con los obtenidos en seres humanos, lo que subraya la necesidad de intervenciones terapéuticas específicas para cada sexo. Estos hallazgos contribuyen a nuestra comprensión de la patología de la enfermedad de Alzheimer y sugieren el valor potencial de las pruebas olfativas como herramienta de diagnóstico precoz.

Resum

Les proves més recents posen en relleu la important variabilitat interindividual observada tant en l'envelliment normal com en la malaltia d'Alzheimer (MA), juntament amb les elevades taxes de mortalitat i la necessitat urgent de comptar amb pronosticadors específics del final de la vida. Tot i això, la investigació d'aquesta heterogeneïtat continua estant limitada en models de ratolins mascles molt vells amb MA a causa de les seves reduïdes taxes de supervivència. En aquesta investigació exhaustiva, examinem 915 ratolins (432 APP23 transgènics i 483 C57BL/6 companys de ventrada) per confirmar les taxes de supervivència superiors observades en els ratolins APP23 mascle en comparació amb les femelles i les seves respectives contraparts de tipus salvatge. Això va permetre la caracterització de firmes conductuals en animals mascles de mitjana edat, vells i longeus. L'anàlisi d'una bateria que comprenia set paradigmes, que abastaven la funció motora (anàlisi de l'activitat i la marxa), símptomes neuropsiquiàtrics i deteriorament cognitiu en un subconjunt de 56 animals que abastaven ratolins APP23 mascle de 12, 18 i 24 mesos d'edat i companys de ventrada de tipus salvatge, va revelar diferències relacionades amb l'edat en diverses variables. Cal destacar que les variables associades amb l'afrontament de l'estrès, la trigmotaxi, la fragilitat, les anomalies de la marxa i el deteriorament cognitiu van distingir eficaçment el fenotip conductual dels ratolins APP23 mascle en els tres grups d'edat en comparació dels controls. Les nostres troballes van revelar patrons conductuals no lineals dependents de l'edat i el genotip en animals longeus, cosa que indica la presència d'interaccions interaccions entre els processos d'envelliment cronològic i biològic/conductual. A més, la trajectòria temporal i la gravetat de la involució del sistema sensorial durant l'envelliment són de vital importància per percebre i reconèixer el món. S'ha demostrat que les deficiències sensorials augmenten significativament el risc de deteriorament biològic, psicològic i social a la gent gran. La pèrdua olfactiva es considera un biomarcador primerenc a la neurodegeneració de la malaltia d'Alzheimer. Per examinar la funció olfactiva tant en l'envelliment típic com en el relacionat amb l'MA, analitzem etogrames olfactius en ratolins C57BL/6 i 3xTg-AD mascles i femelles de mitjana edat, un model genètic d'MA amb deteriorament cognitiu i un fenotip notable tipus neuropsiquiàtric. Es va fer servir un paradigma de privació d'aliments durant un dia per analitzar patrons etològics en la inspecció olfactiva i els

comportaments de cerca d'aliments. Els resultats van revelar que l'MA presentava firmes olfactives diferents de les observades a l'envelliment normal. En particular, els ratolins mascle 3xTg-AD mostraven una signatura olfactiva més precoç que les femelles, que diferia dels patrons observats als ratolins control. A més, l'aïllament social va accentuar els patrons olfactius i va alterar els correlats conductuals, particularment als ratolins mascles 3xTg-AD. Finalment, es va fer servir el paradigma olfactiu Food Finding Test (FFT) per examinar l'olfacció en animals envellits, sense privació d'aliment durant la nit. Es va analitzar meticulosament un etograma de tres comportaments dirigits cap a aliments ocults en ratolins 3xTg-AD mascles i femelles i en ratolins C57BL/6 de la mateixa edat. Aquest nou protocol va demostrar sensibilitat al sexe, genotip i context social, particularment en mascles i ratolins AD, amb modificacions induïdes per l'aïllament a l'etograma i correlacions funcionals. A la llum d'aquestes consideracions, es va fer una avaluació exhaustiva dels fenotips conductuals en ratolins 3xTg-AD, un model transgènic àmpliament utilitzat per a la malaltia d'Alzheimer (MA). Es van emprar diverses avaluacions conductuals, com la prova de la cantonada, la prova del camp obert, el laberint a T, la prova de la caixa fosca i lluminosa i la prova per trobar menjar, per tal d'investigar els dèficits cognitius i emocionals característics d'aquests ratolins. Els resultats indiquen deficiències cognitives significatives dependents de l'edat i comportaments similars a l'ansietat, incloent-hi un augment de la neofòbia, una reducció de l'activitat locomotora i un retard en les respostes de cerca de menjar. Cal destacar que l'estudi també va identificar diferències de sexe, ja que les femelles de ratolins 3xTg-AD mostraven dèficits més pronunciats que els mascles. Aquests resultats coincideixen amb les dades epidemiològiques humanes i posen en relleu la necessitat d'enfocaments terapèutics específics per a cada sexe. Les correlacions observades entre diverses mesures conductuals suggereixen que hi ha disfuncions cognitives i emocionals interconnectades, cosa que aporta una comprensió més profunda de la patologia de l'MA. En resum, investigacions recents subratllen la substancial variabilitat interindividual observada tant en l'envelliment normal com en la malaltia d'Alzheimer (MA), cosa que subratlla la necessitat de desenvolupar pronosticadors específics del final de la vida. L'objectiu d'aquest estudi era investigar les diferències conductuals en funció de l'edat i el genotip en ratolins transgènics APP23 i en companys de ventrada C57BL/6. Els ratolins mascles APP23 van mostrar un comportament similar al dels ratolins transgènics. Els ratolins mascles APP23 van mostrar taxes de supervivència superiors en comparació amb les femelles i ratolins no transgènics, revelant així patrons de comportament dispars

relacionats amb l'edat, particularment en els dominis d'afrontament de l'estrès, fragilitat i deteriorament cognitiu. La funció olfactiva dels ratolins 3xTg-AD i NTg es va avaluar mitjançant el Test de Cerca d'Aliments (Food Finding Test, FFT), que va revelar la presència d'alteracions olfactives primerenques als ratolins mascles AD, que es van agreujar encara més amb l'aïllament social. Una avaluació exhaustiva del comportament va revelar importants dèficits cognitius i emocionals als ratolins 3xTg-AD, i les femelles presentaven símptomes més pronunciats que els mascles. Aquests resultats coincideixen amb els obtinguts en éssers humans, fet que subratlla la necessitat d'intervencions terapèutiques específiques per a cada sexe. Aquestes troballes contribueixen a la nostra comprensió de la patologia de la malaltia d'Alzheimer i suggereixen el valor potencial de les proves olfactives com a eina de diagnòstic precoç.

TABLE OF CONTENTS

*1. **Introduction*** *28*

1.1 ALZHEIMER´S DISEASE *30*

1.1.1. Definition *30*

1.1.2. Neurophysiology of Alzheimer's disease 31

1.1.3. Neuroanatomy of Alzheimer's disease 32

1.1.4. Neuropsychiatric symptoms of Alzheimer´s disease 33

1.1.5. Loss of smell in patients with Alzheimer's disease 34

1.2. TRANSLATIONAL NEUROSCIENCE *35*

1.2.1. Animal models of Alzheimer´s disease 36

1.2.1.1. APP Swedish 38

1.2.1.2. 3xTg-AD 38

1.2.2. Sex and gender medicine in neurocognitive disorders 40

1.2.3. Age factors in neurocognitive disorders 41

1.2.4. Social condition 42

1.3. BEHAVIORAL TEST *43*

1.3.1. Corner test 43

1.3.2. Open field 43

1.3.3. Object recognition 43

1.3.4. T- maze 43

1.3.5. Morris water maze 44

1.3.6. Food finding test 44

1.3.7. Dark and light box 44

*2. **Hypothesis & Objectives*** *46*

*3. **Study 1*** *53*

Survival Bias and Crosstalk between Chronological and Behavioral Age: Age- and Genotype-Sensitivity Tests Define Behavioral Signatures in Middle-Aged, Old, and Long-Lived Mice with Normal and AD-Associated Aging *53*

3.1. MATERIALS AND METHODS 55

3.1.1. Animals 55

3.1.2. Experimental Design 55

3.1.3. Behavioral Assessments 56

3.1.4. Statistics 58
3.2. RESULTS 59
3.2.1. Genotype and Sex-Dependent Increased Mortality Rates 59
3.2.2. Strong Contribution of Age and Specificity of Genotype Factor on Key Variables for AD-Related Phenotype 60
3.3. Discussion 69
3.4. CONCLUSIONS 78
4. ***Study 2*** *81*
Olfactory Signatures in the Food Finding Test in Mice With Normal and Alzheimer's Disease-Pathological Aging With Special Concerns on the Effects of Social Isolation *81*
4.1. MATERIALS AND METHODS 83
4.2. RESULTS 85
4.2.1. Effect of Food Deprivation on Body Weight but Not Related to the Olfactory Signature 85
4.2.2. Sex- and Genotype-Dependent Signatures in the Food Finding Test 86
4.2.3. Food Deprivation and Food Finding Test in Naturalistic Social Isolation of Male 3xTg-AD Mice 88
4.2.4. Correlation Analysis Between the Actions of Olfactory Signatures in the Food Finding Test 89
4.3. DISCUSSION 90
4.4. CONCLUSIONS 93
5. ***Study 3*** *94*
Food Finding Test without Deprivation: A Sensorial Paradigm Sensitive to Sex, Genotype, and Isolation Shows Signatures of Derangements in Old Mice with Alzheimer's Disease Pathology and Normal Aging *94*
5.1. MATERIALS AND METHODS 96
5.1.1. Animals 96
5.1.2.1. Physical Status and Behavioral Assessment 96
5.1.2.1.1. Physical Status 96
5.1.2.1.2. The Food Finding Test of Olfactory Ability without Food Deprivation 97
5.1.2.1.3. Statistical Analysis 97
5.2. RESULTS 98
5.2.1. Physical Status 98

5.2.2. Elicitation of Olfactory Signatures Despite the Lack of Overnight Food Deprivation 98
5.2.3. Time Delays and FFT Behavioral Correlates 100
5.3. DISCUSSION 102
5.4. CONCLUSIONS 105
6. ***Study 4*** *107*
Cognitive, NPS-like and olfactory signatures in mice with normal and AD-pathological aging, with a special focus on the effects of sex. *107*
6.1. MATERIALS AND METHODS 109
6.1.1. Animals 109
6.1.2. Behavioral Assessments 109
6.2. RESULTS 112
6.3. DISCUSSION 128
6.4. CONCLUSION 131
7. ***General discussion*** *133*
8. ***Conclusions*** *149*
9. ***References*** *156*

1. Introduction

1.1 ALZHEIMER´S DISEASE

1.1.1. Definition

Alzheimer's disease (AD) is a neurodegenerative disorder that affects memory and cognitive function (McKhann et al., 1984; World Health Organization [WHO]., 2023). It is characterized by a gradual progression and is associated with neurochemical and neuropathological changes that are similar to those seen in dementia. According to World Health Organization (2023), cognitive decline caused by this disease can affect various domains such as judgment, language, learning, thinking, orientation, and calculation. It can also lead to regression in social behavior, emotional regulation, and motivation. It is important to note that these evaluations are objective and not subjective.

According to the Diagnostic and Statistical Manual for Mental Disorders (DSM-V), approximately 80% of individuals with severe neurocognitive disorders (NCD) resulting from Alzheimer's disease exhibit behavioral and psychological symptoms in specialized clinical settings.(American Psychiatric Association, 2013) These symptoms appear even in the early stages of NCD and can be as distressing as the cognitive symptoms, often prompting individuals to seek medical intervention. In the early stages of Alzheimer's disease, individuals may experience symptoms such as melancholy and apathy. As the disease progresses to a moderately severe stage, these symptoms may become more pronounced and include psychosis, irritability, agitation, combativeness, and wandering. Ultimately, Alzheimer's disease leads to a range of debilitating physical manifestations, including gait disturbances, dysphagia, incontinence, myoclonus, and seizures.

The pathological basis of AD is closely linked to the decline in brain structure and function. AD deviates from the natural aging process and is characterized by an increase in the formation of senile plaques containing β-amyloid peptide (Aβ) ((Cheignon et al., 2018). Additionally, the disease is marked by an accumulation of reactive oxygen species in the brain, which worsens oxidative stress (Cheignon et al., 2018). Individuals with early-onset AD often exhibit mutations in amyloid precursor protein (APP) and presenilin-1 (PS1) genes. This hastens the processing of APP into Aβ and the deposition of Aβ plaques, which correlates with heightened oxidative stress markers within AD-afflicted brains (Guo et al., 1999). Although a significant portion of AD cases are caused by genetic mutations in APP, PS-1, and PS-2 genes, various risk factors including

psychosocial and environmental variables also contribute to the disease's etiology. Despite extensive research efforts, the cause of a significant portion of AD cases remains unknown, with no conclusive evidence implicating a genetic basis for all instances of the disease (Selkoe, 2002)

1.1.2. Neurophysiology of Alzheimer's disease

Alois Alzheimer, a psychiatrist, and neurologist, discovered the histological intricacies underlying Alzheimer's disease (AD) at the beginning of the 20th century. Alzheimer identified changes within the brain characterized by the deposition of plaques known as β-Amyloid plaques and neurofibrillary tangles (NFTs) (Zetzche et al., 2010). Neuronal loss primarily affects regions such as the hippocampus and neocortical association areas, which are crucial for memory and higher cognitive functions (Zetzche et al., 2010). Additionally, Alzheimer's astute observations extended to subcortical regions, revealing a significant decrease in neuronal populations, particularly in the nucleus basalis of Meynert, a vital source of cholinergic neurotransmission that is pivotal for cortical function (Selkoe, 2002)

The main components of β-Amyloid plaques are amyloid peptides, which are derived from the larger amyloid precursor protein (APP). These amyloid deposits are widespread throughout the cerebral cortex, particularly in the cortical gray matter, including layers II and III of the neocortex and the hippocampus. Additionally, β-Amyloid peptides aggregate within the vasculature, leading to amyloid angiopathy (Zetzsche et al., 2010).

In the pathological environment of Alzheimer's disease (AD), neurofibrillary tangles (NFTs) appear as intraneuronal aggregates resulting from the abnormal hyperphosphorylation of tau proteins. Tau proteins typically have a crucial role in preserving neuronal structural integrity and aiding intracellular molecular transport. However, in AD, tau function dysregulation leads to the intertwining of fibrils, ultimately resulting in NFT formation. Therefore, the integrity of neurofilaments and neurotubules is compromised, leading to neuronal death (Zetzsche et al., 2010).

Oxidative stress is a significant factor in the development of AD, caused by an imbalance between pro-oxidant and antioxidant systems. Reactive oxygen species (ROS), which are produced by oxygen and nitrogen-based molecules with unpaired electrons, are the main cause of oxidative stress. These reactive entities scavenge electrons from cellular

constituents, causing molecular destabilization and cellular damage. Lipids, proteins, and nucleic acids are vulnerable to oxidative assaults, disrupting cellular homeostasis and leading to cell death (Chauhan & Chauhan, 2006). Oxidative stress-induced lipid peroxidation is a significant contributor to the neurodegenerative cascades observed in Alzheimer's disease and related disorders (García-Mesa et al., 2016).

The interplay between β-Amyloid plaque deposition, NFT formation, and oxidative stress orchestrates a cascade of neurodegeneration in Alzheimer's disease. Understanding these pathological hallmarks is crucial for unraveling the etiology of AD and developing effective therapeutic interventions to counter its progression.

1.1.3. Neuroanatomy of Alzheimer's disease

Alzheimer's disease (AD) begins by targeting key structures within the limbic system, particularly the medial temporal lobe, which includes the entorhinal cortex and hippocampus. These regions are essential for the Papez circuit, which is involved in memory formation and mood regulation, and are affected by AD pathology, with an early accumulation of plaques and neurofibrillary tangles (NFTs) (Jicha & Carr, 2010). As the disease progresses, it affects subcortical structures such as cholinergic projections from the nucleus basalis of Meynert, which play a crucial role in cortical activation (Braak & Braak, 1996). Additionally, the amygdala, anterior cingulate, and other limbic structures become involved, leading to various neuropsychiatric and behavioral manifestations associated with AD.

Subsequent stages show the continuous progression of AD on the cerebral cortex, leading to impairments in daily activities such as eating, dressing, and personal grooming. Emotional regulation becomes more difficult, adding to the burden on both individuals and caregivers (Jicha & Carr, 2010). Alzheimer's disease (AD) not only affects the brain but also penetrates deeper into the brainstem, impairing the functionality of vital organs such as the heart and lungs. This imperils essential physiological processes crucial for sustaining life (Philips et al., 2015).

As AD progresses, neuronal dysfunction and demise pervade the entire brain, although sensory areas in the frontal and parietal lobes, as well as motor regions, are notably preserved. Alzheimer's disease causes a spatial progression of pathology that leads to a clinical phenotype ranging from intact cognition to cognitive decline and functional impairment (Jicha & Carr, 2010).

The disease relentlessly attacks the neural architecture, dismantling cognitive, emotional, and physiological substrates, ultimately leading to neurodegeneration. Understanding the spatiotemporal dynamics of Alzheimer's disease pathology is crucial for developing targeted therapeutic interventions to reduce its devastating impact and alleviate the burdens it places on affected individuals and their caregivers.

1.1.4. Neuropsychiatric symptoms of Alzheimer´s disease

Individuals with Alzheimer's disease (AD) experience a range of behavioral and psychological symptoms associated with dementia (BPSD). BPSD refers to the non-cognitive symptoms of the disease (Hart et al., 2003). It is important to recognize the importance of thoroughly assessing the cognitive and behavioral features of dementia, given the varied clinical expressions observed in patients with AD (Baeta-Corral, Johansson, et al., 2018) neuropsychiatric symptoms (NPS), also referred to a BPSD.

The prevalence of NPS among individuals with AD ranges from 75-90% (Cummings, 1997a; Reisberg et al., 1987). These symptoms appear throughout the course of the disease, including apathy, depression, anxiety, agitation, hallucinations, delusions, irritability, changes in eating habits, euphoria, and abnormal motor activity (Sorbi et al., 2012). These symptoms greatly reduce the quality of life for patients and their caregivers(Kamiya et al., 2014a), sometimes resulting in unnecessary institutionalization (Vugt et al., 2005)

BPSD is a complex interplay of neurobiological, psychological, social, and environmental factors that act upon biologically predisposed individuals. Although there is variability in the presentation of these symptoms among AD patients, their frequency and severity tend to escalate over time, exacerbating both functional and cognitive decline (Steinberg et al., 2006)

The etiology of BPSD is multifaceted, encompassing genetic, biochemical, and anatomical determinants. Frontal dysfunction is a significant factor in behavioral changes, such as impulsivity and impaired judgment (Olazarán Rodríguez et al., 2012) Sleep disturbances (Cummings, 1997b; Cummings et al., 1994) are common in AD patients and are attributed to degenerative changes in the suprachiasmatic nucleus and cholinergic deficits. Additionally, disruptions in neurotransmitter pathways, including dopamine, noradrenaline, serotonin, and glutamate, are observed (Heilman & Nadeau, 2022).

1.1.5. Loss of smell in patients with Alzheimer's disease

Anosmia, the loss of smell, is emerging as a potential early marker and significant symptom in patients with Alzheimer's disease (AD). Studies have linked anosmia in AD patients to pathological changes in the olfactory system, including the accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) in olfactory regions of the brain.(Attems et al., 2015) Research suggests that olfactory dysfunction may precede cognitive decline and serve as a predictive biomarker for the onset of AD. This emphasizes the significance of exploring olfactory impairment as a potential diagnostic and prognostic indicator in AD.(Wilson et al., 2009a)

Numerous studies have shown a robust correlation between olfactory dysfunction and AD pathology. Postmortem analyses of AD brains have revealed substantial neuronal loss and pathological changes in olfactory regions, including the olfactory bulb and olfactory cortex. Imaging studies using positron emission tomography (PET) have shown a correlation between olfactory deficits and increased Aβ deposition in the brain. These findings suggest that olfactory dysfunction could serve as a non-invasive marker for AD pathology and disease progression.(Attems et al., 2014; Kotecha et al., 2018; Wang et al., 2023)

Recent research has demonstrated that the loss of the sense of smell, more commonly referred to as anosmia, is capable of predicting and indicating an elevated risk of mortality approximately five years prior to that of cardiovascular disease. (Van Regemorter et al., 2020a). There is growing awareness of the association between olfactory impairment and cognitive decline. The recognition of olfactory dysfunction as an indicator for various neurological and neurodegenerative diseases is becoming increasingly prevalent. (Wilson et al., 2009b) The relationship between olfactory impairment, particularly anosmia, and Alzheimer's disease has attracted considerable attention. Research indicates that approximately 90% of individuals with Alzheimer's disease experience some degree of olfactory dysfunction. (Conti et al., 2013)

Furthermore, the reduction in olfactory function becomes apparent as individuals progress from normal aging to dementia, particularly in cases of mild cognitive impairment. Previous research indicates that olfactory dysfunction may serve as a valuable clinical indicator and predictor of these conditions, assisting in the identification of individuals at higher risk.(Invitto et al., 2018a) Further research is required to fully

comprehend the underlying mechanisms of this connection. However, the occurrence of toxic proteins such as beta-amyloid and tau, which are neuropathological markers of Alzheimer's disease, has been linked to olfactory impairment or loss of smell.(Zou et al., 2016) Consequently, olfactory impairment can be used as a valuable tool for early diagnosis of Alzheimer's disease.. (Murphy, 2019) Additionally, The implementation of pre-diagnostic olfactory testing could facilitate the early implementation of preventive strategies, which would ultimately enhance cognitive function, brain health, and general mental health (Brai et al., 2020)

In addition to its diagnostic potential, olfactory dysfunction in Alzheimer's disease (AD) patients has significant implications for their quality of life and daily functioning (Dziechciaż & Filip, 2014). Olfaction plays a crucial role in food perception, social interactions, and emotional well-being. The loss of smell can lead to nutritional deficiencies, decreased appetite, and social withdrawal, which can exacerbate cognitive decline and impair overall health.(Roberts et al., 2016) Addressing olfactory dysfunction in AD patients may improve their quality of life and enhance their ability to engage in meaningful activities. Further research is needed to understand the clinical implications of olfactory dysfunction in AD and to develop interventions aimed at preserving olfactory function and mitigating its impact on patients' lives.(Volkers & Scherder, 2011)

1.2. TRANSLATIONAL NEUROSCIENCE

Translational neuroscience is a crucial link between basic scientific discoveries and clinical applications. Its ultimate goal is to translate laboratory findings into tangible benefits for patients. Animal models are essential in translational neuroscience as they provide valuable tools for studying the pathophysiology of neurological disorders and evaluating potential therapeutic interventions. Characterization of animal models is a crucial aspect of translational neuroscience. The validation and optimization of model systems are necessary to ensure their relevance and reliability for investigating human diseases. It involves rigorous phenotypic and molecular assessment, including evaluation of behavioral, cognitive, and neuropathological endpoints, to establish the validity of animal models and their predictive value for human disease.

A thorough comprehension of animal model characterization is crucial for effectively translating preclinical findings into clinical applications. Researchers can develop

targeted therapeutic strategies and biomarker-based diagnostic tools for neurological disorders by elucidating the molecular mechanisms underlying disease pathogenesis and identifying biomarkers associated with disease progression. Animal model characterization allows for the identification of potential therapeutic targets and evaluation of novel treatment modalities, such as pharmacological agents, gene therapies, and stem cell-based interventions, in preclinical models before clinical trials. It is important to maintain objectivity and avoid biased language when discussing the benefits of animal models in medical research. Translational neuroscience holds the promise of accelerating the development of innovative therapies and improving outcomes for patients with neurological disorders through meticulous characterization and validation of animal models.

1.2.1. Animal models of Alzheimer´s disease

Animal models are essential in Alzheimer's disease (AD) research, providing valuable insights into disease pathogenesis and aiding in the development of new therapeutic strategies. Different animal models have been used to replicate various aspects of AD pathology, including transgenic rodent models and non-human primates (NHPs). Animal models are commonly used in AD research to investigate the molecular mechanisms underlying the disease, evaluate potential treatment interventions, and assess the efficacy of therapeutic compounds. (LaFerla & Green, 2012)

The elucidation of disease mechanisms is one of the primary applications of these models. Transgenic rodent models expressing mutant forms of amyloid precursor protein (APP) and presenilin (PS) recapitulate key pathological features of Alzheimer's disease (AD), including amyloid-beta (Aβ) plaque deposition and tau hyperphosphorylation. (LaFerla & Green, 2012; Meyer-Luehmann et al., 2008)These models have provided valuable insights into the molecular pathways underlying Aβ and tau pathology, synaptic dysfunction, neuroinflammation, and neurodegeneration in AD.

Crucially, the importance of establishing a valid animal model for Alzheimer's disease (AD), stipulating that such models should exhibit progressive AD-like neuropathology and cognitive deficits, a criterion that must be consistently confirmed across different research laboratories. Additionally, transgenic animal models should be capable of distinguishing between the pathogenic effects resulting from familial form mutations and those stemming from transgene overexpression. Moreover, models

generated through microinjection of familial mutation alleles should encompass multiple transgenic lines to enhance reliability.(Janus & Westaway, 2001)

The introduction of genetically modified rodent models in the mid-1990s, most notably exemplified by the PDAPP model, represented a significant turning point in Alzheimer's disease research. (Games et al., 1995) marked a significant milestone in AD research. Subsequent development of various models, including those based on mutations in amyloid precursor protein (APP), presenilin 1 (PS1), and tau, has provided valuable insights into the underlying disease mechanisms. These models have proven indispensable in the preclinical assessment of potential therapeutic interventions, facilitating a deeper understanding of AD pathophysiology and guiding the development



of novel treatment strategies.

Fig 1. The most used AD mouse models. Taken from Alzheimer's Research Forum and can be found at «https://www.alzforum.org/research-models/search».

1.2.1.1. APP Swedish

APP23 transgenic mice (Novartis, Basel, Switzerland) were used in this study. These mice overexpress the APP protein containing the Swedish mutation (K670M/N671L) under the neuronal Thy1 promoter. The heterozygous B6, D2-TgN [Thy-APPSWE]-23-Tg (APP23) mice were provided by the Novartis Institutes for BioMedical Research, Novartis Pharma AG (Basel, Switzerland) and were backcrossed twice to C57BL/6 mice (Janvier). The genotype of the mice was determined by Transnetyx (Cordova, TN, USA) using tail samples. Hemizygous mice and littermates were bred to form the final study cohort.(Bornemann & Staufenbiel, 2000)

Transgenic models expressing wild-type or mutated APP are increasingly utilized in the investigation of the pathogenesis and potential treatment of AD. One such model is the APP23 mouse, initially discussed in 1997 (Sturchler-Pierrat et al., 1997a). In APP23 transgenic mice, extensive β-amyloid pathology is observed. These Aβ deposits first appear at 6-8 months of age. The congophilic plaques increase in size and number with age, occupying up to 25% of the neocortex and hippocampus in 24-month-old mice. Plaques are surrounded by activated microglia and astrocytes containing hyperphosphorylated tau, although oligodendrocytes are not observed. Plaque development is more rapid in females than in males. Neuronal loss is seen in the CA1 region of the hippocampus. In addition, mice develop cerebral amyloid angiopathy (CAA) and microhemorrhages occur at later ages (Sturchler-Pierrat et al., 1997a).

In this model, cognitive deficits begin to appear at three months of age in recognition memory and spatial memory. These deficits are progressive with age, as at 12 months the animals also show cognitive deficits in referential memory (Prut et al., 2007). Cognitive performance on passive avoidance memory tasks deteriorates after between 19 and 20 months of age, as does non-spatial working memory (Webster et al., 2014)

1.2.1.2. 3xTg-AD

In 2003, Professor Frank M. LaFerla's laboratory pioneered the development of the initial cohort of 3xTg-AD mice, incorporating the PS1M146V and APPSwe familial Alzheimer's disease (AD) mutations alongside the introduction of the human tauP301L

transgene. This was achieved through the meticulous technique of microinjection, targeting transgene insertion into unicellular embryos obtained from homozygous PS1M146V knock-in specimens.

This animal model exhibits a gradual manifestation of temporal- and region-specific formations of amyloid β-plaques and neurofibrillary tangles containing tau, mirroring the pathological features observed in the human brain afflicted with Alzheimer's disease (AD).(Belfiore et al., 2019; Oddo et al., 2003) This amalgamation leads to the formation of both amyloid plaques and neurofibrillary tangles (NFTs) in the brain, which are hallmark pathologies of AD. Since its inception, the 3xTg-AD model has garnered substantial attention and adoption within the scientific community.

Brain oxidative stress, neuroinflammation, and alterations in the neurotransmitter system have also been described (Caruso et al., 2013; García-Mesa et al., 2016; Perez et al., 2011) The 3xTg-AD mouse is a commonly utilized model in a homozygous state for both the transgene insertion and the Psen1 M146V familial Alzheimer's disease (AD) mutation. The initial documentation of the 3xTg-AD model delineated the emergence of age-related and progressive amyloid and tau pathologies, with extracellular plaques emerging initially at 6 months of age (Fig 2). However, the replication of plaque appearance in 3xTg-AD mice before 12 months of age has become increasingly challenging in recent years. Subsequently, the manifestation of neurofibrillary tangles (NFTs) becomes discernible at 12 months of age (Oddo et al., 2003). Moreover, at six months of age, 3xTg-AD mice have exhibited localized neurodegeneration, synaptic impairment, and cognitive deficits (Drummond & Wisniewski, 2017). The comprehensive development of both plaques and tangles in 3xTg-AD mice renders them highly esteemed as a comprehensive transgenic mouse model for studying AD pathology (Javonillo et al., 2022).

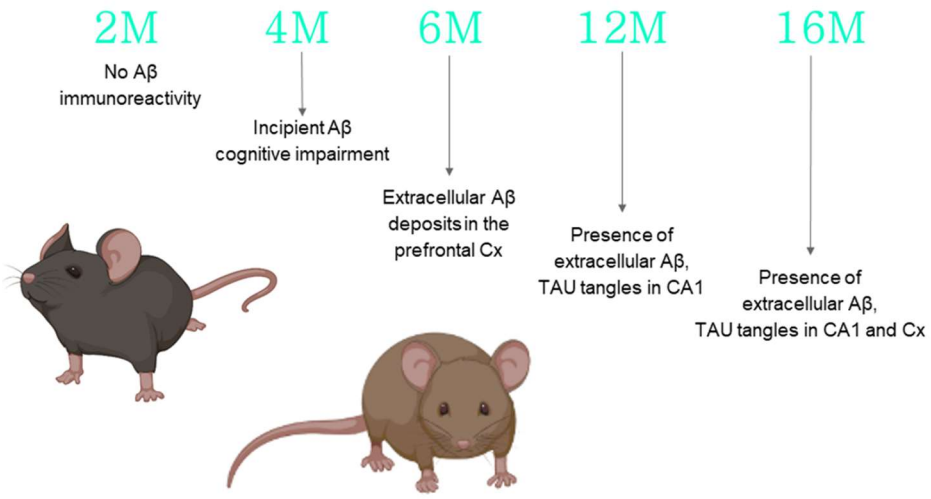


Fig 2. Age-related hallmark of disease

1.2.2. Sex and gender medicine in neurocognitive disorders

Sexual differentiation within the context of medical investigation delineates the biological and physiological distinctions between females and males, primarily influenced by sex chromosomes (XX vs. XY) and gonadal hormones, which significantly shape cellular, organ, and systemic characteristics. Conversely, gender encompasses a blend of environmental, social, and cultural factors that interact with biological elements in females and males. While gender finds its origins in biology, it undergoes further molding through environmental influences and individual experiences. A growing body of evidence indicates that both sex and gender play a significant role in the etiology, clinical presentation, and treatment outcomes of numerous diseases. Despite notable advances in Alzheimer's disease (AD) research, there has been a paucity of attention directed towards exploring the potential influence of sex and gender variations in AD. This has resulted in a substantial knowledge gap and a lack of recognition within the research community regarding the role of sex and gender dynamics in AD (Carter et al., 2012)

To optimize the development of current and forthcoming therapies and interventions for the entirety of the AD continuum, a more comprehensive comprehension and evaluation of sex and gender disparities in AD are imperative (Laws et al., 2016; Mielke et al., 2014a; Miller, 2014). This necessitates that investigations into the distinctions between women and men in AD must encompass not only biological sex but also gender disparities in domains such as educational attainment, caregiving

responsibilities, and other gender-associated roles. Additionally, mental health considerations must be taken into account, given that both biological and societal factors intersect to influence female/male differences (Nebel et al., 2018)

1.2.3. Age factors in neurocognitive disorders

The age factor is a critical element in the development and progression of neurocognitive disorders, particularly Alzheimer's disease (AD)(Akpan et al., 2019; Tahami Monfared et al., 2022). As individuals age, the risk of developing neurocognitive impairments increases significantly. Alzheimer's disease, the most common form of dementia, disproportionately affects older adults, with the incidence rising sharply after the age of 65. This age-related susceptibility is linked to various physiological changes, including the accumulation of amyloid-beta plaques, neurofibrillary tangles, synaptic loss, and chronic inflammation. (Tahami Monfared et al., 2022)Understanding the impact of aging on these pathological processes is essential for developing effective interventions to mitigate cognitive decline. (Silva et al., 2019)

Animal models have been indispensable in elucidating the mechanisms by which aging influences neurocognitive disorders. Transgenic mouse models, such as the APP23 and 3xTg-AD mice, replicate key features of AD pathology, including amyloid-beta accumulation and tau hyperphosphorylation.(Mhillaj et al., 2017; Silva et al., 2019) These models have demonstrated that age exacerbates the deposition of amyloid-beta plaques and the formation of neurofibrillary tangles, thereby accelerating cognitive deficits. For example, studies employing the 3xTg-AD mouse model have demonstrated that older mice exhibit more pronounced cognitive impairments and neuropathological changes compared to their younger counterparts (Baeta-Corral & Giménez-Llort, 2015; Castillo-Mariqueo & Giménez-Llort, 2022; Gimenez-Llort & Alveal-Mellado, 2021; Torres-Lista & Giménez-Llort, 2014)

Translational research that employs animal models provides crucial insights into potential therapeutic targets for age-related neurocognitive disorders. (Barreto Chang et al., 2023; Eaton & Wishart, 2017)Investigations into the role of aging in neuroinflammation have revealed that older animals exhibit heightened microglial activation and increased pro-inflammatory cytokine levels in response to amyloid-beta pathology. (Eaton & Wishart, 2017; Finger et al., 2022)These findings indicate that age-

related changes in the immune system may contribute to the progression of neurodegeneration.

1.2.4. Social condition

Social determinants of health are the conditions in which people are born, work, live, and age, as well as the broader set of forces and systems shaping these daily life conditions. These determinants influence every aspect of life, particularly health and healthcare. There is growing attention on modifiable factors that impact cognition and the risk of Alzheimer's disease and related dementias (ADRDs).(Majoka & Schimming, 2021; World Health Organization [WHO]., 2011)

The effective functioning and communication of the neuro-immune-endocrine systems are crucial for maintaining optimal physical and mental health. (BESEDOVSKY & REY, 1996) Emotional stress and anxiety are detrimental factors that can impair these systems, thereby contributing to or exacerbating various pathological conditions. (Cohen et al., 2007) The capacity to cope with chronic emotional stressors is of particular significance for older adults, given the age-related impairments in their neuroendocrine and immune systems. (Kiecolt-Glaser et al., 2002). This is particularly pertinent to dementia, given the neuro-immunomodulation hypothesis of Alzheimer's disease (AD). This hypothesis posits that alterations in the innate immune system result in dysregulation of the interaction between immune mediators and neuronal functions at both peripheral and central levels.(Giménez-Llort et al., 2010; Giménez-Llort et al., 2012)

Social isolation and loneliness are important emotional stressors in social species, such as humans and group-living like rodents. In recent years, the significance of social relationships on morbidity and mortality has gained increased attention, particularly during the COVID-19 pandemic.(Hwang et al., 2020) In this context, social isolation increases the risk of dementia (Wilson et al., 2007) and exacerbates both its hallmark cognitive decline and neuropsychiatric disorders, which are already prevalent in these patients (Cummings, 1997c; Seitz et al., 2010) The management of neuropsychiatric symptoms (NPS) represents a significant challenge for the quality of life of both patients and caregivers (Kamiya et al., 2014b)

1.3. BEHAVIORAL TEST

1.3.1. Corner test

In the corner test, neophobia is determined as a low level of exploration. The rodents' response to exploring a new place is evaluated based on whether or not they explore for 30 seconds. This test is a single measure and does not require training (Janus and Westwey, 2001) and the current methological version is an adaptation using a home-cage and assessing behavior for 30 seconds (Giménez-Llort, Blázquez, et al., 2007) and/or 1 minute. (Gimenez-Llort & Alveal-Mellado, 2021)

1.3.2. Open field

This test was developed to establish the relationship between emotional behavior and the speed or distance traveled during walking activity, measured per unit of time. In 1934, Calvin Hall observed emotional and non-emotional responses of rodents to an illuminated and new environment in this test. The study identified freezing, defecation, urination, and exploratory activity as responses in rodents. The test is commonly used to evaluate emotional reactivity in rodents.

1.3.3. Object recognition

The novelty of an object or environment can provoke approach behavior in rodents. In the familiarization session, the animal is allowed to freely explore two similar objects. In the subsequent test session, one of the objects is replaced by an unfamiliar object, referred to as the 'new object'. This is a single-task test that does not involve rule learning or require reinforcers. It is based solely on the rodent's innate preference to explore a novel object rather than a familiar one. This is because a rodent that remembers the familiar object will spend more time exploring the new object (Leger et al., 2013).

1.3.4. T- maze

The T-maze has been frequently used since the beginning of the 20th century to evaluate reference and working memory in rodents. It has been identified that these animals use various strategies to carry out tasks through spatial signals. These tasks may include spontaneous discrimination between right and left or forced alternation (Douglas, 1966).

1.3.5. Morris water maze

The Morris Water Maze (MWM) is a widely used behavioral task in neuroscience research, particularly in the fields of cognitive neuroscience, neuropharmacology, and neurobiology. Developed by Richard G. Morris in 1984, the MWM assesses spatial learning and memory in rodents, particularly mice and rats. It is a cornerstone tool for investigating the neural mechanisms underlying spatial navigation, memory formation, and cognitive processes. This text presents an overview of the Morris Water Maze paradigm, its applications, and its significance in contemporary neuroscience research.

1.3.6. Food finding test

As outlined by Deacon et al. (2009), the Food Finding Test (FFT) employs species-specific behaviors and their innate responses to evaluate olfactory function and associated behaviors. This uncomplicated approach offers an assessment instrument that can be readily implemented in any animal research facility, obviating the necessity for supplementary materials, such as the odors commonly utilized in other olfactory tests. Furthermore, it permits experimental designs that circumvent the potential confounding effects of odor carryover from preceding tests.(Coronas-Sámano et al., 2014; Dan et al., 2021; Slotnick & Coppola, 2015; Wersinger et al., 2007). On the other hand, the incorporation of ethologically salient stimuli, such as the search of food, is particularly interesting not only for evalu- ating olfactory function in health and disease throughout the life cycle, but also for the associated cognitive and emotional performance, and in assessing preventive/therapeutic interventions and the impact of risk factors and hazards.

1.3.7. Dark and light box

The Dark/Light Box Test is a crucial tool in behavioral neuroscience for evaluating anxiety-like behavior in rodents. This paradigm is based on the innate aversion of rodents to brightly lit, open spaces and their preference for dark, enclosed environments. It provides a straightforward and robust method for assessing anxiety-related responses. The test apparatus consists of two compartments: a dark, enclosed chamber and a brightly illuminated, open chamber, connected by an aperture. This creates a conflict between the animal's desire to explore novel surroundings and its aversion to light. Researchers gain valuable insights into anxiety-related behaviors by quantifying parameters such as time spent in each chamber and the frequency of transitions between them. This test has a wide range of applications, from studying anxiety disorders and

stress responses to investigating the neurobiological mechanisms underlying emotional regulation. Its simplicity, reliability, and translational relevance make it an indispensable tool for preclinical research aimed at understanding and treating anxiety-related conditions.

2. *Hypothesis & Objectives*

This thesis project is comprised of two principal modules. The initial module is based on Study 1, which employs the APPswe animal model. The results of this study demonstrate that male APP23 mice exhibit prolonged survival compared to APP23 females and respective control wild-type (WT) mice, which were reared and aged under identical living conditions. Subsequently, three cohorts of 12, 18, and 24 months of age male APP23 mice were subjected to a battery of seven behavioral tests for comprehensive screening of exploratory activity and gait analysis, cognitive and NPS-like symptoms. These tests were conducted in comparison to their WT counterparts, the gold-standard C57BL/6 mice strain, with normal aging.

The second module consists of studies 2, 3, and 4 using the 3xTg-AD animal model. We investigated olfactory ethograms in middle-aged male and female C57BL/6J mice and 3xTg-AD mice, a genetic model of Alzheimer's disease (AD) that exhibits cognitive dysfunction and a notable neuropsychiatric-like phenotype, including anxiety, apathy, and depression-like behaviors. Using a paradigm involving 1-day food deprivation, we analyzed ethological patterns during the olfactory inspection of a new cage and the behaviors associated with sniffing, finding, and eating hidden food pellets. These sniffing–find–eat temporal patterns were independent of weight loss and revealed rapid olfactory signatures in AD that contrasted with the slower progressive changes seen in normal aging. Male 3xTg-AD mice displayed an earlier olfactory signature than female mice, in contrast to normal aging where females typically show earlier changes. The sequence of actions was correlated in both male and female 3xTg-AD mice, unlike in control mice.

Social isolation, which naturally occurred in male 3xTg-AD mice due to the death of cage mates, accentuated their distinct olfactory patterns and disrupted the associated behavioral correlates. This paradigm provided distinct contextual, sex, and genotype-specific olfactory ethogram signatures, making it a valuable tool for investigating olfactory function in normal and AD-related pathological aging. Additionally, the impact of isolation was found to enhance the olfactory signature changes, marking this as the first description of such effects in the 3xTg-AD model of Alzheimer's disease. Our study highlights the importance of considering both sex and social environment in understanding the olfactory function and behavioral symptoms in neurodegenerative conditions like AD. For this purpose, we used 3xTg-AD mice and age-matched non-

transgenic mice, both with a C57BL/6J genetic background. The 3xTgAD mouse is a genetic model of AD that presents not only AD cognitive dysfunction but also a striking phenotype which models some “behavioral and psychological symptoms of dementia” (BPSD), including neuropsychiatric symptoms such as anxiety, apathy, and depression-like behaviors. In the last protocol of this module, the animals were subjected to a battery of five behavioral tests for the comprehensive screening of exploratory activity, cognitive and NPS-like symptoms. These tests were conducted in comparison to their NTg counterparts, the gold-standard C57BL/6 mice strain, with normal aging.

With this in mind the hypothesis and objectives of this work are as follows:

1.4. HYPOTHESIS

1.4.1. Module 1

It is hypothesized that 12-, 18-, and 24-month-old APPswe mice will exhibit behaviors analogous to those observed in individuals with dementia when subjected to the corner test, open field, object recognition, and T-maze assays. Additionally, it is anticipated that these subjects will display hyperactivity as a coping strategy in response to stressful situations. The survival curves of APP23 and WT C57BL/6 littermates are expected to confirm genotype- and sex-dependent increases in mortality rates. Long-living males will show an interrelationship between chronological age and biological/behavioral age. This interrelationship will be useful for studying the underlying mechanisms and various trade-offs through natural physiological aging and potentially long-term aging associated with AD in surviving males.

1.4.2. Module 2

It is hypothesized that olfactory deficits associated with Alzheimer's dementia will be modeled in 3xTg-AD mice. The food-finding smell test (FFT) will be able to distinguish the effect of genotype, disease stage, and sex under certain conditions. Furthermore, the olfactory-impaired animal will exhibit behavioral and psychological disturbances (BPSD) characteristic of Alzheimer's dementia and cognitive impairment, which will allow for the identification of a reliable correlation. In the case of isolation, the behavior associated with olfaction will be modified by the exacerbation of anxiety-related symptoms.

1.5. OBJECTIVES

1.5.1. Module 1

General Objective

This research's primary objective is to ascertain age's impact on the. behavioral profile of 12-, 18-, and 24-month-old mice with Alzheimer's disease (AD) and WT C57BL/6 littermates through a battery of tests, including the corner test, open field, object recognition, and T-maze. Furthermore, the study aims to demonstrate which test is more effective for evaluating Alzheimer's transgenic animals, such as APPswe.

Specific objectives:

1. To confirm the better survival curves in male than female APP23 mice and respective wildtypes, providing the chance to characterize behavioral signatures in middle-aged, old, and long-lived male animals.
2. To identify genotype differences in behavioral profile of APPswe animals compared to control animals (Wild Type) in the different tests.
3. To determine age differences between the 12-, 18-, and 24-months groups in APPswe mice and WT C57BL/6 littermates
4. To ascertain the level of interaction between these two factors, age and genotype.

1.5.2. Module 2

1. To validate the sensitivity of the food-finding test to sex and genotype
2. To determine the optimal experimental conditions for optimizing these studies in the 3xTg-AD model. This will entail optimizing the 12-, 13-, and 16-month experimental conditions.
3. To demonstrate the presence of olfactory deficits in 3xTg-AD animals using the experimental food-finding test protocol.
4. To test certain conditions, such as animal age, fasting time, test evaluation time, number of animals to be recorded simultaneously, and environmental conditions (social isolation), for the correct experimental implementation

5. To characterize the olfactory impairment of 3xTg-AD mice and its relationship with cognitive impairment and BPSD profile (especially anxiety) in males and females in different experimental conditions.

MODULE 1



3. Study 1

Survival Bias and Crosstalk between Chronological and Behavioral Age: Age- and Genotype-Sensitivity Tests Define Behavioral Signatures in Middle-Aged, Old, and Long-Lived Mice with Normal and AD-Associated Aging

3.1. MATERIALS AND METHODS

3.1.1. Animals

APP23 transgenic male mice (Hemizygote B6, D2-TgN[Thy-APP23]-23-Tg mice, No- vartis, Basel, Switzerland) (Sturchler-Pierrat et al., 1997b)overexpress the mutant human-type APP protein with the Swedish mutation (K670M/N671L) under the control of the murine brain and neuron- specific Thy-1 promoter (thymocyte antigen-1). Hemizygous APP23 mice were backcrossed with the wildtype (WT) C57BL/6 mice (Janvier Labs, Le Genest-Saint-Isle, France), and the APP genotype was tested by Transnetyx (Cordova, TN, USA). The APP23 mice and their wild-type littermates were aged in the Rodent Platform—Lab Animal Service (LAS), the Vall d'Hebron Research Institute animal facility to obtain the final study cohort. Animals were maintained under standard laboratory conditions of food (SAFE A004, Panlab, S.L., Barcelona, Spain) and water ad libitum, 22 ± 2 ◦C, a 12-h dark/light cycle, 50–60% humidity.

3.1.2. Experimental Design

The experimental design was defined in three steps. First, survival curves were calculated based on the two colonies of 432 APP23 mice and 483 WT C57BL/6 mice. After that, behavioral assessments were performed in 58 male mice, 29 APP23 mice and their 29 WT C57BL/6 mice littermates. Three sets of animals of 12, 18, and 24 months of age, were transferred to Servei d'Estabulari UAB, Universitat Autònoma de Barcelona, which is located at a distance of 17.4 km from Vall d'Hebron Research Institute (a 15 min ride). All the animals were housed three to four per cage and maintained (Makrolon, 35 × 35 × 25 cm3) under standard laboratory conditions (12 h light/dark, cycle starting at 8:00 h, food and water available ad libitum, 22 ± 2 ◦C, 50–60% humidity). One week after arrival, the animals were successively assessed using a battery of seven tests to evaluate four behavioral and functional dimensions: sensorimotor, cognitive, emotional, and daily life activities. All procedures followed Spanish legislation on 'Protection of Animals Used for Experimental and Other Scientific Purposes' and the EU Council directive (2010/63/EU) on this subject. The protocol CEEAH 3588/DMAH 9452 was approved the 8th of March 2019 by Departament de Medi Ambient i Habitatge, Generalitat de Catalunya. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used (Kilkenny et al., 2010a)

3.1.3. Behavioral Assessments

Behavioral assessments (Giménez-Llort, Blázquez, et al., 2007) were performed from 9:00 h to 13:00 h, in a counterbalanced manner and blind to the experiment. Assessments were performed blind to the experiment in a counterbalanced manner. The experimental groups were the following: 12m-WT (n = 10), 12m-APP23 (n = 9), 18m-WT (n = 10), 18m-APP23 (n = 11), 24m-WT (n = 9), and 24m-APP23 (n = 9).

Day 1. Corner and Open-Field Tests (CT and OF)

Animals were individually placed in the center of a clean standard home cage, filled with wood shave bedding. Neophobia was evaluated in the corner test (CT) for 30 s through the number of corners visited (CTc), latency to perform the first rearing (CTlatR), and the number of rearings (CTr). Immediately after, mice were placed in the center of an open-field (OF), beige metal drawer (metalwork, 50 × 40 × 25 cm) and were observed for 5 min. The sequence of behavioral events that define the animal's ethogram was recorded as follows: duration of freezing behavior (LatM, latency to move), latency to leave the central square (LatC) and that of entering the peripheral ring (LatP), as well as latency and total duration of self-grooming behavior (LatG and tG, respectively). Horizontal (crossings of 10 × 10 squares) and vertical (rearings with wall support) locomotor activities were also measured. Bizarre behaviors observed in this test (stereotyped stretching, stereotyped rearing, backward movements, and jumps) were also measured according to previously reported criterion (Baeta-Corral & Giménez-Llort, 2014). During the tests, defecation boli and urination were also recorded. In the present work, we propose gait analysis of locomotion in the open field to measure the number of forward locomotion episodes (walking preceded and followed by a rest) and the number of crossings covered on each, as complementary to measures to horizontal and vertical activity time courses (Giménez-Llort, Blázquez, et al., 2007) and classical total counts.

Day 2. Recognition Tests (OF2 and OR)

The day after, the animals were retested in the open field (OF2) to evaluate the behavioral response when they confronted the same anxiogenic environment again. Analysis of activity was done during the first minute of the test, the time period where we have previously described AD-genotype differences(Torres-Lista et al., 2014). Immediately after, animals were moved to a standard home cage, where they remained for one minute before being reintroduced to the field where two objects were now

allocated to administer the novel object recognition test. The animals were assessed (4 h apart) for their ability to recognize a familiar object (S, sample) from a new one (N). Each of these sessions consisted of a "sample trial" fol- lowed by a "test trial." In the sample trial, the animals were placed in the open-field (a known environment) and left to explore (nose directed to the object not less than 1 cm) two identical objects, S1 and S2 (glass bottles, 15 × 12 cm, 5 cm diameter), equally spaced in the floor of the apparatus until they reached the criteria of exploration of both for 20 s. The time required to reach the criteria was named "time 1." One minute later, animals were reintroduced in the apparatus for 5 min (test trial) where two different non-explored objects were located: an identical copy of the sample object (S3) and a completely new object (N, rectangular aluminum can, 15 × 10 cm, 4 cm high). Preference for the new object was measured through the index TN−TS/TN+TS where TS and TN are the time spent exploring "S3" and "N," respectively.

Day 3. Spontaneous Alternation in the T-Maze (TM)

On the third day, the black T-maze (woodwork) consisted of two short arms of 25 × 8 cm and a long arm of 30 × 8 cm. The animals were placed inside the long arm of the maze with its head facing the end wall. The latencies of the first movement, start walking, arriving at the intersection of the arms, and completing the exploration of the maze were recorded. Finally, defecation boli and urination were also recorded.

Day 4 to 9. Morris Water Maze Test (MWM)

Mice were trained, four trials per day, spaced 30 min apart, to locate a platform (14 cm diameter) in a circular pool for mice (120 cm diameter, 80 cm height, 25 ◦C opaque water), and were covered with a completely black curtain. Mice were gently released (facing the wall) from one randomly selected starting cardinal point and allowed to swim until they escaped onto the platform. On the first day, a cue task (CUE, DAY 1) assessed the visual perceptual learning and memory of a visible platform, elevated 1 cm above the water level in the N position and indicated by a visible striped flag (5.3 × 8.3 × 15 cm). Extra maze cues were absent in the black curtain. During the next four consecutive days (PT1-PT4, DAY2-DAY5), the mice searched for a hidden platform that was located in the middle of the S quadrant. White geometric figures hung on each wall of the room were used as external visual clues. In all trials, mice failing to find the platform were placed on it for 10 s, the same period as the successful animals. On the last day, 2 h after

the last trial of the place-learning task, a probe trial without the platform was administered during 60 s to assess spatial memory for the previously trained platform location.

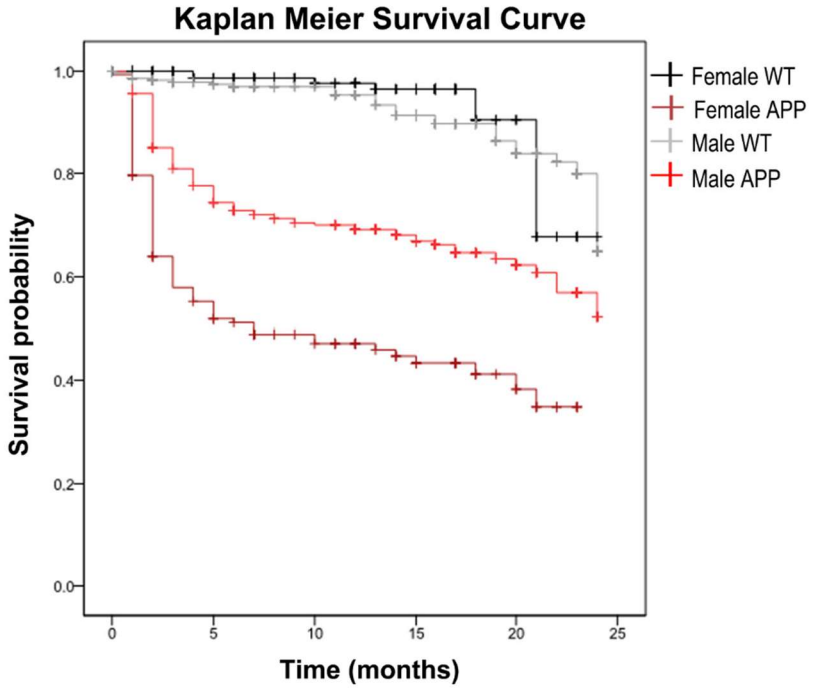
In all the learning tasks, the variables of time (escape latency), distance, and speed were also recorded by a computerized tracking system (ANY-Maze v. 5.14, Stoelting, Dublin, Ireland). The number of crossings over the removed platform position (annulus crossings), the time spent, the distance traveled in each quadrant, and the swimming speed were also analyzed.

3.1.4. Statistics

Statistical analyses were performed using GraphPad Prism 6 and SPSS Statistics 20.0 software. All data are presented as mean ± SEM or percentage. To evaluate the effects of (S) sex, (G) genotype and (A) age factorial analysis design was applied. Differences were studied through multivariate General Lineal Model analysis (mGLM), followed by post-hoc Duncan's test comparisons. Differences between (G) genotype and (A) age × (T) time interval interactions in the different MWM tasks were analyzed by Repeated Measures ANOVA (RMA). Cox regression was used for multivariate analysis of survival. In all cases, statistical significance was considered at $p < 0.05$.

3.2. RESULTS

3.2.1. Genotype and Sex-Dependent Increased Mortality Rates



	Male WT	Male APP23	Female WT	Female APP23
N (survival)	294	275	189	157
Mean lifespan, months (95% CI)	22.3 (21.6–22.9)	17.1 (15.9–18.2)	22.4 (21.5–23.2)	11.6 (9.9 –3.1)
Number (%) of mice, dead	29 (9.9%)	95 (34.5%)	10 (5.3%)	86 (54.8%)
Number (%) of mice, censored	265 (90.1%)	180 (65.5%)	179 (94.7%)	71 (45.2%)

Figure 1. Survival—Effect of age and AD-genotype on survival. Data are expressed by mean and 95% CI, or percentage.

Survival curve (see Figure 1) based on 916 cases and 220 events or natural deaths showed genotype-dependent increased mortality in APP23 mice (G, *** $p < 0.001$), with a severe initial drop of survival, mainly during the first two months of age. Although sex did not influence the risk of mortality in WT littermates, the effect of APP23 genotype in the overall survival was significantly different depending on the sex (S, *** $p < 0.001$). Mortality risk in female APP23 mice was 12.3 times higher than female WT littermates and 2.1 times higher than male littermates with their same AD-genotype. In the case of males, the ratio was 3.6 times higher mortality in the APP23 genotype than male WT littermates.

3.2.2. Strong Contribution of Age and Specificity of Genotype Factor on Key Variables for AD-Related Phenotype

Table 1. Genotype and age factors and interaction effects on behavioral tests and variables from middle-age to long-life.

Behavioral Tests and Variables	Statistics	Genotype	Age	G × A	Significance
Corner test					
Total visited corners	F (2,52) = 7.392	-	A ***	-	$p = 0.001$
	F (2,52) = 4.126	G *	-	-	$p = 0.047$
Total numbers of rearings	F (2,52) = 13.374	-	A ***	-	$p = 0.000$
Latency of rearing (s)	F (2,52) = 10.210	-	A ***	-	$p = 0.000$
Open field test					
Freezing—Latency of first movement (s)	F (2,52) = 13.362	-	A ***	-	$p = 0.000$
	F (2,52) = 3.664	-	-	G × A *	$p = 0.032$
Latency to exit the center (s)	F (2,52) = 6.640	-	A **	-	$p = 0.003$
	F (2,52) = 11.169	G **	-	-	$p = 0.002$
Latency of rearing (s)	F (2,52) = 2.905	-	-	G × A (*)	$p = 0.064$
	F (2,52) = 2.649	-	A (*)	-	$p = 0.080$
Latency of self-grooming (s)	F (2,52) = 5.917	G *	-	-	$p = 0.018$
Total horizontal activity (*n* crossings)	F (2,52) = 12.909	-	A ***	-	$p = 0.000$
	F (2,52) = 6.043	G*	-	-	$p = 0.017$
"in the center (*n* crossings)	F (2,52) = 3.044	-	-	G × A (*)	$p = 0.056$
"in the periphery (*n* crossings)	F (2,52) = 11.655	-	A ***	-	$p = 0.000$
	F (2,52) = 9.404	G **	-	-	$p = 0.003$
Total vertical activity (*n* of rearings)	F (2,52) = 18.966	-	A ***	-	$p = 0.000$
Context and object recognition tests					
Latency to enter into the periphery	F(2,52) = 13.448	-	A ***	-	$p = 0.000$
Total horizontal activity					
"in the center (*n* of crossings)	F(2,52) = 3.325	-	A *	-	$p = 0.044$
"in the periphery (*n* of crossings)	F(2,52) = 3.339	-	A *	-	$p = 0.043$
Time exploring new object (s)	F(2,52) = 3.746	-	A *	-	$p = 0.030$
T-maze					
Latency to cross the intersection (s)	F(2,51) = 27.978	G ***	-	-	$p = 0.000$

Total time exploring both arms (s)	F(2,51) = 6.005	-	A **	-	$p = 0.005$
	F(2,51) = 4.107	G *	-	-	$p = 0.048$
Total time to complete the test (s)	F(2,51) = 19.484	G ***	-	-	$p = 0.000$
Total number of errors (*n*)	F(2,51) = 3.139	-	A (*)	-	$p = 0.052$
Total vertical activity (*n* of rearings)	F(2,51) = 9.700	G **	-	-	$p = 0.003$
	F(2,51) = 3.631	-	-	G × A *	$p = 0.034$

Statistical analysis: Factorial analysis, G, genotype, A, age, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; (*) one-tailed.

As summarized in Table 1, the effect of age was shown in most behavioral variables (A, *** $p < 0.001$) while genotype differences mainly were related to horizontal and vertical activities, thigmotaxis, and coping with stress strategies. Overall, T-maze resulted in being the most sensitive test to genotype effects.

In the corner test, the horizontal locomotor activity, as measured by the number of visited corners (Figure 2A), was higher in APP23 mice as compared to WT animals (G*, $p < 0.05$). However, an age effect pointed at 12 and 24-month animals being more active in comparison with those of 18 months of age ($p < 0.05$, in both cases). In the case of vertical activity, the latency to perform the first rearing (Figure 2C) progressively increased with age (A***, $p < 0.001$). Conversely, the total number of rearings (Figure 2B) recorded decreased as well (A***, $p < 0.001$). Both variables indicated that vertical activity dramatically decreased in the 24-month-old APP23 mice compared to the WT littermates (* $p < 0.05$), unveiling a genotype difference at this age.

The ethogram describing the temporal sequence of behavioral events of animals in the open-field test (Figure 2D) showed that the actions exhibited had a strong effect on age, together with genotype or genotype x age interaction effects. That is, the latency of the first movement (OFlatm) and to leave the central area (OFlatc) were strongly dependent on age (A***, $p < 0.001$ and A**, $p < 0.01$, respectively) but also modulated by genotype (OFlatm, A × G*, $p < 0.05$; OFlatc, $p < 0.01$). The performance of the first vertical exploration in the periphery was modulated by age (A*, $p < 0.05$) and age x genotype interaction (A × G*, $p < 0.05$) with shorter latencies in the APP23 mice as compared to WT littermates due to the presence of stereotyped vertical rearing (without wall support). Self-grooming was affected by genotype with shorter latency.

The total activity exhibited in the open field test on Day 1 (Figure 2E–H) indicated that the main effects were attributed to age factor (A***, $p < 0.001$) in both the total horizontal (Figure 2E) and vertical (Figure 2F) activities, with a progressive reduction of both variables with aging. Genotype differences were also found in the horizontal activity exhibited on the first day (Figure 2E, G*, $p < 0.05$), with a higher total number of crossings shown by APP23 mice than WT littermate mice. Statistically significant differences were mainly shown at 12 and 24 months of age, and they were due to the activity performed in the periphery (Figure 2G, G**, $p < 0.05$). At 12 months of age, the genotype-dependent increase of activity was observed in both the periphery and the central area, while at the older ages, the APP23 mice showed lower activity in the center as compared to WT, albeit that these differences did not reach the statistical significance until the second day of the test (see Figure 3B; G*, $p < 0.05$; A*, $p < 0.05$).

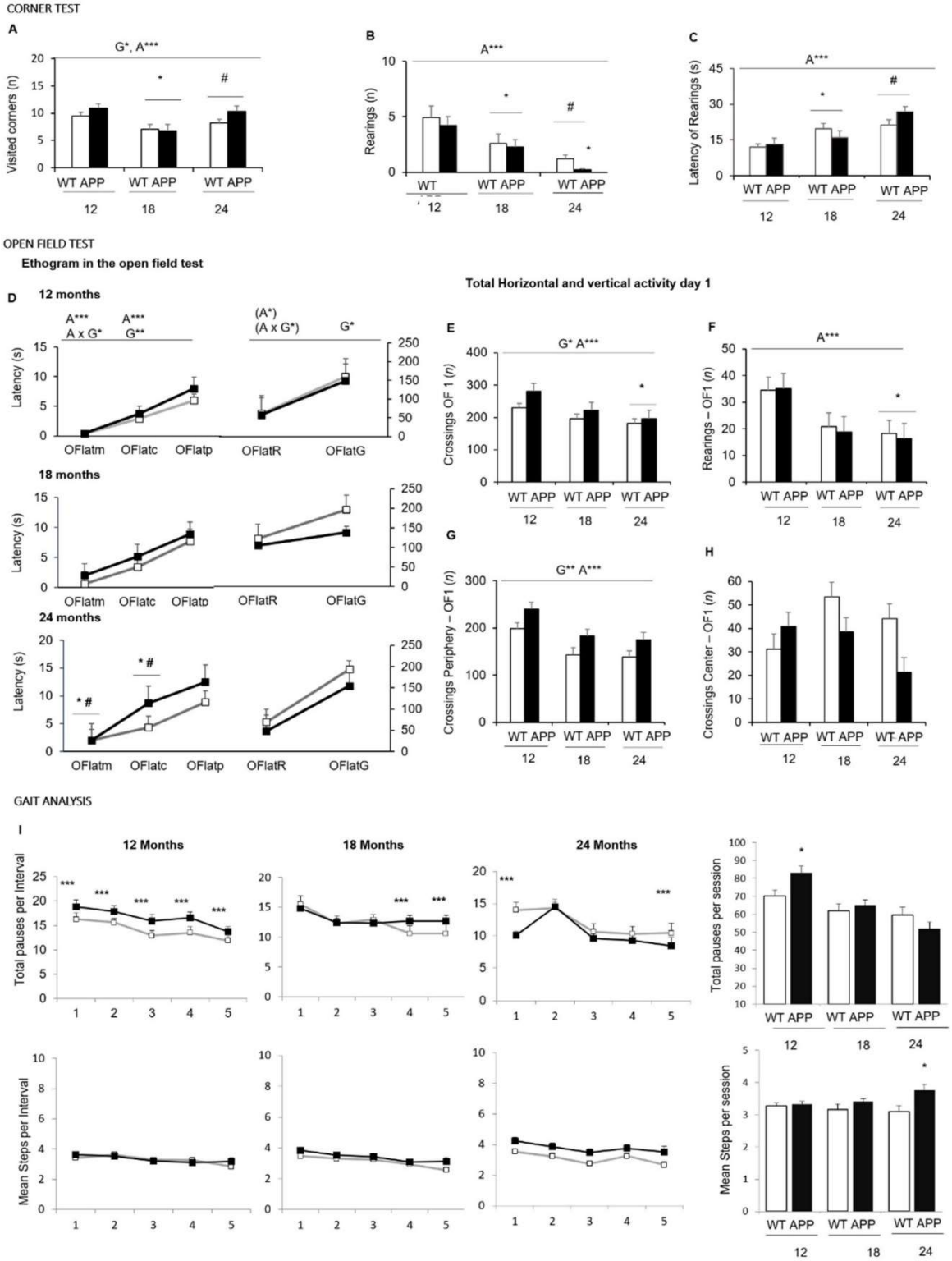
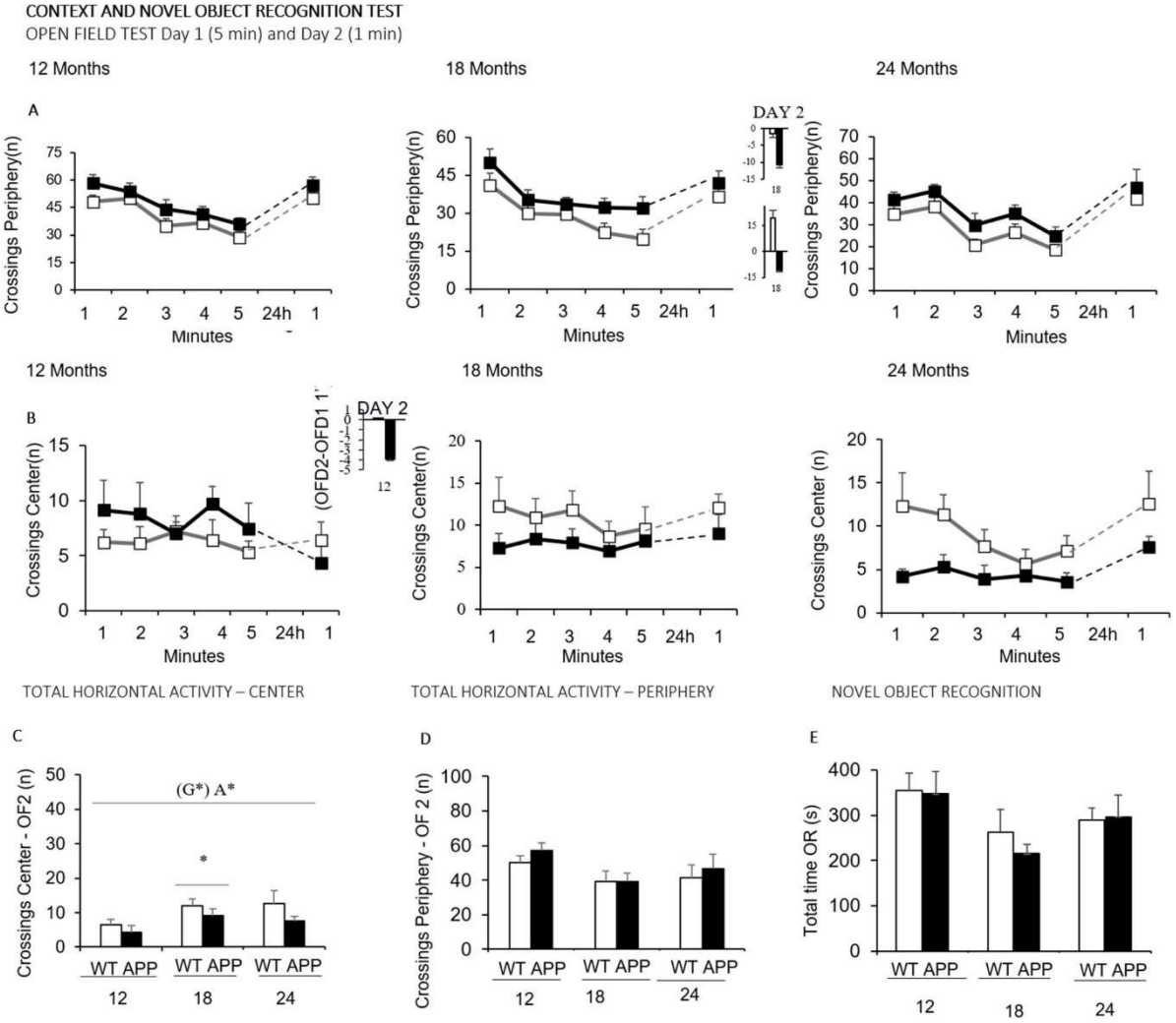


Figure 2. NPS-like behaviors and gait analysis in the corner and open-field test—Effect of age and AD-genotype on the neophobia and exploratory activity elicited in a new home cage (corner test, (A–C)) and the open-field test (D–I) at 12, 18, and 24 months of age. Data are expressed by Mean ± SEM. WT: wildtype mice; APP: APP23 mice. Corner test: Visited corners (A), number (B), and latency of rearings (C). Open field test: Ethogram,

the temporal sequence of behavioral events in the open field test, Variables: OFlatm, latency of the first movement; OFlatc, latency to leave the central area; OFlatp, latency to enter into the periphery; OFlatR, latency of the first rearing; OFlatG, latencies of the first grooming (D). Total horizontal (E) and vertical (F) activity in the open-field test. Total horizontal activity in the periphery (G) and the central area (H). Gait analysis: Total number of pauses (I) and mean number of crossings in each time interval and in the total time of the open field test. Analysis of variance 2 × 3: Effects of genotype (G), Age (A) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Post-hoc analysis: * $p < 0.05$ vs. 12 months of age, # $p < 0.05$, vs. 18 months of age; * $p < 0.05$ vs. the corresponding WT control group.

Figure 3. Context and novel object recognition tasks. Data are expressed by mean ± SEM.



Time course of the horizontal activity (number of crossings) exhibited in the periphery (A) and the center (B) of the open-field test on Day 1 and 2, in 12-, 18- and 24-month-old WT (white squares) and APP23 (black squares). (C) Total horizontal activity (number of crossings) in the periphery on Day 2, in 12-, 18- and 24-month-old WT (white bars) and APP23 mice (black bars), (D) Total horizontal activity (number of crossings) in the

center on Day 2, in 12-, 18- and 24-month-old WT (white bars) and APP23 mice (black bars). (E) Total time of exploration of the novel object (seconds) in the object recognition test in 12-, 18- and 24-month-old WT (white bars) and APP23 mice (black bars). Analysis of variance 2 × 3: Effects of genotype (G), Age (A), * $p < 0.05$. Post-hoc analysis: * $p < 0.05$ vs. 12 months of age.

The increased horizontal activity of APP23 mice compared to WT was further examined through a gait analysis as illustrated in Figure 2I. The performance also showed genotype effects. Thus, at 12 months of age, the increased total activity of APP23 mice did not result from a walking performance covering a greater number of crossings per unit of movement per se, but due to a higher number of walking episodes per interval of time. The total number of episodes was significantly higher as well (G*, $p < 0.05$). With aging, the number of episodes was reduced. At 24 months of age, the variable that evidenced the genotype effects was the number of crossings covered, which was higher in APP23 than their WT littermate group (* $p < 0.05$).

The two recognition tasks performed during the novel object recognition test administered on Day 2 are illustrated in Figure 3. That is, the horizontal activity exhibited when the animals were re-exposed to the open field test (Figure 3A–D) and the discrimination of a novel object in the novel object recognition test (Figure 3E). As shown, when the animals were introduced in the corner of the periphery, both genotypes performed a similar locomotor activity. However, the comparison with their performances on their first experience in the open field test (18 months of age, OFD2-OFD1, minute 1) as well as the before–after activity levels unveiled genotype differences at 12 (Figure 3A) and 18 (Figure 3B) months of age. In the next part, the novel object recognition test showed a low sensitivity to the genotype, only noticed at the oldest age by a lower difference in the latency to explore the novel and familiar objects than WT littermates.

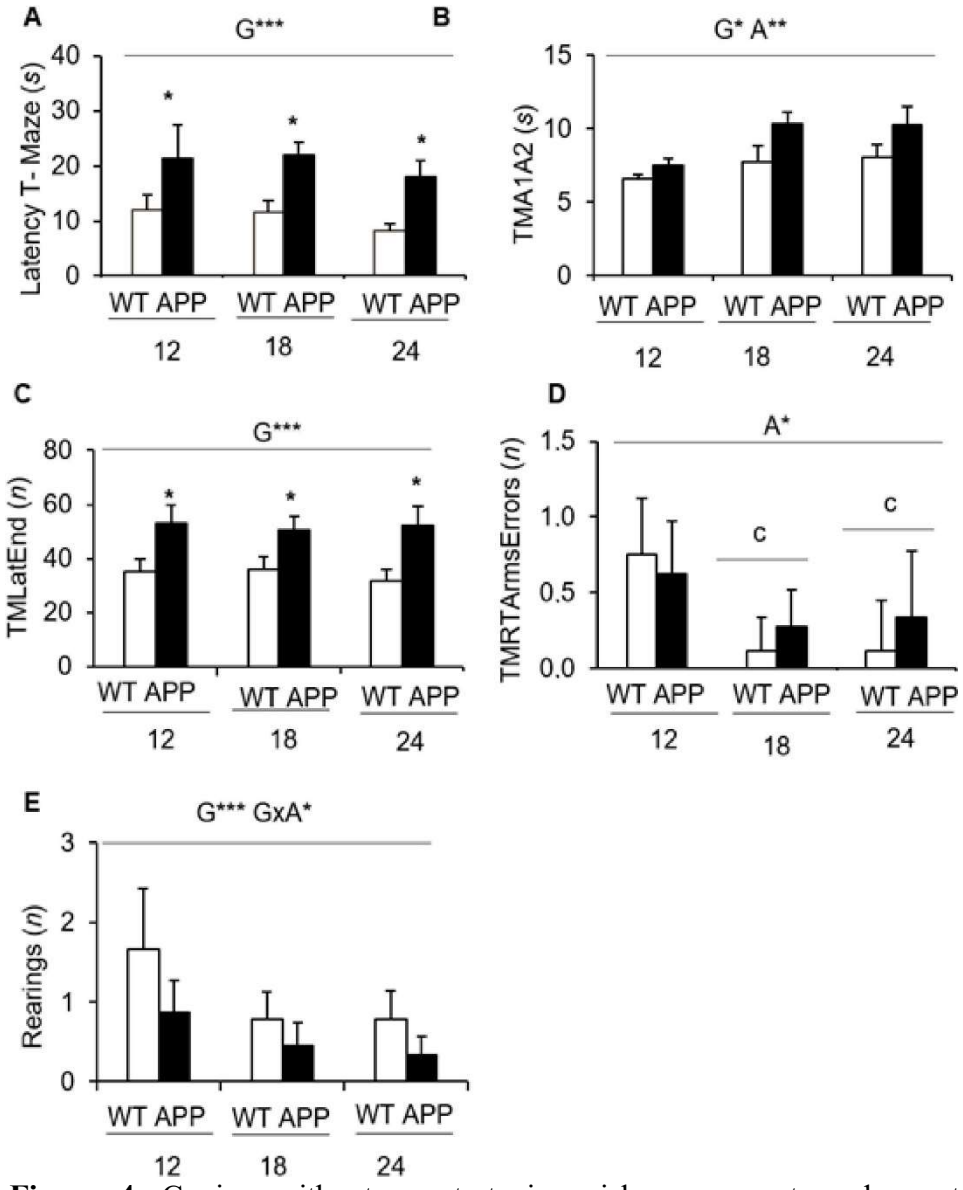


Figure 4. Coping with stress strategies, risk assessment, and spontaneous alternation in the T-Maze. (A) Latency to cross the intersection; (B) Total time invested in the exploration of the left and right arms; (C), Time to complete the exploration of the maze; (D), Total number of errors performed during the exploration of the maze; (E) Vertical exploratory activity. Data are expressed by means ± SEM. WT: wildtype mice; APP: APP23 mice. Analysis of variance 2 × 2: Effects of genotype (G), age (A), genotype × age (G × A), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Post-hoc analysis: c $p < 0.05$ vs. 12 months of age; * $p < 0.05$ vs. the corresponding WT control group

In the T-maze (Figure 4), at all the ages studied and as compared to WT littermates, the APP23 mice took a longer time to reach the intersection of the vertical arm (not shown) and once there to cross it (Figure 4A, G***, $p < 0.001$). In addition, they also spent more time in both arms (Figure 4B, G*, $p < 0.05$) and required more time to

complete the test (Figure 4C, G***, $p < 0.001$). The factor age was also a determinant for the time spent in the arms (Figure 4B, A**, G*, $p < 0.01$). The analysis of errors revisiting the arms that had been already explored indicated a significant age effect (A*, $p < 0.05$) with a decrease at 18 and 24 months of age as compared to 12 months ($p < 0.05$). Furthermore, a decrease in the number of rearings in the APP23 mice, with a genotype x age interaction (G × A*, $p < 0.05$), was found.

To further clarify the results and explain the interrelationships among the various tasks, correlation analyses were performed using data from all the animals in this study (not shown). In the animals' sensorimotor activity measurements, the most prominent correlation was between the open field activity, more specifically on the crossings in the periphery, and the exploration activity obtained in the test corners ($p < 0.005$). Additionally, there was a correlation between the crosses in the periphery of the open field and the exploration time of the arms of the T-maze ($p < 0.005$). On the other hand, a correlation was obtained between the number of corners visited and the amount of exploratory activity performed on the first day of the open field ($p < 0.05$).

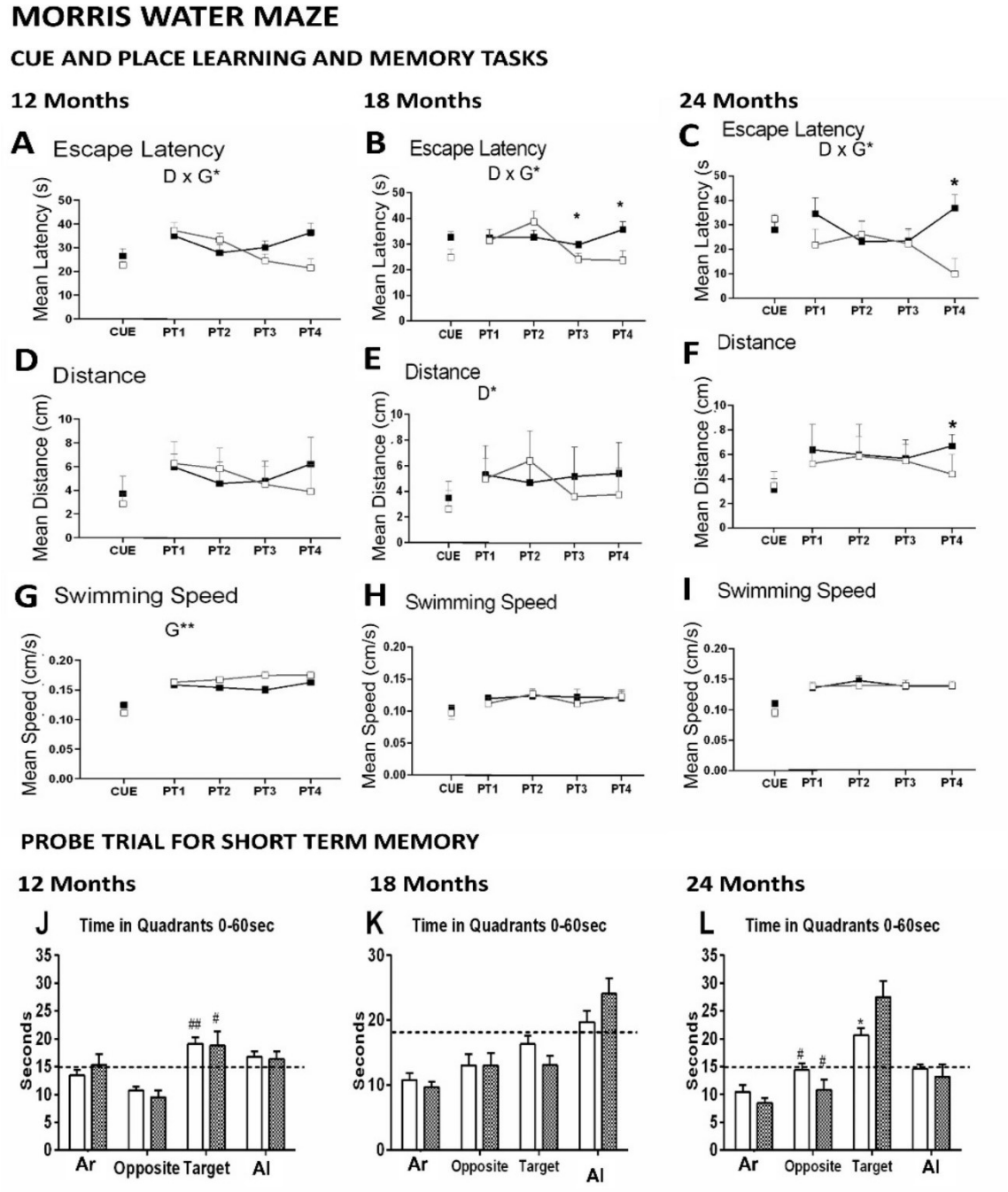


Figure 5. Quantitative analysis of the performance of wildtype (WT) and APP23 mice at 12, 18, and 24 months of age in three paradigms of the MWM. Data are expressed by means ± SEM. Day-by-day quantitative analysis of the CUE and PT place leaning task by means of (A–C) mean escape latency, (D–F) distance and (G–I) swimming speed. Open squares or bars: WT mice; Solid squares or bars: APP23 mice. ANOVA, D, day factor; G, genotype factor, Post-hoc Student t-test; *p < 0.05, ** p < 0.01 vs. the corresponding control group. (J–L) Probe trial for short term memory assessed by means of the time in each quadrant; adjacent right (Ar), Opposite, Target and adjacent left (Al)

quadrants. Statistics: Student t-test; * $p < 0.05$ vs. the corresponding control group. Target vs. opposite vs. # $p < 0.05$, ## $p < 0.01$.

Figure 5 illustrates the performance of animals in three paradigms of the Morris water maze, namely, the cue, place task, and probe trial. No differences between groups were found in the first CUE task for visual perceptual learning in any of the variables studied. However, a trial-by-trial analysis of their first performance in the maze showed distinct behaviors depending on the age. Thus, both groups performed equally at 12 months of age and tended to differentiate at 18 months and finally did it at a very old age. After that, genotype x day interactions were found in the mean escape latencies in the place task for spatial reference memory, at the three ages studied (Figure 5A–C: G × D*, all F's [3,45] = 3.30, $p < 0.05$). Thus, the acquisition curves of both genotypes differed through the four days of the test, with a worse performance of APP23 mice as compared to the WT littermates shown as a longer delay to find the platform on the third (PT3, * $p < 0.05$) and fourth (PT4, * $p < 0.05$) day of the test. Genotype differences in swimming speed were shown at 12 months of age (Figure 5G, G**, F [1,15] = 10.79, $p < 0.01$), with a slower pattern in APP23 mice. Therefore, distances covered to reach the platform were also calculated (Figure 5D) but showed a smother distinction in the temporal course between genotypes. At older ages, both groups of mice slowed their navigation speeds, and genotype differences were lost (Figure 5H, I). Statistically significant differences observed in escape latency translated into the variable of distance only at 24 months of age (Figure 5F, PT4, * $p < 0.05$). In the probe trial (Figure 5J–L), independent of genotype, preference for the target vs. opposed quadrant was only shown at 12 and 24 months of age, while in the 18-month-old groups, the time of permanence was biased to the adjacent left quadrant. Surprisingly, the preference of 24-month-old APP23 mice for the target quadrant was significantly higher than their WT littermate counterparts (Figure 5L, * $p < 0.05$).

3.3. Discussion

In the present work, we determined the age and AD-genotype sensitivity of a battery of behavioral tests in APP23 mice of male sex at middle (12 months), old (18 months), and very old (24 months) ages, and compared them to age-matched C57BL/6 mice with normal aging. First, we confirmed that survival curves of APP23 mice were those suitable to assess long-lived animals, since the scarcity of the literature comparing male animals from middle to very old ages is due to increased mortality risk in this sex,

increased heterogeneity in the aging process, and also in AD at end-of-life dementia stages.

Genetically engineered AD models, primarily based on the overexpression of presinilin1 or APP mutant human genes causing early-onset Familial AD (FAD), are used to study hallmark behavioral and neuropathological mechanisms of SAD, the sporadic and most common form of Alzheimer's disease despite some gaps (Chen et al., 2013; Nardini et al., 2021) For instance, the APP23 mice, overexpressing the mutant human-type APP protein with the Swedish mutation (K670M/N671L), is a widely used animal model of AD since it genetically develops amyloidosis and presents cognitive and behavioral symptoms similar to those found in patients (Lalonde et al., 2003). However, both SAD and FAD models are limited by the difficulty to fully recapitulate the complexity and progression of this neurodegenerative disease in humans (Giménez-Llort, Blázquez, et al., 2007; Nardini et al., 2021; Pietropaolo et al., 2008) Most importantly, this consideration also refers to the challenge to unveil the factors that may underlie the heterogeneity described at end-of-life dementia and the individuals' survival. The neuro-immuno-endocrine hypothesis could explain the morbidity–mortality paradox among sexes that also accounts for AD (Mazure & Swendsen, 2016; Mielke et al., 2014b; Sinforiani et al., 2010) and we have already described it in mice (Giménez-Llort et al., 2010; Giménez-Llort et al., 2012; Gimenez-Llort et al., 2014) However, more recently, distinct genotype and sex differences in life expectancy among different mouse models of AD and inbred strains have been reported in comprehensive reviews (Kane et al., 2018; Rae & Brown, 2015)

Survival—The survival analysis was based on a total of 916 cases from our APP23 and wildtype cohorts with the same gold-standard C57BL/6 genetic background. It showed genotype and sex-dependent increased mortality rates in the mutant mice, with a severe initial drop of survival during the first two months of age, which was more dramatic in APP23 females. Interestingly, APP/PS1 males also exhibit longer lifespans than females (Pugh et al., 2007). Survival curves demonstrated that APP23 males were able to reach a long-life span, rending this animal model suitable for studying the impact of the disease in a male aging scenario. That is, 12- 18- and 24-month-old subjects would serve to study the disease at the middle, old, and very old ages, respectively, and as compared to age-matched C57BL/6 wildtype mice. There is a chance that it is strongly restricted in other genetically engineered AD models where mortality rates of males are

a limiting point to address advanced aging in AD(Torres-Lista et al., 2017, 2019). As mentioned before, the study of the age-matched wildtype mice also contributed to provide data to the scarcely reported normal aging process in the gold- standard C57BL/6 mice strain, from middle, old, and very-old ages.

Factorial effects and battery of tests—In this research, the behavioral profiles of APP23 and WT mice with normal aging were evaluated through a battery of seven tests. Namely, the corner test, open field test, the novel object recognition test, the T-maze, and three paradigms in the Morris water maze determine the effects of genotype and aging process on their motor, emotional, and cognitive functions. On a general basis, aging was shown as the most determinant factor for most behavioral variables studied, achieving the strongest statistically significant values, whereas genotype factor was specific of those variables related to dysfunctional impairment in AD. This is in agreement with previous results reported by our laboratory using another animal model of AD. There, the age factor from adulthood to maturity (7 to 11 months of age) was also a determinant for variables related to sensory and motor function, while genotype differences specifically pointed at cognitive and BPSD-hallmarks of disease (Baeta-Corral & Giménez-Llort, 2014) After the mean life span, the factor of age was shadowing the genetic differences, and a mortality bias was also noted (Torres-Lista et al., 2017, 2019). In the present work, the great majority of the variables that were found to be age-sensitive belong to the corner test and the open field, in agreement with the worsening of fear and anxiety-like symptoms with the progress of the disease. Here, genotype differences selectively pointed at hyperactive horizontal activity patterns, in decrement of vertical exploration, resembling those described in 3xTg-AD mice (Giménez-Llort, Blázquez, et al., 2007).

Corner and open-field tests—Fear and anxiety-like behaviors are the most common neuropsychiatric-like alterations associated with dementia that can be studied in mice models of AD (Giménez-Llort, Blázquez, et al., 2007) since they appear in their early stages, worsen with the progress of the disease, and most standard behavioral tests can record their presence (Gimenez-Llort et al., 2006). Therefore, the battery of tests started with the corner and open-field tests to record the quantitative and qualitative features of these behaviors elicited in these two anxiogenic enclosures. When the APP23 mice were introduced in a new home cage of transference to the open field, the mild neophobia recorded was increased compared to that exhibited by WT littermates and worsened with age. Thus, the mutant mice performed a greater number of visited corners,

and the reduction of vertical activity at 24 months of age was more severe, unveiling genotype differences in this variable.

This response was in agreement with the subsequent behavioral sequence of events observed in the open field, illustrated as an ethogram likeminded with the sequence of 'action programs' described by Lát in 1973 (Lát, 1973) . The APP23 animals showed increased latencies to develop the ethogram immediately after direct exposure to the open and illuminated arena. They differed in the momentary freezing, with a longer fear response when confronting the new environment. Furthermore, they differed in the flight-to-fight copying with a stress strategy developed that elicited the early presence of bizarre vertical behaviors (stereotyped rearing without walk support, as described in (Baeta-Corral & Giménez-Llort, 2014) and anticipatory self-grooming indicative of increased anxiety (Giménez-Llort, Blázquez, et al., 2007). With aging, the overall activity and the thigmotaxis behavior showed a reduction in all the groups. This effect was more notorious in the mutant mice. Thus, while 12-month-old APP23 mice were more active than their WT littermates in all (the central and peripheral) areas of the field, the activity of 18- and 24-month-old APP23 mice was strongly reduced in the center, indicating a worsening of anxiety in the elder ages/stages of the disease. In contrast, the increased allocentric exploration close to the protected areas of the mutants was persistent from middle-age until the oldest age. Age and genotype differences were lost the next day, as assessed in the open-field retest. When confronting the open field for the first time, the hyperactive pattern shown is consistent with previous studies describing hyperactivity in APP23 mice when assessed in this test and a Y-labyrinth (King & Arendash, 2002a). However, other laboratories have reported a hypoactive pattern in this animal model (Dumont et al., 2004). The discrepancies may correspond to the specific anxiogenic conditions elicited by the tests, similarly to hyperactivity of 3xTg-AD mice when assessed under soft light conditions in activity cages. In contrast, they exhibited a clear anxiogenic hypoactive pattern in the open and illuminated open field, the dark lightbox, and the elevated T-maze (Giménez-Llort, Blázquez, et al., 2007; Torres-Lista et al., 2019).

Gait analysis of locomotor activity—The quantitative and qualitative assessment of locomotion, with distinction of inactivity, and slow and fast movements, allows discriminating patterns of hyperactivity (Giménez-Llort et al., 1995). Here, based on that, the analysis of locomotion measured the walking speed (crossings per interval of time) and its time course through the 5 min of the test (Giménez-Llort, Blázquez, et al., 2007)

and contrasted is to the classical total counts. For the first time, in the present work, we propose measuring two more features of ambulation: the pace (mean distance covered in each walk) and the number of walking episodes. These variables allowed for distinguishing the locomotor performance of APP23 mice from the normal gait phenotype of their WT littermate counterparts. Thus, the mean walking distance of a pace during the different minutes of the open-field test was about three–four crossings. This agrees with the number of crossings the animal needs to cover to move from one corner to the closest one or to cross the arena without reaching the center. Here, all through the test, 24-months-old APP23 performed longer walking distances than their age-matched counterparts and the other groups, probably due to their lower activity in the center. It is important to note that the increased total activity of APP23 mice at 12 and 18 months of age did not result from a walking performance covering a greater number of crossings per pace, but due to a higher number of paces, walking episodes per interval of time.

On the other hand, in the three mutant groups, the total paces per interval of time were also increased at several time points of their temporal courses, and the value trend decreased with age. This variable related to kinetics was more sensitive to discriminate mutants at 12 months of age from their age-matched counterparts. The homologous, the swimming speed in the Morris water maze, also allowed unveiling genotype differences at 12 months of age, though in that case, APP23 mice were swimming slower than controls. Interestingly, longitudinal population-based studies have shown a decline in spontaneous or so-called preferred walking speed (PWS) in older people associated with brain structural changes in gray and white matter volumes(Callisaya et al., 2014). At the clinical level, a slower fast-walking speed (FWS) is used as a marker of frailty and mortality (Artaud et al., 2015; Middleton et al., 2016; Sabia et al., 2014; Studenski, 2011). Besides, the slowest PWS and FWS, and a smaller walking speed reserve (the difference between both) have been associated with poorer cognitive stage (Callisaya et al., 2017). Furthermore, recently, an international cross-sectional gait and Alzheimer interaction tracking study has consistently described an abnormal gait phenotype since the earliest stages of dementia (Allali et al., 2016). The Spatio-temporal gait parameters are worse in woman patients and at advanced/severe stages of dementia, although they seem more relevant to non-amnestic forms of cognitive impairment and non-AD dementias (Callisaya et al., 2017).

Cognitive function: Habituation and Recognition tests—In the present work, the hyperactivity pattern of APP23 was consistently apparent from middle to very old ages. Hyperactivity can reflect an altered ability to habituate (Allali et al., 2016), as shown in the psychostimulant effects of caffeine (Giménez-Llort et al., 2005) and A1R knockout mutant mice (King & Arendash, 2002b). In fact, habituation is considered as a form of rudimentary learning and memory process for recent events (Lát, 1973), and where an animal learns to ignore stimulus with no predictive value (King & Arendash, 2002b). Several other aspects of the cognitive profile were analyzed during the following days through different paradigms.

First, we wanted to assess the cognitive function involved in other recognition tasks. As we have previously shown, the performance of young and adult 3xTg-AD animals in the first minute of a repeated open-field test is sensitive to AD-genotype (Torres-Lista et al., 2014). In fact, the re-exposure to a fearful environment is the basis of many conditioned memory tests (i.e., (España et al., 2010)). Therefore, on the second day of tests, we assessed the recognition of the open and illuminated field and, after that, the preference for a novel object from one previously inspected was recognized as familiar. The performance on the first minute of the open field repeated test closely resembled the neophobia response previously shown by the same animals in the corner test. Animals maintained their locomotor activity in the periphery and slightly increased the one performed in the center. Overall, that resulted in the loss of genotype differences previously found on the first exposure to the open field. Concerning the novel object recognition test per se, no significant differences were detected between any of the groups as well. The most found was a genotype difference in 24-months-old APP23 mice, with worse latency to explore the novel object than the familiar, but this was due to a better performance in the WT mice. The lack of AD sensitivity of these recognition tasks regarding their use to assess learning and memory could be related to the animal's anxiety-like phenotype and/or a reduced interest for exploration, the two main potential confounds that can bias the animal's cognitive performance. This would agree with the genotype and age-dependent reduction of vertical exploratory activity already shown on the first experience in the open field test and the hyperactive horizontal locomotor pattern.
T-maze—Whereas the open field allowed us to observe significant differences associated with age, and to a lesser degree with the genotype, the assessment of animals in the T-maze provided the most AD-sensitive variables. This T-shaped labyrinth is a scenario

mainly used to evaluate spatial working memory (Thompson, 1959) with the involvement of the hippocampus, septum, prefrontal cortex, and the basal forebrain (Deacon & Rawlins, 2006; Giménez-Llort, Schiffmann, et al., 2007; Zhang et al., 2004). In addition, the latency to reach the intersection of the maze differs according to coping-with-stress strategies expressed by the animal. This is to the extent that this variable was used for psychological selection of prematurely accelerated aging mice (PAM) and non-PAM mice after demonstrating that it correlates with a worse neuro-immunoendocrine function, indicators of accelerated aging, and premature death (Guayerbas et al., 2002). We have also extensively shown that the spontaneous alternation in the black corridors resembling burrows is able to unveil the nuances of cognitive function and anxious-like profiles in both male and female mice, from young to old age under healthy and diverse neuropathological conditions (Ariza et al., 2014; García-Mesa et al., 2011; Giménez-Llort et al., 2010). More recently, some authors have dissected other cognitive aspects (Sanderson & Bannerman, 2012).

Since the beginning of the test, the ethogram or sequence of behavioral events exhibited by APP23 mice in this maze was strongly affected by genotype. Thus, from a 'facing the wall' starting position, the latencies to turn, reach the T-intersection, and cross it with the four paws were significantly delayed in the mutants. The mean time to explore the two arms was increased with age and the AD-genotype. However, this delay was not due to time invested in the vertical exploratory activity, as it was poor and worse with the AD-genotype already since middle age. As a result, APP23 mice took longer to complete the test as compared to their age-matched wildtype mice, a variable that exhibited a clear and robust genotype effect too. The contribution of errors re-entering an already visited arm was also scarce, with 18- and 24-months-old groups performing slightly better than middle-aged groups. At these ages, there was a trend of worse performance in APP23 mice. In other AD models, such as the 3xTg-AD mice, no differences in the number of execution errors were also found at middle age, but the efficiency to complete the different phases of the test is diminished in 3xTg-AD mice and slightly is affected by long-term treatment with the antipsychotic risperidone(Torres-Lista et al., 2019).

The present results define an altered ethogram in the T-maze and point at the delay to cross the intersection as the most AD-sensitive variable. This variable suggests a worse decision-making process, mainly depending on the prefrontal cortex, which could result from this model's extensive cortical neuropathology. Compared, the delays in the

performance of the 3xTg-AD mice are also found to turn and reach the intersection of the T-maze, but once there, the delay in crossing is not so enhanced, as shown here for the APP23 mice. However, a similar delay suggesting chances in risk assessment is seen in this model when the spontaneous choice of entering an anxiogenic white and lit enclosure is assessed in the dark–light box (García-Mesa et al., 2012; Giménez-Llort, Blázquez, et al., 2007; Gimenez-Llort et al., 2006).. Interestingly, that variable was improved by early postnatal handling, an early life intervention based on a tactile sensorial stimulation experience known to modulate cognitive and anxiety-like behaviors in rodents (Baeta-Corral & Giménez-Llort, 2014). The APP/PS1 (Tempier et al., 2013) and Tg2576 (Rinaldi et al., 2007) mice, as well as selective dopaminergic receptors, cause a blockade in the pre-limbic region of the prefrontal cortex (Rinaldi et al., 2007) or STOP-null mice modeling schizophrenia (Delotterie et al., 2010), also show a reduction of spontaneous alternation, suggesting its relation to frontal hypofunctionality. In the present work, the correlation analysis of the variables of T-maze with other tests indicated that the performance in the maze was strongly related to the variables of the other tests referring to increased emotivity/anxiety (self-grooming, navigation speeds) and apathy (latency of vertical and object exploration).

Cognitive function: Morris water maze—Finally, in the Morris Water maze, short- and long-term spatial reference learning and memory were assessed (Morris, 1984). The performances of animals in two learning and memory tasks administered during five consecutive days were evaluated through the classical mean escape latency and the distance covered to reach the platform since a genotype effect was found in the swimming speed at 12 months of age. All animals performed equally in the cued learning, an easy task that assesses visual perceptual learning. Still, it is interesting to note that the performance in their first experience in the maze differed with aging, as very old APP23 confronted the stressful situation of being immersed in a water tank with faster latencies and shorter distances than their WT littermate counterparts. As mentioned before, the navigation was slower in APP23 at middle age but not after that. Here, the impact of aging could explain that the swimming speed be reduced in old and very old animals as compared to the younger groups. If so, the slow swimming speeds of middle-aged APP23 mice compared to their WT littermate group but similar to the speed shown at 18 and 24 months of age, would suggest that they were performing as older than expected for their chronological age. Although the anxious profile of 3xTg-AD mice explains their faster

swimming speeds from early stages of the disease (Giménez-Llort et al., 2013), we have previously shown a similar situation regarding AD-behavioral performances being similar to those corresponding to WT at older ages, both at the biological (Baeta-Corral & Giménez-Llort, 2015) and behavioral level (Gimenez-Llort et al., 2014). In contrast, the genotype differences become strikingly smoother in long-term AD mice survivors due to a mortality bias (death of the worse animals) on one hand and age-dependent impoverished performances in the WT groups on the other (Torres-Lista et al., 2017). Here, the performances of 24-month-old animals were not as bad as could be expected in a progression from 18 months of age, whereas cognitive impairment was a salient behavior and followed the expected deterioration.

After the removal of the platform, the performance of animals did not indicate differences in short-term memory per se. This lack of genotype differences in the probe trial two hours after the last trial of the place task was surprising as expected from the worse performance of mutants in the last or two last days of this task that involves the contribution of long-term but also short-term learning and memory. However, it is noteworthy to mention that the lack of sensitivity of probe trial to the genotype in APP23 mice is in agreement with previous results reported by other laboratory experts in this animal model (Lalonde et al., 2003, 2005). On the other hand, we have recently demonstrated a mortality bias, with animals that died during the behavioral assessments or those with worse life prognostics who had been excluded from the final analysis, explaining the lack of expected results in 16-month-old 3xTg-AD males (Torres-Lista et al., 2019). However, cognitive deficits in the Morris water maze were the salient behavior in 18-month-old survivor females despite a flat phenotype, not distinguishable from their control strain (Torres-Lista et al., 2017). Here, the distinct preferences for the adjacent areas indicate, for instance, less-focused goal-directed swimming strategies at 18 months of age (Lalonde et al., 2005). This suggests that a swimming strategy analysis would further understand the compensatory behavioral mechanism that resulted in such a paradox probe trial report (Lalonde et al., 2003).

Limitations—The main limitation of the present study is intrinsic to the heterogeneity of the aging process and the extreme difficulties to obtain the desired sample size of old and very old animals, not only of mutants with such high mortality rates, but also of their wildtype littermates with normal aging. In order to counteract the age-related heterogeneity, important efforts were needed to obtain concurrent middle-

aged (12-month- old), aged (18-month-old) and very old (24-month-old) animals with the APP23 and WT genotypes, so they shared the same living conditions; moreover, to adapt research agendas so that the cohorts could be assessed within a narrow frame of time in the calendar. On the other hand, as discussed, the use of a battery of tests must assume that carry-over effects cannot be discarded, despite the benefits that assessing the same construct (i.e., anxiety) in tests differing in the level of anxiogenic conditions has to ensure convergent validity of results. These efforts resulted in a low intra- and inter-group variability, and we have reproduced the findings in subsequent experimental research in a consistent manner. However, interlaboratory reliability would be needed to confirm that the results obtained in our colonies can be reproduced in the colonies of other research institutions. An important limitation would refer to gender medicine demands for the concurrent comparative study of males and females, so future efforts should be devoted to achieve this goal despite the difficulty that it may represent when handling very old ages and survivors.

3.4. CONCLUSIONS

The behavioral profiles at 12, 18, and 24 months of age of male APP23 mice and non-transgenic (WT C57BL/6) littermate counterparts with normal aging were assessed using three independent cohorts but in a narrow temporal frame in the calendar. Animals were successively evaluated in a battery of seven behavioral tests to screen motor, non-cognitive and cognitive-like symptoms comprehensively. The presence of 'Behavioral and Psychological Symptoms of Dementia' (BPSD)–like behaviors in APP23 mice was confirmed in three classical unconditioned tests evaluating locomotion, exploration, anxiety-like behaviors, and emotionality under three different anxiogenic conditions.

The main findings can be summarized in five points as follows:

(1) Survival curves of 920 mice of APP23 and WT C57BL/6 littermates confirmed genotype and sex-dependent increased mortality rates, and long-living males.

(2). Compared to WT littermates, the APP23 mice showed an increased number of visited corners but decreased vertical exploratory activity, evidencing increased neophobia in the corner test. Similarly, APP23 groups showed increased latencies to develop the ethogram of behaviors immediately after the direct exposure to an open and illuminated field (not shown) and increased locomotion, with increased thigmotaxis (or search for the protected peripheral area), primarily noticeable at 12 months of age.

(3).In the T-maze, a black T-shaped corridor resembling burrows, the latency to reach the intersection was consistently shown to be increased at all ages and the longer time required for APP23 mice to complete the exploration of all the arms of the maze. As part of the differences in the status of their cognitive functions exhibited during the exploration of new environments, we assessed APP23 mice for putative cognitive deficits in discriminative tasks (novelty/familiarity) for context (remembering the anxiogenic open field scenario) and object recognition.

(4).The novel object recognition test showed low sensitivity to the genotype, only noticed at the oldest age through the lowest difference in the delay in exploring the novel and familiar objects compared to WT littermates.

(5).Cognitive deficits in Spatial reference learning and memory were assessed in the Morris water maze through a 4-day place learning task using a hidden platform followed by a probe trial for long-term memory where the platform had been removed. Worse performance of 12-month-old APP23 mice was shown as compared to WT littermates during the progress of acquisition (learning and memory) in the place task, while differences were scarce in the older groups. In the probe trial, the worse spatial memory discrimination of the trained quadrant compared to the opposed quadrant was shown at 18 months of age but independently of the genotype. As we have reported in other mice models for AD, a conspicuous BPSD-like profile and age as a biological factor may result in confounding factors in an aquatic maze, considered an anxiogenic environment for mice.



MODULE 2

4. *Study 2*

Olfactory Signatures in the Food Finding Test in Mice With Normal and Alzheimer's Disease-Pathological Aging With Special Concerns on the Effects of Social Isolation

4.1. MATERIALS AND METHODS

4.1.1. Animals

A total number of 97 13-month-old male and female homozygous 3xTg-AD (n = 58, 22 male and 36 female) and non-transgenic (NTg, n = 39, 24 male and 15 female) mice on a C57BL/6J background (after embryonic transfer and backcrossing at least 10 generations) established in the Universitat Autònoma de Barcelona (Baeta-Corral & Giménez-Llort, 2014) were used in this study. The 3xTg-AD mice harboring transgenes were genetically engineered at the University of California Irvine, as previously described (Oddo et al., 2003). The animals were maintained under standard laboratory conditions of food and water ad lib, 22 2◦C, 12-h light/dark cycle with lights on at 8:00 am, and relative humidity of 50–60%.

4.1.2. Social Conditions

Animals of the same genotype and sex were maintained in groups of three to four mice per cage (Macrolon, 35 cm 35 cm 25 cm), which was filled with 5 cm of clean wood cuttings (Ecopure, Chips6, DateSand, United Kingdom; Uniform cross-cut wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm 20 cm, White). In the current work, a natural social isolation scenario was found among male 3xTg-AD mice due to increased mortality rates in male mice of this genotype occurring at this age (Giménez-Llort et al., 2008) Thus, short isolation (2–3 months) was observed in seven of the 22 male 3xTg-AD that recently lost their cage mates after 10 months of living in a standard social environment. In all the cases, the standard home cages covered with a metallic grid allow the perception of olfactory and auditory stimuli from the rest of the colony

4.1.3. Behavioral Assessment

Physical Status

To monitor the effects of fasting used in the food finding paradigm, body weight was measured before (Wpre) and after (Wpost) food deprivation. The recovery of weight was monitored at 24 h later (Wfinal).

4.1.4. The Food Finding Test of Olfactory Ability

To investigate olfactory function, the food finding test (FFT) olfactory paradigm (Deacon et al., 2009) was used. The mice were deprived of food for 14 h before testing (starting with lights off at 2000h), the equivalent to 1-day food deprivation.

The test was performed under dim white light (20 lx) during their light phase of the light/dark cycle (from 10:00 to 11:00 a.m.) using a novel cage (50 cm × 22 cm × 14 cm) with 1 cm of beddings. Eight food pellets (45 mg) were placed in the central zone of the cage and covered by 1 cm of wood chip bedding. The mouse was placed in a corner of the cage, facing the walls, and its behavior was observed. The latencies of three goal-directed behaviors toward hidden food were recorded, namely, sniffing— when the nose makes a direct contact with a surface and the vibrissae are in maximum extension and are in contact with the surface in the same way (Welker, 1964); finding (digging)— "finding a food pellet" was defined as digging, touching, and holding the pellet in the front paws for more than 3 s; and eating the hidden food—holding the pellet in the front paws for more than 3 s of continuous eating. The beddings were renewed between each mouse.

Behavioral assessments were performed in a counterbalanced manner by direct observation by an observer blind to the genotype and the support of video recordings (ViewPoint Behavior Technology, Lion, France) for complementary retrospective analysis. All procedures followed the Spanish legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" and the EU Directive (2010/63/UE) on this subject. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used (Kilkenny et al., 2010b).

4.1.5. Statistical Analysis

The results are expressed as mean SEM. SPSS 20.0 software was used. A 2 × 2 factorial design was used to analyze the effects of (G) genotype and (S) sex factors. Differences were studied through multivariate general linear model analysis, followed by post hoc Duncan's test (multiple comparisons). In analyzing the effects of social isolation, the differences between two independent groups were measured with Student's t-test. In all the tests, $p < 0.05$ was considered statistically significant.

4.2. RESULTS

4.2.1. Effect of Food Deprivation on Body Weight but Not Related to the Olfactory Signature

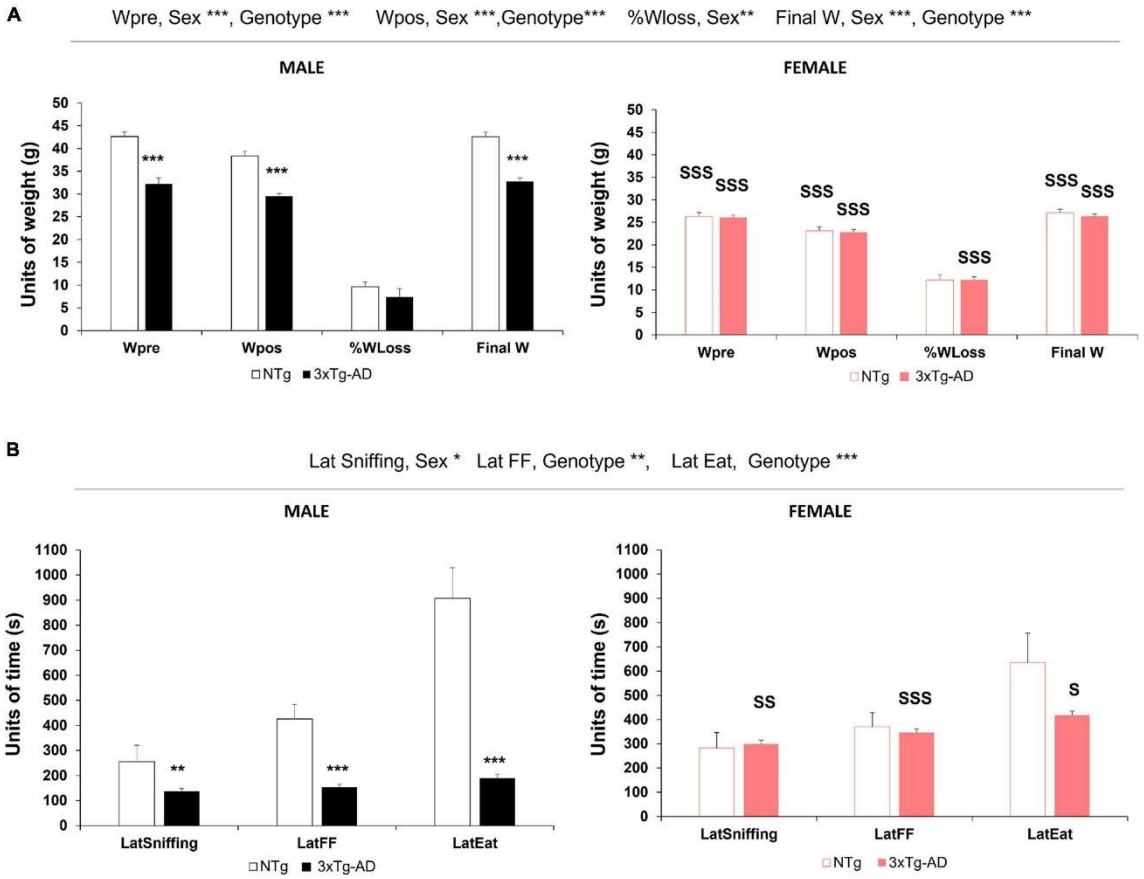


Figure 1. Genotype and sex differences in body weight monitoring and food finding test in 13-month-old NTg and 3xTg-AD mice. The results are expressed as mean ± SEM. (A) Food deprivation: Wpre, initial weight before fasting; Wpost, weight after fasting;%Wloss, percent of weight lost overnight; Final W, final weight at 24 h later. (B) Food finding test—latencies in the food finding test: LatSniffing, latency to sniffing the hidden pellet; LatFF, latency to find the food (hidden pellet); LatEat, latency to eat the pellet. Statistics: **$p < 0.01$, ***$p < 0.001$ vs. NTg mice of the same sex; S, $p < 0.05$; SS, $p < 0.01$; SSS, $p < 0.001$ vs. male of the same genotype.

The body weight of animals was monitored throughout experimental procedures (Figure 1A). At the beginning of the experiments, before the animals were submitted to food deprivation for 14 h, the weight of 3xTg-AD mice was lower than that of controls [Wpre, genotype***, $F(1, 86) = 34.369$, $p < 0.001$]. After the food finding test, all the groups of animals lost weight, but genotype differences persisted [Wpos, genotype***, $F(1, 86) = 36.481$, $p < 0.001$]. Sexual dimorphism was found in the initial body weight [Wpre,

sex∗∗∗, F(1, 86) = 152.694, p < 0.001]. Thus, while the weight of male 3xTg-AD mice was lower than C57BL/6J, the body weight of female mice was independent of the genotype. As expected, food deprivation did not modify sexual dimorphism [Wpos, sex∗∗∗, F(1, 86) = 210.954, p < 0.001]. Regarding the percentage of weight loss [%Wloss, sex∗, F(1, 86) = 6.585, p < 0.05], it was in the range of 5–10% of their weight, independently of the genotype but higher in female mice than in male mice in the 3xTg-AD genotype. At 24 h after fasting, the animals of both genotypes and sexes almost regained their initial weight and showed genotype [Final W, genotype∗∗∗, F(1, 86) = 52.252, p < 0.001] and sex [Final W, sex∗∗∗, F(1, 86) = 223.375, p < 0.001] differences.

The effects of food deprivation and the food finding test in the male sex indicated that the loss of weight, expressed either in units of weight or as a percentage, was not related to the statistically significantly shorter latency of 3xTg-AD mice to find the food and to eat it.

4.2.2. Sex- and Genotype-Dependent Signatures in the Food Finding Test

As indicated in Figure 1B, the FFT paradigm to investigate the olfactory function in normal and AD-pathological aging elicited different sex- and genotype-dependent olfactory ethogram signatures. There was a significant genotype difference between the 3xTg-AD and NTg animals regarding the three actions, albeit only finding [Lat FF, genotype∗∗∗, F(1, 86) = 12.686, p < 0.001] and eating the food [Lat Eating, genotype∗∗∗, F(1, 86) = 41.984, p < 0.001] reached statistical significance. Sex differences were found between male mice (faster) and female mice (slower) just in the actions of sniffing [Lat Sniffing, sex∗, F(1, 86) = 5.558, p < 0.05], but not in the food finding and eating action [Lat FF, n.s., F(1, 86) = 2.811, p = 0.097; Lat Eating, n.s., F(1, 86) = 0.131, p = 0.718].

Male 3xTg-AD mice were faster in all the actions compared to NTg mice, with statistically significant differences in the latency of finding the hidden food [male, Lat FF∗∗∗, F(3, 86) = 8.716, p < 0.001] and eating it once it was found [male, Lat Eat∗∗∗, F(3, 86) = 17.064, p < 0.001], while those in the latency to sniffing the food did not reach statistical significance.

Moreover, very short delays between actions in the male 3xTg- AD mice indicated an immediate execution of actions, which differed from the progressive time increase from one action to the other exhibited by male NTg mice. Besides this, the delays between

actions also indicated a 10-fold faster time pattern in male 3xTg-AD mice than NTg mice (Table 1).

In female mice, both groups exhibited longer latencies compared to male mice, and the time pattern of their ethogram for sniffing–finding–eating food was slower [Figure 2A, female, LatSniffing, S, $p < 0.05$; LatFF, SSS, $p < 0.001$; LatEat, SS, $p < 0.01$]. However, the time delay between actions in the female 3xTg-AD mice was very short, as described for male 3xTg-AD mice, but with two- to threefold increase compared to them. In female NTg, the delays were shorter than those of male NTg, with values that were half of those recorded for them (see Table 1). Therefore, the male–female ratios in NTg and 3xTg-AD mice were in an opposite sense.

Table 1. AD-genotype and sex effects in the time delay between actions.

	NTg Male		NTg Female			3xTg-AD Male			3xTg-AD Female		
Time (s) delay	(n=24)		(n=15)			(n=15)			(n=36)		
	Mean	± SEM	Mean	± SEM	Ratio S	Mean	± SEM	Ratio G	Mean	± SEM	Ratio S
Sniffing - Finding	173.27	± 45.5	87.93	± 27.1	1/2 x	16.75	± 4.9***	10x	47.06	± 19.5	2x
Finding - Eating	488.13	± 134.8	265.13	± 28.4	1/2 x	40.58	± 13.8***	10x	71.67	± 43.2	3x
Sniffing - Eating	661.40	± 137.6	353.07	± 38.4^{S}	1/2 x	57.33	± 13.7***	10x	118.72	± 47.9*	2x

The results are expressed as mean SEM. Time delay (s) between actions: sniffing and finding food, finding food and eating it, and sniffing and eating it. Ratio S, fold increase vs. male mice of the same genotype; Ratio G, fold increase vs. male NTg mice. *$p < 0.05$, ***$p < 0.001$ vs. NTg mice of the same sex; S, $p < 0.05$ vs. mice of the same genotype.

4.2.3. Food Deprivation and Food Finding Test in Naturalistic Social Isolation of Male 3xTg-AD Mice

Table 2. Effects of naturalistic isolation of male 3xTg-AD mice in food deprivation, food finding test, and the time delay between actions.

	NTg Male (*n*=24)		3xTg-AD Male (*n*=15)			3xTg AD-ISO Male (*n*=7)		
	Mean	± SEM	Mean	± SEM		Mean	± SEM	
A) Food deprivation								
Wpre (g)	42.63	± 1.00	32.23	± 1.06***		30.2	± 0.82***	
Wpos	28.49	± 1.11	29.53	± 0.48		28.19	± 0.75	
% Wloss (g)	6.92	± 2.50	7.40	± 1.51		6.66	± 0.28	
Final W (g)	31.74	± 0.91	32.77	± 0.65***		31.33	± 0.75***	
B) Food finding test								
Lat of sniffing (s)	251.00	± 63.88	135.71	± 16.28		85.14	± 14.58	
Lat of finding food (s)	424.27	± 57.92	152.46	± 15.79		111.86	± 17.16***	
Lat of eating (s)	912.40	121.02	193.04	± 17.80		138.14	± 23.62***	
C) Time (s) delay					Ratio G			Ratio G
Sniffing - Finding	173.27	45.50	16.75	± 4.90***	10x	26.71	± 10.77**	6x
Finding - Eating	488.13	134.81	40.58	± 13.85***	10x	26.29	± 14.97***	19x
Sniffing - Eating	661.40	137.57	57.33	± 13.75***	10x	53.00	± 12.92***	12x

The results are expressed as mean SEM. Time (s) between actions: sniffing and finding food, finding food and eating it, and sniffing and eating food. Wpre, initial weight before fasting; Wpost, weight after fasting;%Wloss, percent of weight lost overnight; Final W, final weight at 24 h later; LatSniffing, latency to smell the hidden pellet; LatFF, latency to find the hidden pellet; LatEat, latency to eat the pellet; Ratio G, fold increase vs. male NTg mice. **$p < 0.01$, ***$p < 0.001$.

As detailed in Table 2, the naturalistic social isolation of 3xTg-AD mice did not result in changes in the temporal patterns compared to group-housed mice of the same genotype. On the other hand, the genotype-dependent statistical differences in the ethogram were enhanced in the isolated group, thus the latency of finding the hidden food [Table 2, Lat FF, genotype***, $F(2, 43) = 20.275$, $p < 0.001$] and eating the food [LatEating, genotype***, $F(2, 43) = 10.930$, $p < 0.001$].

4.2.4. Correlation Analysis Between the Actions of Olfactory Signatures in the Food Finding Test

As shown in Figure 2B, the meaningful correlation analysis evidenced that, in the 3xTg-AD mice, the actions were closely related and predictable, mostly in female mice where statistical correlation increased in magnitude. In male mice living in naturalistic isolation conditions, the latency of sniffing was predictive of eating, but not of finding the food, and some of these correlations were lost. In male NTg mice, correlations were found between the finding and eating for male mice, and correlations were enhanced in order of magnitude in female mice.

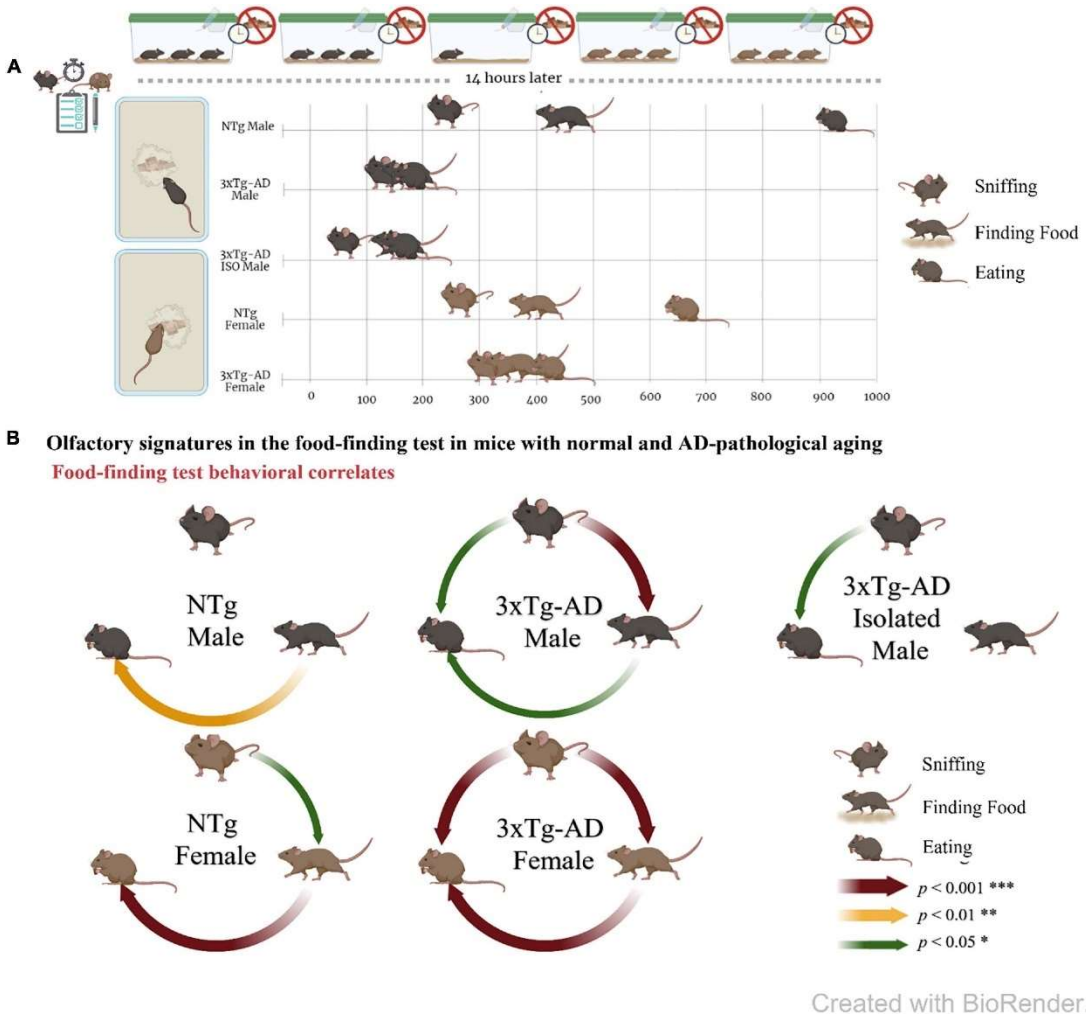


Figure 2. Olfactory signatures in mice with normal and AD-pathological aging in the food finding test. (A) Experimental design and ethogram tablature for "sniffing, finding, and eating the hidden food pellet" by 13-month-old male and female 3xTg-AD and NTg mice and natural isolation male 3xTg-AD mice. (B) Meaningful correlation analysis in food finding test. Graphical representation of the significant Pearson r correlations between the

three olfactory actions. All of them were positive. The figure was created with BioRender.com.

4.3. DISCUSSION

The present work characterizes AD genotype and sex effects on olfactory ethograms in the food finding test that define olfactory signatures in male and female non-transgenic and 3xTg- AD mice.

The food finding test (Deacon et al., 2009) is an easy and ready-to-do test that can be used in any animal department setting without the need to buy anything since it is based on the ethological response of animals to fasting. Therefore, using this behavioral paradigm also allows doing a neuroethological assessment of sniffing function. Approaches that really take into account the specific characteristics of the behavior of the species and use ethological methods will be the most useful to interpret these behavioral findings and to better understand the biological mechanisms in brain function. The use of ethologically relevant stimuli is of particular importance to assess cognitive performance and dissect the effects of preventive/therapeutical interventions or the impact of risk factors and hazards (Giménez-Llort & Torres-Lista, 2021; Torres-Lista et al., 2015, 2019)

Throughout the experimental process in the food finding test, weight is monitored to assess the impact of food finding (Deacon et al., 2009) In the context of aging, body weight also serves as a good indicator of the health status of the animal (Barron et al., 2013). In the case of 3xTg-AD mice, we have shown that weight loss depends on intrinsic factors such as increased nocturnal activity (Baeta-Corral, Johansson, et al., 2018) and hypermetabolic state (Escrig et al., 2020) compared to age- matched NTg mice. We have also shown that weight loss is useful to monitor the aggravation of the AD-pathogenic processes (Torres-Lista et al., 2019)

The exploratory behavior implies an internal conflict between the attraction for the novelty of space and the risk it contains since they could be exposing themselves to potentially dangerous environments (Giménez-Llort et al., 1995). In the case of rodents, most of this behavior depends on olfaction, a sense that allows not only the detection of odors in the surrounding environment necessary to identify food, predators, and sexual partners but also to get the spatial orientation(Coronas-Sámano et al., 2014; Invitto et al., 2018b) being indispensable for survival. Once the animals had inspected the arena, the latencies to sniffing, finding, and eating the food pellets were found to have progressively

increased in male mice with normal aging but consecutively developed in 3xTg-AD mice. It is known that olfactory deterioration during normal aging implies the loss of different olfaction skills, such as recognizing hidden food odor (Murphy et al., 1997; Schiffman, 1997). As referred to in the "Introduction", recent literature on olfaction as a reflection of general health status also unveil a strong link between olfactory impairment and mortality, with underlying mechanisms being suspected to be associated with the impact of olfactory loss on intrinsic (nutrition, accelerated brain aging, and neurodegenerative diseases) and extrinsic (life- threatening situations and social interactions) mechanisms (Van Regemorter et al., 2020b).

In the present work, male mice with normal and AD- pathological aging were equally delayed in their first contact with food pellets, while in the female sex this latency was increased but also depended on the genotype. In contrast, female 3xTg- AD performed the three-action (sniffing–find–eat) ethogram compared to their males cage mates. This could be because the sniffing in female mice is prone to be more affected during this stage of the disease, developing a situation in which the sensitivity to detect odors is more deficient (Mitrano et al., 2021).

Sex differences were found in the latency of the actions, with female mice being delayed compared to male mice, and also in the delay to develop the sequence, in this latter case being in a genotype-dependent manner. Thus, both groups of female mice exhibited longer delays in starting the ethogram than male mice, but the temporal pattern of their ethogram (delays between actions) to sniffing–finding–eating the food was two- to threefold times faster in the 3xTg-AD mice, but 1/2 in the case of NTg mice. This paradoxical genotype and sex modulation of the ethogram could be described as an accordion.

In the present work, the effects of naturalistic isolation (2–3 months) in a subgroup of 3xTg-AD that recently lost their cage mates after 10 months of living in a standard social environment were also assessed. This short isolation was enough to enhance the AD-genotype olfactory patterns shown by group-housed mice of the same genotype. When bearing different explanatory hypotheses, it is important to note that the body weight and the loss of weight after overnight fasting of isolated male 3xTg-AD were similar to those in the group-housed male 3xTg-AD, suggesting similar food motivation. The current report also shows that the ethogram was independent of body weight. Another explanation for their ability to find the hidden food faster could be related to a more intact

olfactory function to detect food and recognize it over the other cage smells. In this case, the results would also suggest these mice as being somehow resilient to insults upon their nervous system, and according to the strong link between olfaction loss and mortality (Van Regemorter et al., 2020b), this could explain why they lived longer than their cage mates. However, 100% of the group-housed 3xTg- AD mice could also be considered survivors with respect to the mean life span of the colony, and the female 3xTg-AD mice—that have better survival than male mice—were delayed compared to male mice. The most plausible interpretation could be that the shorter "inspection" of the new environment performed by 3xTg- AD mice living in social conditions is related to their anxiety-like profile and reduced to a minimum inspection in the isolated animals. In fact, we have recently reported increased anxiety in naturalistic isolated 3xTg-AD mice (Muntsant and Giménez- Llort, 2020) and distinct digging signatures, an ethological behavior of animals whose enhancement in the 3xTg-AD models neuropsychiatric-like behaviors associated with AD (Gimenez-Llort & Alveal-Mellado, 2021) and where the olfactory function is also involved. However, since female 3xTg-AD are more anxious than male 3xTg-AD, other neuronal mechanisms may also be underlying these behavioral observations.

The time interval between actions to inspect the beddings was 10 times faster in the 3xTg-AD animals than in the NTg mice with normal aging. The control animals confirm that the sense of sniffing deteriorates during normal aging, and the different signatures that characterize sniffing, such as the detection of hidden pellet odor, are lost.

Meaningful correlations between the three actions in the food finding test indicate that the actions were correlated in the AD- genotype as could be inferred because the temporal pattern consisted of actions immediately consecutively performed. In the case of 3xTg-AD mice, the accordion effect resulted in correlations with a stronger statistical significance. Interestingly, the lower number of correlations found in the NTg mice, despite the bigger sample size in this group, would suggest that behavioral patterns driven by olfactory function coexisted with behaviors driven by other cognitive functions. It is important to note that the variables were independent of the loss of body weight. In isolated 3xTg-AD mice, the correlations were mainly lost, but we cannot discard this due to the small sample size limitation.

4.4. CONCLUSIONS

the present results on the olfactory signatures in male and female 3xTg-AD mice compared to C57BL/6 non- transgenic mice show the following: (1) the sniffing–find–eat temporal pattern was independent of the loss of weight

(2) olfactory signatures in male and female 3xTg-AD mice were characterized by a fast ethogram and strong behavioral correlates

(3) olfactory signatures in male and female controls, the gold- standard C57BL/6 mice, were slow and progressively developed as part of a more complex ethogram, as also supported by disrupted behavioral correlates between the three actions assessed in the food finding test

(4) social isolation, naturally occurring only in 3xTg-AD mice, enhanced the genotype differences and disrupted the behavioral correlates.

5. *Study 3*

Food Finding Test without Deprivation: A Sensorial Paradigm Sensitive to Sex, Genotype, and Isolation Shows Signatures of Derangements in Old Mice with Alzheimer's Disease Pathology and Normal Aging

5.1. MATERIALS AND METHODS

5.1.1. Animals

A total number of sixty-six 16-month-old male and female homozygous 3xTg-AD (n = 38, 20 male and 18 female) and non-transgenic (NTg, n = 29, 14 male and 15 female) mice on a C57BL/6J background (after embryonic transfer and backcrossing at least 10 generations) established in the Universitat Autònoma de Barcelona (Marín-Pardo & Giménez-Llort, 2021) were used in this study. The 3xTg-AD mice harboring transgenes were genetically engineered at the University of California Irvine, as previously described (Oddo et al., 2003). Animals of the same genotype and sex were grown and maintained in groups of three to four mice per cage (Macrolon, 35 cm × 35 cm × 25 cm), which was filled with 5 cm of clean wood cuttings (Ecopure, Chips6, DateSand, Stockport, UK; Uniform cross-cut wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm × 20 cm, White). The animals were maintained under standard laboratory conditions with food and water ad lib, 22 ± 2 °C, 12-h light/dark cycle with lights on at 8:00 a.m., and a relative humidity of 50–60%.

5.1.2. Social Conditions

At 13 months of age, half of the animals were isolated and the other half remained under standard group housed conditions until their behavioral assessment at 16 months of age, corresponding to normal aging (in the NTg mice) and very advanced stages of the disease in the 3xTg-AD mice. Thus, the experimental groups were as follows: 3xTg-AD (n = 38, 20 male (10 group housing standard conditions, 10 Isolated) and 18 female (7 group housing standard conditions, 10 isolated) and non-transgenic (NTg, n = 28, 14 male (8 group housing standard conditions, 6 Isolated) and 15 female (7 group housing standard conditions, 8 Isolated). In all cases, the standard home cages were covered with a metallic grid allowing for the perception of olfactory and auditory stimuli from the rest of the colony.

5.1.2.1. Physical Status and Behavioral Assessment

5.1.2.1.1. Physical Status

At 16 months of age, the body weight of animals was measured before the FFT behavioral paradigm as an indicator of their health status.

5.1.2.1.2. The Food Finding Test of Olfactory Ability without Food Deprivation

To investigate olfactory function in old animals and advanced stages of AD disease, the Food Finding Test (FFT) olfactory paradigm (Marín-Pardo & Giménez-Llort, 2021) was used without food deprivation. In the present work, we first validate the ability of this modified protocol to elicit the different components of the Food Finding test's goal-directed ethogram. The test was performed under dim white light (20 lx) during the light phase of the light/dark cycle (from 10:00 to 11:00 am) using a novel cage (50 cm × 22 cm × 14 cm) with 1 cm of bedding. Eight food pellets (45 mg of standard food pellets) was placed in the central zone of the cage and covered by 1 cm of wood chip bedding. The mouse was placed in a corner of the cage, facing the walls, and its behavior was observed. The latencies of three goal-directed behaviors toward hidden food were recorded, namely, sniffing—when the nose makes direct contact with a surface and the vibrissae/whiskers are in maximum extension and are in contact with the surface in the same way (Welker, 1964); finding (digging)—"finding a food pellet" was defined as digging, touching, and holding the pellet in the front paws for more than 3 s; and eating the hidden food—holding the pellet in the front paws for more than 3 s of continuous eating. The beddings were renewed between each mouse.

The behavioral assessments were performed using direct observation and video recordings (ViewPoint Behavior Technology, Lion, France) in a counterbalanced manner. The observer was blind to the genotype. A complementary retrospective analysis was conducted. All procedures followed the Spanish legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" and the EU Directive (2010/63/UE) on this subject. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used(Kilkenny et al., 2010b).

5.1.2.1.3. Statistical Analysis

The results are expressed as mean ± SEM. SPSS 20.0 software was used. A 2 × 2 ×2 factorial design was used to analyze the effects of (G) genotype, (S) sex, and (ISO) isolation factors. The differences were studied through multivariate general linear model analysis and post hoc Duncan's test (multiple comparisons). In all the tests, $p < 0.05$ was considered statistically significant.

5.2. RESULTS

5.2.1. Physical Status

According to Table 1, the genotype effect led to a higher body weight in male NTg mice. In contrast, genotype variances among females were associated with decreased weight in isolated animals [S*, $F(1, 58) = 5.309$, $p < 0.05$; G**, $F(1, 58) = 7.140$, $p < 0.01$)].

Table 1. Time delays between goal-directed behaviors in the FFT without overnight food deprivation in 16-month-old male and female NTg and 3xTg-AD mice in group housing conditions or under forced isolation.

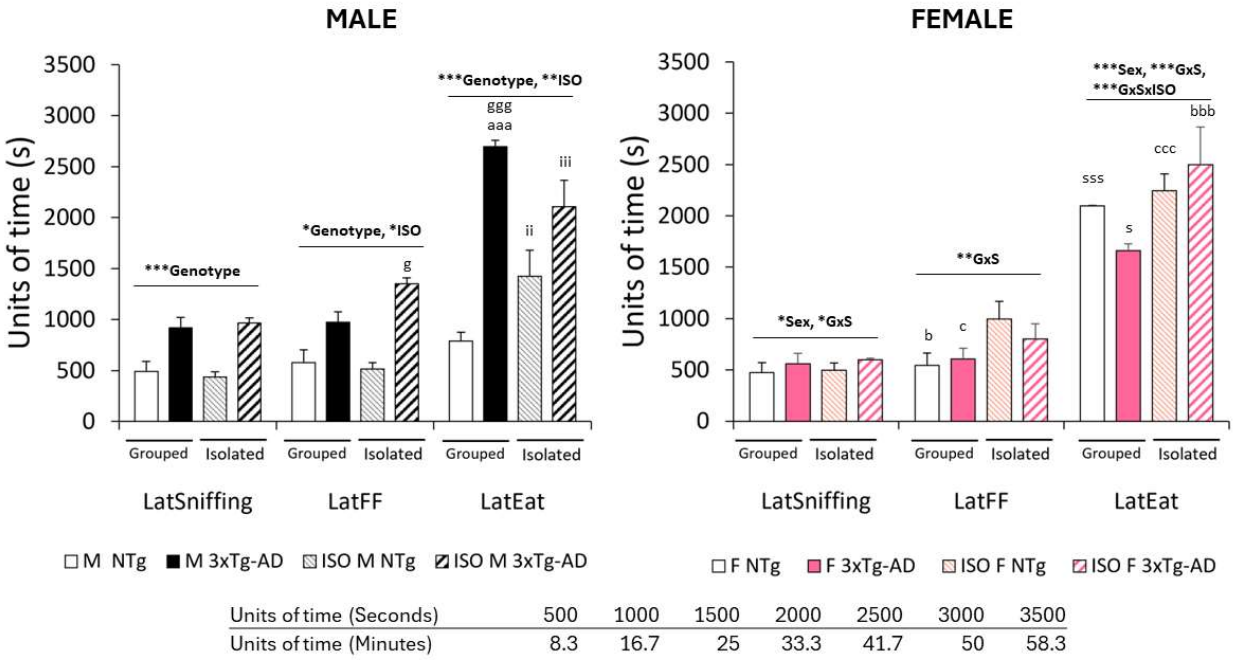
		NTg	**ISO NTg**	**3xTg-AD**	**ISO 3xTg-AD**
		Mean ± SEM	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
Body Weight	Males	46.90 ± 2.18	44.53 ± 1.52	33.60 ± 1.08 ***	30.97 ± 0.50 ***
Factor: G**, S*	Females	30.23 ± 1.77	30.41 ± 1.24	27.89 ± 0.97	24.49 ± 0.76 **
Sniffing—Finding	Males	130.2 ± 83.5	54.3 ± 34.0	80.4 ± 63.4	382.2 ± 192.4
Factor: **ISO	Females	70.0 ± 35.2	498.8 ± 183.4	48.7 ± 42.2	200.4 ± 152.7
Finding—Eating	Males	552.7 ± 112.4	1720.0 ± 142.0	906.6 ± 274.5	757.4 ± 240.5
Factor: **S	Females	1556.6 ± 289.6	1253.1 ± 255.2	1055.3 ± 233.9	1697.0 ± 319.9
Sniffing—Eating	Males	422.5 ± 111.0	1774.3 ± 133.9	987.0 ± 264.0	1139.6 ± 256.0
Factor: **G × S	Females	1626.6 ± 292.5	1751.9 ± 198.2	1104.0 ± 241.1	1897.4± 358.9

Statistics: Factorial analysis of variance 2 × 2 × 2 and post hoc Duncan's test. Effects of genotype (G), sex (S), isolation (ISO) factors and their interactions, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. their respective control group. Male NTg (n = 6), ISO Male NTg (n = 8), Male 3xTg-AD (n = 10), ISO Male 3xTg-AD (n = 10), Female NTg (n = 7), ISO Female NTg (n = 6), Female 3xTg-AD (n = 10), ISO Female 3xTg-AD (n = 7).

5.2.2. Elicitation of Olfactory Signatures Despite the Lack of Overnight Food Deprivation

The ethogram of goal-directed behaviors, sniffing, finding, and eating, was preserved despite the lack of a food deprivation protocol. However, the temporal window to observe this sequence of behaviors exhibited a 3- (control mice) to 10-fold (3xTg-AD mice) amplification compared to the temporal window in our FFT protocol with food deprivation (Marín-Pardo & Giménez-Llort, 2021). Thus, in our previous work, the scale

range had a maximum of 1100 units of time, whereas in the current work, the scale is defined in a maximum range of 3500 units.



Units of time (Seconds)	500	1000	1500	2000	2500	3000	3500
Units of time (Minutes)	8.3	16.7	25	33.3	41.7	50	58.3

Figure 1. Genotype, sex, and isolation effects in the FFT in 16-month-old male and female NTg and 3xTg-AD mice. Results are expressed as mean ± SEM. Latencies in the Food Finding Test: LatSniffing, latency to sniff the hidden pellet; LatFF, latency to find the food (hidden pellet); LatEat, latency to eat the pellet. Statistics: Factorial analysis of variance 2 × 2 × 2: Effects of genotype (G), sex (S), isolation (ISO) and their interactions, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. their respective control group. Post-hoc analysis: genotype, g $p < 0.05$ and ggg $p < 0.001$; genotype × isolation, aaa $p < 0.001$; isolation, ii $p < 0.01$ and iii $p < 0.001$; sex, s $p < 0.05$ and sss $p < 0.001$; genotype × sex, b $p < 0.05$ and bbb $p < 0.001$; sex × isolation, c $p < 0.05$; ccc $p < 0.001$.

As indicated in Figure 1 and depicted in the next sections, the FFT paradigm investigating the olfactory function in normal and AD-pathological aging elicited sex-, genotype- and isolation-dependent olfactory signatures, with alterations being enhanced in male sex.

The three goal-directed behaviors elicited statistically significant genotype differences. However, the effects were exerted in different magnitudes, with eating [Lat Eat, genotype ***, $F(1, 58) = 22.586$, $p < 0.001$] and sniffing [Lat Sniffing, genotype ***, $F(1, 58) = 11.688$, $p < 0.001$] showing the maximum effect, and finding [Lat FF,

genotype *, F (1, 58) = 5.171, p < 0.05] a moderate genotype effect. The effect size to indicate how meaningful the relationship between these variables was further analyzed by means of behavioral correlates, as indicated below.

This gradient was also observed in the effect of sex factor, but not in all behaviors. Thus, a maximum effect of sex was shown in eating [Lat Eating, Sex***, F (1, 58) = 11.415, p < 0.001] and a moderate effect on sniffing [Lat Sniffing, Sex*, F (1, 58) = 4.190, p < 0.05], but not on finding [Lat FF, n.s., F (1, 58) = 0.967, p = 0.329.]. This resulted in genotype × sex interaction effects seen in the three behaviors, with maximum, strong and moderate effects in eating, [LatEat, G × S***, F (1, 58) = 28.254, p < 0.001], finding [LatFF, G × S**, F (1, 58) = 7.859, p < 0.01], and sniffing [LatSniffing, G × S*, F (1, 58) = 5.222, p < 0.026], respectively.

Overall, the eating behavior was the one to exhibit the highest sensitivity to intrinsic factors (genotype and sex), followed by sniffing. The 2.5-fold increase in the latency of eating, compared to previous behaviors (sniffing and finding) in the 3xTg-AD mice, suggests that accumulative effects in latency scores (that is, a delay in one behavior increases the latency of appearance of the subsequent behavior) can be discarded. To further analyze this aspect, the time delays or interval of time between two consecutive behaviors were also calculated (see below).

The effects of isolation emerged in the latencies to find the food with a moderate effect [Lat FF, ISO*, F (1, 58) = 3.932, p < 0.05; but not in the Lat Sniffing, n.s., F (1, 58) = 0.030, p = 0.862] that increased to its maximum in the latency of eating it [Lat Eating, ISO***, F (1, 58) = 7.185, p < 0.001]. In this last goal-directed behavior, genotype and sex factors interacted with isolation [Lat Eating, G × S × I***, F (1, 58) = 16.037, p < 0.001; but not in the Latency of Sniffing, n.s., F (1, 58) = 0.067, p = 0.797; Lat FF, n.s., F (1, 86) = 2.075, p = 0.155] eliciting different behavioral signatures in each of the four experimental groups.

5.2.3. Time Delays and FFT Behavioral Correlates

The time delays between goal-directed behaviors in the FFT was analyzed. The results per each sex and factorial analysis is depicted in Table 1. Significant geno-type-dependent differences between 3xTg-AD male mice and NTg male mice were reported in the food-finding delay and the total time between the start of the ethogram (sniffing) and its end (eating).

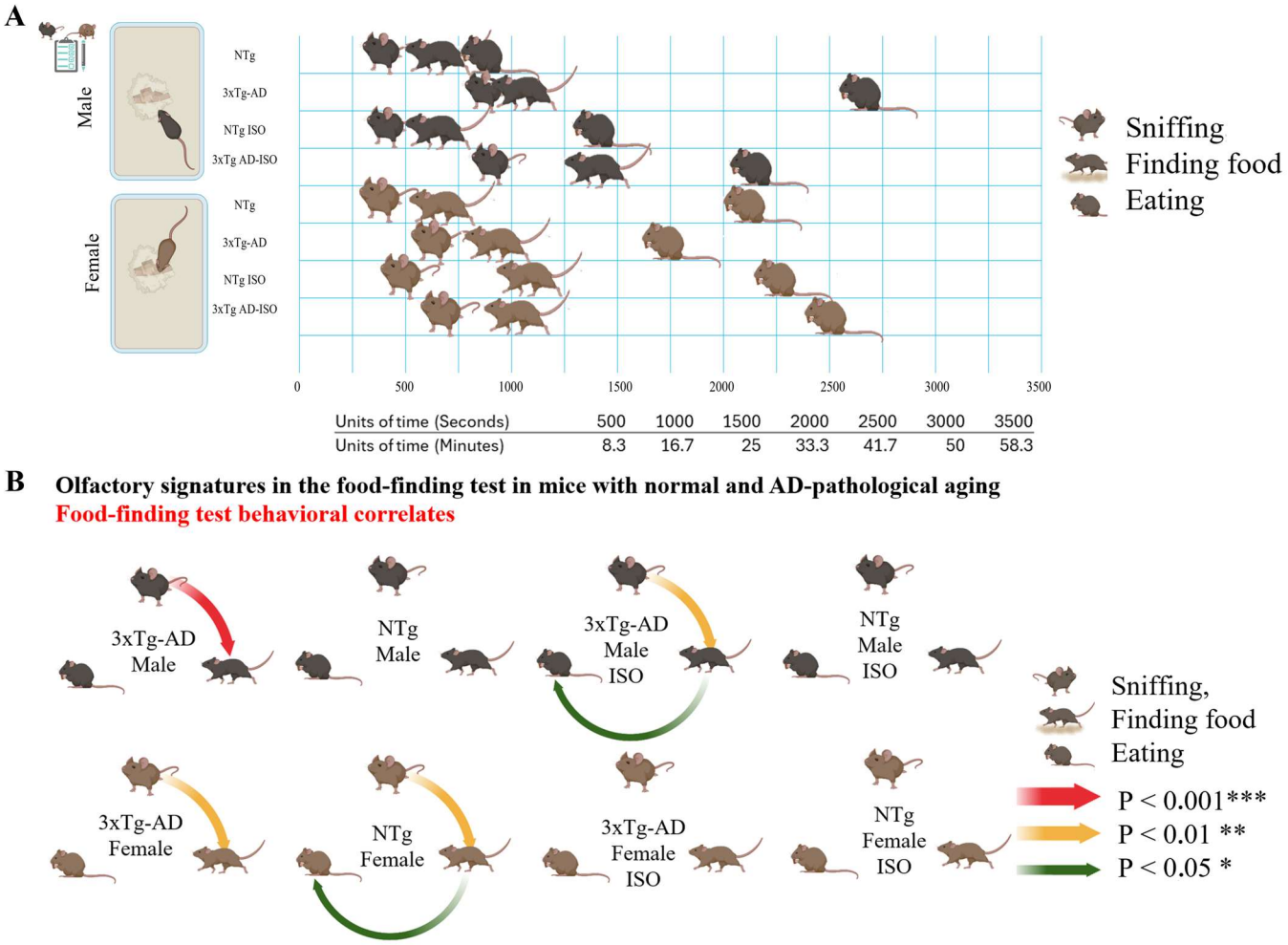


Figure 2. Sex- and genotype-dependent olfactory signatures in mice with normal and AD-pathological aging in the FFT without overnight food deprivation. (A) Experimental design and ethogram tablature for "sniffing, finding, and eating the hidden food pellet" by 16-month-old male and female NTg and 3xTg-AD mice and respective isolated groups. (B) Meaningful correlation analysis in the FFT: Graphical representation of the significant Pearson r correlations * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between the three olfactory actions, all of them positive. The figure was created with BioRender.com, accessed on 20 December 2023.

Figure 2A illustrates the olfactory signatures of 16-month-old animals with normal and AD-pathological aging elicited in the FFT, despite the lack of overnight food deprivation, and under the effects of intrinsic (genotype and sex) and extrinsic (isolation) factors. In addition, as shown in Figure 2B, meaningful correlation analysis revealed that sniffing and finding exhibited a strong and predictable relationship in 3xTg-AD males ($p < 0.001$) and females ($p < 0.01$). Two other groups, in particular, NTg females and ISO

Male 3xTg-AD mice also showed positive correlations between the latencies of subsequent behaviors (sniffing and finding; finding and eating).

5.3. DISCUSSION

The FFT (Deacon et al., 2009), based on species-specific behavioral traits and their ethological responses, facilitates a neuroethological assessment of olfactory function and related behaviors. It presents an easy-to-use assessment tool that can be used in any animal department without needing to purchase additional items such as the odors commonly used in other olfactory tests, and also provides the opportunity to run experimental designs without experimental odor carryover effects (Dan et al., 2021; Muntsant & Giménez-Llort, 2020; Slotnick & Coppola, 2015; Wersinger et al., 2007). On the other hand, the incorporation of ethologically salient stimuli, such as the search of food, is particularly interesting not only for evaluating olfactory function in health and disease throughout the life cycle, but also for the associated cognitive and emotional performance, and in assessing preventive/therapeutic interventions and the impact of risk factors and hazards. Thus, we recently proposed its use as a sensorial paradigm to unveil genotype and sex differences in 13-month-old 3xTg- AD mice in a C57BL/6 genetic background as compared to wildtype mice with normal aging (Marín-Pardo & Giménez-Llort, 2021). In the 3xTg-AD mice, this age corresponds to the advanced stages of the disease with beta-amyloid brain pathology, whereas it is considered middle age in the wildtype mice (Belfiore et al., 2019). In this animal model, more advanced neuropathological stages of AD disease are characterized by concomitant beta-amyloid and tau pathologies, but there is also a strong survival bias (Deacon et al., 2009) and frailty (Muntsant et al., 2023). Therefore, in the present work, we studied the capacity of this test to detect genotype and sex differences in 16-month-old animals when the paradigm is administered without previous overnight food deprivation. In addition, we studied whether their olfactory signatures would be modified by forced isolation, as in our previous work in which we could observe naturalistic isolation due to the death of male cage mates (Marín-Pardo & Giménez-Llort, 2021).

The most important finding is that the current Food Finding Test paradigm for older animals elicited distinct olfactory signatures despite the lack of overnight food deprivation. As expected, genotype was the most important factor, as significant statistical differences were found in the three goal-directed behaviors, with delayed olfactory behavioral responses in the 3xTg-AD mice as compared to mice with normal

aging. Overall, eating behavior was notable as it showed a considerable delay and was sensitive to genotype and sex factors, whereas sniffing and finding were elicited in a quite consecutive manner, a sequence that was found enhanced in 3xTg-AD mice under isolation. Interestingly, sex appeared to be a relevant factor since these effects were mostly due to the behavioral ethogram elicited in males, whereas females showed a strong consistency in the similarity of their latencies in all the three goal-directed behaviors as compared to those of their non-transgenic counterparts with normal aging.

Compared to the previous protocol at 13 months of age, the ethograms of both male and female wildtype mice with the gold-standard C57BL/6 genetic background exhibited a 2-fold increase in recorded latencies. Several intrinsic factors could account for these results. The first consideration is the age factor, as behavioral responses in 16-month-old animals tend to be slower than those observed in their middle- aged counterparts. On the other hand, the lack of overnight food deprivation in the current paradigm for old animals could also reduce the energetic demands of animals, and therefore, reduce the elicitation of fast goal (food)-directed ethograms. However, male NTg mice had lower weights than their transgenic counterparts but showed faster patterns than them. In addition, whilst the latencies of sniffing and finding were similar between males and females, the latency of eating was significantly increased in 16-month-old females, suggesting this final action (eating) was not driven by quick energy production. In addition, the sex factor interaction with genotype and/or isolation also suggests complex interactions in the elicitation of this behavioral ethogram.

Noteworthy, in the current work, the ethograms of 3xTg-AD mice differed from those reported in their congeners at 13 months of age in which fast signatures were observed (Marín-Pardo & Giménez-Llort, 2021). The age/stage-dependent increase of weight in this animal model, mostly in males, could explain the strong delays observed in this sex. However, we consider that the advance in their neuropathology status could explain a different coping-stress strategy when con- fronting a new scenario, being a faster response in beta-amyloid stages but slower when the brain damage severity associated to beta- and tau-pathologies increases. These differences would agree with age/stage-dependent fast/slow responses we have recently reported in the T-maze for this (Muntsant et al., 2023) and other (Giménez-Llort et al., 2021) AD-animal models, and those previously described in mice models with accelerated aging (Guayerbas et al., 2000). In both cases, these factors would explain the ethograms in male sex, whereas the

similarities in females would agree with behavioral convergence we have also described in the old to end-of-life stages in this sex (Muntsant et al., 2023). There- fore, these results support the understanding that male and female sexes can be considered distinct natural scenarios for studying the influence of biological, psychological, and social factors on health and illness across the lifespan, as well as the interconnected dynamics affecting homeostatic networks.

While the comparison between 3xTg-AD and NTg animals revealed substantial genotype disparities across all three olfactory actions, the current protocol also was sensitive to isolation. Thus, in contrast to previous work in which isolation was only naturally occurring in male 3xTg-AD cases (Marín-Pardo & Giménez-Llort, 2021) in the current work, in all experimental groups half of the animals were submitted (forced) to a period of isolation. While social isolation had a relatively modest effect on response latencies, it had distinctive outcomes for males and females. In females, olfactory signatures remained relatively preserved, suggesting a degree of resilience in the face of isolation. However, the situation was quite different for males, to which social isolation disrupted the established patterns of olfactory behavior.

These results highlight the importance of considering gender in the study of olfaction and its susceptibility to external factors. The results also showed that derangements of the FFT-induced olfactory ethograms were also observed in female 3xTg-AD mice and their non-transgenic counterparts. Therefore, here we report for the first-time sex-specific differences within the olfactory domain, a finding undocumented in previous studies con- ducted under naturalistic isolation conditions. Isolated males exhibited slower responses, contrasting with the faster reactions observed in isolated females. This novel finding points at the complex interplay between sex influences and olfactory-related behaviors under positive/negative social conditions.

Meaningful correlation analysis between goal-directed behaviors provided further evidence of the strong and predictable relationship between sniffing and finding in some groups. The lack of significant correlations in male NTg mice highlights genotype-specific differences in the olfactory behavior ethogram. With regards to the impact of social condition on olfactory behaviors, sniff latency emerged as a predictive variable for finding food in isolated male 3xTg-AD mice, yet this correlation did not extend to the act of eating the food. Social isolation led to the disruption of functional correlations among goal-directed behaviors in the other groups, especially pronounced in males, indicating

that the interplay between different behaviors is particularly sensitive to the effects of isolation. The loss of internal correlation among behaviors in the context of isolation adds another layer of complexity to the study of olfactory behavior, highlighting the interconnectedness of different behaviors and also revealing how external factors like social isolation can disrupt these connections. On the other hand, the complexity of their sensory–motor components demand further research on the effect size of variables and differences between groups with respect to other behavioral dimensions. Thus, in the current paradigm it is assumed that food-finding and eating are primarily olfactory behaviors, but the contribution of appetite and consummatory behaviors cannot be excluded. Conversely, bulbectomized (totally anosmic) rodents show robust sniffing. As with literature on measuring olfactory detection and discrimination in rodents (Slotnick et al., 2004; Slotnick & Coppola, 2015), methods like habitation/dishabituation or operant methods should be used to validate the current method.

5.4. CONCLUSIONS

This behavioral research underscores the importance of considering intrinsic (genotype and sex) and extrinsic (isolation) factors when studying olfactory function in the context of aging and AD. Moreover, it demonstrates that this novel paradigm without overnight food deprivation adapted to old animals is sensitive to both intrinsic and extrinsic factors. The elicitation of genotype, sex, and isolation-dependent olfactory signatures contributes to the understanding of the complex aging and AD scenarios, underscores the potential of olfactory behavior as a valuable diagnostic tool, and provides a translational tool to design and assess tailored preventive/therapeutic strategies. Together with our previous work, these findings provide valuable insights into the effects of social isolation, especially in advanced stages of AD disease, with special concern on its impact on males, highlighting the need for specific attention in understanding and addressing these sex-specific challenges.

1. The FFT paradigm without food deprivation elicited longer ethograms than previously reported with the standard overnight food deprivation protocol. However, he did identify genotype-dependent olfactory signatures in normal and pathological aging in AD.
2. The temporal pattern of smell-find-eat differs in females (fast) from males (delayed). 3xTg-AD mice demonstrate olfactory signatures in Alzheimer's disease

3. Food finding latency discriminates normal (advanced) aging from AD (delayed) aging in males only.
4. In males, the ethogram defines normal (progressive) signatures of AD (rapid) aging.
5. Social isolation increased latencies, but olfactory signatures were preserved only in females.
6. A functional disorder was detected as the internal correlation between the three goal-directed behaviors and was lost under isolation in most of the groups

In summary, the integration of genotype-dependent olfactory signatures, sex-specific responses to social isolation, and the disruption of functional correlations underlines the multifaceted nature of olfactory research in aging and AD. As we explore the complexities of olfaction in AD, it becomes increasingly clear that a comprehensive understanding of these interactions is crucial.

6. *Study 4*

Cognitive, NPS-like and olfactory signatures in mice with normal and AD-pathological aging, with a special focus on the effects of sex.

6.1. MATERIALS AND METHODS

6.1.1. Animals

A total number of fifty-one 12-month-old male and female homozygous 3xTg-AD (n = 35, 18 male and 17 female) and non-transgenic (NTg, n = 16, 10 male and 6 female) mice on a C57BL/6J background (after embryonic transfer and backcrossing at least 10 generations) established in the Universitat Autònoma de Barcelona (Marín-Pardo & Giménez-Llort, 2021) were used in this study. The 3xTg-AD mice harboring transgenes were genetically engineered at the University of California Irvine, as previously described (Oddo et al., 2003). Animals of the same genotype and sex were grown and maintained in groups of three to four mice per cage (Macrolon, 35 cm × 35 cm × 25 cm), which was filled with 5 cm of clean wood cuttings (Ecopure, Chips6, DateSand, Stockport, UK; Uniform cross-cut wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 cm × 20 cm, White). The animals were maintained under standard laboratory conditions with food and water ad lib, 22 ± 2 °C, 12-h light/dark cycle with lights on at 8:00 a.m., and a relative humidity of 50–60%.

6.1.2. Behavioral Assessments

Behavioral assessments (Giménez-Llort, Blázquez, et al., 2007) were performed from 9:00 h to 13:00 h, in a counterbalanced manner and blind to the experiment.

Day 1. Corner and Open-Field Tests (CT and OF)

Animals were individually placed in the center of a clean standard home cage, filled with wood shave bedding. Neophobia was evaluated in the corner test (CT) for 30 s through the number of corners visited (CTc), latency to perform the first rearing (CTlatR), and the number of rearings (CTr). Immediately after, mice were placed in the center of an open-field (OF), beige metal drawer (metalwork, 50 × 40 × 25 cm) and were observed for 5 min. The sequence of behavioral events that define the animal's ethogram was recorded as follows: duration of freezing behavior (LatM, latency to move), latency to leave the central square (LatC) and that of entering the peripheral ring (LatP), as well as latency and total duration of self-grooming behavior (LatG and tG, respectively). Horizontal (crossings of 10 × 10 squares) and vertical (rearings with wall support) locomotor activities were also measured. Bizarre behaviors observed in this test (stereotyped stretching, stereotyped rearing, backward movements, and jumps) were also

measured according to previously reported criterion (Baeta-Corral & Giménez-Llort, 2014).

Day 2. Open field test 2

The day after, the animals were retested in the open-field (OF2) to evaluate the behav- ioral response when they confronted the same anxiogenic environment again. Analysis of activity was done during the first minute of the test, the time period where we have previously described AD-genotype differences(Torres-Lista et al., 2014).

Day 3. Spontaneous Alternation in the T-Maze (TM)

On the third day, the black T-shaped maze (woodwork) consisted of two short arms of 25 × 8 cm and a long arm of 30 × 8 cm. The animals were placed inside the long arm of the maze with its head facing the end wall. The latencies of the first movement, start walking, arriving at the intersection of the arms, and completing the exploration of the maze were recorded. Finally, defecation boli and urination were also recorded.

Day 4. Dark–light box test day 1

The dark–light box (Panlab, S.L., Barcelona, Spain) consisted of two compartments (black, 270 × 180 × 270 mm; white, 270 × 270 × 270 mm) connected by an opening (70 × 70 mm). A slight illumination of the white box (red 20 W bulb) was chosen to facilitate animals entering the white compartment. The mice were introduced into the black compartment and observed for 5 min. Latency to enter (all four paws) into the lit compartment, number of crossings, time spent, and distance covered in the lit compartment were recorded. The ratios of time and distance over the number of entries (time and distance indexes, respectively) into the lit compartment were calculated.

Day 5. Dark–light box test day 2

The day after, the animals were retested in the Dark and Lightbox to evaluate their behavioral response when they again confronted the same anxiogenic environment.

Day 6. Food Finding Test

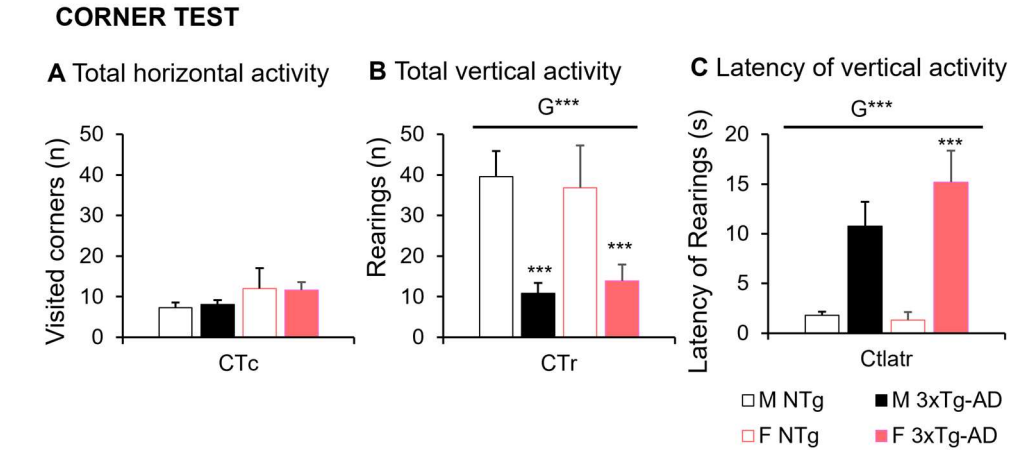
To investigate olfactory function, the food finding test (FFT) olfactory paradigm (Deacon et al., 2009) was used. The mice were deprived of food for 14 h before testing (starting with lights off at 2000h), the equivalent to 1-day food deprivation.

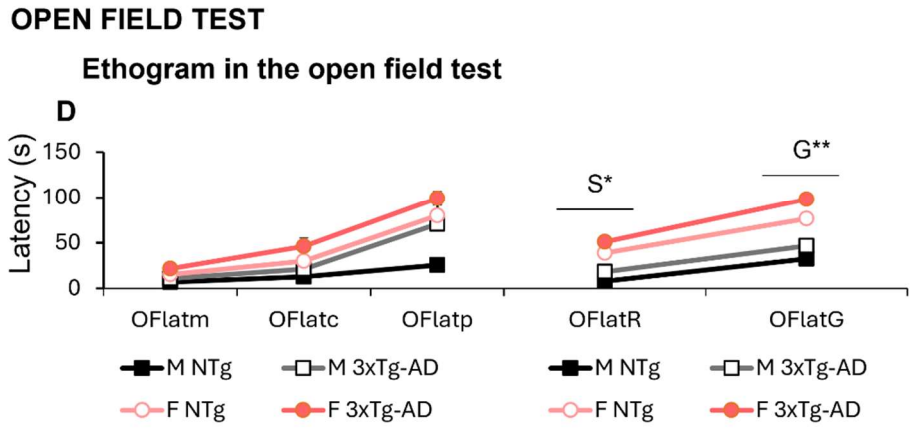
The test was performed under dim white light (20 lx) during their light phase of the light/dark cycle (from 10:00 to 11:00 a.m.) using a novel cage (50 cm × 22 cm × 14 cm) with 1 cm of beddings. Eight food pellets (45 mg) were placed in the central zone of the cage and covered by 1 cm of wood chip bedding. The mouse was placed in a corner of the cage, facing the walls, and its behavior was observed. The latencies of three goal-directed behaviors toward hidden food were recorded, namely, sniffing— when the nose makes a direct contact with a surface and the vibrissae are in maximum extension and are in contact with the surface in the same way (Welker, 1964); finding (digging)— "finding a food pellet" was defined as digging, touching, and holding the pellet in the front paws for more than 3 s; and eating the hidden food—holding the pellet in the front paws for more than 3 s of continuous eating. The beddings were renewed between each mouse.

Behavioral assessments were performed in a counterbalanced manner by direct observation by an observer blind to the genotype and the support of video recordings (ViewPoint Behavior Technology, Lion, France) for complementary retrospective analysis. All procedures followed the Spanish legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" and the EU Directive (2010/63/UE) on this subject. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used (Kilkenny et al., 2010b).

6.1.3. Statistical Analysis

The results are expressed as mean SEM. SPSS 20.0 software was used. A 2 × 2 factorial design was used to analyze the effects of (G) genotype and (S) sex factors. Differences were studied through multivariate general linear model analysis, followed by post hoc Duncan's test (multiple comparisons). In all the tests, $p < 0.05$ was considered statistically significant.


CORNER TEST
A Total horizontal activity
B Total vertical activity
C Latency of vertical activity
Visited corners (n)
Rearings (n)
Latency of Rearings (s)
G***
***
CTc
CTr
Ctlatr
M NTg
M 3xTg-AD
F NTg
F 3xTg-AD


OPEN FIELD TEST
Ethogram in the open field test
D
Latency (s)
S*
G**
OFlatm
OFlatc
OFlatp
OFlatR
OFlatG
M NTg
M 3xTg-AD
F NTg
F 3xTg-AD

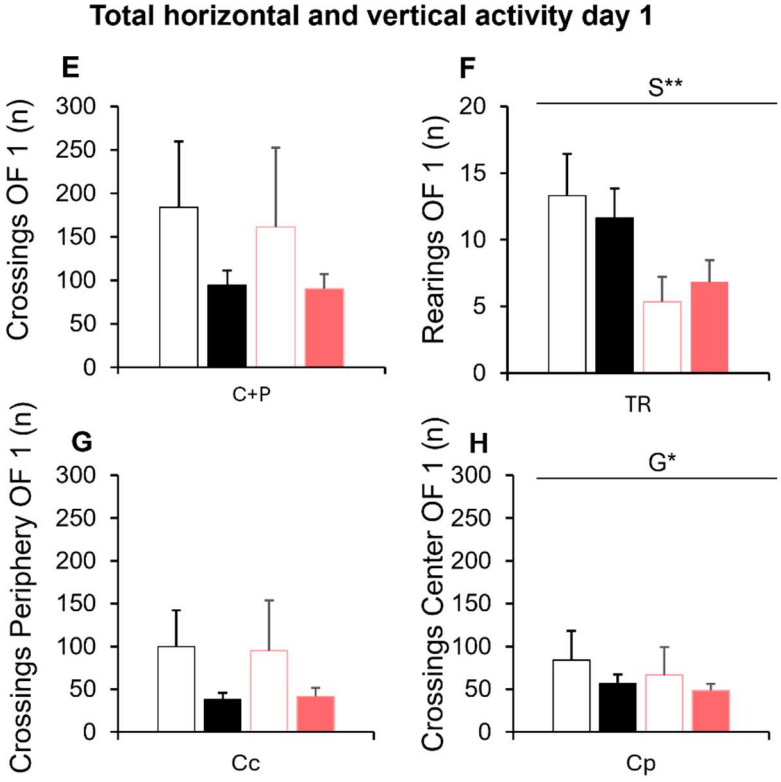

Total horizontal and vertical activity day 1
E
F
S**
Crossings OF 1 (n)
Rearings OF 1 (n)
C+P
TR
G
H
G*
Crossings Periphery OF 1 (n)
Crossings Center OF 1 (n)
Cc
Cp

Fig 1. NPS-like behaviors in the corner and open-field test—Effect of Sex and AD-genotype on the neophobia and exploratory activity elicited in a new home cage (corner test, (A–C)) and the open-field test (D–H). Data are expressed by Mean ± SEM. NTg: wildtype mice; 3xTg- AD: Transgenic mice. Corner test: Visited corners (A), number (B), and latency of rearings (C). Open field test: Ethogram, the temporal sequence of behavioral events in the open field test, Variables: OFlatm, latency of the first movement; OFlatc, latency to leave the central area; OFlatp, latency to enter into the periphery; OFlatR, latency of the first rearing; OFlatG, latencies of the first grooming (D). Total horizontal (E) and vertical (F) activity in the open-field test. Total horizontal activity in the periphery (G) and the central area (H). Analysis of variance 2 x 2: Effects of genotype (G), Sex (S) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Post-hoc analysis: * $p < 0.05$ vs. the corresponding NTg control group.

In the corner test, no significant differences were observed between 3xTg-AD and NTg animals in the horizontal locomotor activity, as measured by the number of visited corners (Figure 1A),. In the case of vertical activity (Figure 1C), significant genotype-dependent differences were observed between the two groups in terms of latency to first rearing (G*** $F(1.47)= 14.097$; $P< 0.000$). Conversely, the total number of rearings (Figure 2B) recorded decreased in the transgenic groups (G*** $F(1.47)= 25.051$; $P<0.000$). Both variables indicated that vertical activity is dependent on genotype.

The ethogram in the open-field test describes the temporal sequence of behavioral events of animals in the open-field test (Figure 1D). That is, the latency of the first movement (OFlatm), to leave the central area (OFlatc) and the latency to arrive to peripheral area, no significant differences were observed between 3xTg-AD and NTg animals and male and female mice. The performance of the first vertical exploration in the periphery was sex-dependent (S* $F(1,47)= 3.954$; $p<0.05$). Self-grooming was affected by genotype (G** $F(1. 47)= 9.838$; $p< 0.01$).

No significant differences was observed between 3xTg-AD and NTg animals in the total activity exhibited in the open field test on Day 1 (Figure 1E–G, total crossings, total rearings and total crossings at the periphery). However, the total crossings in the center were genotype dependent (Figure 1H, G* $F(1. 47)= 5.291$; $p< 0.05$).

Sex differences were also found in the horizontal activity exhibited on the first day (Figure 1F, S**$F(1.47)= 6.162$; $P<0.01$). Statistically significant differences

genotype-dependent were found due to the activity performed in the central area (Figure 1H, G* F(1,47)= 5.291; P<0.05) and are maintained on day 2 (Figure 2C, G* F(1,47)= 3.976; P<0.05)

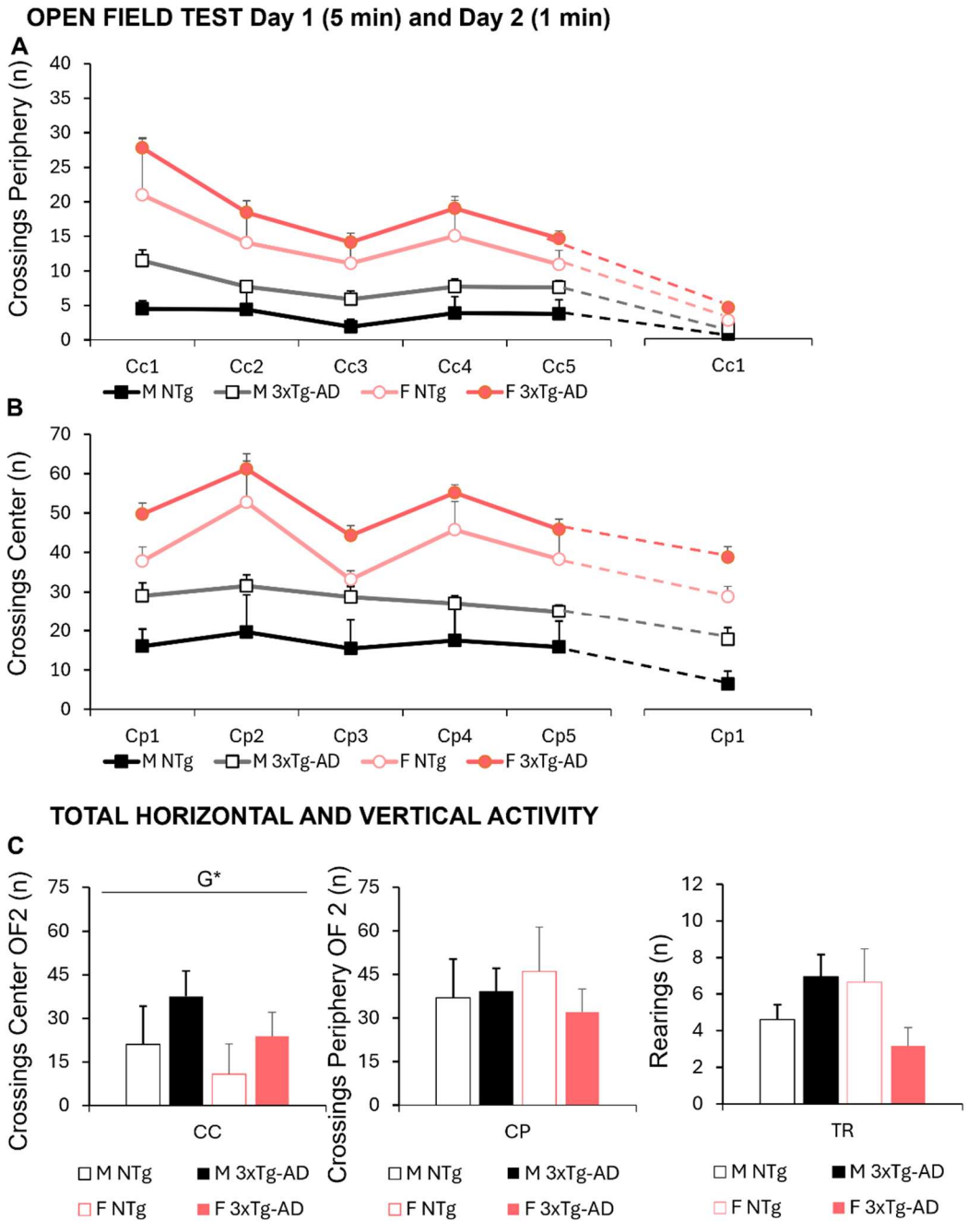


Figure 2. Time course of the horizontal activity (number of crossings) exhibited in the periphery (A) and the center (B) of the open-field test on Day 1 and 2, NTg and 3xTg-AD mice (C) Total horizontal activity (number of crossings) in the periphery on Day 2, (D) Total horizontal activity (number of crossings) in the center on Day 2. Data are

expressed by mean ± SEM. Analysis of variance 2 x 2: Effects of genotype (G), Sex (S) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

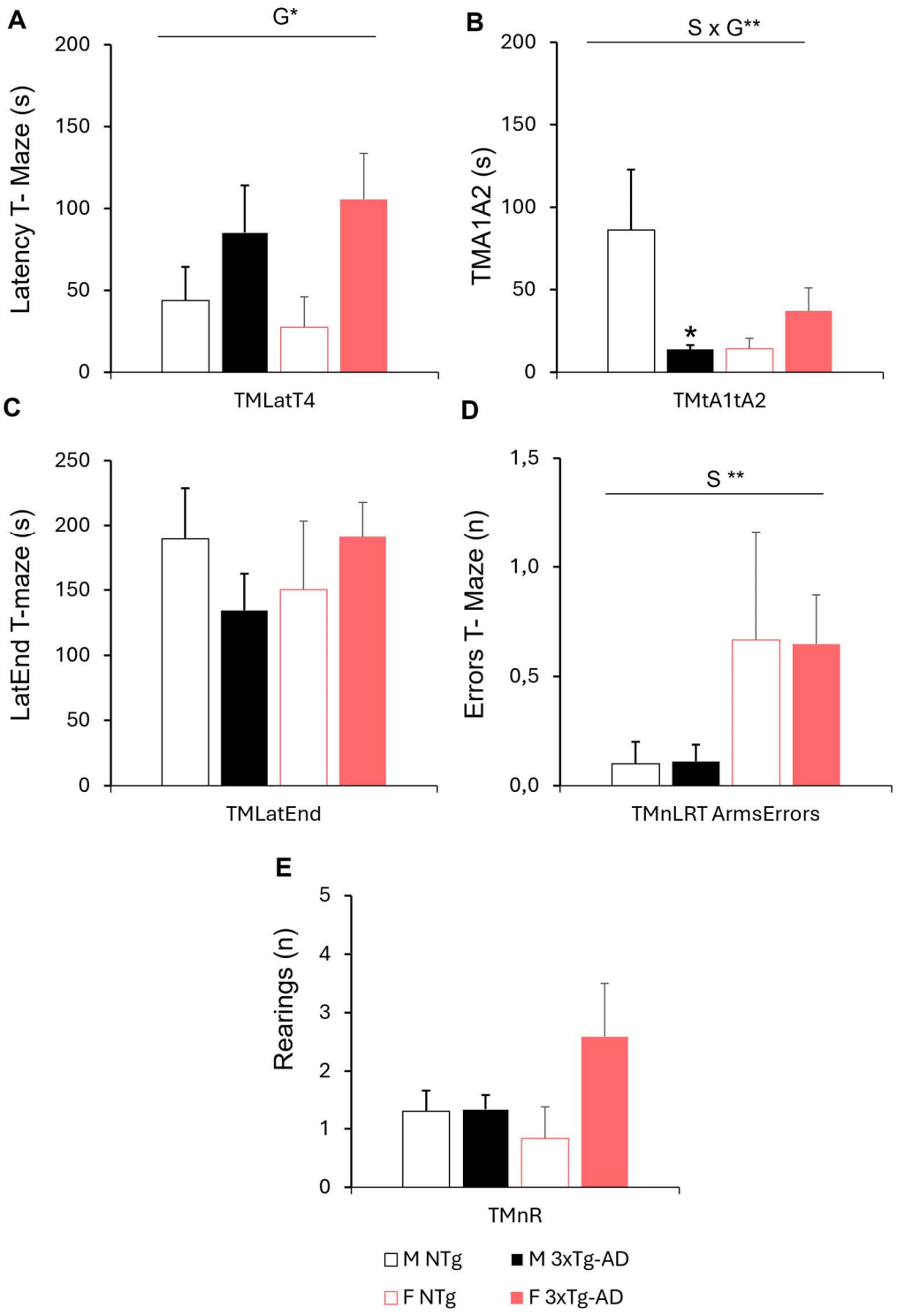


Figure 3. Coping with stress strategies, risk assessment, and spontaneous alternation in the T-Maze. (A) Latency to cross the intersection; (B) Total time invested in the exploration of the left and right arms; (C), Time to complete the exploration of the maze; (D), Total number of errors performed during the exploration of the maze; (E) Vertical exploratory activity. Data are expressed by means ± SEM. Analysis of variance 2 x 2: Effects of genotype (G), Sex (S), Sex X Genotype (S xG), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Post-hoc analysis: * $p < 0.05$ vs. the corresponding NTg control group.

In the T-maze (Figure 3), the 3xTg-AD mice took a longer time to reach the intersection of the vertical arm (not shown) and once there to cross it (Figure 3A, G* $F(1.47)= 3.861$, $p < 0.05$). Statical differences in Sex x Genotype factors interaction were found in the time spent in both arms (Figure 3B, S xG** $F(1,46)= 6.072$; $P< 0.01$). The analysis of errors revisiting the arms that had been already explored indicated a significant sex effect (S* $F(1. 47) = 6.026$, $p < 0.01$).

In the Food Finding Test, the body weight of animals was monitored throughout experimental procedures (Figure 4A). At the beginning of the experiments, before the animals were submitted to food deprivation for 14 h, the weight of 3xTg-AD mice was not different than that of controls, but a Sex x Genotype interaction was found (Wpre, Sex x Genotype* $F(1, 47) = 34.369$, $p < 0.05$]. After the food-finding test, all the groups of animals lost weight, that was in the range of 5–10% of their initial weight. Genotype differences were found in the percentage of this weight loss [%Wloss, G**, $F(1, 47) = 9.849$, $p < 0.01$], with higher percentage in control mice than 3xTg-AD, independently of their sex.

The effects of food deprivation in the food finding test in the male sex indicated that the loss of weight, expressed either in units of weight or as a percentage, was not related to the statistically significantly shorter latency of 3xTg-AD mice to find the food and to eat it.

As indicated in Figure 4B, the FFT paradigm to investigate the olfactory function in normal and AD-pathological aging elicited different sex- and genotype-dependent olfactory ethogram signatures. There was a significant genotype difference between the 3xTg-AD and NTg animals regarding the three actions, albeit only finding [Lat Sniffing, genotype*, $F(1, 47) = 3.937$, $p < 0.05$] and Food finding [LatFF, Genotype**, $F(1, 46) = 9.527$, $p < 0.01$] reached statistical significance. Sex differences were found between male

mice (faster) and female mice (slower) just in the actions of sniffing [Lat Sniffing, sex*, $F(1, 47) = 5.959$, $p < 0.05$] and eating action [Lat Eating, Sex *., $F(1, 46) = 5.678$, $p < 0.05$] but not in the food action.

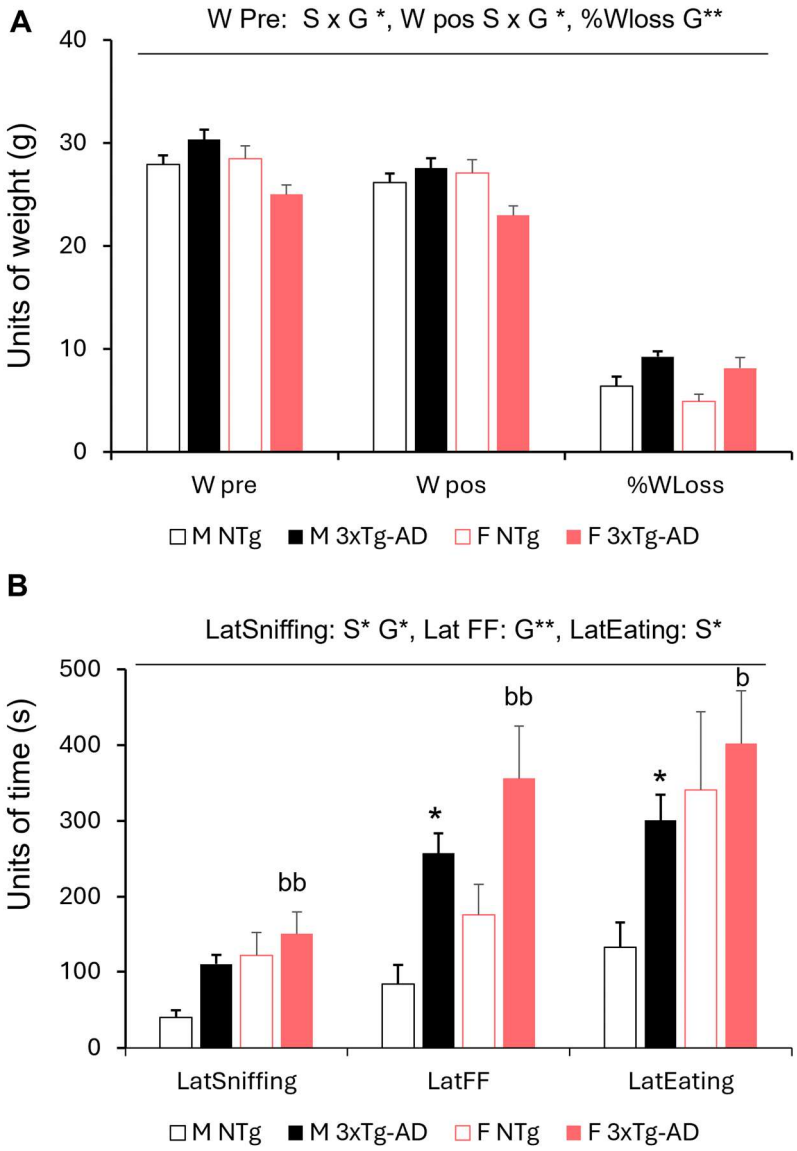


Figure 4. Genotype and sex differences in body weight monitoring and food finding test in 12-month-old NTg and 3xTg-AD mice. The results are expressed as mean ± SEM. (A) Food deprivation: Wpre, initial weight before fasting; Wpost, weight after fasting; %Wloss, percent of weight lost overnight; Final W, final weight at 24 h later. (B) Food finding test—latencies in the food finding test: LatSniffing, latency to sniffing the hidden pellet; LatFF, latency to find the food (hidden pellet); LatEat, latency to eat the pellet.

Statistics: *p < 0.05, **p < 0.01, ***p < 0.001 vs. NTg mice of the same sex; S, p < 0.05; SS, p < 0.01; SSS, p < 0.001 vs. male of the same genotype; genotype × sex, b p < 0.05 and bb p < 0.01.

Male 3xTg-AD mice were slower in all the actions compared to NTg mice, with statistically significant differences in the latency of finding the hidden food [male, LatFF * p < 0.05] and eating it once it was found [male, Lat Eat * p < 0.05], while those in the latency to sniffing the food did not reach statistical significance.

Table 1. Time delays between goal-directed behaviors in the FFT without overnight food deprivation in 12-month-old male and female NTg and 3xTg-AD mice in group housing conditions

	NTg Male (n=10)		3xTg-AD Male (n=18)			NTg Female (n=6)		3xTg-AD Female (n=17)		
	Mean	± SEM	Mean	± SEM	Ratio G	Mean	± SEM	Mean	± SEM	Ratio G
Smelling - Finding. Factor: S*, G*	44.40	± 18.46	147.33	± 23.83	3x	53.67	± 21.48	206.06	± 63.04	3x
Finding - Eating. Factor: G*, SxG*	48.50	± 19.98	43.33	± 23.07 b		165.67	± 67.95	46.12	± 15.59 *	
Smelling - Eating.	92.90	± 28.14	190.67	± 32.61	2x	219.33	± 78.31	252.18	± 62.84	

Note: Statistics: Factorial analysis of variance 2 × 2 and post hoc Duncan's test. Effects of genotype (G), sex (S) factors and their interactions, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. their respective control group. genotype × sex, b p < 0.05.

Time Delays and FFT Behavioral Correlates

The time delays between goal-directed behaviors in the FFT were analyzed (Table 1). The results per each sex and factorial analysis are depicted in Table 1. Significant genotype- dependent differences between 3xTg-AD male mice and NTg male mice were reported in the food-finding delay and the total time between the start of the ethogram (sniffing) and Finding the food (finding) and the total time between the finding the food and its end (eating)

Figure 5A illustrates the olfactory signatures of 12-month-old animals with normal and AD-pathological aging elicited in the FFT, despite the lack of overnight food deprivation, and under the effects of intrinsic (genotype and sex) factors. In addition, as shown in Figure 2B, meaningful correlation analysis revealed that finding and eating exhibited a strong and predictable relationship in 3xTg-AD males (p < 0.001) and females

($p < 0.01$, $p<0.01$). Two other groups, NTg animals showed positive correlations between the latencies of subsequent behaviors (sniffing and finding; finding and eating).

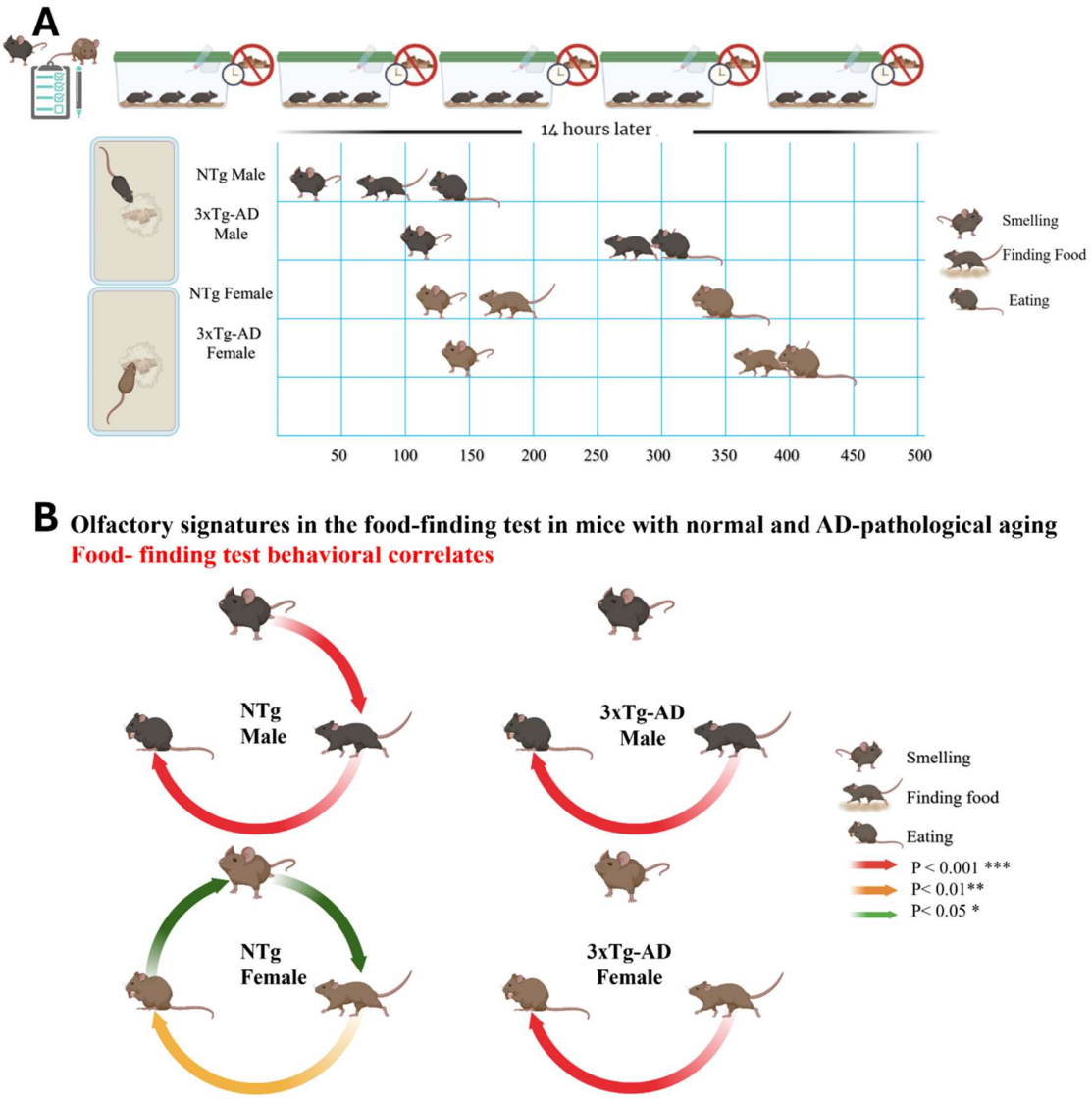


Figure 5. Sex- and genotype-dependent olfactory signatures in mice with normal and AD-pathological aging in the FFT without overnight food deprivation. (**A**) Experimental design and ethogram tablature for "sniffing, finding, and eating the hidden food pellet" by 16-month-old male and female NTg and 3xTg-AD mice and respective isolated groups. (**B**) Meaningful correlation analysis in the FFT: Graphical representation of the significant Pearson r correlations * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between the three olfactory actions, all of them positive. The figure was created with BioRender. com, accessed in July of 2022.

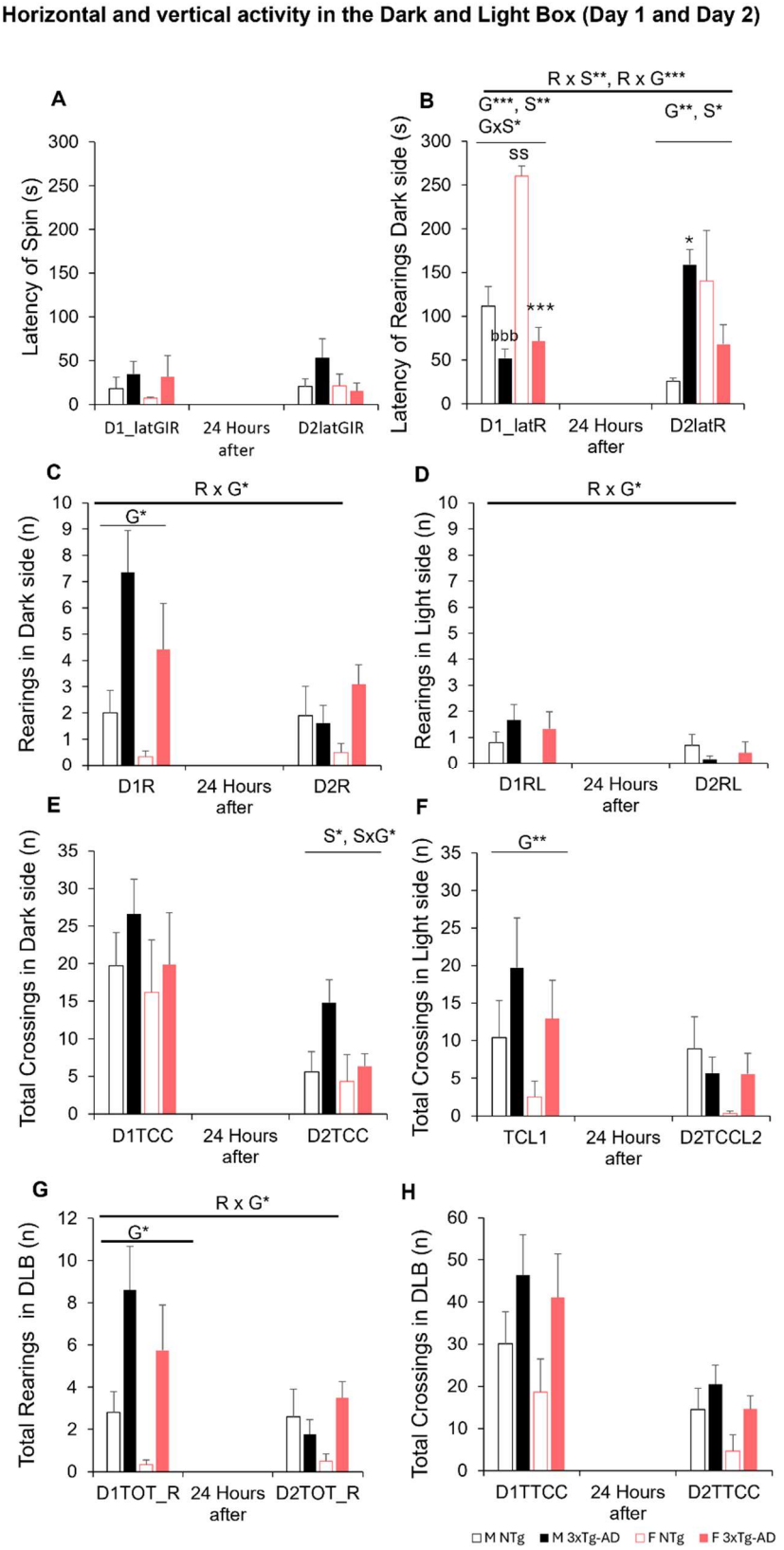


Figure 6. Horizontal and vertical activity in the dark and lightbox on day 1 and day 2 in 12-month-old NTg and 3xTg-AD mice. The results are expressed as mean ± SEM. (**A**) Latency of turn: The interval of time required for the animal to complete a full 360-degree

turn and start a period of exploration (**B**) Latency of rearings in the dark and lightbox on day 1 and day 2: The interval of time required for the animal to make the first vertical exploration (**C**) Rearings in the dark compartment on day 1 and day 2: The frequency with which the animals engage in vertical exploratory activity (**D**) Rearings in the light compartment on day 1 and day 2: The frequency with which the animals engage in vertical exploratory activity. (**E**) Total crossings in the dark compartment on day 1 and day 2: The total horizontal activity performed on the dark compartment (**F**) Total crossings in the light compartment on day 1 and day 2: The total horizontal activity performed on light compartment (**G**) Total rearings in the dark and lightbox on day 1 and day 2 (**H**) Total crossings in the dark and lightbox on day 1 and day 2: The total horizontal activity performed by animals in the dark and lightbox. Statistics: Effects of genotype (G), sex (S) and (G x S) genotype x sex factors and their interactions, (R x G)genotype and re-test effects Rx G* $p< 0.05$, ***$p < 0.001$, (R x S) sex and re-test effects Rx S** $p< 0.01$; Post-hoc analysis: *$p < 0.05$, ***$p < 0.001$ vs. NTg mice of the same sex; genotype × sex, b $p < 0.05$ and bb $p < 0.01$.

In the DLB test the latency of turn (Fig 6A), there was no significant differences in the latency of turn between the groups. The time required for the mice to start exploring by spinning does not appear to be significantly affected by genotype or sex. The latency of rearings (Fig 6B) 3xTg-AD mice show a higher latency to rearing in the dark compartment on both days, with significant effects of genotype (G*** $F(1.18)= 19.756$, $P< 0.000$ Day 1 and G** $F(1.18)=10.202$, $P<0.01$ Day 2) sex (S** $F(1.24)= 10.049$, $P<0.01$ Day 1 and S* $F(1,18)=6.511$, $P<0.05$ Day 2) Genotype x Sex (G x S*$F(1.18)= 5.954$, $P< 0.05$ Day 1) and re-test effects (R x G *** [$F(1.18)= 25,802$, $P<0.000$] and R x S **[$F(1.18)=8.915$, $P<0.01$) Day 1 latency shows significantly higher values in 3xTg-AD groups compared to NTg groups, indicating increased hesitancy in exploring the dark compartment. This hesitancy may be reflective of altered anxiety levels, with 3xTg-AD mice exhibiting more anxiety-like behavior. The significant interaction effects (G***, S**, GxS*) indicate genotype and sex both influence these behaviors.

In the rearings in Dark compartment (Fig 6C) There was a significant re-test effect (RxG* $F(1,41)= 3.921$, $P< 0.05$) and genotype effect (G* $F(1,18)= 4.836$, $P< 0.05$ Day 1), with 3xTg-AD mice showing reduced rearing in the dark compartment. Significant differences based on genotype, with 3xTg-AD mice showing reduced rearing in the dark

compartment on Day 1. This reduction in exploratory behavior further supports the presence of increased anxiety in the 3xTg-AD mice.

In the rearings on the Light compartment (Fig 6D) significant differences was observed in the re-test just in the genotype (RxG* $F(1,41)=4.400$, $P<0.05$) the frequency of rearing on the Light compartment remains consistently low across all groups, reflecting the general aversion to bright, open spaces. No significant differences was observed between groups for rearings in the light compartment. Rearings in the light compartment generally indicate lower anxiety. The lack of differences suggests similar levels of avoidance behavior in the lit area across all groups.

About the horizontal exploration activity in the DLB test, the total crossings in Dark compartment (Fig 6E) Significant sex (S* $F(1,25)= 4.521$, $P< 0.05$ Day 2) and sex × genotype (SxG* $F(1,25)= 5.923$, $P<0.05$ Day 2) effects, with females showing fewer crossings. Female 3xTg-AD mice exhibit fewer crossings in the dark compartment, indicating higher anxiety levels, as they are less willing to move around in the perceived safe space just in the second day. 3xTg-AD mice might show reduced crossings, indicating increased anxiety and a preference for staying in one place rather than exploring. The total crossing in the light compartment (Fig 6F). Significant genotype (G** $F(1,18)= 29.003$, $p< 0.01$ Day 1) effects were observed. Reduced crossings in the light compartment for 3xTg-AD mice suggest increased anxiety-like behavior, as these mice are less inclined to enter and explore the aversive lit environment.

In the total vertical exploration activity in the DLB test (Fig 6G))Significant differences was observed in the re-test just in the genotype (RxG* $F(1,41)=4.589$, $P<0.05$) and genotype (G* $F(1.47)=5.291$, $P<0.05$ Day 1). Total rearings are lower in 3xTg-AD mice, consistent with the hypothesis of increased anxiety-like behavior and reduced exploratory activity.

At the total horizontal exploration activity in the DLB test (Fig 6H) No significant differences was observed between groups. Total crossings might be less sensitive to the anxiety-related differences or might reflect overall activity levels rather than anxiety per se.

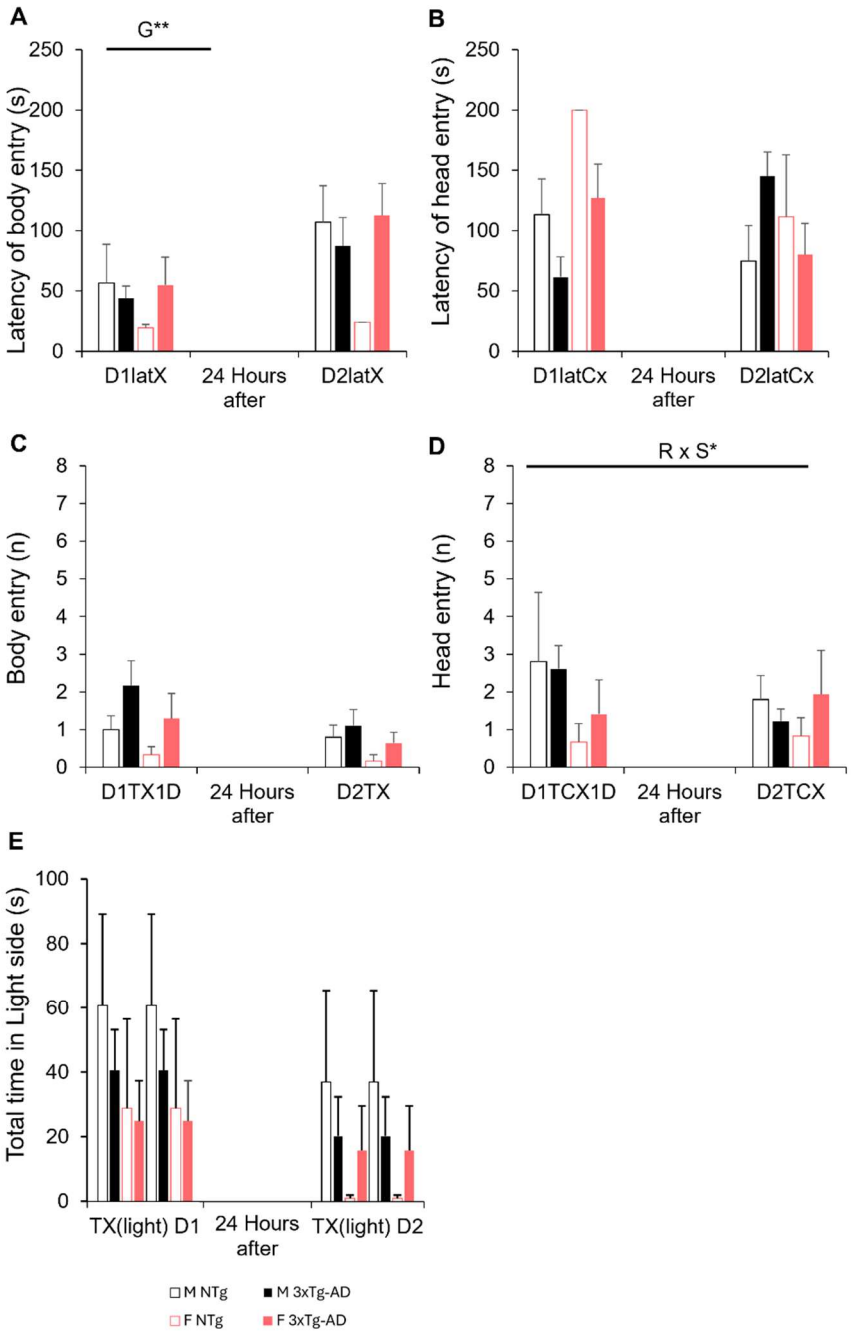


Figure 7. Assessment of 12-month-old 3xTgAD and NTg male and female mice in a series of tests for BPSD-like behaviors on the dark and light box on day 1 and day 2. (**A**) Latency of body entry: Equal latency to leave the black compartment (no light) and through an opening, entering the white compartment. (**B**) Latency of head entry: Equal latency of peeking through the compartment between the dark and light compartment (**C**) body entry: The number of times the animal crosses between compartments (**D**) Head entry: The number of times the animals peeking through the compartment between dark and light compartments (**E**) Total time in the light compartment: The total amount of time

spent by the animals exploring the light area. Statistics: Effects of genotype (G), (R x S) Sex and re-test effects Rx S * $p< 0.05$.

In the figure 7A Significant differences was observed in the genotype (G** $F(1.18)=11.950$, $P< 0.01$) indicating that the genetic background influences the latency to body entry. Both NTg and 3xTgAD mice show a significant reduction in latency on day 2 compared to day 1 but no significant differences was observed in the re-test.

In the figures 7B and 7C No significant differences was observed related to re-test, genotype, or sex effect,

In the number of times the animals peeking through the compartment between dark and light compartments (Fig 7D) Significant differences was observed in the re-test just in sex (RxS* $F(1,41)= 4.048$, $P<0.05$) indicating that the sex influences the number of head entries because de females maintains the patron of behavior in both days.

In the figures 7E No significant differences was observed related to re-test, genotype, or sex effect in the total amount of time spent by the animals exploring the light area

Correlation analysis

The heat map shows various correlations between behavioral measures. Positive correlations are indicated in blue and negative in red, with significant interactions noted (Fig 8) This map highlights relationships between different behavioral metrics across tests, providing insights into how certain behaviors may co-vary. Correlations between food-finding tests and other behaviors show significant relationships in both 3xTg-AD and NTg mice, as shown in Figure 9A the Latency of finding food correlates with total vertical activity in the corner test, the scatter plot shows a weak positive correlation ($R^2 = 0.132$) between the latency of finding food and total vertical activity in the corner test for 3xTg-AD mice, This suggests that mice taking longer to find food also exhibit increased vertical activity in the corner test.

The Latency of eating food correlates with total vertical activity in 3xTg-AD mice (Fig 9B) Another scatter plot reveals a similar weak positive correlation ($R^2 = 0.156$) between the latency of eating food and total vertical activity in the corner test for 3xTg-AD mice. Mice with longer eating latencies tend to have higher vertical activity levels.

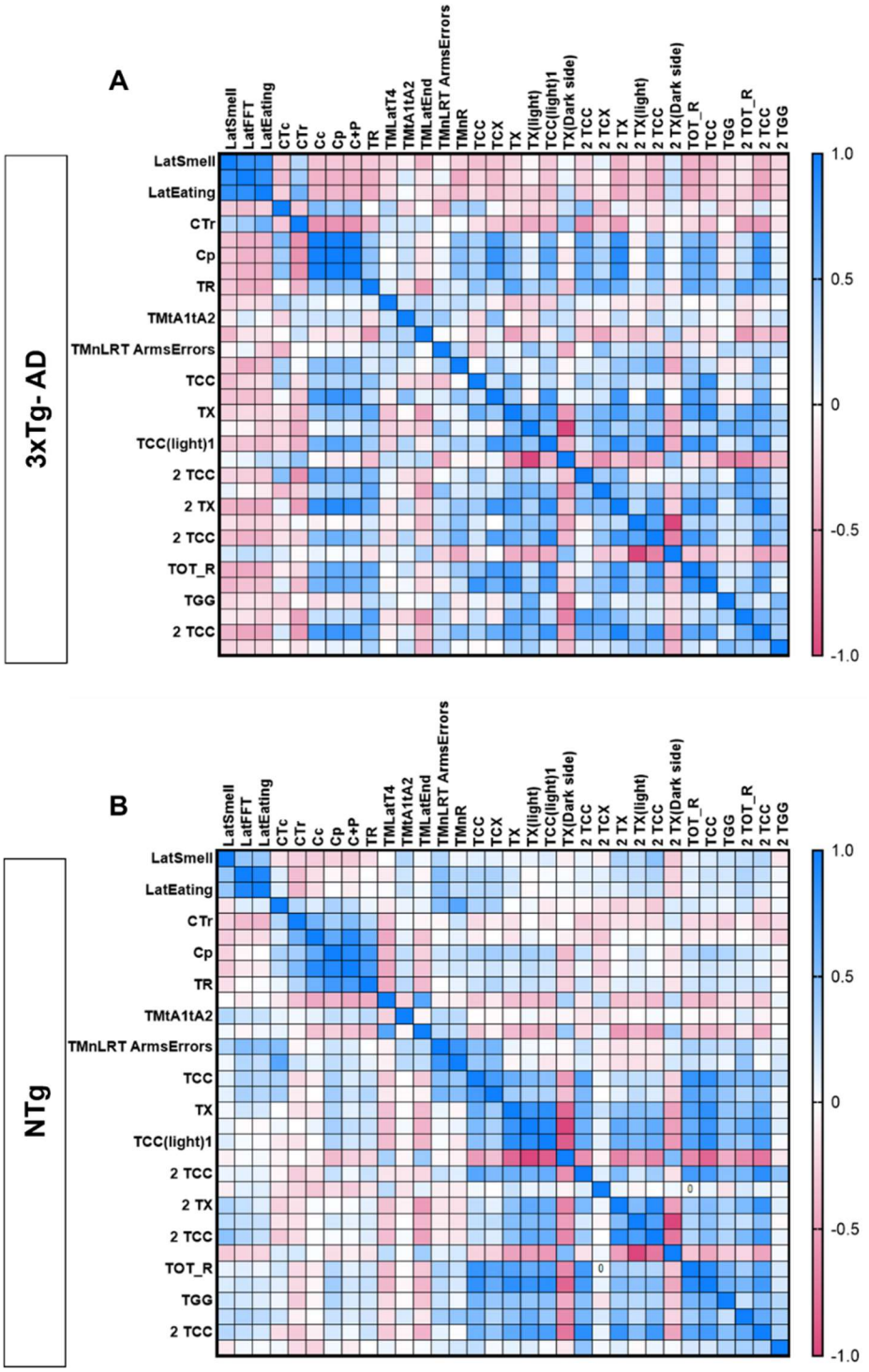


Figure 8. Heat map of correlations between the studied variables in all the study protocols in 12-month-old 3xTg-AD and NTg mice. The color scale shows the correlation intensity between 1 and −1, with positive correlations in blue and negative correlations in red. The statistical analysis was based on Pearson's test.

Similar correlations are seen in NTg mice in Figure 9C, in NTg mice, there is positive correlation ($R^2 = 0.356$) between the latency of eating food and total vertical activity in

the corner test. This suggests that the relationship observed in 3xTg-AD mice holds for the non-transgenic group.

The errors in T-Maze correlate with latencies of finding and eating food in 3xTg-AD mice (Fig 9D-9E) The scatter plots demonstrate low positive correlations ($R^2 = 0.251$ and $R^2 = 0.218$) between total errors in the T-Maze and the latencies of finding and eating food in 3xTg-AD mice. The mice making more errors in the T-Maze tend to have longer latencies for finding and eating food. These significant correlations suggest that cognitive and exploratory behaviors are interrelated. Impaired olfactory or cognitive functions (as indicated by food finding latency and T-Maze errors) are linked to reduced exploratory activity.

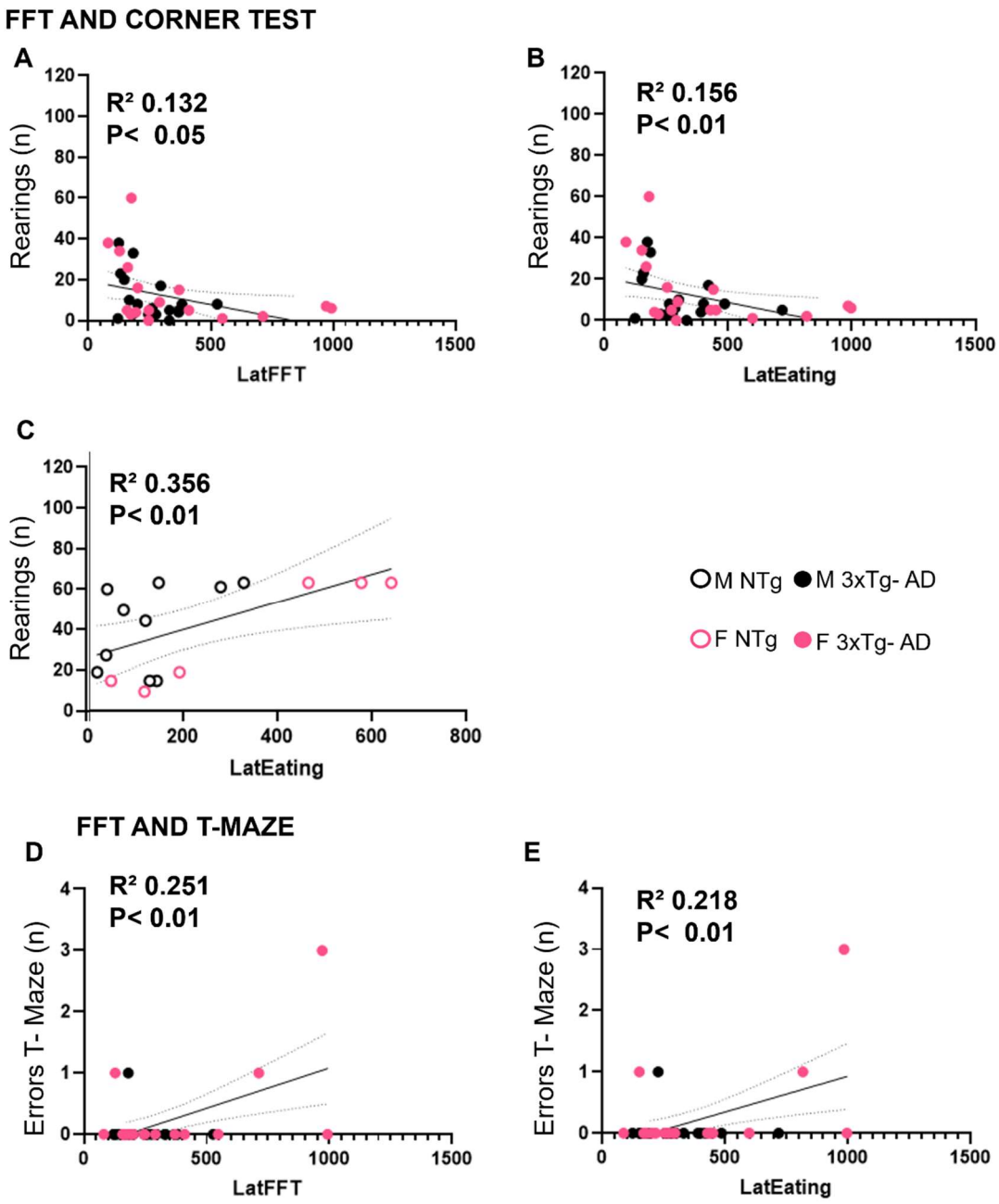


Figure 9. The scatter plot depicts the most significant correlations between the food finding test and the other tests in the study. (**A**)Latency of finding food and total vertical activity in the corner test in 3xTg- AD mice (**B**) Latency of eating the food and total vertical activity in the corner test in 3xTg- AD mice (**C**) Latency of eating the food and total vertical activity in the corner test in NTg mice (**D**-**E**) Total errors in T-Maze and Latency's of finding and eating food in 3xTg- AD mice. Each linear regression includes the correlation index (R^2) and the significance level of the correlation *p < 0.05, ***p < 0.001.

6.3. DISCUSSION

The current study provides a comprehensive evaluation of behavioral phenotypes in 3xTg-AD mice, offering Alzheimer's disease (AD). Utilizing a variety of behavioral assessments, the research delineates the cognitive and emotional deficits observed in this transgenic model. By examining the results from the Corner Test (CT), Open-Field Test (OF), T-maze (TM), Dark-Light Box Test, and Food Finding Test (FFT), this study enhances our understanding of the intricate behavioral changes associated with AD and offers a robust framework for future research and therapeutic interventions.

The neurobehavioral phenotyping of 3xTg-AD mice, which harbor PS1, APP, and tau transgenes, reveals a progressive phenotype closely mirroring that observed in humans with AD. In accordance with previous studies, our findings demonstrate that these mice develop age-dependent cognitive impairments and anxiety-like behaviors. (Javonillo et al., 2022b; Muntsant & Giménez-Llort, 2021)The comprehensive battery of behavioral tests employed enables a detailed analysis of specific aspects of these deficits, providing a nuanced understanding of how AD pathology manifests behaviorally. The 3xTg-AD mice exhibited significant differences in various tests compared to non-transgenic (NTg) mice, particularly in measures of anxiety and exploratory behavior. On the first day of testing, the Corner Test (CT) and Open-Field Test (OF) revealed notable differences between 3xTg-AD and NTg mice. The 3xTg-AD mice exhibited increased neophobia, as evidenced by a reduced number of corners visited and a delayed latency to perform the first rearing. These findings align with previous reports indicating heightened anxiety-like behavior in 3xTg-AD mice. (Baeta-Corral, Johansson, et al., 2018; Giménez-Llort et al., 2010)Additionally, in the open-field, these mice showed increased latency to move and decreased exploratory behavior, further supporting the presence of anxiety and reduced locomotor activity. Such behavioral alterations may be attributed to the progressive accumulation of amyloid-beta and tau pathology, particularly in brain regions associated with anxiety and movement (Giménez-Llort et al., 2010; Gimenez-Llort & Alveal-Mellado, 2021; Muntsant & Giménez-Llort, 2021; Torres-Lista et al., 2017)

On the second day, the open-field was re-exposed to assess the persistence of anxiety-like behavior. The 3xTg-AD mice demonstrated reduced habituation compared to the NTg mice, as evidenced by consistent anxiety-related measures such as latency to move and reduced locomotor activity. This finding at 12 months of age, mimicking advanced

stages of disease, complements those found at 2, 4 and 6 months of age (Torres-Lista et al., 2014) modeling asymptomatic, prodromal and early stages of disease, respectively. The new data, also verified in the current work using the dark-light box (see below), provides further evidence that suggests that the 3xTg-AD mice may have impairments in cognitive flexibility and adaptability, which are critical in novel environments. Interestingly, we have recently reported this phenomena at this advanced age in other behavioral paradigms such as the water maze (Alveal-Mellado & Giménez-Llort, 2024; Baeta-Corral & Giménez-Llort, 2015)The inability to habituate to a previously encountered environment is indicative of potential disruptions in neural circuits involved in learning and memory.(Baeta-Corral, Johansson, et al., 2018; Baeta-Corral & Giménez-Llort, 2015; Battaglia et al., 2024)

The T-maze (TM) conducted on the third day further highlighted the cognitive deficits in 3xTg-AD mice. These mice exhibited significant impairments in spontaneous alternation, indicating deficits in working memory and executive function. This is consistent with the progressive accumulation of amyloid-beta and tau pathology observed in the hippocampus and prefrontal cortex of these mice, regions that are critically involved in working memory. (Muntsant et al., 2023).The T-maze performance underscores the utility of 3xTg-AD mice in modeling the cognitive deficits seen in human AD, providing a valuable tool for investigating the underlying mechanisms of these impairments.

The dark-light box tests conducted on days four and five provided further evidence of heightened anxiety-like behavior in 3xTg-AD mice. These mice displayed increased latency to enter the lit compartment and reduced time spent in the lit area, which reinforces the notion of increased anxiety and aversion to bright, open spaces. The consistency of these findings across consecutive days highlights the robustness of the anxiety phenotype in 3xTg-AD mice. The dark-light box test is a reliable measure of anxiety-related behavior, further validating the relevance of the 3xTg-AD model to human AD.(Baeta-Corral, Johansson, et al., 2018; Baeta-Corral & Giménez-Llort, 2014).

On the sixth day, the Food Finding Test (FFT) evaluated olfactory function, a critical sensory modality often impaired in AD. The 3xTg-AD mice demonstrated increased latency in locating hidden food, suggesting deficits in olfactory function. This aligns with clinical observations of early olfactory deficits in AD patients, suggesting that the FFT may serve as a preclinical biomarker for the disease. The FFT provides insights into the

olfactory impairments in 3xTg-AD mice, offering a non-invasive measure to assess early sensory deficits associated with AD.(Marín-Pardo & Giménez-Llort, 2021, 2024)

The observed correlations between food-related behaviors and vertical activity suggest that anxiety-like behaviors may influence food-seeking responses. Mice exhibiting heightened anxiety, as observed in the 3xTg-AD mice, may take longer to explore and find food. The lack of such correlation in NTg mice highlights potential genotype-specific effects, suggesting that the altered behavior in 3xTg-AD mice could be related to neurodegenerative processes. (Marín-Pardo & Giménez-Llort, 2021, 2024) These findings support previous results (Muntsant & Giménez-Llort, 2021) and further underscore the importance of considering both genotype and behavior in neuroscientific research, as they provide insights into the complex interplay between AD-genotype (or genetic risk factors) and behavioral manifestations in AD.

The study's design, which incorporated both male and female mice, allowed for the analysis of sex differences in behavioral phenotypes. Interestingly, sex differences were observed in several tasks, with female 3xTg-AD mice generally displaying more pronounced deficits compared to males. (Barber et al., 2024; Barron et al., 2013; Muntsant et al., 2021; Santana-Santana et al., 2021)This finding aligns with epidemiological data indicating a higher prevalence and severity of AD in women. The underlying mechanisms for these sex differences may involve interactions between sex hormones and AD pathology, warranting further investigation. (Oddo et al., 2003)It is therefore of great importance to gain a deeper understanding of these sex-specific differences if we are to develop targeted therapeutic interventions that address the unique needs of male and female AD patients.(Baeta-Corral, Castro-Fuentes, et al., 2018a, 2018b; Muntsant et al., 2021)

The comprehensive behavioral phenotyping of 3xTg-AD mice presented here provides valuable insights for the development of therapeutic interventions. By identifying specific cognitive and emotional deficits, targeted therapies can be designed to ameliorate these symptoms. (Barber et al., 2024; Dennison et al., 2021)For instance, interventions aimed at enhancing synaptic plasticity or modulating neurotransmitter balance may hold promise in mitigating the observed impairments, at cognitive, NPS and sensorial levels. The comprehensive behavioral assessment including these dimensions further demonstrates the potential of 3xTg-AD mice as a robust platform for preclinical testing of therapeutic strategies, paving the way for translational research aimed at

improving the lives of individuals affected by AD.(Baeta-Corral & Giménez-Llort, 2015; Barber et al., 2024; Cañete & Giménez-Llort, 2021; Revilla et al., 2014)

The study's design, which incorporated both male and female mice, allowed for the analysis of sex differences in behavioral phenotypes. Interestingly, sex differences were observed in several tasks, with female 3xTg-AD mice generally displaying more pronounced deficits compared to males. This finding aligns with epidemiological data indicating a higher prevalence and severity of AD in women. The underlying mechanisms for these sex differences may involve interactions between sex hormones and AD pathology, warranting further investigation.(Santana-Santana et al., 2021) It is therefore of great importance to gain a deeper understanding of these sex-specific differences if we are to develop targeted therapeutic interventions that address the unique needs of male and female AD patients.

6.4. CONCLUSION

In conclusion, the comprehensive neurobehavioral characterization of 3xTg-AD mice substantiates their utility as a model for investigating Alzheimer's disease. The observed cognitive impairments and anxiety-like behaviors, along with significant sex differences, mirror the complexity of human AD pathology. These insights enhance our understanding of the phenotypical expression of the disease and inform the development of targeted therapeutic interventions. The study's findings emphasize the necessity for considering both genetic and sex-specific factors in future AD research and therapeutic strategies.

The main findings can be summarized in five points as follows:

1) 3xTg-AD mice displayed significant age-dependent cognitive impairments and anxiety-like behaviors. Increased neophobia, reduced locomotor activity, and anxiety-like behavior were evident in the CT and OF tests, demonstrated by a reduced number of corners visited and delayed latency to perform the first rearing.
2) The 3xTg-AD mice exhibited decreased exploratory behavior and increased anxiety, as indicated by increased latency to move, and reduced vertical and horizontal activities.
3) In the FFT, 3xTg-AD mice showed prolonged latencies in finding and eating food, correlating with total vertical activity in the corner test. These correlations indicate

that anxiety-like behaviors influence food-seeking responses, suggesting impaired olfactory or cognitive functions.

4) The study revealed notable sex differences in behavioral phenotypes, with female 3xTg-AD mice displaying more pronounced deficits compared to males.
5) Correlations between rearings in the CT and latencies in finding and eating food.
6) Correlations between latencies in finding and eating food and errors in the T-Maze.

7. *General discussion*

Module 1

Cognitive Function and Habituation in APP23 Mice: The hyperactivity pattern observed in APP23 mice was maintained from middle to very old ages in our study. Hyperactivity may be indicative of altered habituation ability (Allali et al., 2016), as evidenced by the psychostimulant effects of caffeine (Giménez-Llort et al., 2005) and A1R knockout mutant mice (King & Arendash, 2002b). Habituation, considered a rudimentary form of learning and memory (King & Arendash, 2002b), involves an animal learning to ignore stimuli lacking predictive value (King & Arendash, 2002b). Additionally, other cognitive aspects were explored using various paradigms over subsequent days.

The objective of our study was to evaluate cognitive function through the use of recognition tasks. Prior research has demonstrated that the performance of young and adult 3xTg-AD mice during the initial minute of a repeated open-field test is susceptible to alterations in AD genotype (Torres-Lista et al., 2014). Re-exposure to a familiar environment is a fundamental aspect of many conditioned memory tests (España et al., 2010). On the second day of testing, the recognition of both the open and illuminated field was assessed. Furthermore, the preference for a novel object in comparison to a previously inspected familiar object was evaluated. It is noteworthy that the performance during the initial minute of the repeated open-field test exhibited a resemblance to the neophobia response observed in the corner test. While the animals exhibited sustained locomotor activity in the periphery, there was a slight increase in central activity. It was unexpected that the genotype differences observed during the initial exposure to the open field were no longer evident during the repeated test. With regard to the novel object recognition task, no significant differences were observed among the groups. However, 24-month-old APP23 mice exhibited a genotype-related delay in exploring the novel object, likely attributable to superior performance in wild-type mice. The absence of AD sensitivity in these recognition tasks may be influenced by the animals' anxiety-like phenotype or reduced interest in exploration. These factors could potentially bias cognitive performance. This aligns with the genotype-dependent reduction in vertical

exploratory activity observed during the initial open field experience and the hyperactive horizontal locomotor pattern.

The open field test permitted the observation of age-related differences and, to a lesser extent, genotype-related variations. However, the T-maze assessment yielded the most AD-sensitive variables. The T-shaped labyrinth in the T-maze is primarily utilized for the evaluation of spatial working memory, engaging a multitude of brain regions, including the hippocampus, septum, prefrontal cortex, and basal forebrain (Thompson, 1959; Deacon & Rawlins, 2006; Giménez-Llort, Schiffmann, et al., 2007; Zhang et al., 2004). Furthermore, the latency to reach the maze intersection is indicative of the animal's coping strategies in response to stress. It is noteworthy that this variable has been employed for the psychological selection of prematurely accelerated aging mice (PAM) and non-PAM mice, as it correlates with impaired neuro-immunoendocrine function, indicators of accelerated aging, and an increased risk of premature death (Guayerbas et al., 2002). Moreover, our research has demonstrated that spontaneous alternation behavior in the black corridors, which resembles burrowing, reveals cognitive nuances and anxious-like profiles in both male and female mice across various ages and neuropathological conditions (Ariza et al., 2014; García-Mesa et al., 2011; Giménez-Llort et al., 2010). Additionally, recent studies have explored other cognitive aspects (Sanderson & Bannerman, 2012).

During the T-maze test, the ethogram (sequence of behavioral events) exhibited by APP23 mice was strongly influenced by genotype. Starting from a ‘facing the wall’ position, the latencies to turn, reach the T-intersection, and cross it with all four paws were significantly delayed in the mutants. Interestingly, the mean time spent exploring the two arms increased with age and AD genotype. However, this delay was not due to vertical exploratory activity, which was poor and worsened with the AD genotype since middle age. Consequently, APP23 mice took longer to complete the test compared to their age-matched wildtype counterparts, a variable that also showed a clear and robust genotype effect. The occurrence of errors when re-entering an already visited arm was scarce, with 18- and 24-month-old groups performing slightly better than middle-aged groups. At these ages, APP23 mice trended toward worse performance. In other AD models, such as 3xTg-AD mice, no differences in the number of execution errors were found at middle age. However, the efficiency to complete different phases of the test was

diminished in 3xTg-AD mice and slightly affected by long-term treatment with the antipsychotic risperidone (Torres-Lista et al., 2019).

The results demonstrate an altered ethogram in the T-maze, with the delay to cross the intersection emerging as the most AD-sensitive variable. This delay indicates impaired decision-making processes, which are primarily dependent on the prefrontal cortex. It is noteworthy that while delays in turning and reaching the intersection were also observed in 3xTg-AD mice, once they reached the intersection, the delay in crossing was not as pronounced as what was observed in APP23 mice. Nevertheless, a comparable delay associated with risk assessment was observed in this model when evaluating spontaneous choices to enter an anxiogenic white and lit enclosure in the dark-light box (García-Mesa et al., 2012; Giménez-Llort, Blázquez, et al., 2007; Giménez-Llort et al., 2006). Notably, this variable exhibited improvement with early postnatal handling, an intervention based on tactile sensorial stimulation that has been demonstrated to modulate cognitive and anxiety-like behaviors in rodents (Baeta-Corral & Giménez-Llort, 2014). Furthermore, other models, such as APP/PS1 mice (Tempier et al., 2013) and Tg2576 mice (Rinaldi et al., 2007), as well as selective dopaminergic receptor manipulations, have been observed to exhibit reduced spontaneous alternation, which suggests a potential link to frontal hypo functionality. The results of the correlation analysis indicate a strong relationship between T-maze performance and other test variables associated with increased emotivity/anxiety (self-grooming, navigation speeds) and apathy (latency of vertical and object exploration).

The Morris Water Maze (MWM) was employed to assess both short- and long-term spatial reference learning and memory (Morris, 1984). Over a period of five consecutive days, the animals were required to complete two learning and memory tasks. Performance was evaluated using the classical mean escape latency and the distance covered to reach the platform. It is noteworthy that a genotype effect was observed with regard to swimming speed at 12 months of age. All animals demonstrated comparable performance in the cued learning task, which assesses visual perceptual learning. However, during their inaugural experience in the maze, the APP23 mice exhibited markedly reduced latencies and distances when compared to their wild-type (WT) littermates. Although middle-aged APP23 mice initially exhibited slower swimming speeds, their swimming velocity normalized with age. It is noteworthy that the anxious profile of 3xTg-AD mice is responsible for their accelerated swimming speeds from the

early stages of disease onset (Giménez-Llort et al., 2013). It is noteworthy that the behavioral performances of AD mice in long-term survival studies have been observed to resemble those of WT mice at older ages. This resemblance has been demonstrated at both the biological and behavioral levels (Baeta-Corral & Giménez-Llort, 2015; Gimenez-Llort et al., 2014). The mortality bias (death of the worst-performing animals) and age-dependent impoverished performances in WT groups result in genotype differences becoming smoother in long-term AD survivors (Torres-Lista et al., 2017). Nevertheless, the 24-month-old animals demonstrated performance levels that exceeded expectations, given their progression from 18 months of age. Conversely, the cognitive impairment exhibited a deterioration that aligned with the anticipated trajectory.

Following the removal of the platform, the probe trial performance did not reveal any significant differences in short-term memory as a standalone construct. It was unexpected that no genotype differences were observed in the probe trial conducted two hours after the last place task trial. This unexpected result contrasts with the mutants' inferior performance during the final one or two days of the place task, which requires both long-term and short-term learning and memory. It is noteworthy that the lack of genotype sensitivity observed in the probe trial for APP23 mice is consistent with the findings reported by other experts using this animal model (Lalonde et al., 2003, 2005). However, it should be noted that a mortality bias was recently identified, whereby animals that died during behavioral assessments or had poor life prognostics were excluded from the final analysis. This may explain the lack of expected results in 16-month-old 3xTg-AD males (Torres-Lista et al., 2019). Despite a phenotype that was indistinguishable from that of the control strain, cognitive deficits in the Morris water maze were evident in 18-month-old female survivors (Torres-Lista et al., 2017). It is noteworthy that distinct preferences for adjacent areas indicate a lack of focused, goal-directed swimming strategies at 18 months of age (Lalonde et al., 2005). Further analysis of the swimming strategies employed could shed light on the compensatory behavioral mechanisms that underpin this paradoxical outcome of the probe trial (Lalonde et al., 2003).

Module 2

The Food Finding Test (FFT)(Deacon et al., 2009) is a neuroethological assessment tool that allows for the evaluation of olfactory function and related behaviors in animals. It is particularly useful for evaluating cognitive and emotional performance, assessing preventive/therapeutic interventions, and assessing the impact of risk factors and hazards. In this study, the FFT was used as a sensorial paradigm to uncover genotype and sex differences (Marín-Pardo & Giménez-Llort, 2021) in 12, 13 and 16 -month-old 3xTg-AD mice in a C57BL/6 genetic background compared to NTg mice with normal aging.

The study "*Olfactory Signatures in the Food Finding Test in Mice With Normal and Alzheimer's Disease-Pathological Aging With Special Concerns on the Effects of Social Isolation",* investigates the effects of Alzheimer's disease (AD) genotype and sex on olfactory ethograms in the food finding test, which defines olfactory signatures in 13-month-old male and female non-transgenic and 3xTg-AD mice. The food finding test is an easy and ready-to-do behavioral paradigm that allows for neuroethological assessment of sniffing function. The use of ethologically relevant stimuli is crucial to assess cognitive performance and dissect the effects of preventive/therapeutical interventions or the impact of risk factors and hazards. (Giménez-Llort & Torres-Lista, 2021; Torres-Lista et al., 2015, 2019)

The exploratory behavior of rodents involves an internal conflict between the attraction for novelty and the risk it contains, as they could be exposed to potentially dangerous environments. (Giménez-Llort et al., 1995) Olfaction, a sense that allows not only the detection of odors in the surrounding environment necessary to identify food, predators, and sexual partners but also to get the spatial orientation Coronas-Sámano et al., 2014; Invitto et al., 2018b), is essential for survival. In the case of 3xTg-AD mice, weight loss depends on intrinsic factors such as increased nocturnal activity (Baeta-Corral, Johansson, et al., 2018) and hypermetabolic state (Escrig et al., 2020) compared toto age-matched NTg mice. Weight loss is useful to monitor the aggravation of AD-pathogenic processes (Torres-Lista et al., 2019).

In the work, male mice with normal and AD- pathological aging were equally delayed in their first contact with food pellets, while in the female sex this latency was increased but also depended on the genotype. Female 3xTg-AD performed the three-action (sniffing–find–eat) ethogram compared to their male cage mates. This could be because the sniffing in female mice is prone to be more affected during this stage of the disease, developing a situation in which the sensitivity to detect odors is more deficient (Mitrano et al., 2021). Sex differences were found in the latency of the actions, with female mice being delayed compared to male mice and in the delay to develop the sequence, in this latter case being in a genotype-dependent manner. Both groups of female mice exhibited longer delays in starting the ethogram than male mice, but the temporal pattern of their ethogram (delays between actions) to sniffing–finding–eating the food was two- to threefold times faster in the 3xTg-AD mice, but 1/2 in the case of NTg mice. This paradoxical genotype and sex modulation of the ethogram could be described as an accordion.

The effects of naturalistic isolation (2-3 months) in a subgroup of 3xTg-AD that recently lost their cage mates after 10 months of living in a standard social environment were also assessed. This short isolation was enough to enhance the AD-genotype olfactory patterns shown by group-housed mice of the same genotype. The body weight and loss of weight after overnight fasting of isolated male 3xTg-AD were similar to those in the group-housed male 3xTg-AD, suggesting similar food motivation. The current report also shows that the ethogram was independent of body weight (Van Regemorter et al., 2020b).

Meaningful correlations between the three actions in the food finding test indicate that the actions were correlated in the AD- genotype, as could be inferred because the temporal pattern consisted of actions immediately consecutively performed. In the case of 3xTg-AD mice, the accordion effect resulted in correlations with a stronger statistical significance. Interestingly, the lower number of correlations found in the NTg mice, despite the bigger sample size in this group, would suggest that behavioral patterns driven by olfactory function coexisted with behaviors driven by other cognitive functions.

In isolated 3xTg-AD mice, the correlations were mainly lost, but the small sample size limitation cannot be dismissed

Continue with the next study, "*Food Finding Test without Deprivation: A Sensorial Paradigm Sensitive to Sex, Genotype, and Isolation Shows Signatures of Derangements in Old Mice with Alzheimer's Disease Pathology and Normal Aging*" the FFT was used as a sensorial paradigm to uncover genotype and sex differences in 13-month-old 3xTg-AD mice in a C57BL/6 genetic background compared to wildtype mice with normal aging (Marín-Pardo & Giménez-Llort, 2021).

The results showed that the current FFT paradigm for older animals elicited distinct olfactory signatures despite the lack of overnight food deprivation. As expected, genotype was the most important factor, as significant statistical differences were found in the three goal-directed behaviors, with delayed olfactory behavioral responses in the 3xTg-AD mice as compared to mice with normal aging. Overall, eating behavior was notable as it showed a considerable delay and was sensitive to genotype and sex factors, whereas sniffing and finding were elicited in a quite consecutive manner, a sequence that was found enhanced in 3xTg-AD mice under isolation. Interestingly, sex appeared to be a relevant factor since these effects were mostly due to the behavioral ethogram elicited in males, whereas females showed a strong consistency in the similarity of their latencies in all the three goal-directed behaviors as compared to those of their non-transgenic counterparts with normal aging.

Compared to the previous protocol at 13 months of age (Marín-Pardo & Giménez-Llort, 2021). the ethograms of both male and female wildtype mice with the gold-standard C57BL/6 genetic background exhibited a 2-fold increase in recorded latencies. Several intrinsic factors could account for these results, such as the age factor, as behavioral responses in 16-month-old animals tend to be slower than those observed in their middle-aged counterparts. However, the lack of overnight food deprivation in the current paradigm for old animals could also reduce the energetic demands of animals, and therefore, reduce the elicitation of fast goal (food)-directed ethograms.

The ethograms of 3xTg-AD mice differed from those reported in their congeners at 13 months of age in which fast signatures were observed (Marín-Pardo & Giménez-Llort, 2021). The age/stage-dependent increase of weight in this animal model, mostly in males, could explain the strong delays observed in this sex. However, the advance in their neuropathology status could explain a different coping-stress strategy when confronting a new scenario, being a faster response in beta-amyloid stages but slower when the brain damage severity associated with beta- and tau-pathologies increases. These differences

align with age/stage-dependent fast/slow responses in other AD-animal models and those previously described in mice models with accelerated aging (Giménez-Llort et al., 2021, Guayerbas et al., 2000, Muntsant et al., 2023). The results support the understanding that male and female sexes can be distinct natural scenarios for studying the influence of biological, psychological, and social factors on health and illness across the lifespan, as well as the interconnected dynamics affecting homeostatic networks.

These results highlight the importance of considering gender in the study of olfaction and its susceptibility to external factors. The results also showed that derangements of the FFT-induced olfactory ethograms were also observed in female 3xTg-AD mice and their non-transgenic counterparts. This novel finding points at the complex interplay between sex influences and olfactory-related behaviors under positive/negative social conditions.

Meaningful correlation analysis between goal-directed behaviors provided further evidence of the strong and predictable relationship between sniffing and finding in some groups. The lack of significant correlations in male NTg mice highlights genotype-specific differences in the olfactory behavior ethogram. With regards to the impact of social condition on olfactory behaviors, sniff latency emerged as a predictive variable for finding food in isolated male 3xTg-AD mice, yet this correlation did not extend to the act of eating the food. Social isolation led to the disruption of functional correlations among goal-directed behaviors in the other groups, especially pronounced in males, indicating that the interplay between different behaviors is particularly sensitive to the effects of isolation. The results support the understanding that male and female sexes can be distinct natural scenarios for studying the influence of biological, psychological, and social factors on health and illness across the lifespan, as well as the interconnected dynamics affecting homeostatic networks.

The last study of this thesis is the "*Cognitive, NPS-like and olfactory signatures in mice with normal and AD-pathological aging, with a special focus on the effects of sex"* The study examines the behavioral phenotypes of 3xTg-AD mice, which are transgenic models of Alzheimer's disease (AD). The research uses various assessments to understand the cognitive and emotional deficits observed in this transgenic model. The results from the Corner Test (CT), Open-Field Test (OF), T-maze (TM), Dark-Light Box Test, and Food Finding Test (FFT) provide a comprehensive

understanding of the intricate behavioral changes associated with AD and offer a robust framework for future research and therapeutic interventions.

The neurobehavioral phenotyping of 3xTg-AD mice reveals a progressive phenotype closely mirroring that observed in humans with AD. These mice develop age-dependent cognitive impairments and anxiety-like behaviors, which may be attributed to the progressive accumulation of amyloid-beta and tau pathology, particularly in brain regions associated with anxiety and movement (Giménez-Llort et al., 2010; Gimenez-Llort & Alveal-Mellado, 2021; Muntsant & Giménez-Llort, 2021; Torres-Lista et al., 2017). The open-field test revealed significant differences between the 3xTg-AD mice and non-transgenic (NTg) mice, particularly in measures of anxiety and exploratory behavior.

On the second day of testing, the open-field was re-exposed to assess the persistence of anxiety-like behavior (Baeta-Corral, Johansson, et al., 2018; Giménez-Llort et al., 2010). The 3xTg-AD mice demonstrated reduced habituation compared to the NTg mice, as evidenced by consistent anxiety-related measures such as latency to move and reduced locomotor activity. This finding at 12 months of age, mimicking advanced stages of disease, complements those found at 2, 4, and 6 months of age (Torres-Lista et al., 2014)

The T-maze (TM) conducted on the third day further highlighted the cognitive deficits in 3xTg-AD mice, indicating deficits in working memory and executive function. This is consistent with the progressive accumulation of amyloid-beta and tau pathology observed in the hippocampus and prefrontal cortex of these mice, which are critically involved in working memory (Muntsant et al., 2023) The T-maze performance underscores the utility of 3xTg-AD mice in modeling the cognitive deficits seen in human AD, providing a valuable tool for investigating the underlying mechanisms of these impairments.

The study aimed to investigate the anxiety-like behavior in 3xTg-AD mice, which were found to be more robust than those in other animals. The dark-light box tests on days four and five showed increased latency to enter the lit compartment and reduced time spent in the lit area, indicating increased anxiety and aversion to bright, open spaces (Baeta-Corral, Johansson, et al., 2018; Baeta-Corral & Giménez-Llort, 2014). These

findings are consistent across consecutive days, confirming the relevance of the 3xTg-AD model to human Alzheimer's disease (AD) (Oddo et al., 2003)

On the sixth day, the Food Finding Test (FFT) evaluated olfactory function, a critical sensory modality often impaired in AD. The 3xTg-AD mice demonstrated increased latency in locating hidden food, suggesting deficits in olfactory function. This aligns with clinical observations of early olfactory deficits in AD patients, suggesting that the FFT may serve as a preclinical biomarker for the disease. The observed correlations between food-related behaviors and vertical activity suggest that anxiety-like behaviors may influence food-seeking responses (Marín-Pardo & Giménez-Llort, 2021, 2024)

The study's design, which incorporated both male and female mice, allowed for the analysis of sex differences in behavioral phenotypes (Barber et al., 2024; Barron et al., 2013; Muntsant et al., 2021; Santana-Santana et al., 2021) Female 3xTg-AD mice generally exhibited more pronounced deficits compared to males, which aligns with epidemiological data indicating a higher prevalence and severity of AD in women. The underlying mechanisms for these sex differences may involve interactions between sex hormones and AD pathology, warranting further investigation.

The comprehensive behavioral phenotyping of 3xTg-AD mice provides valuable insights for the development of therapeutic interventions. By identifying specific cognitive and emotional deficits, targeted therapies can be designed to ameliorate these symptoms. (Barber et al., 2024; Dennison et al., 2021). Interventions aimed at enhancing synaptic plasticity or modulating neurotransmitter balance may hold promise in mitigating observed impairments at cognitive, NPS, and sensorial levels. The comprehensive behavioral assessment includes these dimensions further demonstrates the potential of 3xTg-AD mice as a robust platform for preclinical testing of therapeutic strategies, paving the way for translational research aimed at improving the lives of individuals affected by AD (Baeta-Corral & Giménez-Llort, 2015; Barber et al., 2024; Cañete & Giménez-Llort, 2021; Revilla et al., 2014)

In light of the three most recent studies, which examine olfactory signatures in the context of the three behavioral goal-directed (namely, sniffing, finding food, and eating), a unified analysis of the factors that influence these behavioral responses can be facilitated. The first factor to be considered is the age of the subjects. Two studies were conducted with subjects aged 12 and 13 months, in a fasting state. In the first study, at 13

months of age, it was observed that the transgenics exhibited a behavioral signature characterized by the agglomeration of the three behaviors consecutively. This behavioral signature enabled the transgenics to be distinguished from the non-transgenics.

In the second work, which involved a more advanced age (16 months), in addition to the beta-amyloid pathology characteristic at 12 and 13 months of age (the first study), there was also tau pathology. Furthermore, the behavioral functional evaluation of the animals was assessed in terms of their physical, cognitive, and neuropsychiatric-like profiles. At this level, the primary distinguishing feature observed in the olfactory signatures of the 3xTg-AD and NTg was the simultaneous occurrence of the first two behaviors, sniffing and finding, in the ethogram, with a notable temporal distance from the third behavior, eating. In examining the results of this study, two potential explanations were considered for the observed change in the olfactory signature. The first is that the animals' age may have contributed to this shift, as they were 16 months old, which is a period when pathological states associated with both beta-amyloid and tau pathologies tend to become more pronounced. The second is that the absence of overnight food deprivation may have influenced the animals' eating behavior, which may have been less urgent than their sniffing and finding responses. Additionally, the neuropathological affectation characteristic of this disease may have continued to affect the animals' behavioral patterns, as reflected in the olfactory signature. The animals were not deprived of food, and thus the urge to eat was not as pressing for them. However, the sniffing and finding responses were rapid, likely due to the neuropathological affectation that will continue to be reflected at the behavioral level in the ethogram profile and therefore in the olfactory signature.

The third work enables us to dismiss some of these hypotheses. In this third work, the animals were 12 months old, yet their behavior, as observed in the ethogram, was equivalent to that of an animal 16 months old. Therefore, the neuropathological profile that gives rise to the observed behavioral phenotype allows us to say that in light of these findings, it can be concluded that the pathological profile observed at 12 months, which is primarily characterized by beta-amyloid, is already associated with this profile of two consecutive behaviors and a delay in eating behavior. Initially, it was hypothesized that this delay may have been caused by fasting. However, the animal were also fasting in this third protocol, but it can be seen that the delay persists.

It is important to note that the weight loss observed during fasting was not correlated with the latencies of these behaviors, thus eliminating this third explanatory hypothesis. Moreover, in this third study, the observed association with a battery of behaviors is accompanied by a detailed analysis of different types of anxious profiles in various anxiogenic environments, including the corner test, the open field, the dark and light box, and the T-maze. Additionally, the tests are repeated from one day to the next, allowing for the examination of cognitive processes in the different paradigms. The T-maze incorporates the working memory, allowing us to conclude that the finding behavior, significantly dependent on genotype and sex factors, was correlated with the anxious behavior of neophobia (of encountering novelty in the corner test) and with the errors in the T-maze, that is, the cognitive part of involving working memory.

It can be concluded that, from a behavioral perspective, sniffing behavior does not allow the differentiation of genotypes. This is likely due to the brief latency associated with this behavior. However, in the case of other latencies such as that of freezing associated to the neophobic behavior, despite the brief duration of this behaviors, the latency of freezing allows the genotype differentiation. Therefore, it is likely that sniffing behavior is nourished or triggered by a multitude of stimuli, not solely olfactory but also exploratory of the environment in order to safeguard itself. This is a fundamental and ubiquitous survival mechanism observed in a wide range of animals. The food finding behavior is the first to reach statistical significance with respect to genotype, and in some cases, also with respect to sex. It is possible that a confounding factor is present, or that the latencies are cumulative, meaning that a delay in the sniffing behavior would imply a delay in the finding behavior. To address this issue, the temporal difference between one behavior and the next was also calculated and analyzed in all studies to rule out the possibility of a cumulative phenomenon. In instances where discrepancies arise, the finding behavior was identified as the first significant one. The third study demonstrated that this behavior correlates more clearly with neophobia, as well as with the running test and working memory of the T-maze. Concerning eating behavior, it presents a challenge for analysis in the context of the three studies, as the three behaviors are expressed consecutively in some scenarios, as in the first study, while in the other two studies (which were conducted over 16 and 12 months, with food deprivation), the results are expressed with a time delay for eating. The underlying reasons for this behavior warrant further investigation. It is essential to ascertain whether this behavior is distinct or influenced by

different conditioning elements compared to the previous two behaviors, sniffing and finding, where the olfactory signature is well-preserved for a certain age considering the other factors genotype, sex and social condition.

8. *Conclusions*

Module 1

1. **To confirm the better survival curves in male than female APP23 mice and respective wildtypes, providing the chance to characterize behavioral signatures in middle-aged, old, and long-lived male animals.**
    1) The survival analysis was based on a total of 916 cases from our APP23 and wildtype C57BL/6, showed genotype and sex-dependent increased mortality rates in the mutant mice. A severe initial drop of survival during the first two months of age, which was more dramatic in APP23 females. Interestingly, APP/PS1 males also exhibit longer lifespans than females.
    2) Survival curves demonstrated that APP23 males were able to reach a long-life span, rending this animal model suitable for studying the impact of the disease in a male aging scenario. That is, 12- 18- and 24-month-old subjects would serve to study the disease at the middle, old, and very old ages, respectively, and as compared to age-matched C57BL/6 wildtype mice.
2. **To identify genotype differences in behavioral profile of APPswe animals compared to control animals (Wild Type) in the different behavioral tests.**
    3) Compared to WT littermates, the APP23 mice showed an increased number of visited corners but decreased vertical exploratory activity, evidencing increased neophobia in the corner test.
    4) Similarly, APP23 groups showed increased latencies to develop the ethogram of behaviors immediately after the direct exposure to an open and illuminated field and increased locomotion.
3. **To determine age differences between the 12-, 18-, and 24-months groups in APPswe mice and WT C57BL/6 littermates**
    5) In the T-maze, a black and narrow T-maze, the latency to reach the intersection was consistently shown to be increased at all ages and the APP23 mice required longer time to complete the exploration of all the arms of the maze.
    6) Increased thigmotaxis was noticeable already since 12 months of age.
    7) As part of the differences in the status of their cognitive functions exhibited

during the exploration of new environments, we assessed APP23 mice for putative cognitive deficits in discriminative tasks (novelty/familiarity) for context (remembering the anxiogenic open field scenario) and object recognition. However, the novel object recognition test showed low sensitivity to the genotype, only noticed at the oldest age through the lowest difference in the delay in exploring the novel and familiar objects compared to WT littermates.

8) The novel object recognition test showed low sensitivity to the genotype, only noticed at the oldest age through the lowest difference in the delay in exploring the novel and familiar objects compared to WT littermates.

4. **To ascertain the level of interaction between these two factors, age, and genotype.**

9) Worse performance of 12-month-old APP23 mice was shown as compared to WT littermates during the progress of acquisition (learning and memory) in the place task, while differences were scarce in the older groups.

10) In the probe trial, the worse spatial memory discrimination of the trained quadrant compared to the opposed quadrant was shown at 18 months of age but independently of the genotype

11) As we have reported in other mice models for AD, a conspicuous BPSD-like profile and age as a biological factor may result in confounding factors in a Morris maze, considered an anxiogenic environment for mice.

12) Most of the variables analyzed in the Corner test, Open field, Object recognition, and T-maze were able to show age-related differences, and those presented here are among those that, under our experimental conditions and in our hands, better discriminate the behavioral phenotype of female APP23 mice through their aging process, from middle-age (12 months of age) and old (18 months of age) to very old (24 months of age), as compared to their respective WT littermates.

13) Most importantly, non-linear age- and genotype-dependent behavioral signatures were found in 24-month-old mice, suggesting the existence of a crosstalk between chronological and biological/behavioral ages in long-lived animals. This finding is useful to study underlying mechanisms and distinct compensations through natural physiological aging and, maybe also, long-term AD-associated aging male survivors.

Module 2

1. **To validate the sensitivity of the food-finding test to sex and genotype**

   Study 2. At 13 months of age

   1) The temporal pattern of sniffing-find-eat differs in females (delayed) from males (fast). 3xTg-AD mice demonstrate olfactory signatures in Alzheimer's disease.
   2) Olfactory signatures in male and female 3xTg-AD mice were characterized by a fast ethogram and strong behavioral correlates.
   3) Olfactory signatures in male and female controls, the gold- standard C57BL/6 mice, were slow and progressively developed as part of a more complex ethogram, as also supported by disrupted behavioral correlates between the three actions assessed in the food finding test.

   Study 3. At 16 months of age

   4) The FFT paradigm without food deprivation elicited longer ethograms than previously reported with the standard overnight food deprivation protocol. However, it did identify genotype-dependent olfactory signatures in normal and pathological aging in AD.
   5) The temporal pattern of sniffing-find-eat differs in females (fast) from males (delayed).
   6) 3xTg-AD mice in Alzheimer´s disease, exhibit olfactory signatures.

2. **To determine the optimal experimental conditions for optimizing these studies in the 3xTg-AD model. This will entail optimizing the 12-, 13-, and 16-month experimental conditions.**

Study 2. At 13 months of age

   7) Food finding latency discriminates normal (advanced) aging from AD (delayed) aging in females only.

Study 3. At 16 months of age

8) In males, the ethogram defines normal (progressive) signatures of AD (rapid) aging.

3. **To demonstrate the presence of olfactory deficits in 3xTg-AD animals using the experimental food-finding test protocol.**

Study 3. At 16 months of age

9) The temporal pattern of sniffing-find-eat differs in females (fast) from males (delayed). 3xTg-AD mice demonstrate olfactory signatures in Alzheimer's disease
10) Food finding latency discriminates normal (advanced) aging from AD (delayed) aging in males only.

Study 4. At 12 months of age

11) Food Finding Test (FFT) evaluated olfactory function, a critical sensory modality often impaired in AD. The 3xTg-AD mice demonstrated increased latency in locating hidden food, suggesting deficits in olfactory function.

4. **To test certain conditions, such as animal age, fasting time, test evaluation time, number of animals to be recorded simultaneously, and environmental conditions (social isolation), for the correct experimental implementation**

Study 2. At 13 months of age

12) Social isolation, naturally occurring only in 3xTg-AD mice, enhanced the genotype differences and disrupted the behavioral correlates.

Study 3. At 16 months of age

13) Social isolation increased latencies, but olfactory signatures were preserved only in females. A functional disorder was detected as the internal correlation between the three goal-directed behaviors and were lost under isolation in most of the groups.

5. **To characterize the olfactory impairment of 3xTg-AD mice and its relationship with cognitive impairment and BPSD profile (especially anxiety) in males and females in different experimental conditions.**

Study 4. At 12 months of age

14) In the FFT, 3xTg-AD mice showed prolonged latencies in finding and eating food, correlating with total vertical activity in the corner test. These correlations indicate

that anxiety-like behaviors influence food-seeking responses, suggesting impaired olfactory or cognitive functions.

15) The study revealed notable sex differences in behavioral phenotypes, with female 3xTg-AD mice displaying more pronounced deficits compared to males.
16) Correlations were found between rearings in the CT and latencies in finding and eating food.
17) Correlations were found between latencies in finding and eating food and errors in the T-Maze.

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