Universitat Autònoma de Barcelona

Refinement of Marble Burying Test to Model Behavioral and Neuropsychiatric Symptoms: Studies in Two Animal Models

MIKEL ENRIQUE SANTANA SANTANA

Refinement of Marble Burying Test to Model Behavioral and Neuropsychiatric Symptoms: Studies in Two Animal Models

DOCTORAL THESIS

MIKEL ENRIQUE SANTANA SANTANA

Sep. 2022

PhD. Lydia Giménez Llort, Thesis Director

PhD. José Ramón Bayascas Ramirez, Thesis Director

Doctorat en Neurociències

Institut de Neurociències

Departament de Psiquiatria i Medicina Legal, Facultat de Medicina

Universitat Autònoma de Barcelona (Catalunya)

Agradecimientos

Cuando me preguntaban que quería ser de mayor respondía que millonario. Mi segunda respuesta era decir científico. Caprichos de la vida aquí estoy. Como dijo Tyler Durden ¿Y ahora qué?

Creo que este trabajo representa una victoria de la pequeña ciencia. Aquella que se hace con escasos recursos y con objetivos humildes. Que no busca dar grandes pasos, sino dar uno pequeño y seguro. Que aún tiene su espacio y es relevante. En una academia gobernada por la competitividad, la prisa, la inestabilidad laboral, la escasez de recursos y la necesidad publicar, este tipo de ciencia tiene cada vez menos espacio para sobrevivir. Víctimas de este sistema no podemos permitirnos fallar, parar o examinar si lo que estamos haciendo está bien. Entramos pues en un juego peligroso: ya no buscamos falsar nuestras hipótesis, sino que buscamos demostrarlas. Leia hace poco un estudio de mostraba que, aunque un experimento fuesa falseado por otro trabajo, el articulo original seguía siendo más citado que aquel que lo rebatía. De todo esto trata este trabajo. De romper con corrientes. De “paren el mundo que me quiero bajar”. De cuestionarnos. De escuchar a las voces discordantes.

Esta tesis supone también una reivindicación de la conducta dentro de la neurociencia. De que esta no es un mero instrumento para apoyar los hallazgos en otras ramas.. Como me dijo una vez mi directora todo el mundo nos quiere, pero nadie se casa con nostrosos, Que es válida y suficiente por sí misma

A nivel personal estos 6 años han sido un viaje de autodescubrimiento, pero no de los bonitos. De bajar a lo más profundo. De llegar a mis limites. Como me decía mi madre de pequeño: hasta que no lo rompas no estas tranquilo. Y vaya si me rompí. Cuatro mudanzas, cuatro trabajos, dos master, una pandemia, un accidente laboral con despido improcedente, etc. La vida nunca se ha parado. Por suerte, siempre se me ha dado bien aprender. Así, que todo lo que aprendí en esos 6 años me ha hehecho avanzar y salir de ahí

Quiero agradecer a toda la gente que me ha acompañado en este viaje, ya sea de principio hasta el fin o solo parcialmente. A mi familia, quienes probablemente estatan mas orgullos de mi trbajao que yo. Especialmente a mis padres, Ma y Pa, por sacrificarse por mi. A mis tíos, Tata y Claymore, poque tuve la suerte de nacer con otro par de padres. A mis hermanos y a mis primos, por siempre verme como el pequeño de la familia y nunca dejar de cuidarme. A mi Sancho, porque el poder reírnos juntos siempre fue mi refugio. A Bea, porque sin su estabilidad hubiera caído mucho más profundo. A Delgui, por ser es un grande. A mis amiccis, por hacerme el trabajo sucio. A Bri por ponerme una vela. A Andrea, porque la paz que me das me ha ayudado a cerrar esta etapa. Y por último, a mis tutores, Lydia y Jose,,por la oportunidad, la confianza, la responsabilidad y independencia, pero sobre todo, la paciencia que han tenido estos últimos dos años.

List of contents

Abstract

Marble Burying Test (MB) is a commonly used in neuroscience to assess burying behavior in rodents. Initially, this test was pharmacologically validated for its use to measure anxiety-related behaviors and screening for anxiolytic pharmacological. However, controversy existed regarding its specificity as it was also proposed to model better repetitive and/or perseverative behaviors manifested in obsessive-compulsive disorder (OCD) symptoms. Currently, is also proposed to model repetitive behavior exhibited in autism spectrum disorders (ASD).

But the debate is no longer whether marble burying mimics anxiety-like or compulsive-like disorders. Nowadays, the major concern is whether MB is a reliable test to model any behavioral and/or neuropsychiatric symptom (BNPS). Their major are methodological inconsistencies, a wide variety of BNPS modeled based solely on a single variable and a lack of robust hypothesis to justify the attribution of such constructs, contradictory drug effects, and an absence of well-defined and probed neurocircuitry explaining burying behavior. Hence, MB's reliability in modeling any BNPS is severely questioned. But not the test itself but how it is applied, executed, and reported.

In this scenario, in this thesis we provide a proposal to overcome this issue based in the implementation of methodological changes, new variables, and multicriteria hypothesis. Our objective is to experimentally validate this proposal to improve MB BNPS screening capabilities and providing then a practical demonstration of the application of such modifications to facilitate and encourage their use.

This thesis is divided into two independent experimental phases that address the application of MB as a BNPS screening in two different scenarios: 1) a mutant mice model with unknown behavioral phenotype which high possibilities of exhibiting an anxious phenotype, the PDK1 K465E KI mice, that was described here for the first time; and 2) a transgenic mice model for Alzheimer Disease with anxious phenotype and also increased burying, the 3xTg-AD mice. Results obtained in each phase answered specific questions of the respective animal models employed.

Subsequently, the collected evidence in the two phases was integrated to address the general objectives of this thesis, providing the following conclusions: 1) Genotype is the most influential factor for the appearance of burying phenotypic differences, appearing

sex and age/ageing effects in specific circumstances or in interactions.; 2) MB burying behavior features make it incompatible to model an anxiety-like behavior, even considering differentiated coping-strategies; 3) MB burying behavior features make it a normal repetitive behavior by itself, fitting better to model psychiatrics repetitive-like behaviors; 4) In non-related OCD/ASD animal models, MB burying could be accurate to model impulsivity and apathy; 5) Mice that perform a certain of burying activity are likely also to present a similar burrowing activity; the initiation of both behaviors is intentional, exclusive, and correlated; and both are the manifestation of closely related goal-directed diggings; 6) Employing multicriteria hypothesis in the MB, including core features of the BNPS to model, facilitates the interpretation of the results and provides robustness to it BNPS screening capabilities; 7) Transforming the MB in a multivariable test, through methodological changes and new variables, provides meaningful burying behavior insights to refute or accept multicriteria hypotheses; 8) The combined application of the two-zone analysis of digging activity variables and the time-course of marbles buried along the tests provide the most profitable burying information to reject or accept multicriteria hypotheses; 9) MB repeated trial should be employed depending on which BNPS needs to be modeled.

Abbreviations

3xTg-AD: Triple Transgenic mice of Alzheimer's Disease
AD mice: 3xTg-AD mice
AD: Alzheimer's Disease
ADL: Activities of Daily Living
AKT: Protein kinase B (PKB)
AKT2 KO mice: AKT isoform 2 knock-out mice
AKT3 KO mice: AKT isoform 3 knock-out mice
ASD: Autism Spectrum Disorders
Aβ: amyloid-β
bADL: Basic ADL
BB: Brief Burrowing Test
BDNF: Brain-derived neurotrophic factor
BNPS: Behavioral and neuropsychiatric symptoms
BPSD : Behavioral and psychological/neuropsychiatric symptoms of dementia
BDNF: brain-derived neurotrophic factor
cADL: Complex ADL
CT: Corner Test
DB: Deacon´s Burrowing Test
ECM: Extended classification of marbles
ERK: Extracellular signal-regulated kinase 2
MAPK: Mitogen-activated protein kinase 1
GSK3: Glycogen synthase kinase-3
H-motif: Hydrophobic-motif
iADL: Instrumental ADL
MB: Marble Burying Test
mTOR: Mammalian target of rapamycin
NTg: Non-transgenic
OCD: Obsessive-compulsive disorders
OF: Open Field Test
PDK1 Hm mice: PDK1 hypomorphic mice
PDK1-/- : Homocigous PDK1 K465E knock-in mice
PDK1+/- : Heterozygous PDK1 K465E knock-in mice
PDK1+/+: PDK1 K465E wild-type mice
PH-domain: Pleckstrin homology domain
PI3K: Phosphatidylinositol 3-kinase
PIP2: Phosphatidylinositol-4, 5-
PIP3: Phosphatidylinositol-3, 4, 5-triphosphate
PIF-pocket: Hydrophobic motif biding pocket
PKB: Protein kinase B
PKC: Protein kinase C
RSK: Ribosomal protein S6 Kinases
RT: Repeated trial
SGK: Serum and glucocortioid-induced kinases
T-loop: Activation loop
TZA: Two-zone analysis
TZC: Two-zone configuration
WT: Wild-Type

Organization and List of publications

The content of this thesis was developed in two phases, four publications:

Phase I. Studies in the PDK1 K465E Knock-In Mice

Study 1:

Giménez-Llort, L., Santana-Santana, M., & Bayascas, J. R. (2020). The impact of the PI3K/Akt signaling pathway in anxiety and working memory in young and middle-aged PDK1 K465E knock-in mice. *Frontiers in Behavioral Neuroscience*, *14*, 61. https://doi.org/10.3389/fnbeh.2020.00061

This article belongs to the Research Topic: The modification of working memory function.

Study 2:

Santana-Santana, M., Bayascas, J. R., & Giménez-Llort, L. (2021). Fine-Tuning the PI3K/Akt Signaling Pathway Intensity by Sex and Genotype-Load: Sex-Dependent Homozygotic Threshold for Somatic Growth but Feminization of Anxious Phenotype in Middle-Aged PDK1 K465E Knock-In and Heterozygous Mice. *Biomedicines*, *9*(7), 747. https://doi.org/10.3390/biomedicines9070747

This article belongs to the Special Issue Crosstalk between Depression, Anxiety, Dementia, and Chronic Pain: Comorbidity in Behavioral Neurology and Neuropsychiatry 2.0.

Phase II. Studies in the 3xTg-AD Mice

Study 3:

Santana-Santana, M., Bayascas, J. R., & Giménez-Llort, L. (2021). Sex-Dependent Signatures, Time Frames and Longitudinal Fine-Tuning of the Marble Burying Test in Normal and AD-Pathological Aging Mice. *Biomedicines*, *9*(8), 994. https://doi.org/10.3390/biomedicines9080994

This article belongs to the Topic: Emerging Translational Research in Neurological and Psychiatric Diseases: from In Vitro to In Vivo Models.

Study 4:

Santana-Santana, M., Bayascas, J. R., & Giménez-Llort, L. (2022). Burying and Burrowing Behavior in Male and Female Normal and 3xTg-AD Mice: A New

Comprehensive Study Based on the Two-Zone Configuration. *Biomedicines*: This original research article is under review process (minor revisions) pending editorial decision.

This article may belong to the Topic: Emerging Translational Research in Neurological and Psychiatric Diseases: From In Vitro to In Vivo Models, from Animals to Humans, from Qualitative to Quantitative Methods 2.0

List of figures

Chapter 1. Introduction

1.1. The Marble Burying Test

According to the latest World Health Organization (WHO) data, 1 in every 8 people worldwide suffer from a mental disorder. A mental disorder is a condition characterized by cognitive and emotional disturbances, abnormal behaviors, impaired functioning, or any combination of these (APA, 2014). There are many different types of mental disorders, but all the disorders have one thing in common: they cause suffering, distress, or impairment in critical areas in a person's life and those around them.

In neuroscience, mental disorders are studied collectively from various disciplines in an attempt to discover new treatments. One of the many ways to expand our knowledge about them is by employing animal models. Nowadays, one method consists of using rodents and evaluating them in a behavioral test (Fernández-Teruel et al., 2001; Dixit et al., 2020). Usually, these mice are subjected to genetic, selective breeding, lesional, or pharmacological interventions, among others, to elicit in them some trait that mimics or models features of a specific mental disorder. In this context, both behavioral tests and mice models can be used to study the etiology, neurobiology, and cognitive and non-cognitive deficits behind the modeled disorder. Also, it can be employed to evaluate the therapeutical effect of pharmacological or non-pharmacological treatments in them (de Brower et al., 2019). A test or mice model must fulfill several requirements to be considered reliable. The more they meet, the more valid they will be (Fernández-Teruel et al., 2001). First, the mice's behaviors or characteristics must share similar features to the modeled disorder. This is called face validity. Both the behavior elicited in the test or the mice themselves also should possess construct validity, demonstrating that neurobiological and/or neurocognitive systems involved in the human expression of the disorder are manifested in them too. The last requisite is predictive validity; effective pharmacological or non-pharmacological interventions in the human condition should be effective in the model, whereas those without demonstrable efficacy should be inactive (Belzung & Lemoine, 2011; de Brower et al., 2019; Dixit et al., 2020).

1.1.1. The Marble Burying Test

Behavioral neuroscience uses several tests to assess possible behavioral- and neuropsychiatric-like symptoms (BNPS) in mice resembling symptoms presented in mental disorders. The Marble Burying test (MB) is one of them. Since its first appearance (Broekkamp et al., 1986), its experimental use has not ceased, being a widely used tool

in pharmacology due to its high sensitivity to various drugs. Currently, its use goes beyond that and is often incorporated into large test batteries and used to perform behavioral phenotyping of many animal models. It is an unconditioned test, easy to perform, and economically affordable. Usually, MB is applied in the following way. A number of small objects, commonly glass marbles, are placed in a cage with the sufficient substrate to allow their burial; usually, the same type of substrate as their home cage. Once the animal is in the cage, it is left to interact freely with them for a certain period. At the end of the test, the animal is retired, and the buried marbles are counted, following a criterion established by the investigator (usually, the most common is buried by 2/3). Initially, this test was pharmacologically validated for its use to measure anxiety-related behaviors and screening for anxiolytic pharmacological drugs (Broekkamp et al., 1986; Njung'e & Handley, 1991a). However, controversy existed regarding its specificity as it was also proposed to model better repetitive and/or perseverative behaviors manifested in obsessive-compulsive disorder (OCD) symptoms (e.g., Gyertyán, 1995)

1.1.2. Validity Concerns

Although the controversy over whether BNPS are modeled in the MB has existed since the 90s, many publications have addressed this topic in recent years. However, the debate is no longer whether marble burying mimics anxiety-like or compulsive-like disorders. The major concern is whether MB is a reliable test to model any BNPS (Thomas, 2009; Wolmarans et al.,2016, de Brower et al., 2019, Dixit et al., 2020).

Here, one must highlight the review by Brower et al. (2019), entitled: “A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward”. Their work, an important work of reference in this thesis, is a comprehensive and critical review that challenges both the anxious-like and OCD-like modeling of the MB. In general terms, the main criticisms of the MB can be summarized in the following aspects (Thomas et, 2009; Alonso et al., 2015; Torres-Lista et al., 2015; Çalişkan et al., 2017; de Brower et al., 2019; Dixit, 2020): 1) Methodological inconsistencies: there is no standard application consensus leading to incongruences in methodological aspects, as the number of marbles, cage size or bedding material, that can alter the results; 2) Wide variety of BNPS modeled based solely on a single variable, the number of buried marbles, and a lack of robust hypothesis to justify the attribution of such constructs; 3) indiscriminate effect of anxiolytics and anticonvulsive drugs, whereas in humans anxiolytics do not reduce OCD symptoms, and pharmacological contradictions; 4) absence of well-defined

and probed neurocircuitry explaining burying behavior, provoking a lack of construct validity independently of the BNPS modeled.

In this scenario, MB's reliability in modeling any BNPS is severely questioned. But not the test itself but how it is applied, executed, and reported (de Brouwer et al., 2019). In consonance with this vision, we will provide a proposal to overcome this issue in this thesis. But first, we will address what burying behavior is from an ethological perspective, and then which are the strengths and flaws of the BNPS proposed to be modeled by this test.

1.1.3. Digging, Burying, and Burrowing

Digging, burying, and burrowing are normal in the behavioral repertoire of mice. In nature or our labs, mice dig using their forepaws to displace substrate (Tomas et al., 2009). With such simple behavior, they can perform many different things, such as digging in the ground to find food, hoarding food, creating a refuge from predators or cold, and making a safe nursery area for the young (Deacon, 2006). Digging can be explained as a primary action to perform a more complex task. Then, both burying and burrowing are goal-directed diggings. When mice dig to displace substrates to make a tunnel for habitation, it is called burrowing (Deacon, 2012). Whereas burying would be digging to displace substrates to either cover or move something (de Brouwer et al., 2009). In nature, mice bury both harmful (e.g., predators) and non-harmful things (e.g., food).

At an experimental level, burying could be divided into four categories: defensive burying, neophobic burying, inherent burying, and induced burying. Defensive burying is the act of burying anxious, threatened, and harmful things, such as predators, bad testing or smelling food, or electrified pods (Homma & Yamada, 2009). Neophobic-burying can be defined as the act of burying novel but harmless and non-reactive objects (Torres-Lista et al., 2015). This is the type of burying that was thought to measure in the MB, but as we will see later, this is not the case. What seems genuinely expressed in the MB is the inherent burying of the mouse (Thomas et al., 2009). It could be defined as their behavioral burying pattern or phenotype triggered by an investigative drive (Londei et al., 1998). Inherent burying is highly dependent on strain (Deacon, 2006). This is probably why MB is so widely used in genetic mice models since inherent burying behavior is strongly influenced by the mice's genetics or strain (de Brouwer et al., 2019). Regarding the factor of sex, studies are scarce and report no differences (e.g., Taylor, 2017).

Additionally, the estrous cycle can influence burying behavior (e.g., Schneider & Popik, 2007). However, only 17% of the studies employ the two sexes, while only 8.33% are conducted in females. Regarding age, no studies address this question specifically, and cohort studies directed at other interests must be used (e.g., Deacon et al., 2008). It is inferred that mice younger than one year of age will show a higher burying than those of older ages (Deacon, 2006). Undoubtedly, the effect of sex and age needs further study, especially when there may be an interaction due to genetic modifications. Lastly, induced burying results from a relatively new approach, and its concept is not well defined. Briefly, after a non-pharmacological stressful intervention, mice burying is altered (Barnum et al.,2012; Kedia, & Chattarji, 2014; Yohn, & Blendy, 2017), and, conversely, it can be modulated by non-pharmacological anxiolytic interventions (Torres-Lista et al., 2015). Therefore, it could be defined as the modification of inherent mice burying due to an induced anxiety state. Although these protocols are scarce and recent, they appear to be a more translational way to test possible treatments for anxiety (de Brouwer, 2019).

1.1.4. Marble Burying Test as a Model of Anxiety-like Behavior

The use of burying behavior as a model of anxiety dates back to the 1980s before the MB existed. This was born as a "child" of conditioned tests to measure defensive burying as a model of anxious responding. Historical work by Pinel & Treit (1981) demonstrated that rats, when threatened by electrified pods, perform vigorous burying towards them. Furthermore, they showed that the administration of anxiolytics reduced this behavior. Nowadays, this model still has a good face and construct validity. In 1986, Broedkamp first presented the MB and demonstrated that anxiolytic drugs such as meprobamate, clonazepam, and flunitrazepam reduced burying without changes in locomotor and self-grooming responses. From that moment on, the MB gained predictive validity, and its use became popular as a pharmacological screening test.

The use of burying behavior as a model of anxiety dates back to the 1980s before the MB existed. This was born as a "child" of conditioned tests to measure defensive burying as a model of anxious responding. Historical work by Pinel & Treit (1981) demonstrated that rats, when threatened by electrified pods, perform vigorous burying towards them. Furthermore, they showed that the administration of anxiolytics reduced this behavior. Nowadays, this model still has a good face and construct validity. In 1986, Broedkamp first presented the MB and demonstrated that anxiolytic drugs such as meprobamate, clonazepam, and flunitrazepam reduced burying without changes in locomotor and self-

grooming responses. From that moment on, the MB gained predictive validity, and its use became popular as a pharmacological screening test.

The American Psychological Association (APA, 2014) defines anxiety as an emotion featured by feelings of tension, worried thoughts, and physical changes. People with anxiety disorders usually have recurring intrusive thoughts or concerns, avoid certain situations and/or experience physical symptoms such as sweating, trembling, dizziness, or a rapid heartbeat. Under normal conditions, anxiety is a normal body response that helps us prepare to cope with threats (Salaberría et al., 1995). Briefly, to model this type of behavior in rodents, we look for changes in their behavior when faced with certain aversive situations/stimuli (e.g., electrified pods in a defensive burying test) or when exposed to novel and unfamiliar situations/stimuli (e.g., an open an unlimited field like the Open Field test) (Hall & Ballachey, 1932; La-Vu et al., 2020). The second one is the core concept of MB as a model of anxiety-like behavior. Marbles in this context are harmless but unknown objects, so the animal responds by performing burying towards them, as they do in nature. This unconditioned fear of unknown situations or stimuli is called neophobia. An important characteristic of anxiety responses is that mice could act with different coping- strategies in such situations. In the MB, mice could respond with both active burying or active avoidance against marbles as a sign of anxiety (Bruins Slot et al., 2008; Kinsey et al., 2011). Another important trait is that neophobic-anxiety responses tend to habituate. Habituation could be defined as a decremental change in the anxiety response after repeated exposures to the situation or stimuli (van der Goot et al., 2021). Therefore, for burying elicited in the MB to be considered a model of anxiety, mice must display an excessive active avoidance or active burying against marbles and habituation in their respective coping strategies among repeated exposures.

However, a large body of evidence clearly shows that an anxiety-like behavior of this type is not modeled in the MB. First, burying marbles is probably not driven by neophobia since rodents also show similar burying of familiar objects or non-reactive objects like food pellets (e.g., Gyertyán, 1995, Thomas et al., 2009). In fact, it has been shown that changes in burying behavior do not occur over repeated trials, even when mice can avoid them completely or there have been habituated to them (e.g., Njung'e & Handley, 1991b; Thomas et al., 2009; Taylor et al., 2017). Moreover, the number of buried marbles correlates poorly with anxiety tests such as the Dark-Light box or the Open Field test. (Thomas et al., 2009; Savy et al., 2015; Sanathara et al., 2018). Additionally, in recent years, the test's predictive validity has been reduced since marble-burying behavior can be reduced with non-anxiolytic drugs (Matsushita et al., 2005; Nicolas et al., 2006; Bruins

Slot et al., 2008; Torres-Lista et al.,2015). Finally, MB studies with two zones (Torres-Lista et al., 2015), where the animal can completely avoid the interaction of marbles, did not show mice avoidance of marbles, even when a divider separates the two zones (e.g., Thomas et al., 2009, de Brouwer & Wolmarans, 2018). This collides with the notion that marble burying is induced by novelty/anxiety and suggests that burying is motivated by the need to investigate novel surroundings (de Brower et al., 2019). Altogether, the behavioral pattern manifested in MB hardly mimics a neophobic anxiety-like behavior.

1.1.5. Marble Burying Test as a Model of Compulsive-like Behavior

Historically, the notion that marble buying mimics OCD was born in the 90s (e.g., Njung'e & Handley, 1991a; Gertyán, 1995). Obsessive-compulsive disorder is characterized by the presence of obsessions and compulsions, although they can be manifested independently (APA, 2014). Obsessions are recurrent thoughts, urges, or impulses experienced as intrusive and unwanted, causing anxiety or distress. Compulsions are repetitive behaviors or mental acts that the person applies as a ritual to prevent o reduce anxiety or distress. For obvious methodological limitations, only compulsions can be modeled in rodents models.

Compulsions are modeled through repetitive behaviors, mostly stereotypies. In terms of face validity, marble burying resembles compulsion features such as repetitiveness, persistence, and resistance to habituation. However, OCD modeling by burying behavior also has important validity problems. First, those studies placing marbles spread all over the cage (one zone) cannot demonstrate that marble buying was goal-directed. Hence animals are more likely to bury them due to investigative or explorative behavior rather than compulsive-like behavior (de Brower, 2019). Since compulsions are considered a non-normal repetitive behavior, to probe that burying is a compulsion, it should appear even when the rodent can avoid them. In addition, doubts about predictive validity are increasing. The main reasons are the indiscriminate reduction of burying by anxiolytics and anticompulsant drugs or the absence of increased burying with proconvulsant drugs (de Brower, 2019; Dixit et al., 2020). Moreover, independence between burying and stereotyped behaviors as pharmacological responses have been reported. In deer-mouse with high stereotypy, burying behavior was not responsive to citalopram, which is used to trait both anxiety and OCD, but reduced their stereotypy (Wolmarans et al., 2016). Finally, the neurocircuitry implied in burying has not been described yet. The closest that exists are hypotheses relying on the similarity of burying and other repetitive behaviors with well-documented neurocircuitry (Dixit et al., 2020). In summary, although

the burying behavior resembles better a repetitive behavior, it does not guarantee that compulsion is modeled.

1.1.6. Marble Burying Test as a Model of Autism-Like Disorder

Additionally, MB has also been used to model ASD repetitive- and/or perseverative-like behavior (Gal & Yirmiya, 2021). ASD is a heterogeneous group of disorders characterized by deficits in social communication and social interaction and the presence of restricted and repetitive behavioral patterns, interests, or activities. Their repetitive behavior is featured as stereotyped, insistent and inflexible, highly restricted, and hyper- or hyporeactive to external stimuli (APA, 2014).

Multiple mice models, including transgenic and non-transgenic mice, have shown enhanced burying behavior (Angoa-Pérez et al., 2013; Bey & Jiang, 2014; Kim, H., Lim & Kaang, 2016; Chang et al., 2017). However, it is necessary to mention that some mice models of ASD manifest decreased burying albeit expressing ASD-like phenotypes (Lim & Kaang, 2016). For example, Shank1 KO mice manifested decreased burying, whereas their self-grooming, a repetitive or stereotyped behavior, was enhanced (Sungur et al.,2014). Thus, there may be a disparity between the models used. Pharmacologically, a treatment with mGluR5-antagonist, a potential therapy for ASD, reduced burying behavior among other ASD-like phenotypes in a non-transgenic model of autism and a transgenic mouse to model Fragile X Syndrome, highly comorbid with ASD (Mehta et al.; 2011; Gandhi et al., 2014). Overall, ASD modeling by MB seems reasonable in terms of face and predictive validity. However, the major problem is that MB is employed using a one-zone configuration. Therefore, mice burying activity toward the marbles cannot be characterized as goal-directed. This is detrimental when attributing perseverance, insistence, inflexibility, or highly restrictive features to their burying behavior.

1.1.7. Our Perspective

Before explaining our approach, we will quote a few ideas from Cerejeira & cols. (2012) that we believe reflects the essence of this thesis:

> *"The first step to better understanding the psychiatric manifestations of dementia is to appropriately recognize and describe the psychopathology and accurately distinguish between similar symptoms (e.g., depression vs. apathy). This can be*

challenging considering the overlap between symptoms and the lack of proper definitions and consensus criteria for their diagnosis.

The assessment of neuropsychiatric symptoms requires a thorough examination to collect specific and detailed information about the clinical history, patient's subjective experiences, and objective behavior. Information from a reliable family member or caregiver is essential to obtain adequate characterization of neuropsychiatric disturbances from the patient's own ecological context as many abnormal symptoms cannot be elicited during the clinical interview".

In behavioral neuroscience, as unfortunately happens with dementia patients, we cannot communicate with our mice. We must observe their behavior carefully, patiently, deeply, and objectively as if they were our patients. We must use all possible sources of information, such as different tests or several behavioral variables. Observe how the mice behave in their natural context and how a possible behavioral- or neuropsychiatric-like symptom impacts their daily lives because this is the only way to truly understand how a mental disorder affects a person's life and, thereby, the source of their suffering.

In this thesis, we will objectively observe the MB test just as it is, a test to measure the inherent burying behavior of mice. That allows characterizing a behavioral phenotype highly sensitive to their genetic background and always considering relevant factors such as sex and age. Through methodological changes and variables in the MB, we will try to extract as much information as possible about their behavior. We will analyze their burrowing phenotype as alternative and complementary information sources. Subsequently, we will use all the information to meticulously contrast if our mice fulfill the necessary requirements to model a concrete BNPS. In this way, we will improve the BNPS screening capability of MB, and providing then a practical demonstration of how to employ the MB to study BNPS in mice models.

1.1.7. Methodological changes, New Variables, and Multicriteria Hypotheses

Briefly, the methodological changes and new variables will be described and justified. It is important to note that not all modifications have been used in all studies. The distribution is reflected in the methodology (see page 39).

The first methodological modification consists of placing the marbles only in one half of the cage, a procedure described as two-zone configuration (TZC) used by our (Torres-

Lista et al., 2015) and other laboratories (reviewed by de Brouwer & cols., 2019) as indispensable to be able to infer if burying behavior towards the marbles is goal-directed, both in an anxious or in a repetitive response. The next tool is the extended classification of marbles (ECM). Once the test is completed, the marbles will be classified into three categories: intact, changed of position, and buried (Torres-Lista et al., 2015). With this measure, we qualitatively assess the degree of interactions with the marbles. Also, a repeated trial (RT) of the MB was used. This condition allows us to observe whether or not there is habituation in the burying behavior. Time-course (TC) of the buried marbles was applied. This approach allows us to monitor the number of marbles buried throughout the test and to appreciate differences that might otherwise go unnoticed. In addition, justifying interpretations only using the last score of the MB could lead to incorrect conclusions. The last one is the two-zone analysis (TZA). It is based on using the TZC distribution to record variables relevant to burying behavior concerning the zone in which it is performed. The variables recorded are the latencies of appearance of digging and the number of digging in each zone. These variables were chosen because they are direct measures of digging activity and, by then, burying. The intention is to be able to perform a deeper analysis of the behavioral pattern and obtain correlations between the different variables of the test (intra-test) and with other tests (inter-test).

Once we collect all the information, we will use the following multicriteria hypotheses, based on core concepts of the construct to moderate, to interpret the results. To prove that buying reflects an anxiety-like behavior, will be necessary that: 1) mice exhibit active avoidance against marbles or active burying towards them; 2) their response habituates in a MB repeated trial; 3) MB variables present meaningful and consistent correlations with variables from other tests to assess anxiety-like behavior. As mentioned in previous sections, points 1 and 2 are core elements in anxiety-like behavior. In point 3, correlations with other tests are used to check if the burying pattern is related to the expression of anxiety in another context. Consequently, an excessive anxiety trait could be translated to other anxious scenarios, providing more robustness to the resulting conclusions (do-Rego et al., 2006). To prove that burying reflects a repetitive-like behavior, will be necessary that: 1) mice exhibit active interaction with marbles, even with the possibility of avoiding it; 2) burying behavior remains stable in a MB repeated trial; 3) Mice manifest increased burying behavior, acquiring an “excessive” nuance. Both points 1 and 2 are core symptoms of repetitive-like behaviors, albeit non-pathological. Point 3 dismisses between non-pathological and pathological.

This thesis is divided into two independent phases that address the application of MB as a BNPS screening in two different scenarios: 1) studies in the PDK1 K465E KI mice, a mutant mouse with unknown behavioral phenotype which high possibilities of exhibiting an anxious phenotype; 2) studies in the 3xTg-AD mice, a transgenic mouse model with anxious phenotype and also increased burying. Results obtained in each phase will solve specific questions of their respective animal models. Subsequently, the collected evidence will be integrated to address the general objectives of this thesis.

1.2. The PDK1 K465E PH-Domain Knock-In Mice

1.2.1. The PI3K Pathway

The phosphatidylinositol 3-kinase (PI3K) is an enzyme that acts near the membranes in the cell to regulate a wide range of signaling pathways as well as membrane trafficking and metabolic processes (Jeans & Kiger, 2014). These signaling pathways respond to insulin, growth factors, and numerous other agonists and play fundamental roles in regulating virtually every physiological process related to cell growth, proliferation, survival, and metabolism (Mora et al., 2004; Bayascas 2010). After PI3K activation, phosphatidylinositol-4,5-bisphosphate (PIP2) its directly phosphorylated by PI3K to generate the phosphatidylinositol-3,4,5-trisphosphate (PIP3) second messenger. Then, PIP3 located at the inner layer of the cell membrane promotes the recruitment and activation of the protein kinase B (PKB), a member of the AGC family of protein kinases, also known as AKT.

For decades, AKT was considered the major and almost the only effector of PI3K signaling response until the 3-phosphoinositide-dependent protein kinase-1 (PDK1) was discovered (Alessi et al., 1997). This study proved that PDK1 mediates the activation of AKT in a PIP3-dependent manner and could, therefore, trigger many of the actions of the PI3K pathway. Decades of studies not only confirmed this finding but also established PDK1 as a major transducer of the PI3K pathway.

1.2.2. PDK1, the Major Transducer

Currently, it is well known that PDK1 acts by reading out the levels of PIP3 and regulating the activation of as many as 23 AGC kinase family-members besides Akt, including, among others S6K, RSK, SGK, and PKC isoforms (Mora et al., 2004). The essentiality of this enzyme in the PI3K pathway has been widely demonstrated by the severe phenotypes reported in different PDK1-deficient mice models and the early lethality of PDK1 knock-out models (reviewed in Bayascas, 2010).

PDK1 is ubiquitously expressed in the cell, and its intrinsic catalytic activity is not directly altered by agonist stimulation, which converts the different PDK1 targets into forms that can be recognized, phosphorylated, and activated by PDK1 (Bayascas, 2010). The AGC kinases are activated trough the phosphorylation of two residues that are each located

in two highly conserved motifs: the activation loop (T-loop), present in the catalytic domain of the majority of protein kinases, and the hydrophobic-motif (H-motif), a structural signature of most AGC kinases that is positioned C-terminal to the kinase domain (Bayascas, 2010). Regulation of the H-motif phosphorylation is quite distinct among the different PDK1 targets since some of these AGC kinases are phosphorylated by mTOR complex 1, namely S6K and classical PKC isoforms. In contrast, mTOR complex 2 phosphorylates AKT, SGK, and conventional PKCs and ERK/MAPK phosphorylate RSK isoforms. When the H-motif of these AGC kinases is phosphorylated, it serves as a docking site for the biding of PDK1, which owns a hydrophobic motif biding pocket (PIF-pocket) located in its catalytic domain, enabling in this manner the phosphorylation of the T-loop and the activation of the downstream enzyme (reviewed in Bayascas, 2010). This is the common activation mechanism of these AGC kinases, except for AKT isoforms. In this case, both PDK1 and AKT possess PIP3-binding pleckstrin homology domains (PH-domains). The activation of PKB/AKT is independent of the phosphorylation in its H-motif since, upon agonist stimulation, the generated PIP3 recruit AKT and PDK1 to the plasma membrane via the interactions of their PH-domains with the second messenger, and then PDK1 can gain access to phosphorylate AKT (adapted from Bayascas, 2010).

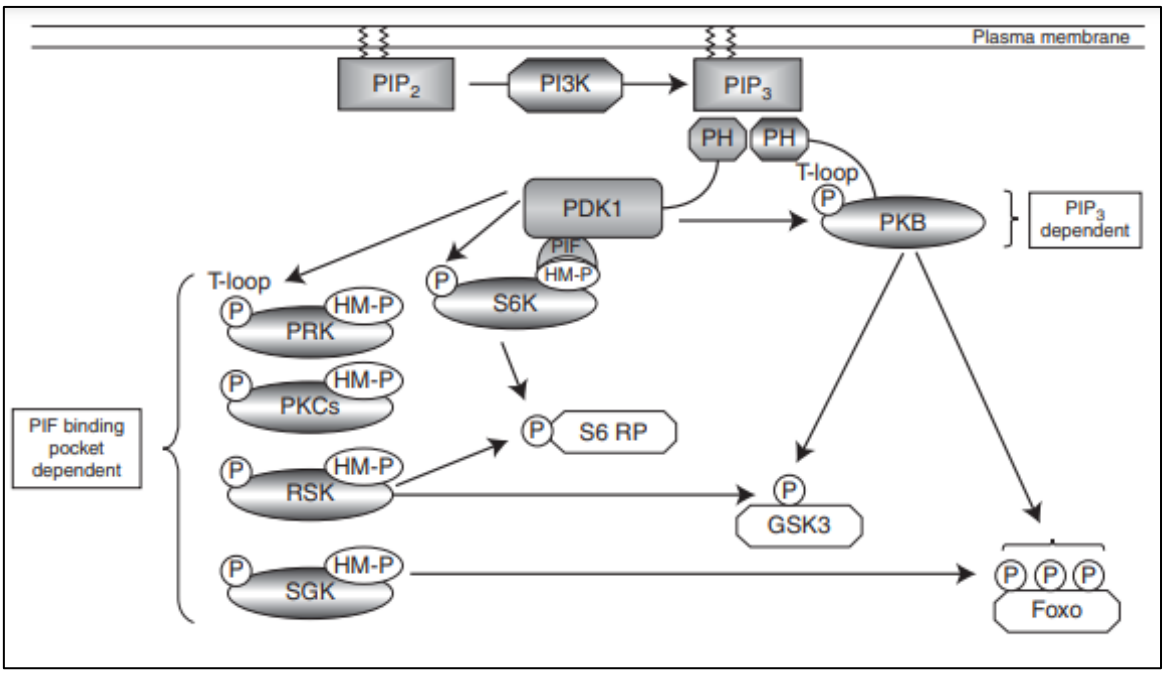

Figure 1. Differentiated PDK1 signaling mechanisms involved in the phosphorylation of AGC kinases (Finlay & Cantrell; 2011)

1.2.3. A Pathway to Psychiatric Diseases

Although the PI3K/PDK1/AKT pathway functional roles have been mainly and largely explored in cellular biochemistry, physiology, as well as cancer and metabolism areas (Bayascas, 2010), the literature have proven that it also is extensively involved in controlling neuronal development and function (Waite & Eickholt, 2010). As a result, in recent years further evidence have been generated about the role of PI3K in psychiatric diseases such as bipolar disorder, depression, anxiety, and schizophrenia, and thereby positioning this pathway as a promising therapeutic target for such diseases (e.g., Jope & Roh, 2006; Freyberg et al., 2010; Beurel et al., 2015).

However, most works have focused on the study of GSK3, a kinase which is regulated downstream of AKT and RSK, among others. GSK3 is dysregulated in patients with depression, bipolar disorder and schizophrenia; relevant drugs like lithium inhibit GSK3, and behavioral phenotypes in gain-of-function GSK3 transgenic rodent models mimics such psychiatric diseases. In addition, brain-derived neurotrophic factor (BDNF), which acts as an agonist in the PI3K signaling pathway, plays an important role in cellular responses linked to conditionate fear, anxiety and depression (Ou & Gean, 2006; Martinowich et al., 2007). Moreover, BDNF stimulation is necessary for the fast antidepressant effect of various drugs (e.g., Shi et al, 2012; Tao et al., 2016). Since PDK1 and AKT transduce the BDNF signal to GSK3, it is probable that these two kinases were also involved in BDNF responses. This is empirically supported by many of the studies previously cited, that found dysregulated AKT activity in psychiatric pathology, but also by the relationship between AKT dysregulation and the expression psychiatric-like phenotypes. As an example, mice selected by the high anxiety-related behavior in an anxiety test presented stronger acquisition, slower extinction, and more spontaneous recovery of learned fear in conjunction with enhanced phosphorylation of AKT in the amygdala. Also, in recent years, some studies have also proven that PI3K signaling pathway mediates in the therapeutic effect of non-pharmacological interventions. Treadmill exercise attenuated the cognitive deficit and depressive/anxiety-like behaviors induced by a stressor through the recovery of hippocampal AKT activity and ameliorated the contextual fear conditioning and anxiety-like behavior in a post-traumatic demoralization syndrome model due to upregulation of BDNF and the related PI3K/AKT pathway, among other adaptations (Sun et al., 2020). In addition, mice treated with an early life enrichment environment after postnatal maternal separation showed

ameliorated depressive and anxiety-like behavior through enhanced phosphorylated AKT in the hippocampus (Huang et al., 2021).

Taken the relevance of the PI3K signaling pathway in the manifestation and treatment of psychiatric disorders, the specific role of PDK1 and AKT in its topic must be addressed. Hence mutant mice with specific alteration of PDK1 and AKT are necessary experimental tools to depict the nuances of their biological and behavioral roles in mental illnesses.

1.2.4. Specific PDK1 and AKT Mice Models

Conditional knockout and knock-in strategies are many times required to avoid prenatal mortality and generate viable mutant mice (Bayascas, 2010), that allows to explore the functional role of PDK1 (and AKT) in the expression of psychiatric disorders. Over the years, various models have been generated with increasingly selective PDK1 or AKT modifications.

The first mutant model behaviorally phenotyped was the PDK1 hypomorphic mice (PDK1 Hm), which express up to 10-25% highly reduced PDK1 protein levels when compared to the controls (Ackermann et al., 2008). Their behavioral phenotyping revealed increased anxiety-like behavior in various task in conjunction with reduced serotonin, GABA and taurine and more noradrenaline in the amygdala. Similar changes were manifested in the olfactory bulb. Later, the AKT isoform 2 knock-out mice (AKT2 KO) manifested a higher anxiety-like and depressive-like behaviors (Leibrock et al., 2013). Soon after, AKT isoform 3 knock-out mice (AKT3 KO) showed a schizophrenic, depressive and anxiety-like phenotypes (Bergeron et al., 2017). Due to this genetic alteration, inhibition of GSK3 by AKT-mediated phosphorylation was reduced; re-inhibiting GSK3 with lithium rescued the depressive and anxiety-like behaviors but not the schizophrenic phenotype. Likewise, another study revealed cognitive dysfunction and schizophrenic like phenotype, but no manifested enhanced anxiety-like behavior, in the AKT3 KO mice (Howell et al., 2017). Recently, Wong et al. tested the three AKT knock-out mice models, proving that their anxiety-like phenotype depended on which AKT isoform was altered (Wong et al 2020). Lastly, tissue–specific PDK1 L155E (PDK1fl / fl CRE+) conditional knock-in mutant mice expressing a mutant form of PDK1 with a disruption in the PIF-pocket were generated. This mutation abolishes the activation of several AGC kinases by PDK1, but left AKT activation intact (Biondi et al., 2002; Collins et al., 2003; Bayascas et al., 2006). In the brain-specific PDK1 L155E mice, ablating the activation of most PDK1-regulated AGC kinases with the exception of AKT resulted in

sensorimotor problems, exacerbated disruptive behavior and cognitive deficits (Cordón-Barris et al., 2016). These mice model is especially relevant, since it highlights the importance of AKT-independent actions in the PDK1 signaling for brain development and function.

Despite differences in the behavioral outputs, it is clear that specific modifications of both PDK1 and AKT have consequences in the PI3K signaling pathway, causing diverse but consistent expression of anxiety-, depression-, and schizophrenic-like behaviors.

1.2.5. The PDK1 K465E PH-Domain Knock-In Mice

In this thesis, we work with the PDK1 K465E PH-domain knock-in mice (Bayascas et al., 2008), whose behavioral phenotype was at the time uncharacterized. This mutation in the PH-domain was meant to affect exclusively the phosphorylation and activation of AKT isoforms, but leave intact the activation of the other AGC-kinase family members regulated by PDK1. However, mice still showed low levels of AKT activation, whereas the activation of some substrates shared by PDK1 and AKT, namely S6K, was moderately reduced. These mice exhibited a smaller body size, insulin resistance but no differences in the total number of neurons in the brain. By contrast, mild deficient neurogenesis, abnormal cell polarization and reduced axonal outgrowth was present (Zurashvili et al 2013). The selective alteration of such cellular processes hints the possibility that AKT activity regulates them in a threshold-manner, leading then to diverse physiological responses depending on the level of AKT activation (Zhou et al., 2014). Recently, our group reported that the deficits in the AKT activation are pronounced both in the cortex and the hippocampus during young adulthood (3–4 months) but tend to be attenuated at middle age (11–14 months) in these mutant mice (Yang et al., 2018).

Due to the specificity of the PDK1 PH-domain mutation, mice homozygous for the PDK1 K465E mutation (from now termed PDK1-/- for simplicity) represented a promising model to investigate the in vivo relationship between specific reduction of AKT phosphorylation and anxiety-like behavior. The lack of knowledge of these mice in conjunction with its high potential to display and anxious phenotype provided a valuable opportunity to illustrate how helpful the methodological refinement of the MB can be in similar scenarios. In order to assess if genotype-load could mediates in vivo the behavioral responses, PDK1 K465E heterozygous mice (PDK1+/-) were also employed in one of our studies, mimicking the distinctive AKT activity levels necessary for the activation of differentiated physiological responses.

1.3. The 3xTg-AD Mice Model for Alzheimer Disease

1.3.1. Alzheimer Disease

Alzheimer's disease (AD) is a neurodegenerative disorder that leads to a progressive decline in the brain functionally and morphologically (Knopman et al., 2021). It is one of the most important healthcare challenges of current times since it is the main cause of dementia, and there is not yet a cure for it (Philip et al., 2016; Morley et al., 2018). The causes of the disease are not yet completely understood, but several risk factors partly explain the disease's development.

Age is the major risk factor for developing AD (Konopman et al., 2021). In their work, Ritichie and Kildea (1995) showed that "for every 5-year increase in age, AD incidence rates triple before age 64, double before age 75, and drop down to an increase of 1.5 times around age 85". Therefore, AD is age-related, where the relationship to age is simply an expression of other biological risk factors and not age-dependent since the increase slows as age increases (Ritichie and Kildea, 1995; Sujuan et al.,1999, Niu et al., 2017). In recent years, the relevance of sex and gender as risk factors has been highlighted (Mielke,2014; Ferretti et al., 2018). The literature suggests that women are more likely to develop AD, experiencing worse pathology and faster cognitive decline in the later stages of the disease, whereas men are more affected in the early stages (Niu et al., 2017; Ferretti et al., 2018). These differences are partially explained by sex- and gender-specific risk factors (Mielke et al., 2014; Ferretti et al., 2018). In this scenario, the practice of sex-approach AD research is recommended since it is necessary to develop better and more efficient therapeutic strategies (Dennison et al., 20221).

Although genetics represent only a modest part of the risk factors for developing AD, its study has been crucial to further understanding the disease. The most studied gene mutations have been those that cause the dominantly inherited AD, which onset is approximately 40 years earlier than sporadic AD (Knopman et al., 2021). Most patients with dominantly inherit AD present mutations in APP (encoding amyloid precursor protein), PSEN1 (encoding presenilin 1), and PSEN2 (encoding presenilin 2) genes. (Thambisetty & Tanaka, 2013). Currently, more than 600 genes have been investigated as risk factors for AD (Knopman et al., 2021). Lastly, lifestyle factors such as low educational attainment, metabolic disorders, hearing loss, traumatic brain injury, alcohol abuse, smoking, depression, low physical activity, social isolation, and air pollution can

potentially increase the risk of AD development (Philip et al., 2016; Knopman et al., 2021).

Histopathologically, AD is distinguished by the presence of extracellular plaques of β-amyloid peptide (Aβ-plaques) and intraneuronal neurofibrillary tangles (NFTs), which are aggregates of hyperphosphorylated tau protein (Knopman et al., 2021). Aβ-plaques formation occurs in the early stages of the disease, slowly accumulating at neocortical association areas and medial temporal lobe structures. In more advanced stages, they appear in subcortical nuclei such as the thalamus and striatum (Arnold et al., 1991; Serrano-Pozo et al., 2011). In contrast, NFTs begin to appear at later stages, emerging first in the entorhinal cortex, hippocampus, and neocortical association areas. In the later stages, NFTs appear in primary cortical areas and subcortical structures such as the striatum and substantia nigra. (Arnold et al., 1991; Serrano-Pozo et al., 2011). The accumulation of Aβ-plaques and NFTs eventually leads to neuronal death in the brain areas leading to the clinical symptoms of the disease and aggravating it over time.
At the clinical level, undoubtedly, the most notable hallmark of AD is the cognitive deterioration that patients gradually experience. Memory loss is one of the most characteristic symptoms of the cognitive deterioration of the disease. From the early stages, patients begin to experience difficulties acquiring new memories and forget the most recent ones more quickly; as the disease progresses, they lose older and more remote memories (Albert, 1996; Arnaiz and Almkivist, 2003). Executive function impairment also occurs in the early stages of AD (Baudic et al., 2006; Webster-Cordero & Giménez-Llort, 2022). This cognitive loss is evidenced in tasks that require concurrent manipulation of information or the rapid and simultaneous integration of multiple types of information. Other cognitive impairments such as aphasia, apraxia and/or agnosia) and abstract reasoning ability also appear in later stages (Buaudic et al., 2006; Kirova et al., 2015).

1.3.2. Behavioral and Neuropsychiatric Symptoms in Alzheimer Disease

Another relevant AD clinical manifestation is the neuropsychiatric symptoms, also referred to as ‘Behavioral and Psychological Symptoms of Dementia’ (BPSD). BPSD are an offset of perceptual, behavioral, and emotional disturbances similar to those manifested in psychiatric disorders. Nowadays, commonly BPSD manifested in AD patients such as depression, apathy, aggression, agitation, psychosis, and sleep

disruption, are now recognized as core symptoms of the disease (Lyketsos et al., 2011; Li et al., 2014; Cloak & Khalili, 2019; Giménez-Llort & Johansson, 2020).

These non-cognitive problems affect 50–90% of people with AD decreasing their quality of life, causing an important source of distress to them and their caregivers, deteriorating the relationship between patient and caregiver, frequently leading to premature institutionalization, and increasing their risk of mortality (Hope, et al., 1998; deVugt et al., 2003; Shin et al., 2005, Ballard & Corbertt, 2010; Keszyck et al., 2019). Moreover, its noteworthy that, in many cases, these symptoms appear before cognitive impairment and their appearance in the early stages predicts a worse and more rapid cognitive decline (Cerejeira et al., 2012).

The appearance of BPSD is explained by the damage caused by the disease to different brain regions, so their appearance depends on the affected area and changes in neurotransmitter systems involved in AD (Lyketsos et al., 2011; Cerejeira et al., 2012). The complex and diverse manner in which BPSDs manifest themselves makes their treatment challenging for clinicians (Ballart & Corbett, 2010, Giménez-Llort and Johansson, 2022). Their expression has a high degree of variation and affects each individual distinctively (Cerejeira et al., 2012). Moreover, they are also differentially manifested between sexes (Ferretti et al., 2018). Likewise, the presence of up to 4 simultaneous symptoms occurs in 50% of patients (Frisoni et al., 1999). This complex scenario causes, in part, the absence of a clear and concise classification of BPSD. We found useful the one put forward by Cloak & Khalili (2019), which divides them into 5 categories: cognitive/perceptual (delusions, hallucinations), motor (e.g., pacing, wandering, repetitive movements, physical aggression), verbal (e.g., yelling, calling out, repetitive speech, verbal aggression), emotional (e.g., euphoria, depression, apathy, anxiety, irritability), and vegetative (disturbances in sleep and appetite. Another promising classification is the one proposed by van der Linde & cols. (2014), through a cluster analysis of 62 studies, obtained 5 categories: affective domain, apathy domain, psychosis domain, euphoria domain, and hyperactivity-impulsivity-irritibility-disinhibition-aggression-agitation (HIDA) domain.

Currently, pharmacological treatments for BPSD are not effective and some may pose a risk to patients (Liketsos et al., 2011, Giménez-Llort and Johansson, 2022), a fact that has been reproduced in animal models (Torres-Lista et al., 2019). The most recommended clinical practice is the application of non-pharmacological interventions

and the use of the least harmful medication for the shortest possible time (Cerejeira et al., 2012; Giménez-Llort & Johansson, 2022).

1.3.3. Activities of Daily Living

As cognitive and non-cognitive impairment progresses, the patients lose their ability to interact with others and their environment. Gradually, they begin to experience difficulties in activities that they used to do with ease, eventually becoming unable to fend for themselves in simple tasks such as eating or bathing. Currently, almost 80% of people suffering from AD live in their homes, being taken care of by their families (Opara, 2012). The loss of functional independence, so precious in later life, diminishes patients' self-esteem and impoverishes the quality of life of both patients and caregivers (Potkin, 2002; Cipriani, 2020). This is the cruelest effect that AD has on people's life.

The loss of functionality to perform activities of daily living (ADL) is a requirement to diagnose dementia (Slachevsky et al., 2019). These are classified into instrumental ADL (iADL) and basics ADL (bADL). iADL are those essential to maintain independent living and maintaining life in the community, such as managing finances, shopping, handling medications, or using public transport. Its deterioration usually manifests in mild cognitive impairment, a phase prior to dementia, and its presence is associated with worse prognostic (Marshal et al., 2012; Slachevsky et al., 2019). As the disease progresses, the iADLs deterioration continues until a certain point when bADL deterioration appears too. bADL consists of activities like eating, dressing, grooming, bathing, and toileting, and their deterioration appears in the moderate/severe stages of AD dementia (Mioshi et al., 2007). In recent years, complex ADL (cADL) has been proposed (Marshal et al., 2012, Slachevsky et al., 2019). These activities require a high cognitive, physical, and social skill level. Their deterioration goes unnoticed by those affected and their families, as they remain fully independent, but their presence could hint at a subtle cognitive impairment (Marshall, 2012). This category is composed of activities requiring complex interpersonal or social functioning, such as using smartphones, planning a holiday, practicing hobbies, working, etc. Anatomically, impaired bADL is related to frontal atrophy, iADL with widespread frontal, temporal and occipital atrophy, whereas aADL with occipital and temporal atrophy (Slachevsky et al., 2019).

The impairment of ADL, especially iADL, is related to the deterioration of executive functions (Norton et al., 2001; Cipriani et al., 2020). This cognitive function includes the

capability to goal-planning, initiating and executing actions, multitasking, switching between tasks, monitoring, and inhibiting habitual behaviors (Cipriani et al., 2020). However, impaired ADL is not only caused by cognitive impairment; BPSD play a critical role too. Both bADL and IADL impairment has been linked with symptoms such as apathy, depression, agitation, irritability, disinhibition, and anxiety (Norton et al., 2001; Ikeda et al., 2020). Although more research is needed, cADL could also be sensible to BPSD since it has been demonstrated that apathy is a predictor of their impairment (Delgado et al., 2019). Due to their critical role in AD, ADL measures emerge as valuable tools to predict and monitor disease progression, assess the functional impact of cognitive and BPSD, and evaluate the efficacy of pharmacological and non-pharmacological treatments (Green et al.,1993; Marsha et al., 2012; Ikeda et al., 2020).

In this scenario, natural species-typical behaviors can be excellent ethological scenarios to mimic the impairment of ADL in AD patients (Deacon, 2012; Torres-Lista & Gimenez-Llort, 2013; Jirkof, 2014; Si et al., 2022) and to assess their welfare and disease progression (Muntsant &Giménez-Llort, 2021; Giménez-Llort &and Torres-Lista, 2021). They could be important as preclinical tools for drug design, development, and assessment, but also to investigate pharmacological and non-pharmacological strategies before they can be effectively translated into clinical scenarios. Social interactions, nesting, burying, and borrowing are rodent-typical behaviors commonly affected in a cross-sectional manner among mice models of AD (e.g., Deacon et al., 2009; Torres-Lista & Gimenez-Llort, 2013; Kemppainen et al., 2015; Si et al., 2022). As products of the disease, changes in their typical behaviors mirror the ADL deterioration in humans. From a translational perspective, these behaviors also represent an important opportunity to model BPSD, since they are a core aspect of ADL impairment, and both patients' and caregivers' lives are greatly affected by them. Consequently, our research in 3xTg-AD mice is committed to such an approach.

1.3.4. The 3xTg-AD Mice Model

The triple transgenic mouse model for AD (3xTg-AD) possesses the human transgenes PS1/M146V, APPswe, and tauP301L (Oddo et al., 2003a). Amyloid-beta and tau development in brain regions such as the cortex, hippocampus, and amygdala progressively develop in their brain (Oddo et al. 2003b). The temporal progression of the disease and anatomical structures affected in this model reproduces a similar pattern observed in AD patients (Oddo et al. 2003a; Mesulam 2000).

The onset of symptoms is noted at 4-6 months, although at that age, they only present intraneuronal Aβ immunoreactivity (Oddo et al., 2003a). Even so, they exhibit deficits in electrophysiological in the hippocampus, learning and memory problems, cholinergic deficiencies, and emotional disturbances (Oddo et al., 2003a; Giménez-Llort et al., 2007). At 12 months of age, βA deposits and NFTs can be seen in their brain, displaying neuropathological parallelism with the advanced stages of AD in humans (Oddo et al., 2003a). Currently, several studies report cognitive, emotional, and motor deficits in the different stages of AD (e.g., Giménez-Llort et al., 2007, Sterniczuk et al., 2010; Belfiore et al., 2019; Castillo-Mariqueo et al., 2021). It is very important to report that, in recent years, a delay in the development of brain pathology and the appearance of sex differences have been warned and assessed (Belfiore et al., 2019; Javonillo et al., 2022). Results vary among the different colonies, but, as a general pattern, AD pathology begins to be more relevant around 12 months old or later. In addition, females tend to show a more marked pathology, while males sometimes fail to show Aβ-plaques or NFTs. Even so, it is still a valid model to study the disease as it continues yielding valuable findings (e.g., Chiquitita et al., 2019), but it's necessary to choose wisely the age and sex of the mice and be careful in comparing results between colonies. This delay can also be exploited as an opportunity to study the progression of AD in a more accurate and human-like manner (Javonillo et al., 2022).

Throughout the years, our lab has conducted in-depth research about ADL-like impairment in 3xTg-AD mice, taking into account sex factor. This is not only because of the relevance of sex in AD but also because we consider males and females as two natural biological scenarios where genotype-phenotype translations are divergent-convergent depending on the level of study and temporal frames of lifespan and/or disease progression (Giménez-Llort et al., 2012). Nesting behavior was impaired in 3xTg-AD mice at 6 and 12 months of age in individual and parental structures (Torres-Lista & Giménez-Llort, 2013). However, the impaired ability to build the nest was only shown with paper material because it is more difficult to manipulate than cotton making the task more demanding. When cotton was used, the variable of latency to start to build the nest was the only one that discriminated the performance per genotype, suggesting impairment of goal-directed behaviors influenced by their apathy- and anxiety-like profiles, as it worsened with them and the progress of the disease. In addition, nesting behavior was more impaired depending on the genotype and sex (3xTg-AD females worse than in males) and stage of disease (at advanced (12 months of age) than at the onset of cognitive symptoms (6 months of age)). Social nesting (Giménez-Llort & Torres-Lista, 2021), was also impaired in the 3xTg-AD mice, and the phenotype was attenuated

by pharmacological (Van der Jeugd et al., 2018) and non-pharmacological (early postnatal handling, Giménez-Llort & Torres-Lista, 2019) interventions. In the social interaction test (Torres-Lista & Giménez-Llort, 2019), 3xTg-AD mice presented a different pattern of social interaction from NTg mice, showing sex differences in their phenotype. These phenotypes were replicated in a second study, and also their profile was attenuated by risperidone (Torres-Lista et al., 2019), an antipsychotic often used to treat BPSD symptoms (Yunusa & Helou, 2020). Finally, burying behavior was enhanced in 12-month-old 3xTg-AD males and their phenotype was reduced with risperidone, handling, and caffeine (Torres-Lista et al., 2015; Baeta-Corral, et al., 2018; Torres-Lista et al., 2019) and increased by social isolation (Giménez-Llort & Alveal-Mellado, 2021).

Altogether, the 3xTg-AD mice model mimics relevant features present in ADL impairment, such as the presence or not of difficulties depending on task complexity, sex-dependent differences, deterioration through time, and response to drug or non-pharmacological interventions. However, we consider that there are still many unknowns. Regarding burying behavior, we do not know 1) how the phenotype of 3xTg-AD females is, 2) if burying further deteriorates at older ages, and 3) most importantly, we are not confident on which BPSD is modeled. About burrowing behavior, a relevant test to measure ADL in AD, the behavioral phenotype of 3xTg-AD mice is still unknown. Therefore, these questions will be addressed in the second part of this thesis using the 3xTg-AD mice.

Chapter 2. Hypothesis and Objectives

2.1. Hypothesis

This thesis is guided by the following hypotheses:

1. Burying behavior is sensitive to genotype, sex, age/ageing, and its interactions.

2. Standard MB application is insufficient to provide empirical justification for any proposed BNPS modeled by the test.

3. MB methodological changes and new variables can enhance the interpretability of the test outcome.

4. Burying behavioral patterns in the MB will be similarly reflected in burrowing behavior.

5. Considering the multicriteria hypothesis can improve the modeling of BNPS

6. If burying reflects an anxiety-like behavior, it is necessary that:
   - Mice exhibit active avoidance against marbles or active burying towards them.
   - Their response habituates in a MB repeated trial.
   - MB variables present meaningful and consistent correlations with variables from other tests to assess anxiety-like behavior.

7. If burying reflects a repetitive-like behavior, it is necessary that:
   - Mice exhibit active interaction with marbles, even with the possibility of avoiding it.
   - Mice manifest increased burying behavior, acquiring an “excessive” nuance.
   - Burying behavior remains stable in a MB repeated trial.

2.2. Objectives

The main objectives of this works are:

1. To validate methodological changes, new variables, and multicriteria hypothesis in the MB to improve its BNPS screening capabilities using the two described models.

2. To provide a practical demonstration of the application of such modifications to facilitate and encourage their use.

2.3. Specific Objectives

The thesis is divided into two differentiated phases, one for each animal model. Therefore, the specific objectives are accordingly presented.

2.3.1. Studies in PDK1 k465E Knock-In Mice

The specifics objectives in this phase are:

1. To provide a behavioral and functional phenotype of the PDK1-/- mice.

2. To assess the burying behavior in these mutant mice, evaluate if their behavior is altered by genotype, genotype-load, sex, age/ageing, and the interactions of these variables.

3. To implement methodological changes and new variables in the MB for a better understanding of the burying behavior:
   - To employ the two-zone configuration (TZC) to ensure that marble interaction is goal-directed and voluntary.
   - To use the extended classification of marbles (ECM) to assess the degree of interactions with them in a qualitative manner.

4. To examine the correlations between variables from other tests to assess anxiety and the MB outcome

5. To ponder if burying behavior could mimic some BNPS in the PDK1-/- mice.

2.3.1. Studies in the 3xTg-AD Mice

The specifics objectives of this phase are:

1. To replicate and confirm the increased burying behavior of 3xTg-AD male mice previously described by our research group, to establish the burying behavior pattern of 3xTg-AD female mice, and to assess if burying behavior is affected by AD-pathological aging.

2. To implement methodological changes and new variables in the MB for a better understanding of the burying behavior:
   - To employ the two-zone configuration (TZC) to ensure that marble interaction is goal-directed and voluntary.
   - To check, through the time-course of marbles buried (TC), if differences in the number of marbles buried are present along the test.
   - To verify if burying behavior is habituated through a repeated trial (RT).
   - To develop a more comprehensive profile of the displayed burying behavior by recording and analyzing behavioral variables related to each zone of the MB (TZA), the zone with marbles and the zone without marbles.

3. To examine, through behavioral correlations, the relationship between tests to model anxiety and the MB outcome.

4. To stablish the burrowing behavioral pattern of the 3xTg-AD mice and determine if it corresponds to the burying profile shown in the MB.

5. To employ the results to discuss the possible BPSD modelled by burying behavior in the 3xTg-AD mice.

Chapter 3. Methodology

3.1. Methodological Changes and New Variables Distribution

The methodological changes and new variables are distributed across studies in the following manner:

- **Study 1:** TZC and ECM
- **Study 2:** TZC and ECM
- **Study 3:** TZC, TC and RT.
- **Study 4:** TZC, TC and TZA

3.2. Studies in the PDK1 K465E Knock-In Mice

As indicated in the section on organization and list of publications, Phase I comprises Study 1 and Study 2. The methodology employed can be consulted in each published original research article.

3.3. Studies in the 3xTg-AD Mice

As indicated in the section on organization and list of publications, Phase II comprises Studies 3 and 4. The methodology employed in Study 3 can be consulted in the published original research article. Study 4, included as an annex, is in review and pending of academic editor's decision. The methodology employed will be addressed here.

3.3.1. Study 4.

3.3.1.1. Animals

A total number of sixty-four 12-month-old male and female mice, homozygous 3xTg-AD (males n=20, “AD males”; females n=16, “AD females”) and NTg (males n=18, “NTg males”; females n=10, “NTg females”) 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. The 3xTg-AD mice harboring transgenes were genetically engineered at the University of California Irvine, as previously described (Belfiore et al., 2019). Animals were maintained in groups of 3-4 mice per cage (Macrolon, 35 × 15 × 15 cm) filled with a 5 cm thick layer of clean woodchips that were the same used for behavioral testing (Eco-pure, Chips6, DateSand, UK; Uniform cross-cut wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 × 20 cm, White). All animals were maintained under

standard laboratory conditions of food and water ad libitum, 22 ± 2°C, 12 h light: dark cycle with lights on at 8:00 am, and relative humidity 50–60%.

3.3.1.2. Experimental Design

As illustrated in figure 1, animals were behaviorally assessed for four consecutive days in a counterbalanced manner using a factorial design genotype (G) x sex (S).

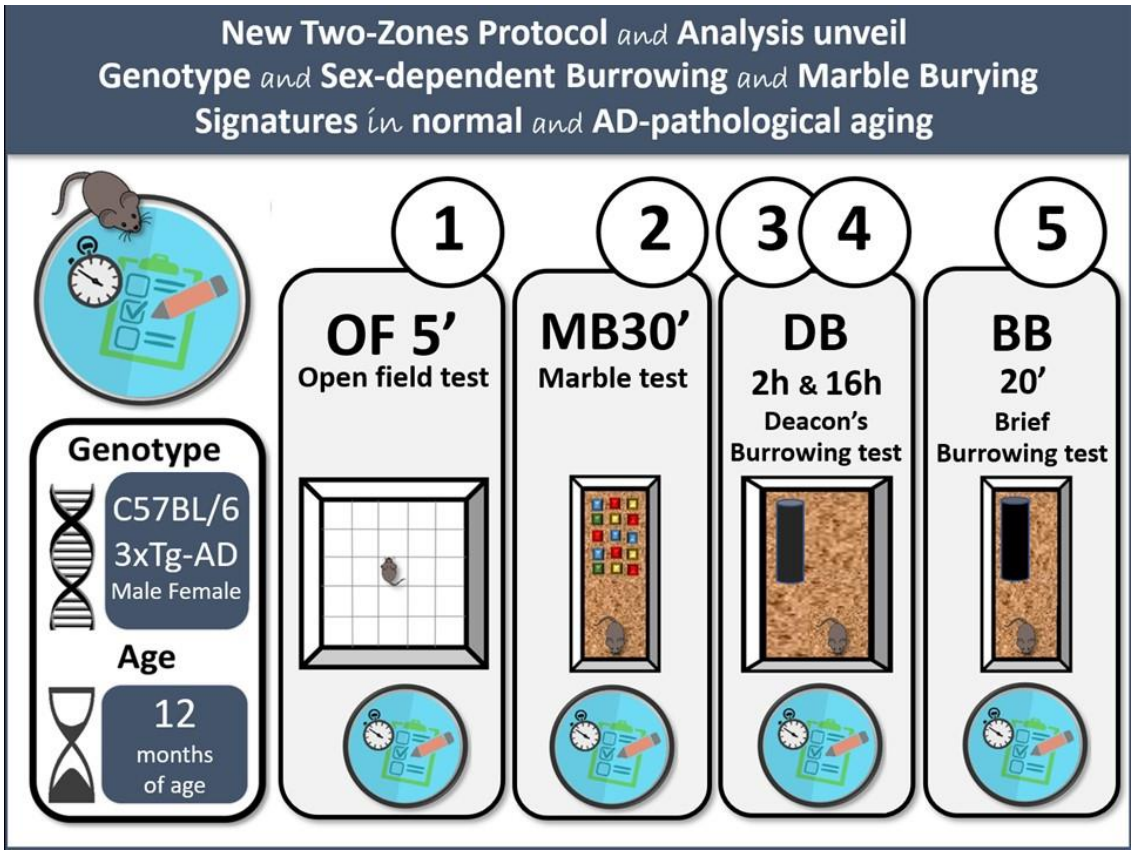

Figure 2. Graphical abstract. Experimental design: a 5-day battery of behavioral tests consisting of an open field test (OF) test on day 1, a two-zones marble test (MB) on day 2, a Deacon's burrowing test (DB) on day 3 until day 4, and a two-zones brief burrowing test (BB) on day 5.

3.3.1.3. Behavioral Assessment

Behavioral assessments in the different tests were conducted under dim white light (20 lx) and during the light phase of the light: dark cycle, in the morning (from 10 am to 1 pm) except for the Deacon's burrowing test that started at 3 pm and ended on the next day at 9 am, as detailed below. A trained observer performed direct observation assessments, blind to the genotype and with a camera's support. All procedures were in accordance with 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 protocol CEEAH 3588/DMAH 9452 was approved on the 8th of March 2019 by the 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 (Kilkeny et al, 2010) .

Day 1— Open field test (OF)

This classical anxiety test was used to evaluate the ethogram of anxiety-like behaviors and exploratory activity. The animal was placed in the center of an open and illuminated field (homemade woodwork, white box, 55 × 55 × 25 cm) and observed for 5 minutes. First, the ethogram of action programs (sequence of behavioral events) was recorded. Thus, the duration of freezing behavior (OFlatM) and the latency of the behavioral events that follow it were recorded: leaving the central square (OFlatC), reaching the periphery zone (OFlatP), performing the first rearing (OFlatR) and the first grooming (OF-latG). Additionally, the number of rearings (OFnR), the number of grooming episodes (OFnG), the distance traveled (OFd), the number of entries in the center zone (OFeC), the time spent in the center zone (OFtC), the distance traveled in the center zone (OFdC), the time spent in the periphery zone (OFtP) and the distance traveled in the periphery zone (OFdP) were also recorded.

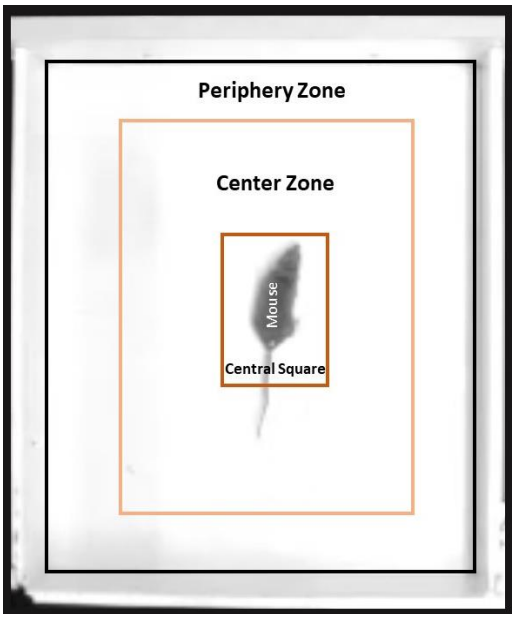

Figure 3. Open Field Test. The apparatus was divided into three zones: central square, center zone, and periphery.

Day 2—Marble Burying test (MB) with Two-Zones analysis (TZA)

The Marble Burying test (marbles equally spaced in a cage) is usually used to evaluate burying behavior. In the present work, we propose using our two-zone configuration (8) and a dual analysis, evaluating marble burying and digging behaviors.

The two-zones protocol consisted of virtually dividing a standard home cage (Macrolon, 35 × 15 × 15 cm), with a 5 cm thick layer of clean woodchips, into two zones: with marbles (w/MB) and without marbles (w/oMB). In this way, we allow the animals to avoid interacting with the marbles if they do not want to. In this work, fifteen glass marbles were placed evenly spaced (five rows of three) in one-half of the cage (zone w/MB), and the test was video recorded. Then, the mouse was introduced in the zone w/oMB facing the wall and left to interact with the cage freely. After 30 minutes, the mouse was gently removed from the cage, and the buried marbles were counted (MB30). Later, to assess the buried marbles time-course, the number of buried marbles was counted every 5 minutes in the video recording (MBx, x=minute). In all the measures, the number of marbles buried was transformed into a percentage for further statistical analysis. The burying criteria was strict: marbles were counted as buried when their surface was covered at least 90% with bedding material.

Additionally, TZA for a better understanding of digging intentionality was applied, the latency of dig-ging appearance and the number of diggings episodes were registered, taking into account the area in which it was made (MBlatDw/ and MBnDw/, in the zone with marbles) (MBlatDw/o and MBnDw/ in the zone without marbles). Subsequently, regardless of the zone, the latency of digging appearance in the test was established (MBlatD), and the number of total digging episodes was calculated (MBnD). All these variables were counted through the video recording. Digging was defined as using front legs and/or hind legs to displace the substrate of the cage.

Day 3 and 4— Deacon's Burrowing Test (DB)

Burrowing behavior was measured using this test (Deacon, 2009). A burrowing tube (PVC, 20 cm) filled with 200 grams of food pellets was introduced into a big home cage (Macrolon, 50 × 22 × 14 cm) with a 3 cm thick layer of woodchips. At 3 pm, mice were placed in the cage facing the wall opposite the tube and left to explore freely. After two hours, the tube was retired to be weighed and refilled. Then, the tube was reintroduced and left until the following day. Sixteen hours later, at 9 am, the tube was retired and weighed again. Finally, the animals were returned to their home cage until the following day. The amount of food out of the tube was calculated and converted into a percentage

in both the 2 hours measure (short, DB%s) and the overnight measure (overnight, DB%o).

Day 5— Brief Burrowing Test (BB) with Two-Zone Analysis (TZA)

To assess burrowing behavior in a format easily comparable to the data obtained in the MB, here we propose a two-zone approach of the protocol proposed by Deacon and a dual analysis, that is, evaluating burrowing and digging behaviors. This test was performed the day after completing the Deacon's test.

A burrowing tube (PVC, 20 cm) filled with 80 g of woodchip bedding material was weighed and introduced into a standard home cage (Macrolon, 35 × 35 × 25 cm) with a 5 cm thick layer of woodchips. Then, the mouse was placed in the cage facing the wall opposite the tube and left to explore freely. After 20 minutes, the mouse was gently removed from the cage, and the tube was weighed. Thus, the amount of wood chips out of the tube was calculated and converted into a percentage (BB%).

Digging was defined as using front legs and/or hind legs to displace the substrate of the cage. The latency of digging appearance and the number of diggings episodes were recorded for each zone: outside the tube (BBlatDout and BBnDout) and inside the tube (BBlatDin and BBnDin). Afterward, regardless of the zone, the latency of digging appearance in the test was established (BBlatD), and the number of total digging episodes was calculated (BBnD). All these variables, except for diggings inside the tube, were counted through the video recording.

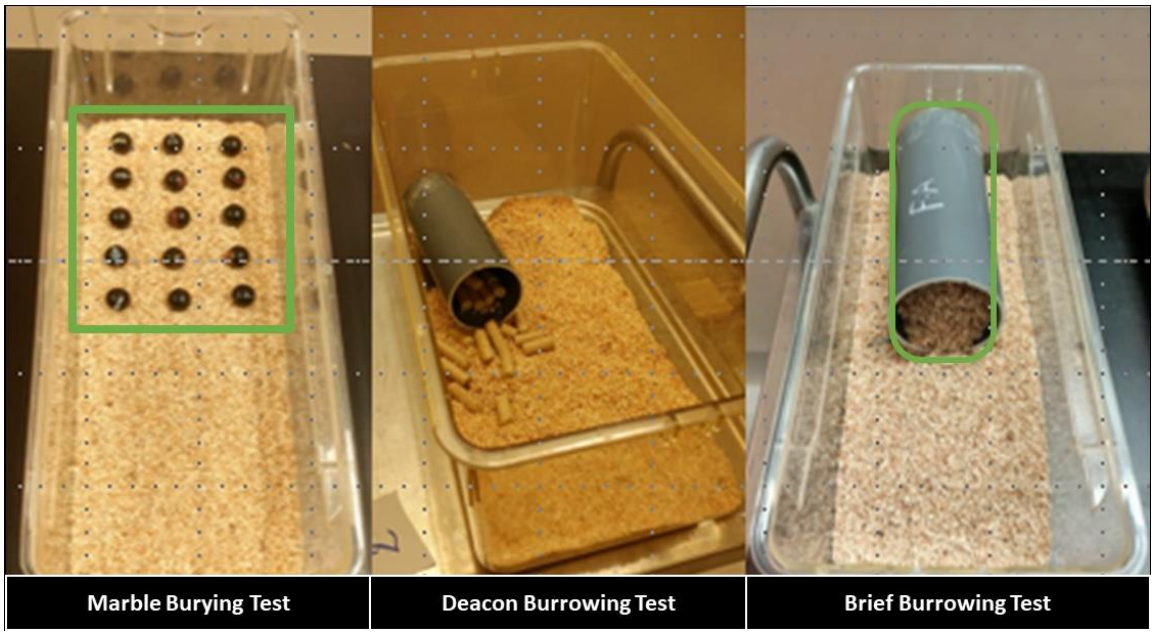

Figure 4. Marble Burying Test, Deacon Burronwing Test, and Brief Burrowing Test. In both, Marble Burying test (MB) and Brief Burrowing test (BB) a two-zone analysis was carried. MB was virtually divided into two zones: a zone with marbles and a zone without marbles. BB was virtually divided into two zones: inside the tube and outside the tube. In both tests, the latency to dig and de number of digging were recorded in each zone,

3.3.1.4. Statistics

Statistical analyses were performed using SPSS 23.0 software. In all the tests, variables were analyzed by ANOVA split-plot analysis, with (G) genotype and (S) sex as the main factors, in a G(2) × S(2) design. In the case of the percentage of marbles buried, the time (T) was included as a within factor according to the experimental design G(2) × S(2) × T(7). Post-hoc comparisons were run with Bonferroni corrections. Spearman correlations were made to analyze behavioral correlates. Due to genotype and sex being significant factors in almost every variable in MB and both burrowing tests, correlation analyses towards OF were performed accordingly. Correlates between MB, DB, and BB were generated without any categorial division to assess their general relationship. Correlation coefficients (r) are indicated. A p-value < 0.05 was considered statistically significant. Graphics were made with GraphPad Prism 6.

Chapter 5. Discussion

5.1. Studies in the PDK1 K465E Knock-In Mice

5.1.1. PDK1 K465E Knock-In Mice Behavioral and Functional Phenotype

This work explores, for the first time, the behavioral phenotype of the PDK1−/− mice. In Study 1, it was found that these mice exhibited increased response of fear- and anxiety-like behaviors. In the CT that measures mild neophobia in a familiar environment, an increased mild neophobia was exhibited as a delayed and reduced number of rearings. However, this phenomenon was present exclusively in young adults (3-4 months old). It is worth mentioning that mature PDK1−/− mice (11-14 months old) exhibited reduced latency of rearing compared to WT mature mice, but this was probably due to the presence of an outlier with extremely higher latency in such a group. The increased neophobia in the CT was confirmed in a more anxiogenic test such as the OF. There, young PDK1−/− mice presented a delayed sequence of events in their behavioral ethogram, suggesting an enhanced fear of facing a novel environment (Lát.,1979). In addition, the higher number of stretch attendance reflects enhanced risk assessment or vigilance when facing a threatening situation. Lastly, the reduction of vertical activity, mainly in the first minutes, resembles an inhibition of the exploratory behavior due to anxiety that gradually increase as mice become more comfortable with the environment. This phenotype is present in other animal models with anxious-like profiles (Baeta-Corral & Giménez-Llort, 2014). Lastly, mature adult PDK1−/− mice emotionality was enhanced, as evidenced by the higher urination incidence. This differentiated expression of anxiety-like behavior at both age stages could be explained by the age-dependent different levels of deficit in AKT signaling described for this model. As stated in the introduction, deficits in Akt signaling are pronounced both in the cortex and the hippocampus during young adulthood (3-4 months of age) but tend to be attenuated by middle age (16-21 months of age) (Yang et al. 2018). To our knowledge, a similar effect has not been reported in the other mutant models reported for this pathway, although it is true that most of them were analyzed only at 3-4 months old mice, which is precisely the age stage in which enhanced anxiety-like behavior is observed in our mutant. The only study testing a mutant model at older ages was conducted in the PDK1fl/fl CRE+ mice, which were evaluated at 12 months of age, thereby matching the mature age reported here. It would be interesting to study if other mutant models also show a loss of the behavioral phenotype with aging.

In the T-maze, PDK1−/− mice explored already visited areas of the maze, which are considered errors attributed to working memory problems and prefrontal cortex dysfunction (Goldman-Rakic, 1994). Alternation behavior has been shown to reflect short-term habituation in responding to stimuli based on relative familiarity because of recent exposure (Sanderson and Bannerman, 2012). Importantly, similarly to the rewarded alternation and win–shift behavior on the radial arm maze, spontaneous alternation is sensitive to hippocampal lesions (Deacon et al., 2002). Here again, the worse performance was mostly observable in young mutant mice, whose brains exhibited more pronounced deficits in Akt signaling in both the cortex and the hippocampus (Yang et al., 2018), key neuroanatomical areas for these behaviors. Although this event was most prominent in young adults, it is important to note the higher number of errors done by a young mutant mouse since it could be and outlier. Altogether, these findings could indicate the presence of small cognitive deficits in the PDK1 model analyzed, although more research is needed since only one test was employed to measure cognition. Cognitive deficits mainly manifest in the AKT3 KO mice, the AKT isoform most expressed in the brain (Bergeron et al., 2017; Howell ey al., 2017; Wong et al., 2020). Since activation of all three AKT isoforms is equally affected in the PDK1-/- mice, reduced levels of AKT3 could cause the observed cognitive deficits. Additionally, PDK1fl/fl CRE+ mice also manifested severe cognitive deficits (Cordón-Barris et al., 2016).

The results in Study 2 do not unveil behavioral differences attributable to anxious behavior caused by genotype-load. Statistical differences emerged in the OF ethogram, but these must be dismissed because the presence of an outlier causes them. Then, we fail to behaviorally detect the activation of differentiated physiological responses in response to distinctive AKT activity levels. This is perhaps because the study was performed in the mature age, where the anxiety phenotype disappears concomitantly to the attenuated AKT activity deficits. In view of our results, it would have been preferable to do this research at 3-4 months of age, but at the time the experiment was performed, age-dependent attenuation of the Akt activity deficits was yet not reported. In the literature, only homozygous PDK1/AKT transgenic mice have been behaviorally evaluated, and both showed similar deficits in cognitive tests (Howel et al., 2017). Hence, the genotypic-load induction of differentiated behavioral phenotypes seems unlikely, although further research is needed. Nevertheless, the complex interplay between genetic-load and sex was revealed in our studies. Although no evidence of increased anxiety is detected, behavioral differences did emerge depending on the genetic load and sex interaction. At a mature age, both PDK1−/− and PDK1+/− male mice showed

and increased bizarre rearing zone similar to that expressed by both PDK1+/+ wild type males and PDK1+/− females, whereas PDK1−/− females expressed a reduced a bizarre behavior comparable to that displayed by PDK1-/- males. These results cannot be interpreted as a sign of excessive anxiety, but they serve to illustrate how, even at normal levels of anxiety, there can be nuances in the behaviors elicited. Only one previous study has included both sexes, and divergences could not explain the behavioral differences in AKT expression or activation between sexes. Given our scarce results and the limited research available, studies on the influence of sex should be continued in the PDK1/AKT mutant mice.

In summary, the PDK1-/- mice manifested enhanced anxiety-like behavior in concordance with other PDK1/AKT mutant mice models. The presence of this phenotype is age-dependent, probably explained by the recovery of AKT activity levels at a mature age. The mutant also manifested signs of cognitive deficit, but further research is needed to conclude it definitively. At a mature age, genotype-load and sex do not cause relevant alterations in the animal's behavior, but it will be recommendable to probe it also at a young age.

5.2.2. PDK1 K465E Knock-In Mice Burying Phenotype

Despite showing increased anxiety-like behavior, the PDK1−/− mice burying behavior does not differ from PDK1+/+ mice. This absence of differences persists at both young and mature ages. Moreover, burying behavior was not influenced by genotype-load. At a mature age, PDK1−/−, PDK1+/−, and PDK1+/+ mice presented similar numbers of marbles buried in the MB. In addition, no differences between male and female mice were observed, even when the genotype-load was considered. However, it can be observed that mature mice (11-14 months) showed a reduction in the number of buried marbles compared to young mice (3-4 months old). This event occurred regardless of the genotype, although it affected the PDK1−/− mice more prominently.

The absence of genotype differences has relevance regarding the convenience of using the PDK1−/− mice to study further and validate new variables and methodological strategies in the MB. We conclude that it does not seem to be an appropriate transgenic strain to continue this research.

5.2.3. Insights from the TZC and ECM Application

Despite the absence of differences in burying behavior, the combined application of the TZC and the EC of marbles provides valuable information for a more detailed understanding of the behavior exhibited.

First, the TZC allows us to infer that both the interaction and the non-interaction with marbles are goal-directed and voluntary. With this methodological approach, the mice can avoid interacting with marbles if they wish to. In view of our results, we can state that PDK1−/−, PDK1+/− and PDK1+/+ mice show some reticence to bury the marbles. In this scenario, we could hypothesize whether this reticence to bury marbles would reflect an aversion to interacting with them. Here is where EC provides helpful insights to clarify this issue.

The EC allows qualitatively classifying three levels of interaction with marbles (buried, changed of position, and intact). We can observe that, regardless of how we group the mice, most of the marbles are categorized as changed of position (approximately 4 of 6 marbles). Meanwhile, the remaining marbles are distributed in the buried or intact categories. This distribution is similar to the exhibited by WT mice employed in a previous work of our lab (Torres-Lista et al., 2015), although in that study the number of marbles employed was higher. The changed of position category included those marbles that were partially buried, turned and/or displaced by the mice. If we combine the buried marbles and positions changed, we can conclude that the interaction with marbles is the most dominant behavior. However, this interaction does not solely reflect burying, as they can interact with them in other ways (e.g., by displacing them when moving through the cage or burying them when running over them). Therefore, we can conclude that our mice's actions are insufficient to completely bury the marbles rather than avoid interacting with them. In view of the results, it would be more useful to record direct measurement of burying behavior. Since burying is the application of digging to a complex task (de Brouwer et al., 2019), the number of diggings made to bury marbles would be a promising alternative. In addition, the EC facilitates interpreting the reduction of buried marbles in mature-age mice. With aging, along with the reduced buried marbles, we could observe an increase in the number of marbles left intact. This increase is even statistically significant. Those are marbles that have been unaltered along the test. However, although the number of intact marbles surpasses the number of buried marbles, it does not exceed those changed of position. Therefore, despite the increase of marbles left intact and the reduction of marbles buried, the interaction with marbles is

still the main activity displayed by our mice, independently of genotype. This reduction of marbles buried could correlate the loss of the anxiety phenotype with aging, although in this case, it cannot be attributed to changes in AKT activity levels since it was also manifested in WT mice.

Among PDK1/AKT mutant mice, only one model has been tested in the MB, which was applied with a TZC and EC methodological modifications. The PDK1fl/fl CRE+ mice exhibited a similar phenotype, where most marbles were changed of position accompanied by a low number of marbles left intact or buried. In this case, the mutant mice showed an enhanced number of marbles changed of position compared to their WT, without differences in the other categories.

Despite the lack of MB differences, the TZC and EC have allowed us to interpret better their burying behavioral pattern. Both can be useful to extend the BNPS screening power of the test, but their explanatory utility may be limited. It would be relevant to incorporate and analyze variables that allow us to capture more directly the burying activity and the intentionality behind this behavior.

5.1.4. Anxiety Tests and MB Correlations

The analysis of the relationship between both OF and CT variables to MB yields poor and incongruent results in both, PDK1−/− and PDK1+/+ mice. Due to the greater latencies in the behavioral ethogram of the OF and CT denoted in the PDK1−/− mice as a sing of increased anxiety-like behavior, it would be expected that the number of buried marbles would correlate inversely with these measures. In addition, the delay in this behavioral ethogram causes a reduced number of rearings, so marbles buried should correlate positively with them. In contrast, we observed that the PDK1+/+ mice buried more marbles at the same time that it took them longer to both perform rearing in the CT and to leave the central square in the OF. In PDK1−/− mice, those individuals that buried fewer marbles were those that performed fewer rearings in the CT. However, in this genotype, it is interesting to note the positive correlation between the number of intact marbles and the latency of rearing in the CT. Thus, PDK1−/− mice interacting less with the marbles showed a more anxiety-like phenotype in the CT. In view of this, we might question whether it would be better to use the number of intact marbles as a measure of anxiety rather than buried marbles, although this data would probably be highly misleading. Since even the displacement of the mice can modify the marble's state, the apparition of marbles left intact could reflect better exploratory/locomotor activity rather

than burying activity. The poor correlations between MB and the outcome of other tests to measure anxiety have been previously described by other studies (Njung'e & Handley, 1991b; Thomas et al., 2009; Savy et al.; 2015)

5.1.5. Could be some BNPS modeled by the K465E Knock-In Mice Burying Behavior?

Although in these studies we do not employed the necessary methodological changes and new variables to fullfil all the critea included in our hypotheses, the results provide valuable information to discuss the possible BNPS modeled by the PDK1$^{-/-}$ mice burying behavior.

Regarding to prove a repetitive-like behavior, due to their low burying outcome, we can reject the "excessive" character criteria. the other criteria cannot be tested. The presence of anxiety-like behavior in our mutant mice and other related PDK1/AKT mice models would encourage us to hypothesize that this low burying activity could mimic an avoidant response, thus depicting an anxiety-like behavior. However, as previously explained, the data obtained do not support this hypothesis.

First, the PDK1$^{-/-}$ mice buried marbles did not differ from those buried by the PDK1$^{+/+}$ mice, so their response, even being anxious, would not be "clinically" different from that made by the "normal population". Second, TZC and EC show a voluntary interaction of the PDK1$^{-/-}$ mice with the marbles. The low number of buried marbles indicates that their activity in the MB is insufficient to bury the marbles completely rather than reflecting an aversion to interacting with them since most marbles are changed of position and are not intact. Finally, buried marbles correlations with other tests to measure anxiety are scarce and inconsistent.

For all these reasons, the MB modeling of anxiety-like behavior in the PDK1$^{-/-}$ mice is quite questionable. We do not deny some type of influence of anxiety on the MB performance, but it is clearly not the main BNPS modeled. Furthermore, it is unlikely that any BNPS will be modeled on these mutant mice. Thus, the PDK1$^{-/-}$ mice burying behavior most likely solely represents an inherent behavioral phenotype of the animal (Thomas et al., 2009), which is not modified by their alterations in the PI3K/PDK1/AKT signaling pathway.

5.2. Studies in the 3xTg-AD Mice

5.2.1. Genetic, Sex and Aging Effects in Burying Behavior

In the present work, we corroborated the previously described increased burying in 3xTg-AD males at 12 months of age (Torres-Lista & Giménez-Llort, 2015; Torres-Lista et al., 2019). In our laboratory, this phenotype has been consistently and robustly manifested over years of research and independently of variations in the number and material of marbles employed. Altered burying behavior has also been found in other AD mice models, although depending on the model used, it was described as reduced or increased (e.g., Kemppainen et al., 2015; Zhang et al., 2018; Si et al., 2022). Hence, this rodent-typical behavior is strongly sensitive to AD pathology in mice, but the pattern of impairment varies among the different mice models, suggesting that different genetic and neuronal substrates may mediate nuances in the phenotype.

Here, for the first time, we provide the burying phenotype of 12 months old 3xTg-AD female mice. The percentage of marbles buried by AD females was similar to that of NTg females. This was proved in two independent studies. Although in Study 4, both AD and NTg females presented lower percentages than the values shown in Study 3, the relationships between the two groups remained the same, as NTg females also presented lower percentages. This event could be due to changes in the number and material of marbles employed between studies and/or the implicit variability in spontaneous behaviors such as burying (Deacon et al., 2008). Despite the absence of statistically significant differences in both studies, it is noteworthy that marbles buried by AD females were slightly higher than those buried by their NTg counterparts. Moreover, in Study 4, the marbles buried by AD males were statistically higher than those buried by AD females. The differentiated burying phenotypes manifested by male and female AD mice warn about the presence of sexual dimorphism. This finding joins the list of sexual differences manifested by 3xTg-AD mice as Aβ plaque load, immune response, lifespan, memory, non-memory-related behavior, and therapeutic response (Dennison et al., 2021). Due to sex-differentiated impairment in AD patients (Ferreti et al., 2018, studying and modeling this phenomenon in AD animal models is necessary to develop treatments that can be clinically translated. As far as we know, this is the first time that sex differences in burying behavior have been studied in a transgenic mice model of AD.

Our longitudinal approach allows us to understand how normal aging and AD-pathological can influence burying behavior. For this purpose, we retested 12 months mice at 16 months old in the MB. Regarding normal aging, NTg mice buried marbles remained stable at 16 months of age. However, sexual dimorphism was exhibited in AD-pathological aging. Whereas AD males burying remains intact, AD females showed impaired burying at 16 months. This deterioration led to a significantly reduced burial of marbles compared to NTg females and accentuated the previous differences regarding AD males. This worsening of burying behavior with the disease's temporal progression mimics AD's progressive nature. Due to the naturalistic approach of the MB, we could be modeling a loss of capability to do and ADL. In this manner, pathological AD aging can worsen AD female mice's capability to perform a typical and necessary behavior, similar to those patients that lose the capability to perform certain activities.
But why is there no sign of aging effect in AD males? A possible explanatory hypothesis would be that in AD males, it was already impaired, showing excessive traits, whereas AD females showed burying levels comparable to those shown by NTg mice. Also, this event could be partly explained by the delayed and sex-dependent apparition of brain pathology (Belfiore et al., 2019). Our future directions are to study further burying behavior in previous age stages to assess the temporal progression of the behavior and clarify this issue.

In addition, the sex-dependent affectation by AD-pathological aging could partially explain the differences in burying percentages in AD-females between Studies 3 and 4. The AD females in Study 4 could be affected earlier by the disease and thus impaired burying at 12 months. Also, due to the longitudinal design of Study 3, a survivor bias may be present as the animals that survived 16 months were probably the healthiest mice. However, normal aging could not explain the lower burying shown by NTg females in Study 4.

To our knowledge, this is the first time a longitudinal study has been conducted assessing burying in AD mice models. A few examples in the literature analyze the deterioration of burying along AD-pathological aging, but these use a cohort approach (e.g., Deacon et al., 2008; Si et al., 2022). In Si & cols. works, Double Knock-Out mice burying worsens as age increases, even to the extreme of not burying marbles at 6 months old. In contrast, in Deacons & cols. work, Tg2576 female mice showed no signs of deterioration even at 23 months of age, although they did not show differences from their controls in all the age cohorts evaluated. Therefore, the worsening of burying behavior as AD advances is a promising topic that could mimic ADL's deteriorative

progression, albeit the modeling of this event is subject to the mice model employed. To our knowledge, this is the first time that a burying ADL-like impairment has been proven through a longitudinal study.

Altogether, our work reveals that burying behavior in 3xTg-AD is sensitive to the complex interplay between genotype, sex, and pathological aging and its interactions, confirming our hypothesis.

5.2.2. Methodological Changes and New Variables: Insights of 3xtTg-AD Burying Behavior

The studies in this work reveal important findings that allow an in-depth understanding of the burying phenotype manifested by our mice. The most relevant will be discussed below.

First, it should be emphasized the utility of the TZC configuration. This methodological approach allows us to ensure that mice interaction with marbles is goal-directed and voluntary, regardless of their burying phenotype. As will be discussed in later sections, such a simple modification is fundamental to interpreting the BNPS modeled by burying behavior in our model.

The TC of marbles buried provides valuable information about the behavioral pattern of the animal through the test, helping to establish a more accurate profile of the animals and giving robustness to the differences between groups. In AD- males, it is noteworthy that the greater percentage of buried marbles appeared since the initial 10-15 minutes of the test, suggesting that their burying behavior appears earlier and is more frenetic than that shown by ADfemales and NTg-males. In addition, it can be noticed that the impaired burying of AD females at 16 months is also manifested in the initial 15 minutes of the test, which makes the results even more robust. Therefore, the TC allows perceiving differences in the pattern throughout the test without solely relying on the final test score.

If we consider only the last score, we could erroneously conclude that there are significant differences (false positive) or not (false negative) between the groups when in fact, throughout the test, this was not the case. In our experiment, we can see an example of each. As a false positive in the final measurement, we have that the 16-month-old AD-females burying percentage is significantly different regarding their score

on the previous testing, but only in that score. While as a false negative, we see that in the last measurement of the re-test, the significant difference between AD-males and NTg-males at 12 months disappears when there have been significant differences in all the previous scores.

The RT unveils the stability of MB buying percentages between the test and retests, independently of the genotype, sex, and age. Thus, there are no apparent hallmarks of habitation for both NTg and AD mice. The only significant difference that appears is a lower percentage of marbles buried by NTg females at 16 months in the retest, although, as we commented in the previous section, with the data provided by the TC it could be considered a false positive since no further significant differences appeared along the test. The dual application of the TC and the RT provides robustness to support the stability between testings since we can appreciate that there are not even performance differences in the intermediate scores. In the retest, it is noteworthy that the percentage curves of females displayed a greater discrepancy than that shown in the previous testing, which denotes a greater variability that causes certain differences that existed the previous day to disappear. This stability between test and retest has important implications for discussing the anxiety-like behavior modeling by burying behavior since it seems to indicate that there is no habituation. A possible hypothesis to support that anxiety-like behavior is still modeled could be that the inherited anxiety trait of these mice could make their response to marbles resistant to habituation (Stein et al., 1997; Steimer, 2011) and thereby invoking either active burying or active avoidance behavior as coping strategies (e.g., Koolhas et al., 2007). Since 3xTg-AD mice present higher baseline anxiety (Baeta-Corral & Giménez-Llort, 2014), which produces differentiated anxious responses depending on the test (Torres-Lista et al., 2019), the previous hypothesis is still possible. Interestingly, we have already reported that other animal models for anxiety, such as the A1 receptor knock-out mice, also show reduced habituation (Giménez-Llort et al., 2005). This issue will be addressed later.

The insight from the new variables included in the TZA complements the analysis of the percentages and allows us to elaborate more complete behavioral profiles of our mice, thus increasing and improving our understanding of how they behaved in the MB. First, both NTg males and females showed lower percentages of buried marbles, which would agree with the lower number of diggings in the marble zone. Since they started later to dig in the marble zone and their digging episodes in the zone without marbles were clearly higher than their diggings in the marble zone, this would suggest that these animals show a preference for digging in the zone without marbles and avoiding, to a

certain extent, digging in the area with marbles. On the other hand, the behavioral pattern exhibited by 3xTg-AD mice would be sex-dependent. As well as NTg mice, AD females would also prefer digging in the zone without marbles and avoiding burying in the area with marbles, but even so, their burying percentage is slightly higher than NTg females. This could be due to a more efficient burying in the marble zone and/or “contamination” from the activity in the zone without marbles, as they manifested a slightly higher number of diggings compared to NTg-females, albeit not statistically significant. Meanwhile, AD males exhibited an earlier and higher activity in the zone with marbles. However, this increased activity was not detrimental to the activity in the zone without marbles since they showed similar latencies and activity in both zones and similar to the shown by the other groups.

In addition, the intra-test correlations of the MB provide valuable insights into how variables are related to each other. First of all, the absence of a relationship between the latencies of diggings of the two different zones could indicate that the occurrence of digging in each zone, with marbles, and without marbles, were independent events subjected to the mouse will. Of these two latencies, only the one done in the area with marbles was related to the percentage of marbles buried at the test's beginning and end. This suggests that it does not matter whether the animals start digging earlier or later in the zone without marbles because the buried marbles only depend on how long it takes to start digging in the marble zone. Furthermore, the idea that the animal's behavior in each zone is voluntary and independent of each other is suggested when we observe that the latency in each one of the zones correlates only with the number of diggings of their respective zone and not with de diggings of the other zone. Finally, it’s important to consider that both the number of diggings in the zone with-out marbles and the zone with marbles correlated with the percentage of marbles buried at the beginning and the final of the test, albeit being bigger for the marbles-zone digging. This implies that the burying percentage does not depend solely on the diggings in the marbles-zone, indicating a certain "contamination" from the non-marbles zone activity to the marble zone. This could be caused by the absence of physical separation between the two areas in two different ways: throwing woodchips over the marbles and covering them from the zone without marbles when they dig, and/or shifting the marbles to the zone without marbles and then burying there.

5.2.3. In-Depth Review of the Relationship Between Anxiety Tests and Burying Behavior in 3xTg-AD Mice

The results allow further examination of the relationship between other tests used to assess anxiety and the MB outcome.

First, we can observe how in Study 3, despite the lack of genotype differences in neophobia in the CT, the differences in burying phenotype are conserved. This absence of differences in the CT is surprising since, in other studies, we have shown enhanced latencies of rearing and a lower number of rearings in AD mice (Torres-Lista et al., 2015; Giménez-Llort & Alveal-Mellado, 2021). In contrast, in Study 4, we can appreciate an enhanced anxiety behavior in AD mice since they showed higher latencies along their OF ethogram. These situations make us suspect that the burying behavioral pattern of AD mice is still preserved regardless of whether or not they manifest anxiety-like behavior in other tests.

Before discussing the analysis of correlations, it is necessary to consider some issues. It is not enough that correlations exist between the anxiety test and the MB; they must show coherent relationships between their variables. The use of increased latencies of the ethogram as a signal of anxious behavior in the CT and OF conditions the type of relationship (positive or negative) that they should have with the different MB variables. In addition, the sexual dimorphism manifested by AD mice in their burying phenotype implies that we must establish differentiated relationship patterns. In view of this, we will discuss the correlations.

If we hypothesize that animals with low burying (NTg male and female, AD female) actively avoid marbles because of anxiety, then we should expect negative correlations in both CT and OF latencies regarding direct and indirect measures of burying (percentages, number of diggings in the zone with marbles) and positive correlations with the latency of digging in the zone with marbles. In Study 3, 12-month-old AD females showed increased latency to do rearing in the CT and a lower percentage of marbles buried at 5 minutes. However, no significant correlations were found in NTg mice, at other ages, or in other measures in the MB. Additionally, the reduced burying behavior shown by AD females at 16 months of age is not correlated with CT, which will have important implications in their interpretation of their behavioral pattern as an anxiety-like behavior. Also, NTg mice and AD females did not show correlations in Study 4.

In AD males, the hypothesis would be the opposite. If increased burying is related to anxiety, there should be positive correlations between both CT and OF latencies with direct and indirect measures of burying and negative correlations with the latency digging in the marbles zone. The results were not like that but were incongruous. In Study 3, AD males did not present any statistical relationship between tests. In study 4, MB percentages in the first 15 minutes were negatively related to OF latency of movement and leaving the central square. The latency of digging in the marble zone was positively related to the latency of leaving the central square in the OF. There was some congruence in the negative relationship between OF latency to leave the central square and the number of diggings in the zone without marbles. Although it is not a direct measure, due to contamination, it influenced the final burying percentage, although it had less relevance than other variables. In our team's previous work (Torres-Lista et al., 2015), the absence of correlations between the CT and OF variables and the number of buried marbles was also exhibited in AD-males.

In view of the results, the relationship between anxiety tests and MB performance could be described as poor, incoherent, and inconsistent. It is quite questionable to relate the performance on other anxiety tests to the MB outcome, even hypothesizing different coping-strategies. These poor correlational data fit with those obtained in other studies. We have observed how the burying phenotype is preserved whether or not the anxious phenotype is present. Although it has not been discussed here, the pattern of correlations between the OF and the burrowing tests also shows a poor relationship. Given the common behavioral substrate of burrowing and burrowing (de Brouwer, 2019), it is not surprising that this occurs.

5.2.4. Unveiling the 3xTg-AD Mice Burrowing Phenotype

This work represents the first description of the 3xTg-AD burrowing behavior. It has been measured with the DB and the BB, which we have elaborated. In both tests, AD males show higher burrowing than NTg males and AD-females, while AD-females show similar burrowing to those shown by NTg mice.

In conjunction with the analysis of percentages, the insight obtained from the TZA of the BB allows us to elaborate a comprehensive behavioral profile of burrowing behavior displayed by AD mice and their NTg counterparts. First, both NTg males and females showed lower percentages of burrowed material, which would agree with the lower

number of diggings done inside the tube. Since they started later to dig inside the tube and their digging episodes outside were higher than their diggings inside, this would suggest that these animals prefer to dig outside the tube. Therefore, they manifest some hesitation to dig inside the tube. On the other hand, the new variables were analyzed to support the sex-dependent burrowing pattern exhibited by 3xTg-AD mice. As well as NTg mice, AD females preferred to dig outside the tube and showed similar activity inside, which translated into a low burrowing percentage. Meanwhile, AD males exhibited an earlier and higher activity inside the tube, which translated into a higher percentage of burrowing. However, this increased activity was only manifested inside the tube since both the latency to dig and the number of diggings outside were similar to that performed by NTg mice.

Intra-test correlations of the BB provide valuable insights into how variables are related. First, the absence of a relationship between the latencies of diggings of the two zones, inside and outside the tube, could indicate that digging in each zone was an independent event subjected to the mouse's will. Of these two latencies, only the one done inside the tube is related to the percentage of woodchip outside the tube. This suggests that it does not matter whether the animals start digging earlier or later outside the tube because the material only depends on how long it takes to start digging inside.
Furthermore, the latency in each one of the zones correlates only with the number of diggings of their respective zone and not with de diggings of the other zone. This supports the idea that the animal's behavior in each zone is voluntary and independent of each other. Finally, it’s important to note that only the number of diggings inside the tube correlated with the burrowing percentage. This implies that the burrowing percentage did not depend on the diggings done outside the tube. Hence there is no contamination between the activity done in each zone. This is probably due to the physical separation between the two zones caused by the tube walls.

Although this is the first time that burrowing behavior has been assessed in the 3xTg-AD mice, this is not the first time that burrowing has been studied in rodent models of AD. Contrary to our results, the majority of AD transgenics mice models tested in the DB or similar protocols have shown decreased burrowing (Deacon et al.,2008; Deacon et al., 2009; Sagare al., 2013; Janus et al., 2015; Lippi et al., 2018; Si et al., 2022). In contrast, Wistar rats injected with amyloid-beta peptides in the hippocampus manifested enhanced burrowing behavior (Salgado-Puga et el. 2015). The differences in the onset and progression of AD brain pathology in the different AD transgenic mouse models is a topic well documented (e.g., LaFerla & Green, 2012; Janus & Westaway, 2001; Lippi et

al., 2018; Götz, et al., 2018). However, differences in behavioral phenotypes do not receive the same depth of study. Some examples in the bibliography show how these behavioral phenotypes do or do not manifest themselves or do so distinctly depending on the mouse model employed (Kobayashi & Chen, 2005; Bryan et al., 22011; Puzzo et al., 2014; Si et al.,2022). As we mentioned, the incongruence between AD mice models also occurred in burying behavior. In addition, it is important to remember that burrowing behavior is sensitive to strain differences (e.g., Contet et al., 2001). Therefore, the AD mice model employed could influence the type of differences that appear, but it seems clear that, as happen with burying, is a rodent typical behavior sensible to AD pathology.

5.2.5 Are Burrowing and Burying Behavior in 3xTg-AD Mice Similarly Impaired?

The methodological design of the BB allows better comparability between the burrowing pattern and the MB burying pattern. Moreover, applying the TZA in both tests enables a deeper analysis of the relationship between such behaviors.

Overall, the burrowing behavioral pattern exhibited by the 3xTg-AD mice was remarkably similar to their burying phenotype. AD males showed increased percentages of burying and burrowing, shortened latencies to initiate the digging in the zone with marbles/inside the tube, and increased episodes of diggings in such zones. In Figure 5, included in the supplementary material, the MB and BB temporal progression in one example of both AD male and NTg male mice. It can be observed how the AD mice do both bury the marbles and burrow the tube earlier. However, there were also some performance differences in the AD males. Perhaps the most relevant one was that AD males displayed increased diggings in the area without marbles, in contrast to diggings outside the tube in the BB. This could be because there is no physical separation between the two MB zones, and the MB and BB have different durations.

AD females burying and burrowing phenotypes were identical and did not differ from those shown by the NT mice. Similarly, the NTg mice's behavioral patterns were also transferred between tests. Therefore, both behaviors are coherently manifested in both MB and the BB, whether impaired or not.

The correlations reported in this work provide valuable information on how burying and burrowing behavior are related. First, the burying percentage and all the burrowing percentages from both DB and BB, are positively correlated. This supports the idea that the performance shown in one test is, to a certain extent, transferable to other tests. This finding has important implications. Thus, an animal that performs a low burying is likely also to present a similar level of burrowing and vice versa. Furthermore, the latencies of digging in the zone with marbles and inside the tube are positively related. This relationship is exclusive, as they do not correlate with other latencies. Surprisingly, the latencies performed in the zone without marbles and outside the tube do not correlate. In addition, we can observe how the latencies of digging in the zone with marbles and inside the tube are negatively related to the percentages of the other tests. However, each latency is uniquely related to a different percentage in the DB. Then, the initiation of both burying and burrowing is intentional, exclusive, and closely related. Moreover, the number of diggings in both zones of the MB is positively correlated with diggings done inside the tube in the BB. However, diggings done outside the tube are unrelated to both digging measures in MB. This pattern is also observed when the burying percentage is compared to diggings measures in the BB, and conversely. This pattern mirrors the contamination effect previously described in the MB and absents in the BB. Therefore, burying and burrowing behavior is the manifestation of goal-directed digging, which in turn are deeply and closely related to each other. Finally, only the number of diggings in the zone with marbles and inside the tube are correlated with both DB burrowing percentages. Contrary to the BB, the contamination effect of the MB is not transferred to the DB percentages. This may be due to the methodological differences between MB and DB, as MB and BB present a similar methodological design.

In resume, burying and burrowing are two goal-directed digging behaviors coherently interconnected to each other through correlations of direct and indirect behavioral variables, although they are not entirely alike. The burying behavior of the animals, regardless of whether it is altered or not, is equally mirrored in its burrowing behavior. Thus, in both tests, in AD-males, behavior is increased, whereas in AD-females is not. From a neuroethological perspective, it could be said that AD-males manifest an impaired capability to modulate their digging towards a goal, thus causing it to appear both burying and burrowing excessively.

5.2.6. Is 3xTg-AD Mice Burying Behavior in the MB a Model of Anxiety-Liker Behavior?

In order to model an anxiety-like behavior, the following criteria must be met: 1) Mice exhibit active avoidance to interact with marbles or active burying towards them; 2) Avoidance response decrease in a MB repeated trial and 3) MB variables present meaningful and consistent correlations with variables from other tests to assess anxiety-like behavior. These criteria will be discussed below.

First, due to the utilization of the two-zone configuration (TZC) we can assume that the interaction with the marbles is, to some extent, voluntary. In our work, AD females showed no robust evidence of avoidance-like response. Their behavioral burying pattern was not statistically different from NTg mice in all the variables analyzed. This lack of avoidance-like response in AD mice is replicated in two independent studies, although there were differences in the percentage of buried marbles manifested between the two studies, prominently in AD females. These would be consistent with the results shown by other studies using a TZC, where mice still interact with marbles and bury them even when they have the opportunity to avoid them completely (e.g., Njung'e & Handley, 1991b; Thomas et al., 2009; de Brower and Wolmarans, 2018). It could be argued that the early onset of digging in the zone without marbles, in contrast to the zone with marbles, could reflect and avoid interacting with marbles. But this gap between the appearance of digging in the two zones is manifested by all the groups, although reduced in AD males. Then, it is expected and initial hesitation to begin digging in the marbles zone, which is not enhanced in the AD females. Therefore, this initial hesitation is not "clinically" and statistically distinctive from the displayed by NTg mice, and labeling it as an avoidance-like response would be an overstatement. The same reasoning could be applied to interpreting the higher number of diggings in the non-marbles zone compared to the marble zone as a sing of avoidance. This "preference" is a phenomenon manifested in all the groups, although in minor terms in AD males. In contrast, AD males manifested a reduced latency to dig and an increased number of diggings in the zone with marbles, accompanied by a higher percentage of buried marbles, meeting the criteria of active burying. Additionally, smaller gaps between both latencies and diggings would support this claim. However, more criteria are needed to label it as and anxiety-like behavior.

Second, the data obtained in the RT would not indicate that habituation to a stressful situation is produced. One could either assume two different fight-to-flight scenarios: a case where the animals were so frightened of the marbles that they used an active burying defensive strategy (AD-males), or they actively avoided their interaction with them (AD-females). In addition, there is no sign of habituation in NTg mice either. Although, due to variability, some of the genotype, sex, and aging differences could disappear between trials, here we probe the lack of differences in the TC of marbles buried. Therefore, the performance of the mice does not even vary statistically along the test form one trial to another. It seems pretty clear that regardless of whether they bury more or fewer marbles, their performance in this test is persistent and stable over time, in concordance with other studies with repeated MB application (e.g., Gyertyán, 1995; Thomas et al., 2009; Taylor et a. 2017). Then, marble burying is behavior resistant to habituation, and this phenomenon is also present in AD mice. It could be argued that only two trials are insufficient to elicit habituation in the MB. This is probably true, but it does not invalidate our statement. Thomas & cols. (2009) proved unaltered burying in C57BL/6J mice in five consecutive days of repeated testing. In the same work, in five successive MB trials with one hour of rest between them, only differences were found between the first and the las trial. It seems unlikely that AD mice would manifest a habitation even if the number of trials was increased, although this should be verified in future studies.

Third, the relationships between the behavior manifested in other tests to assess anxiety and the MB are scarce and inconsistent. The burying behavioral pattern is still preserved regardless of whether or not the AD mice manifested anxiety-like behavior in other tests, as revealed the fact that AD males manifested enhanced burying behavior even without differences in the CT. In addition, the MB outcome correlations with CT and OF are poor and even incongruent. Even with hypothesizing differentiated coping strategies, the criteria are not met. In addition, the age-dependent reduction of burying in Study 3 can not be attributed to anxiety since both the absence of CT differences at 16 months of age and meaningful correlations do not support this claim. Given the lack of evidence to support enhanced anxiety, the possibility of apathy-like behavior arises, a BPSD present in AD patients (Cloak & Khalili, 2019) and manifested in 3xTg-AD mice (e.g., Pardossi-Piquard et al., 2016). Moreover, this hypothesis will make sense given the investigative motivation driving burying inherent behavior (Londei et al., 1998). However, our data are not sufficient to answer this question. In general, it should be noted that a poor and incongruent relationship is not only manifested with the number of buried marbles but also with direct variables of digging behavior employed in the TZA in Study 4. Moreover,

the OF variables also show a poor and scarce relationship with the behavior shown in the burrowing tests, although we have not included them in this thesis.

Definitively, the burying behavioral pattern of AD females does not meet the criteria of active avoidance, habituation, and correlation with other anxiety tests. In contrast, AD males showed active burying towards marbles, but the criteria of habituation and correlations are not met. Then, AD mice burying patter do not model an anxiety-like behavior. We do not deny that anxiety may mediate or influence the behavioral outcome of MB, but anxiety-like behavior is not modeled by burying behavior manifested by AD mice.

5.2.7. Could be 3xTg-AD Mice Burying Behavior in the MB a Model of Repetitive-Like Behavior?

Given the extensive evidence in support of the use of MB as a model of repetitive-like behavior, we considered the possibility that AD mice burying behavior could mimic this BNPS. We established the following criteria to probe which hypothesis: 1) Mice exhibit active interaction with marbles, even with the possibility of avoiding it; 2) Mice manifest increased burying behavior, acquiring an “excessive” nuance; 3) Burying behavior remains stable in a MB repeated trial.

Our results show that NTg mice an AD female only meets criteria 1 and 3. Thus, their burying acquires a repetitive character that does not reach excessive connotations. However, AD male burying behavioral pattern fulfills all the criteria. Therefore, we can affirm that AD male burying behavior reflects an excessive repetitive-like behavior. The next question to ask us is what kind of “excessive” or pathological repetitive-like behaviors are modeled, and that is not an easy question to answer.

Under the label of repetitive-like behavior, we can include a highly heterogeneous set of responses associated with a wide range of conditions, including normative development (Whitehouse & Lewis, 2015). We are going to focus on three possibilities: repetitive behavior, stereotypy, and perseveration. These behaviors have been extensively studied in ASD (e.g.; Lewis & Kim, 2009; Tian et al., 2022), but they are not exclusive to this pathology. Repetitive-like behaviors such as repetitive behavior, stereotypy, and perseveration are manifested in AD patients (Neistein & Seial, 1997; Nyatsanza et al., 2003; Pekkala ey al., 2008; Cipriani et al., 2013, Deardoff & Grossberg, 2019), and our

research group has also described them in the 3xTg-AD mouse (Baeta-Corral & giménez-Llort, 2014; Torres-Lista & Giménez-Llort, 2014; Baeta-Corral & Gimenez-Llort, 2015). In the following, we will define these constructs and discuss how our results conform to these. However, before discussing them, we must define what we mean by repetitive, stereotypical, and perseverative. This is not an easy task, even though they are terms that we usually handle in the field of psychology, psychiatry, and neuroscience. Depending on the source consulted, we can find definitions for the same term with notable differences, the belonging of these to different classifications, and the use of different terms as synonyms (e.g. Ridley, 1994; Garner (2005; Garner, 2006; Lewis & Kim, 2009; Cipriani et al., 2013). All this confuses and makes the interpretation of the results difficult. Our intention is not to redefine these terms or to create a theoretical framework but to specify, as far as possible, what these constructs mean to us. In this way, we intend to give clarity to the conclusions, avoiding confusion and misunderstanding of our results so that other researchers can transfer the conclusions obtained to their field.

First, we will define repetitive behavior as that behavior or response that occurs in an excessive repeated manner. This behavior may be functional in the situation in which it appears, but it occurs in individuals in greater quantities than under normal conditions. This definition would align with what Ridley (1994) refers to as productive stereotypy. The higher number of diggings and the higher percentage of burying along the test shown by the AD males with respect to their NTg and female counterparts in the MB confer an excessive character to such behavior. Moreover, this phenomenon not only occurs in the MB but is also present in the two tests used to measure burrowing. Digging being the primary behavior behind burying and burrowing, we can say that this is a repetitive and persistent behavior, not only resistant to habituation but also consistently manifested in the different contexts that facilitate its occurrence.

Stereotyped-like behavior can be defined as abnormal repetitive movements or behaviors. They are considered maladaptative and/or malfunctional (Garner, 2005). They are usually present in captive animals and can even lead to self-injurious behavior. It would be the equivalent of what Ridley (1994) defined as deprivation-stereotypies and confinement-stereotypy. In mice, these behaviors have been widely studied and include behaviors such as grooming, jumping, barbering or circling (e.g. Baeta-Corral & Gimenez-Llort; Masuda, 2016). First, digging is not a maladaptive or malfunctional behavior per se in our context because even if excessive, a test that needs digging to be performed is not abnormal, nor is it unrelated to the context. Second, there is no

correlation of any kind between the grooming observed in the OF with any of the variables of the other tests. This is important, as grooming is a deeply studied and well-documented rodent-typical behavior for studying stereotypic behaviors (Shepherd et al., 2021). It is important to note, however, that in the OF there were no differences between genotypes in this behavior and it occurred in very low numbers. However, on other occasions, we have documented the presence of this type of behavior in the 3xTg-AD model (Baera-Corral & Giménez-Llort, 2014). Therefore, attributing this construct to excessive digging by the animals in these tests is neither theoretically nor empirically supported.

Finally, we would define perseverance as the performance of a behavior or strategy several times that, although it may make sense in a given situation, is not adapted to the current demand. It is demonstrated by the inability to shift, change or cease a behavior pattern once started (Millan et al., 2002). In our opinion, this construct is difficult to test with the tests used, or at least with the methodology employed. First, we observed that AD-male mice present a greater number of diggings both in the zone without and in the zone with marbles. We could make parallelism and say that the digging done in the zone without marbles would be synonymous with this perseverance. A reflection of the animal's insistence to continue burying or make a burrow when it is impossible to do so. However, it has been proven that the mere presence of bedding material in the cage is sufficient to elicit this behavior in mice (Thomas et al., 2009). Therefore, performing the digging behavior makes sense from a neuroethological perspective, whether the marbles are there or not. Moreover, we should not forget that digging in the area without marbles is the most common behavior in the other groups. These show a certain reluctance to diggings in the area with marbles, as corroborated by the greater latency to bury the marbles. Therefore, it is difficult to prove with the current protocol that the digging behavior present is perseverative in nature.

Due to the repetitive nature of digging behavior in the 3xTg-AD male mice, this could be a consequence of the presence of impulsivity. Impulsivity is a BPSD manifested in patients with AD (Kszycki, et al., 2019) and animal models of the disease (e.g., Adriani et al.; 2006; Masuda, 2016; Shepherd et al., 2021). Also, some authors have argued and employed the MB as a model of impulsive behavior (Gyertyán; 1995; Millan et al.m 2002; Llaneza & Frye, 2009; Taylor et al., 2017, Shepherd, 2021). Garner (2006) defined impulsive behavior as a form of repetitive behavior that usually varies in the form and motor pattern and is goal-directed. In our view, given the investigative drive underlying the burying behavior AD male mice could be overwhelmed by this impulse. Once they

begin to dig in the non-marble zone, they cannot contain their drive to continue exploring, starting to bury the marbles much earlier and with great intensity in the marbles zone, as is evidenced by the significant differences in the number of marbles buried at 15 minutes. Also, the reduced gap between latencies supports this claim. NTg mice, and even AD females, start to dig at the same time as AD males but hesitate to start digging in the marble zone. In addition, since burying and burrowing are both enhanced in AD males, it can be stated that this impulsivity is capable of being displayed in analogous scenarios.

Furthermore, the fact that this impulsivity to explore only is exhibited in the MB but does not in the OF support our idea. In the OF, AD males spent more time frozen, stayed more time in the central square, and took longer to reach the periphery. Due to this pattern, they spent less time in the periphery. Altogether, they manifest enhanced anxiety. In Figure 6, included in the supplementary material, the behavioral pattern of both AD males and NTg males. It can be observed how they shown a more center-oriented and slower trajectories and how spent more time in the center zone contrary NTg males. This shows how AD males perform a slower and more limited exploration than NTg mice in the OF. These differences in their response are probably because MB is a more naturalistic-non-anxious environment, whereas the OF is an environment more anxiogenic. Then, in different situations, AD male mice showed differentiated BPSD-like behaviors. This presence of both neuropsychiatric categories mimics the comorbidity of disorders manifested in BPSD in human AD patients (García-Alberca et al., 2008).

In summary, burying behavior in 3xTg-AD male mice represent a repetitive behavior, understood as excessive in quantity but with functionality and directed to a goal. It implies performing excessive digging towards a specific task. However, it cannot be attributed to stereotypical properties. It is a persistent behavioral pattern that does not change in repeated trials and manifests itself in different tests involving such behavior. To prove the presence of perseverance, it would be necessary to devise methodological modifications or other experimental protocols. This enhanced burying resembles enhanced impulsivity.

5.3. General Discussion

This thesis provides a practical demonstration of how to employ the MB for BNPS screening in two different scenarios: 1) a mutant mouse model with unknown behavioral phenotypes that had high possibilities of exhibiting an anxious phenotype, as described here for the first time; 2) a transgenic mouse model with an anxious phenotype and also increased burying. The following sections will discuss the most relevant findings in general terms.

5.3.1 Genotype, Sex, and Age Effects in Burying Behavior.

Burying behavior has been shown to be sensitive to genotype, but not all. As demonstrated in our studies, there may or may not be differences depending on the model. The fact that a model animal is genetically modified does not imply that differences will emerge. Regarding sex, the results seem to support that there are no sex differences unless it interacts with the genotype, as in the 3xTg-AD mice. In none of the four studies, control mice showed sex differences in burying behavior. This is mainly reflected in Study 4, where we used the TZA, since there are no differences in any of the included variables. This result would be in line with that shown by Taylor and cols. (2017). Regarding age, in the WT of Study 1, we can observe a reduction in the number of marbles. Young adult mice of 3-4 months of age buried more than middle-aged adults, those of 11-14 months of age. The same occurred with PDK1 K465E KI mice. Therefore, it is likely that burying behavior may be reduced at higher age stages relative to younger ages. It would be interesting to perform a longitudinal study to test this, in the same set of animals, at several time points. However, in Study 3, no impairment of burying was observed in NTg at 16 months f age, although more variability in the number of buried marbles was seen throughout the test. In the 3xTg-AD mice, we could observe how pathology and aging interact, causing deterioration of burying, although only in females.

In summary, it seems that the most influential factor for the appearance of differences in the burying phenotype is the genotype, with sexual and age/ageing effects appearing in specific circumstances or interactions.

5.3.2. Promoting Changes

The usefulness of the methodological changes and new variables employed is based on the results obtained in the studies, their usefulness in interpreting the animal behavior, and the achievement of the specific objectives within each animal model. This provides more than enough validation to recommend its use. As a whole, the methodological changes and new variables yielded meaningful burying behavior insights to refute or accept the multicriteria hypotheses . Below we will discuss the most important findings for each of them, their strengths and limitations , and their level of recommendability.

Although it is the most basic and simple approach, TZC is an indispensable methodological change. It makes it possible to ensure that the animal's behavior is goal-directed, regardless of which BNPS we want to model. But the fact that it is indispensable does not imply that it is sufficient. As a major negative criticism, this approach on its own cannot discriminate differentiated behavioral patterns that generate a similar number of buried marbles. In other words, it is unable to discriminate between two mice that bury, or do not bury, the same number of marbles but do it differently. For example, the marble test result of a mouse that avoids burying in the marble zone but buries in the non-marble zone can be the same as a mouse that does not dig in both zones. The result is the same, but the features of the behavior emitted are different. Both responses will be goal-directed but can be attributed to totally different causes. This has important consequences when we try to model BNPS. Not burying marbles because of anxiety is not the same that not burying marbles because of apathy. The result is likely to be the same, a low number of marbles buried, but they will probably differ in other variables. Therefore, we consider it necessary to go a step further and record variables related to the two zones as we did with TZA. As a recommendation, it might be physically dividing the two zones to prevent the non-marbles zone diggins from influencing the number of buried marbles. That way, we would avoid the contamination effect we observed in the correlations. Even so, the burying behavior itself also implies throwing substrate from a certain distance to the object to be buried, so ethologically, this contamination is an expected phenomenon.

The ECM is perhaps the most limited variable of all. Even so, its application would be useful in strains/mutants that do not bury many marbles. As was the case with PDK1 K465E KI mice, allowed to discriminate when mice actions are insufficient to bury marbles completely, a high number of marbles changed of position, and when they avoided interacting with them completely, resulting in a high number of marbles left

intact. Although it does not have the interpretive power of the TZA, it may be a convenient solution if we do not have the possibility of recording the behavior of the animals during the test.

The TC of marbles may be the most efficient methodological change in terms of cost/benefits. First of all, its application is easy and affordable. It can be done through photography or video, not interfering with the normal development of the test. Moreover, the intervals can be easily adapted to the needs of the study, although it would be advisable to include at least one measurement in the middle of the test. This is because, at least in our model, differences usually appear at 10–15 min, and the score at that time does not differ significantly from the score obtained at 30 min (analysis not shown). To us, the most important reason for its use is that it gives us valuable information about the behavioral pattern of the animal throughout the test, helping us to establish a more accurate profile of the animal and the possible differences between them. This may be especially important in pharmacological and non-pharmacological interventions as well, as such interventions could modify the pattern of the animal and not just the final test score. Concerning the latter, the TC could save many nightmares for researchers using the MB. If we consider only the final measurement, we could erroneously conclude that there are significant differences (false positive) or not (false negative) between the two groups when in fact, throughout the test, this was not the case. Although the counting of marbles through the test has existed for a long time (Gyertián et al., 1995), its use is not widespread. It is difficult to find examples of its use in the literature, although they certainly exist (Sugimoto et al., 2007; Kedia & Chattarji, 2014). As in our experiment, the differences found at the end of the test usually manifest from the first measurements and are relatively stable over the time course. Considering the foregoing, we consider that its application's advantages far outweigh its implementation's costs.

The RT of the MB could be indispensable depending on which BPNS we are trying to model. To prove the modeling of anxiety-like behavior, it would be necessary that the response in the burying abates in a RT. In contrast, the burying behavior should be stable among trials to probe a repetitive behavior. In Study 3, it was critical to demonstrate the non-habituation of marble burying. Moreover, the stability between trials could strengthen the conclusions in pharmacological and non-pharmacological studies. The fact that burying behavior is stable among trials allows behavioral monitoring through the different stages of the disease and, most importantly, to test the same mice before and after treatment to compare their performance, knowing with certainty that our results are

derived from the intervention and not caused by an effect of test repetition. This can save both economic costs and mice's lives.

The TZA, that includes the TZC, is probably the most valuable approach. It enables to perform a comprehensive study of the mouse's behavior. In this manner we can test more complex and elaborate hypotheses, containing multiple criteria that capture the core features of the BNPS that we are trying to model. The TZA ensures that the animal's behavior toward marbles is goal-directed but also, we previously mentioned, enables to discriminate differentiated behavioral patterns that produce similar numbers of buried marbles. Regarding which variable to use for this analysis, we consider both the number of diggins and the latency of diggins in each zone as the minimum-indispensable measures. From there onwards, any extra measure used is welcome and will add richness to the interpretation of the results, but these cannot be substituted by others, such as the time spent by the animal in each zone. Both measures have strong correlations with the percentage of burying or burrowing. The number of diggings within each zone is particularly relevant since it represents a direct measure of the behavior to be captured. Calculating times in each zone or digging latencies are indirect measures, as is counting the number of marbles. The inclusion of the latency record is justified, in our opinion, by the ease of obtaining this measure and provides information on the on-set of burrowing behavior, which we believe is a more direct variable than the time in each zone. Counting the number of diggings can be laborious, especially in investigations with very large samples or very long protocols. However, we believe that the gains in interpreting the results far outweigh the costs We consider this approach especially relevant in the study of burying, but we also encourage implementing tests to measure burrowing since it is a behavior that is increasingly implemented in behavioral evaluations, and we would avoid making the historical mistakes made with the MB.

In general, all the adaptations provide robustness to better interpretation of the results. We cannot recommend the application of only one. In our opinion, the best combination is to employ a TZA with TC, similarly to what we did in Study 4, as they provide the most profitable information that can be used to refute or accept multicriteria hypotheses. A helpful combination, although with less interpretative capability, would be to apply TZC, TC and ECM. Depending on which BNPS we want to model, at least one RT would be indispensable.

5.3.3. Burying and Burrowing Relationship: A Question of Diggins

The results obtained on burying and burrowing behavior in our work confirm the close relationship between both behaviors. The burrowing behavioral patterns exhibited by both 3xTg-AD and NTg mice were remarkably similar to their burying phenotype. However, the best results were obtained when the relationship between MB and BB was examined. Three major findings arise from Study 4: 1) mice that perform a low burying are likely also to present a similar level of burrowing and vice versa; 2) the initiation of both burying and burrowing is intentional, exclusive, and closely related; and 3) both burying and burrowing behaviors are the manifestation of goal-directed diggings, which in turn are deeply and closely related to each other.

To our knowledge, this is the first time that the relationship between burying and burrowing behaviors has been studied through correlations. However, other authors have explored, in the same study, how both behaviors are manifested. However, the number of studies is scarce since, most often, only one of these behaviors is tested. Below, we will examine those studies in which both burying and burrowing have been included. Burying and burrowing were investigated in both the 5-HTT overexpressing mice (5-HTT OEs) and the 5-HTT knockout mice (5-HTT KOs) (Line et al., 2011). Each of them was compared with their respective wild-type mice. 5-HTT OEs mice manifested an enhanced burrowing behavior while the burying was unaffected. However, the unaffected burying could be caused by a ceiling effect, as both the 5-HTT OEs mice and their wild types bury almost all the marbles (approximately 9 out of 10). Besides this, reduced burying and burrowing behavior is exhibited in the 5-HTT KOs mice. In other research, Konsolaki and colleagues (2016) studied the burying and burrowing in mice lacking high-affinity nicotinic receptors (β2) and their wild-type mice at two different ages, adult (4-6 months) and old (22-24 months). Older β2-/- showed reduced burrowing. The other groups did not present any differences in both behaviors. Finally, a Double Knock-out mice model of AD displayed decreased burying and burrowing behavior (Si et al. 2022). The review of these studies yields the following conclusions. Examples of burying and burrowing showing reversed patterns (e.g., increased burying and decreased burrowing) do not exist. Normally, either both behaviors are altered, or only one of them does. Therefore, the similar burying and burrowing behavioral pattern displayed by the 3xTg-AD mice is not an exclusive event of this transgenic mice model.

Overall, it is clear that burying and burrowing behaviors were closely related. This is not only based on the mere correlation of percentages but also the interconnection of inter-test latencies and diggings variables. The burying behavior of the animals, regardless of whether it is altered or not, is equally mirrored in its burrowing behavior. Furthermore, it is confirmed that burying and burrowing percentages result from goal-directed diggings. This means that the indiscriminate use of digging did not cause them. Therefore, depending of the necessity of the research, both test could be used as interchangeable but always considering the peculiarities of each test. The BB is a useful test to study the relationship between burying and burrowing and, then, produce comparable results.

5.3.4. What is Modeled with the Mable Burying Test?

Answering this question is not an easy job. Neither do we consider that with the results obtained, we can provide a definitive answer to a debate that has been going on for almost 40 years. But we do consider that the results obtained in this thesis can help to corroborate and reinforce certain issues.

But first, we will address another more straightforward question to answer. What is not modeled by the MB? Our results and the evidence cited throughout the manuscript make it clear that the burying response does not model an anxiety-like behavior. Several results in our paper, both from model mice and control mice, support this view. First, there is independence between the occurrence or not of the anxious phenotypic appearance in other tests and the one displayed in burying. In young PDK1 K465E KI mice, we described phenotypic anxiousness using classical tests, but it was not reflected in the MB because despite presenting a lower number of buried marbles, they interact with them. A similar phenomenon is observed in the 3xTg-AD studies. In Study 3, the AD males do not present anxious phenotypes in the TC but still present enhanced buried marbles. Conversely, Study 4 exhibited an anxious phenotype in the OF and an enhanced marbles burying. Second, the correlations between anxiety tests and burying behavior were poor, incoherent, and inconsistent. Even establishing differentiated relationships based on two differentiated coping strategies: active avoidance of marbles or active burying towards them. In addition, the burrowing behavior manifested in the BB also presented a poor, incoherent, and inconsistent relationship. Third, the data obtained in both 3xTg-AD studies show that regardless of which mice burrow more or less, their response does not fall into the criteria for being considered a neophobic response, active avoidance, and habituation. The absence of habituation is especially important since even NTg mice do not habituate. Therefore, attributing anxious features to burying

behavior does not stand up in any manner. And what happens when anxiolytic treatment reduces burying? In the words of Njung'e & Handley (1991b): "Inhibition of marble burying may, therefore, constitute a correlational model for detecting anxiolytics rather than an isomorphic model of anxiety". Therefore, it is important to state that unless an induced burying protocol is used, future researchers who apply treatments with suspected anxiolytic effects reducing burying in MB would best be described as: "Treatment X produced an anxiolytic effect in burying" rather than "Treatment X reduced anxiety-like behavior".

As for what we model with the MB, burying behavior features make it a normal repetitive behavior by itself, fitting fits better to model psychiatrics repetitive-like behaviors. From the 3xTg-AD studies, we can conclude that it is resistant to habituation, occurs in multiple similar contexts, and is maintained over time unless there is some interaction with the mouse pathology. All this is shown in both AD mice and NTg mice. Moreover, the TC results confirm that there is not even variation in any of the measurements throughout the test. Therefore, this repetitive behavior is not pathological. Unless there are significant differences associated with some kind of genetic, lesional, pharmacological, etc. alteration. And, even then, this can certainly be questionable. The alteration of repetitive behavior is not exclusive to any behavioral and psychiatric disorder. It is clear that in animal models related to OCD and ASD it makes sense to treat them for compulsions and stereotypies, respectively.

But what do we do in models that do not present this type of alteration where the emission of excessive repetitive behaviors does not play a central role? In our opinion, it seems more accurate to speak of impulsivity when there is an excess of burying and apathy when there is a reduction. This view fits us more within the ethological characteristics of the burying behavior itself. The MB assesses a mouse's inherent burying, and it is a behavior motivated by the need to investigate. So, in a sense, we could be measuring a mouse's motivation for inherent burying. Impulsivity can be defined as displaying behavior characterized by little or no forethought, reflection, or consideration of the consequences of an action, particularly one that involves taking risks. In contrast, apathy is the lack of motivation or goal-directed behavior and indifference to one's surroundings (APA, 2014). From a naturalistic perspective, an excess of impulsivity to investigate through burying could put the animal in some dangerous situation, while not doing so may deprive it of favorable opportunities in its environment. In this sense, the results obtained in the AD male fit that modeling of impulsivity. As for apathy, as we mentioned in the previous discussion, we suspect it may be behind the reduced burying of AD

females at 16 months of age. But we cannot test this with certainty, as we did not employ a TZA in that study. We hypothesize that they would probably show a reduced number of diggings in both areas of the MB. This would be an interesting unknown to explore in future studies, in this or other models.

5.3.4. Problems with your BNPS Screening Capability? Here is your Prescription: Multicriteria Hypothesis and Multivariable Analysis

To de Brouwer & Wolmarans (2018):

> *"The MB test is a widely-applied screening tool used to assess the behavioral manifestations of a number of putative neurocognitive constructs in rodents,… Although the burying of marbles, irrespective of the construct it is intended to mimic, may seemingly resemble some of the said clinical symptomology, the purported mono-dimensional architecture of the action and its quantification deserve consideration. Indeed, MB has been reported to respond to various pharmacological agents that together constitute a therapeutic framework that interfere in most aspects of central nervous system functioning . Taken these findings into consideration, two questions arise. First, how can one behavioral phenotype be sensitive to an array of drugs altering a number of divergent neurobiological constructs? Consequently, if such therapeutic sensitivity is accurate, can MB truly be applied as a behavioral measure of specific neuropsychological symptomology? Indeed, although these questions have been raised previously, and considering that burying is a natural response within the normal behavioral repository of rodents translational research that apply the MB test as a measure of specific psychiatric constructs…"*
>
> *"However, the relevance of the MB test as a screening tool for behavioral modification cannot simply be disregarded. Previous investigations that applied the MB test with careful consideration of the purported construct it is to measure, are proof of this. It is therefore not burying behavior per se that prove to be the root of skepticism, but rather its manner of application. If standardized and targeted MB protocols for the assessment of the various behavioral symptoms of different neuropsychological constructs have existed, the questions asked here would have been addressed – this is not the case"*

For us, this text reflects what we believe we have addressed with this thesis. The need to convert the MB, a test in which only one variable is used to "diagnose" mice with multiple disorders, into a multivariate test. Because only through this will we be able to refute or accept hypotheses with multiple criteria that contain the core features of the BNPS we are trying to model. We have been "diagnosing" mice and validating drugs based on a single variable for almost 40 years. It is time to stop this dynamic. To do so, we need to start using multicriteria hypotheses because by analyzing multiple variables,

we can address questions such as: do two drugs that reduce the number of marbles buried elicit the same behavioral response? Do the two areas behave in the same way? All these details go unnoticed. Because it is perhaps in these nuances that the potential of MB to model BNPS or to test drugs lies. We consider that our methodological proposal, TZA and TC, may allow us to answer many of these similar questions.

Due to MB controversial predictive validity and almost inexistent construct validity, face validity is the most "robust" of the three validity criteria (Dixit, et al., 2020), despite, as we have mentioned, it is not exempt from problems. However, as Dixit et cols. cited (2022):

> *"...the worst error committed in the name of models is to forget that at best a model represents only a part — and usually only a small part — of the thing being modeled… Models, in a word, are judged by criteria of usefulness; theories, …".*

Therefore, we need to take this aspect seriously, especially in the area of translation to psychiatry, and for us, the only way to do this is to incorporate the MB multicriteria hypothesis and multivariate analysis. Because perhaps, with this approach, the greatest disadvantage of the marble test, its high sensitivity to drugs and its wide use for BNPS modeling, may one day become its greatest strength.

Chapter 6. Conclusions

6.1. Studies in the PDK1 K465E Knock-In Mice

1. PDK1 K465E KI mice exhibit an anxious behavioral phenotype, concretely at young age, and hint a possible cognitive impairment.

2. PDK1 K465E KI mice menifest a low burying behavior, which is not influenced by genotype, genotype-load or sex differences.

3. Mature mice show a reduction in the number of buried marbles compared to young mice.

4. The combined application of the two-zone congiruation (TZC) in conjution with the extended classification of marbles (ECM) unveils that PDK1 K465E KI mice actions are insufficient to completely bury marbles rather than they avoid interacting with them.

5. Anxiety test performance correlates poorly and icoherently to MB outcome, in both PDK1 K465E KI and control mice.

6. PDK1 K465E KI mice low buryig is better explained by a reduced inherent burying rarther than by a BNPS.

6.2. Studies in the 3xTg-AD Mice

1. 3xTg-AD males enhanced burying is consistent across all studies.

2. At 12 months old, 3xTg-AD females burying is not different from controls mice.

3. Only 3xTg-AD females MB outcome was reduced by AD-pathological ageing, mimicking an AD-related impairment in activities of dalyly livings.

4. Time cours of marble buried show differences are present early in the test.

5. Burying behavior remains stable in a MB repeated trial (RT)

6. Two-zone analysis unveils differentiated behvioral patterns onguently related to the MB outcome, regardless of genotype or sex.

7. Anxiety tests performance correlates poorly, incoherently, and inconsistently to both the MB outcome and behavioral pattern, in both AD and control mice, and even considering their different coping-strategies.

8. Independently of the genotype or sex, burying and burrowing behavioral patterns are alike.

9. 3xTg-AD males burying features resembles an excessive repetitive behavior rather than both a stereotypie or a perseverative behavior.

10. 3x-Tg-AD males enhanced burying, burying behavioral phenotype, and similar burrowing outcome and phenotype, suggest a modelling of impulsivity.

11. 3xTg-AD males exhibition of impulsive- and anxiety-like behaviors mimics the comorbidity of BPSD manifested in human AD patients.

12. 3xTg-AD females age-dependent reduction of MB outcome could imply a presence of enhanced apathy, although further research is needed.

6.3. General Conclusions

1. Genotype Is the most influential factor for the appearance of burying phenotypic differences, appearing sex and age/ageing effects in specific circumstances or in interactions.

2. MB burying behavior features make it incompatible to model an anxiety-like behavior, even considering differentiated coping-strategies.

3. MB burying behavior features make it a normal repetitive behavior by itself, fitting fits better to model psychiatrics repetitive-like behaviors.

4. In non-related OCD/ASD animal models, MB burying could be accurate to model impulsivity and apathy.

5. Mice that perform a certain of burying activity are likely also to present a similar burrowing activity; the initiation of both behaviors is intentional, exclusive, and correlated; and both are the manifestation of closely related goal-directed diggings.

6. Employing multicriteria hypothesis in the MB, including core features of the BNPS to model, ease the interpretation of the results and provides robustness to it BNPS screening capabilities.

7. Transforming the MB in a multivariable test, through methodological changes and new variables, provides meaningful burying behavior insights to refute or accept multicriteria hypotheses.

8. The combined application of the two-zone analysis (TZA) of digging activity variables and the time-course (TC) of marbles buried along the tests provide the most profitable burying information to reject or accept multicriteria hypotheses.

9. A MB repeated trial (RT) should be employed depending on which BNPS needs to be modeled.

References

1. Ackermann, T. F., Hörtnagl, H., Wolfer, D. P., Colacicco, G., Sohr, R., Lang, F., … Lang, U. E. (2008). Phosphatidylinositide dependent kinase deficiency increases anxiety and decreases GABA and serotonin abundance in the amygdala. *Cellular Physiology and Biochemistry, 22*(5–6), 735–744. http://doi.org/10.1159/000185557

2. Albert, M. S. (1996). Cognitive and neurobiologic markers of early Alzheimer disease. *Proceedings of the National Academy of Sciences, 93*(24), 13547-13551. https://doi.org/10.1073/pnas.93.24.13547

3. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., & Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current Biology, 7*(4), 261–269. https://doi.org/10.1016/S0960-9822(06)00122-9

4. Alonso, P., López-Solà, C., Real, E., Segalàs, C., & Menchón, J. M. (2015). Animal models of obsessive–compulsive disorder: utility and limitations. *Neuropsychiatric disease and treatment, 11*, 1939. https://doi.org/10.2147/NDT.S62785

5. American Psychiatric Association (APA) (2014). DSM-5: Manual diagnóstico y estadístico de los trastornos mentales.

6. Angoa-Pérez, M., Kane, M. J., Briggs, D. I., Francescutti, D. M., & Kuhn, D. M. (2013). Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *JoVE (Journal of Visualized Experiments),* (82), e50978. https://dx.doi.org/10.3791/50978

7. Arnáiz, E., & Almkvist, O. (2003). Neuropsychological features of mild cognitive impairment and preclinical Alzheimer's disease. *Acta Neurologica Scandinavica, 107*, 34-41. https://doi.org/10.1034/j.1600-0404.107.s179.7.x

8. Arnold, S. E., Hyman, B. T., Flory, J., Damasio, A. R., & Van Hoesen, G. W. (1991). The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in the cerebral cortex of patients with Alzheimer's disease. *Cerebral cortex, 1*(1), 103-116. https://doi.org/10.1093/cercor/1.1.103

9. Baeta-Corral, R., & Giménez-Llort, L. (2014). Bizarre behaviors and risk assessment in 3xTg-AD mice at early stages of the disease. *Behavioural brain research, 258*, 97-105. https://doi.org/10.1016/j.bbr.2013.10.017

10. Baeta-Corral, R., & Giménez-Llort, L. (2015). Persistent hyperactivity and distinctive strategy features in the Morris water maze in 3xTg-AD mice at advanced stages of disease. *Behavioral neuroscience, 1*29(2), 129-137. https://doi.org/10.1037/bne0000027

11. Baeta-Corral, R., Johansson, B., & Giménez-Llort, L. (2018). Long-term treatment with low-dose caffeine worsens BPSD-like profile in 3xTg-AD mice model of Alzheimer's disease and affects mice with normal aging. *Frontiers in Pharmacology, 9*, 79. https://doi.org/10.3389/fphar.2018.00079

12. Ballard, C., & Corbett, A. (2010). Management of neuropsychiatric symptoms in people with dementia. *CNS drugs, 24*(9), 729-739. https://doi.org/10.2165/11319240-000000000-00000

13. Barnum, C. J., Pace, T. W., Hu, F., Neigh, G. N., & Tansey, M. G. (2012). Psychological stress in adolescent and adult mice increases neuroinflammation and attenuates the response to LPS challenge. *Journal of Neuroinflammation, 9*(1), 1-15. https://doi.org/10.1186/1742-2094-9-9

14. Baudic, S., Dalla Barba, G., Thibaudet, M. C., Smagghe, A., Remy, P., & Traykov, L. (2006). Executive function deficits in early Alzheimer's disease and their relations with episodic memory. Archives of clinical neuropsychology, 21(1), 15-21. https://doi.org/10.1016/j.acn.2005.07.002

15. Bayascas, J. R. (2010). PDK1: the major transducer of PI 3-kinase actions. *Phosphoinositide 3-kinase in Health and Disease,* 9-29. https://doi.org/10.1007/82_2010_43

16. Bayascas, J. R., Sakamoto, K., Armit, L., Arthur, J. S. C., & Alessi, D. R. (2006). Evaluation of approaches to generation of tissue-specific knock-in mice. *Journal of Biological Chemistry, 281*(39), 28772-28781. https://doi.org/10.1074/jbc.M606789200

17. Bayascas, J. R., Wullschleger, S., Sakamoto, K., García-Martínez, J. M., Clacher, C., Komander, D., ... & Logie, L. (2008). Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. *Molecular and cellular biology, 28*(10), 3258-3272. https://doi.org/10.1128/MCB.02032-07

18. Belfiore, R., Rodin, A., Ferreira, E., Velazquez, R., Branca, C., Caccamo, A., & Oddo, S. (2019). Temporal and regional progression of Alzheimer's disease-like pathology in 3xTg-AD mice. *Aging cell, 18*(1), e12873. https://doi.org/10.1111/acel.12873

19. Belzung, C., & Lemoine, M. (2011). Criteria of validity for animal models of psychiatric disorders: focus on anxiety disorders and depression. *Biology of mood & anxiety disorders, 1*(1), 1-14. https://doi.org/10.1186/2045-5380-1-9

20. Bergeron, Y., Bureau, G., Laurier-Laurin, M. É., Asselin, E., Massicotte, G., & Cyr, M. (2017). Genetic deletion of Akt3 induces an endophenotype reminiscent of psychiatric manifestations in mice. *Frontiers in molecular neuroscience, 10*, 102. https://doi.org/10.3389/fnmol.2017.00102

21. Beurel, E., Grieco, S. F., & Jope, R. S. (2015). Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacology & therapeutics, 148*, 114–131. https://doi.org/10.1016/j.pharmthera.2014.11.016

22. Bey, A. L., & Jiang, Y. H. (2014). Overview of mouse models of autism spectrum disorders. Current protocols in pharmacology, 66(1), 5-66. https://doi.org/10.1002/0471141755.ph0566s66

23. Biondi, R. M., Komander, D., Thomas, C. C., Lizcano, J. M., Deak, M., Alessi, D. R., & van Aalten, D. M. (2002). High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site. *The EMBO journal, 21*(16), 4219-4228. https://doi.org/10.1093/emboj/cdf437

24. Broekkamp, C. L., Rijk, H. W., Joly-Gelouin, D., & Lloyd, K. L. (1986). Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. *European journal of pharmacology, 126*(3), 223-229. https://doi.org/10.1016/0014-2999(86)90051-8

25. Çalişkan, H., Şentunali, B., Özden, F. M., Cihan, K. H., Uzunkulaoğlu, M., Çakan, O., ... & Zaloğlu, N. (2017). Marble burying test analysis in terms of biological and non-biological factors. *Journal of Applied Biological Sciences, 11*(1), 54-57. Https://Www.Jabsonline.Org/Index.Php/Jabs/Article/View/528

26. Castillo-Mariqueo, L., Pérez-García, M. J., & Giménez-Llort, L. (2021). Modeling functional limitations, gait impairments, and muscle pathology in Alzheimer's disease: studies in the 3xTg-AD mice. *Biomedicines, 9*(10), 1365. https://doi.org/10.3390/biomedicines9101365

27. Cerejeira, J., Lagarto, L., & Mukaetova-Ladinska, E. B. (2012). Behavioral and psychological symptoms of dementia. *Frontiers in neurology, 3*, 73. https://doi.org/10.3389/fneur.2012.00073

28. Chang, Y. C., Cole, T. B., & Costa, L. G. (2017). Behavioral phenotyping for autism spectrum disorders in mice. *Current protocols in toxicology, 72*(1), 11-22. https://doi.org/10.1002/cptx.19

29. Chiquita, S., Ribeiro, M., Castelhano, J., Oliveira, F., Sereno, J., Batista, M., ... & Castelo-Branco, M. (2019). A longitudinal multimodal in vivo molecular imaging study of the 3xTg-AD mouse model shows progressive early hippocampal and taurine loss. *Human molecular genetics, 28*(13), 2174-2188. https://doi.org/10.1093/hmg/ddz045

30. Cipriani, G., Danti, S., Picchi, L., Nuti, A., & Fiorino, M. D. (2020). Daily functioning and dementia. *Dementia & neuropsychologia, 14*, 93-102. https://doi.org/10.1590/1980-57642020dn14-020001

31. Cipriani, G., Vedovello, M., Ulivi, M., Nuti, A., & Lucetti, C. (2013). Repetitive and stereotypic phenomena and dementia. *American Journal of Alzheimer's Disease & Other Dementias®*, *28*(3), 223-227. https://doi.org/10.1177/1533317513481094

32. Cloak, N., & Al Khalili, Y. (2019). Behavioral and psychological symptoms in dementia. https://www.ncbi.nlm.nih.gov/books/NBK551552/

33. Collins, B. J., Deak, M., Arthur, J. S. C., Armit, L. J., & Alessi, D. R. (2003). In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *The EMBO journal, 22*(16), 4202-4211. https://doi.org/10.1093/emboj/cdg407

34. Contet, C., Rawlins, J. N. P., & Deacon, R. M. (2001). A comparison of 129S2/SvHsd and C57BL/6JOlaHsd mice on a test battery assessing sensorimotor, affective and cognitive behaviours: implications for the study of genetically modified mice. *Behavioural brain research, 124*(1), 33-46. https://doi.org/10.1016/s0166-4328(01)00231-5

35. Cordón-Barris, L., Pascual-Guiral, S., Yang, S., Giménez-Llort, L., Lope-Piedrafita, S., Niemeyer, C., Claro, E., Lizcano, J. M., & Bayascas, J. R. (2016). Mutation of the 3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1) Substrate-Docking Site in the Developing Brain Causes Microcephaly with Abnormal Brain Morphogenesis Independently of Akt, Leading to Impaired Cognition and Disruptive Behaviors. *Molecular and cellular biology, 36*(23), 2967–2982. https://doi.org/10.1128/MCB.00230-16

36. De Brouwer, G., & Wolmarans, D. W. (2018). Back to basics: A methodological perspective on marble-burying behavior as a screening test for psychiatric illness. *Behavioural processes, 157*, 590-600. https://doi.org/10.1016/j.beproc.2018.04.011

37. De Brouwer, G., Fick, A., Harvey, B. H., & Wolmarans, D. W. (2019). A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive–compulsive disorder: Mapping the way forward. *Cognitive, Affective, & Behavioral Neuroscience, 19*(1), 1-39. https://doi.org/10.3758/s13415-018-00653-4

38. De Vugt, M. E., Stevens, F., Aalten, P., Lousberg, R., Jaspers, N., Winkens, I., ... & Verhey, F. R. (2003). Behavioural disturbances in dementia patients and quality of the marital relationship. *International journal of geriatric psychiatry, 18*(2), 149-154. https://doi.org/10.1002/gps.807

39. Deacon, R. (2012). Assessing burrowing, nest construction, and hoarding in mice. *JoVE (Journal of Visualized Experiments)*, (59), e2607. http://dx.doi.org/10.3791/2607

40. Deacon, R. M. (2006). Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nature protocols, 1*(1), 122-124. https://doi.org/10.1038/nprot.2006.20

41. Deacon, R. M. J. (2009). Burrowing: a sensitive behavioural assay, tested in five species of laboratory rodents. *Behavioural brain research, 200*(1), 128-133. https://doi.org/10.1016/j.bbr.2009.01.007

42. Deacon, R. M. J., Cholerton, L. L., Talbot, K., Nair-Roberts, R. G., Sanderson, D. J., Romberg, C., ... & Rawlins, J. N. P. (2008). Age-dependent and-independent behavioral deficits in Tg2576 mice. *Behavioural brain research, 189*(1), 126-138. https://doi.org/10.1016/j.bbr.2007.12.024

43. Deacon, R. M. J., Koros, E., Bornemann, K. D., & Rawlins, J. N. P. (2009). Aged Tg2576 mice are impaired on social memory and open field habituation tests. *Behavioural brain research, 197*(2), 466-468. https://doi.org/10.1016/j.bbr.2008.09.042

44. Deacon, R. M., & Rawlins, J. N. P. (2005). Hippocampal lesions, species-typical behaviours and anxiety in mice. *Behavioural brain research, 156*(2), 241-249. https://doi.org/10.1016/j.bbr.2004.05.027

45. Deardorff, W. J., & Grossberg, G. T. (2019). Behavioral and psychological symptoms in Alzheimer's dementia and vascular dementia. *Handbook of clinical neurology, 165*, 5–32. https://doi.org/10.1016/B978-0-444-64012-3.00002-2

46. Delgado, C., Vergara, R. C., Martínez, M., Musa, G., Henríquez, F., & Slachevsky, A. (2019). Neuropsychiatric symptoms in Alzheimer's disease are the main determinants of functional impairment in advanced everyday activities. *Journal of Alzheimer's Disease, 67*(1), 381-392. https://doi.org/10.3233/jad-180771

47. Dennison, J. L., Ricciardi, N. R., Lohse, I., Volmar, C. H., & Wahlestedt, C. (2021). Sexual dimorphism in the 3xTg-AD mouse model and its impact on pre-clinical research. *Journal of Alzheimer's disease, 80*(1), 41-52. https://doi.org/10.3233%2FJAD-201014

48. Dixit, P. V., Sahu, R., & Mishra, D. K. (2020). Marble-burying behavior test as a murine model of compulsive-like behavior. *Journal of Pharmacological and Toxicological Methods, 102*, 106676. https://doi.org/10.1016/j.vascn.2020.106676

49. Do-Rego, J. C., Viana, A. F., Le Maître, E., Deniel, A., Rates, S. M., Leroux-Nicollet, I., & Costentin, J. (2006). Comparisons between anxiety tests for selection of

anxious and non anxious mice. *Behavioural brain research, 169*(2), 282-288. https://doi.org/10.1016/j.bbr.2006.01.018

50. Fernández-Teruel, A., Giménez-Llort, L., Aguilar, R., & Tobeña, A. (2003). Modelos animales en Psiquiatria. In *Tratado de Psiquiatría* (1 ed., pp. 341-356). https://portalrecerca.uab.cat/en/publications/modelos-animales-en-psiquiatria-2

51. Ferretti, M. T., Iulita, M. F., Cavedo, E., Chiesa, P. A., Schumacher Dimech, A., Santuccione Chadha, A., ... & Hampel, H. (2018). Sex differences in Alzheimer disease—the gateway to precision medicine. *Nature Reviews Neurology, 14*(8), 457-469. https://doi.org/10.1038/s41582-018-0032-9

52. Finlay, D., & Cantrell, D. (2011). The coordination of T-cell function by serine/threonine kinases. Cold Spring *Harbor perspectives in biology, 3*(1), a002261. https://doi.org/10.1101/cshperspect.a002261.

53. Freyberg, Z., Ferrando, S. J., & Javitch, J. A. (2010). Roles of the Akt/GSK-3 and Wnt signaling pathways in schizophrenia and antipsychotic drug action. *The American journal of psychiatry, 167*(4), 388–396. https://doi.org/10.1176/appi.ajp.2009.08121873

54. Frisoni, G., Rozzini, L., Gozzetti, A., Binetti, G., Zanetti, O., Bianchetti, A., ... & Cummings, J. (1999). Behavioral syndromes in Alzheimer's disease: description and correlates. *Dementia and geriatric cognitive disorders, 10*(2), 130-138. https://doi.org/10.1159/000017113

55. Gal, E., & Yirmiya, N. (2021). *Repetitive and Restricted Behaviors and Interests in Autism Spectrum Disorders*. Springer International Publishing.

56. Galasko, D., Schmitt, F., Thomas, R., Jin, S., Bennett, D., & Ferris, S. (2005). Detailed assessment of activities of daily living in moderate to severe Alzheimer's disease. *Journal of the International Neuropsychological Society, 11*(4), 446-453. https://doi.org/10.1017/s1355617705050502

57. Gandhi, R. M., Kogan, C. S., & Messier, C. (2014). 2-Methyl-6-(phenylethynyl) pyridine (MPEP) reverses maze learning and PSD-95 deficits in Fmr1 knock-out mice. *Frontiers in Cellular Neuroscience, 8*, 70. https://doi.org/10.3389/fncel.2014.00070

58. García-Alberca, J. M., Pablo Lara, J., González-Barón, S., Barbancho, M. A., Porta, D., & Berthier, M. (2008). Prevalencia y comorbilidad de síntomas neuropsiquiátricos en la enfermedadde Alzheimer. A*ctas espanolas de psiquiatria, 36*(5), 265–270. PMID: 18597200

59. Gimenez-Llort, L. & Alveal-Mellado, D. (2021). Digging Signatures in 13-Month-Old 3xTg-AD Mice for Alzheimer's Disease and Its Disruption by Isolation Despite Social Life Since They Were Born. *Frontiers in Behavioral Neuroscience, 14*. https://doi.org/10.3389/fnbeh.2020.611384

60. Giménez-Llort, L. & Johansson, B. (2021). Editorial: Pharmacology of BPSD (Behavioral and Psychological Symptoms of Dementia). *Frontiers in Pharmacology, 12*. http://dx.doi.org/10.3389/fphar.2021.704421

61. Giménez-Llort, L., & Torres-Lista, V. (2021). Social nesting, animal welfare, and disease monitoring. *Animals, 11(*4), 1079. https://doi.org/10.3390/ani11041079

62. Gimenez-Llort, L., Blazquez, G., Canete, T., Johansson, B., Oddo, S., Tobena, A., ... & Fernandez-Teruel, A. (2007). Modeling behavioral and neuronal symptoms of

Alzheimer's disease in mice: a role for intraneuronal amyloid. *Neuroscience & Biobehavioral Reviews, 31*(1), 125-147. https://doi.org/10.1016/j.neubiorev.2006.07.007

63. Giménez-Llort, L., Masino, S. A., Diao, L., Fernández-Teruel, A., Tobeña, A., Halldner, L., & Fredholm, B. B. (2005). Mice lacking the adenosine A1 receptor have normal spatial learning and plasticity in the CA1 region of the hippocampus, but they habituate more slowly. *Synapse, 57*(1), 8-16. https://doi.org/10.1002/syn.20146

64. Giménez-Llort, L., Maté, I., Manassra, R., Vida, C. & De la Fuente, M. (2012). Peripheral immune system and neuroimmune communication impairment in a mouse model of Alzheimer's disease. *Annals of the New York Academy of Sciences, 1262*(1), 74-84. https://doi.org/10.1111/j.1749-6632.2012.06639.x

65. Goldman-Rakic, P. S. (1994). Working memory dysfunction in schizophrenia. *The Journal of Neuropsychiatry and Clinical Neurosciences, 6(*4), 348–357. https://doi.org/10.1176/jnp.6.4.348

66. Götz, J., Bodea, L. G., & Goedert, M. (2018). Rodent models for Alzheimer disease. Nature Reviews *Neuroscience, 19*(10), 583-598. https://doi.org/10.1038/s41583-018-0054-8

67. Green, C. R., Mohs, R. C., Schmeidler, J., Aryan, M., & Davis, K. L. (1993). Functional decline in Alzheimer's disease: a longitudinal study. *Journal of the American Geriatrics Society, 41*(6), 654-661. https://doi.org/10.1111/j.1532-5415.1993.tb06740.x

68. Gyertyán, I. (1995). Analysis of the marble burying response: marbles serve to measure digging rather than evoke burying. *Behavioural pharmacology*. https://doi.org/10.1097/00008877-199505001-00026

69. Haass, C., Kaether, C., Thinakaran, G., & Sisodia, S. (2012). Trafficking and proteolytic processing of APP. *Cold Spring Harbor perspectives in medicine, 2*(5), a006270. https://doi.org/10.1101/cshperspect.a006270

70. Hall, C., & Ballachey, E. L. (1932). A study of the rat's behavior in a field. A contribution to method in comparative psychology. University of California *Publications in Psychology, 6*, 1–12. https://psycnet.apa.org/record/1932-04321-001

71. Homma, C., & Yamada, K. (2009). Physical properties of bedding materials determine the marble burying behavior of mice (C57BL/6J). *The Open Behavioral Science Journal, 3*(1). http://dx.doi.org/10.2174/1874230000903010034

72. Hope, T., Keene, J., Gedling, K., Fairburn, C. G., & Jacoby, R. (1998). Predictors of institutionalization for people with dementia living at home with a carer. *International journal of geriatric psychiatry, 13*(10), 682-690.. https://doi.org/10.1002/(sici)1099-1166(1998100)13:10%3C682::aid-gps847%3E3.0.co;2-y

73. Howell, K. R., Floyd, K., & Law, A. J. (2017). PKBγ/AKT3 loss-of-function causes learning and memory deficits and deregulation of AKT/mTORC2 signaling: Relevance for schizophrenia. *PloS one, 12*(5), e0175993. https://doi.org/10.1371/journal.pone.0175993

74. Huang, H., Wang, Q., Guan, X., Zhang, X., Zhang, Y., Cao, J., & Li, X. (2021). Effects of enriched environment on depression and anxiety-like behavior induced by early life stress: A comparison between different periods. *Behavioural Brain Research, 411*, 113389. https://doi.org/10.1016/j.bbr.2021.113389

75. Ikeda, Y., Han, G., Maruta, M., Hotta, M., Ueno, E., & Tabira, T. (2020). Association between daily activities and behavioral and psychological symptoms of dementia in community-dwelling older adults with memory complaints by their families. *International journal of environmental research and public health, 17*(18), 6831. https://doi.org/10.3390/ijerph17186831

76. Javonillo, D. I., Tran, K. M., Phan, J., Hingco, E., Kramár, E. A., Da Cunha, C., ... & LaFerla, F. M. (2022). Systematic phenotyping and characterization of the 3xTg-AD mouse model of Alzheimer's Disease. *Frontiers in neuroscience, 1829*. https://doi.org/10.3389/fnins.2021.785276

77. Jean, S., & Kiger, A. A. (2014). Classes of phosphoinositide 3-kinases at a glance. *Journal of cell science, 127*(Pt 5), 923–928. https://doi.org/10.1242/jcs.093773

78. Jirkof, P. (2014). Burrowing and nest building behavior as indicators of well-being in mice. *Journal of neuroscience methods, 234*, 139-146. https://doi.org/10.1016/j.jneumeth.2014.02.001

79. Jope, R. S., & Roh, M. S. (2006). Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. *Current drug targets, 7*(11), 1421–1434. https://doi.org/10.2174/1389450110607011421

80. Kedia, S., & Chattarji, S. (2014). Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice. *Journal of neuroscience methods, 233*, 150-154. https://doi.org/10.1016/j.jneumeth.2014.06.012

81. Kemppainen, S., Lindholm, P., Galli, E., Lahtinen, H. M., Koivisto, H., Hämäläinen, E., ... & Tanila, H. (2015). Cerebral dopamine neurotrophic factor improves long-term memory in APP/PS1 transgenic mice modeling Alzheimer's disease as well as in wild-type mice. *Behavioural brain research, 291*, 1-11. https://doi.org/10.1016/j.bbr.2015.05.002

82. Keszycki, R. M., Fisher, D. W., & Dong, H. (2019). The hyperactivity–impulsivity–irritiability–disinhibition–aggression–agitation domain in Alzheimer's disease: current management and future directions. *Frontiers in pharmacology, 10*, 1109. https://doi.org/10.3389/fphar.2019.01109

83. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS biology, 8*(6), e1000412. https://doi.org/10.1371/journal.pbio.1000412

84. Kim, H., Lim, C. S., & Kaang, B. K. (2016). Neuronal mechanisms and circuits underlying repetitive behaviors in mouse models of autism spectrum disorder. *Behavioral and Brain Functions, 12*(1), 1-13. https://doi.org/10.1186/s12993-016-0087-y

85. Kinsey, S. G., O'Neal, S. T., Long, J. Z., Cravatt, B. F., & Lichtman, A. H. (2011). Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay. *Pharmacology Biochemistry and Behavior, 98*(1), 21-27. https://doi.org/10.1016/j.pbb.2010.12.002

86. Kirova, A. M., Bays, R. B., & Lagalwar, S. (2015). Working memory and executive function decline across normal aging, mild cognitive impairment, and Alzheimer's disease. *BioMed research international, 2015.* https://doi.org/10.1155/2015/748212

87. Kitamura, T., Kitamura, M., Hino, S., Tanaka, N., & Kurata, K. (2012). Gender differences in clinical manifestations and outcomes among hospitalized patients with

behavioral and psychological symptoms of dementia. *The Journal of clinical psychiatry, 73*(12), 7633. https://doi.org/10.4088/jcp.11m07614

88. Knopman, D. S., Amieva, H., Petersen, R. C., Chételat, G., Holtzman, D. M., Hyman, B. T., ... & Jones, D. T. (2021). Alzheimer disease. Nature reviews Disease primers, 7(1), 1-21. https://doi.org/10.1038/s41572-021-00269-y

89. Konsolaki, E., Tsakanikas, P., Polissidis, A. V., Stamatakis, A., & Skaliora, I. (2016). Early signs of pathological cognitive aging in mice lacking high-affinity nicotinic receptors. *Frontiers in aging neuroscience, 8*, 91. https://doi.org/10.3389/fnagi.2016.00091

90. LaFerla, F. M., & Green, K. N. (2012). Animal models of Alzheimer disease. *Cold Spring Harbor perspectives in medicine, 2*(11), a006320. https://doi.org/10.1101/cshperspect.a006320

91. Lát J. (1973). The analysis of habituation*. Acta neurobiologiae experimentalis, 33*(4), 771–789. https://pubmed.ncbi.nlm.nih.gov/4781921/

92. La-Vu, M., Tobias, B. C., Schuette, P. J., & Adhikari, A. (2020). To approach or avoid: an introductory overview of the study of anxiety using rodent assays. *Frontiers in behavioral neuroscience, 14*, 145. https://doi.org/10.3389/fnbeh.2020.00145

93. Leibrock, C., Ackermann, T. F., Hierlmeier, M., Lang, F., Borgwardt, S., & Lang, U. E. (2013). Akt2 deficiency is associated with anxiety and depressive behavior in mice. *Cellular Physiology and Biochemistry, 32*(3), 766–777. http://doi.org/10.1159/000354478

94. Lewis, M., & Kim, S. J. (2009). The pathophysiology of restricted repetitive behavior. *Journal of neurodevelopmental disorders, 1*(2), 114-132. https://doi.org/10.1007/s11689-009-9019-6

95. Li, X. L., Hu, N., Tan, M. S., Yu, J. T., & Tan, L. (2014). Behavioral and psychological symptoms in Alzheimer's disease. *BioMed research international, 2014*. https://doi.org/10.1155/2014/927804

96. Line, S. J., Barkus, C., Coyle, C., Jennings, K. A., Deacon, R. M., Lesch, K. P., ... & Bannerman, D. M. (2011). Opposing alterations in anxiety and species-typical behaviours in serotonin transporter overexpressor and knockout mice. *European Neuropsychopharmacology, 21*(1), 108-116. https://doi.org/10.1016/j.euroneuro.2010.08.005

97. Lippi, S. L., Smith, M. L., & Flinn, J. M. (2018). A novel hAPP/htau mouse model of Alzheimer's disease: inclusion of APP with Tau exacerbates behavioral deficits and zinc administration heightens tangle pathology. *Frontiers in aging neuroscience, 10*, 382. https://doi.org/10.3389/fnagi.2018.00382

98. Llaneza, D. C., & Frye, C. A. (2009). Progestogens and estrogen influence impulsive burying and avoidant freezing behavior of naturally cycling and ovariectomized rats. *Pharmacology Biochemistry and Behavior, 93*(3), 337-342. https://doi.org/10.1016/j.pbb.2009.05.003

99. Londei, T., Valentini, A. M., & Leone, V. G. (1998). Investigative burying by laboratory mice may involve non-functional, compulsive, behaviour. *Behavioural brain research, 94*(2), 249-254. https://doi.org/10.1016/s0166-4328(97)00162-9

100. Lyketsos, C. G., Carrillo, M. C., Ryan, J. M., Khachaturian, A. S., Trzepacz, P., Amatniek, J., ... & Miller, D. S. (2011). Neuropsychiatric symptoms in Alzheimer's disease. *Alzheimer's & Dementia, 7*(5), 532-539. https://doi.org/10.1016/j.jalz.2011.05.2410

101. Marshall, G. A., Amariglio, R. E., Sperling, R. A., & Rentz, D. M. (2012). Activities of daily living: where do they fit in the diagnosis of Alzheimer's disease?. *Neurodegenerative disease management, 2*(5), 483-491. https://doi.org/10.2217/nmt.12.55

102. Marshall, G. A., Rentz, D. M., Frey, M. T., Locascio, J. J., Johnson, K. A., Sperling, R. A., & Alzheimer's Disease Neuroimaging Initiative. (2011). Executive function and instrumental activities of daily living in mild cognitive impairment and Alzheimer's disease. *Alzheimer's & Dementia, 7*(3), 300-308. https://doi.org/10.1016/j.jalz.2010.04.005

103. Martinowich, K., Manji, H., & Lu, B. (2007). New insights into BDNF function in depression and anxiety. *Nature neuroscience, 10*(9), 1089-1093. https://doi.org/10.1038/nn1971

104. Masuda, A., Kobayashi, Y., Kogo, N., Saito, T., Saido, T. C., & Itohara, S. (2016). Cognitive deficits in single App knock-in mouse models. *Neurobiology of learning and memory, 135,* 73-82. https://doi.org/10.1016/j.nlm.2016.07.001

105. Matsushita, M., Egashira, N., Harada, S., Okuno, R., Mishima, K., Iwasaki, K., ... & Fujiwara, M. (2005). Perospirone, a novel antipsychotic drug, inhibits marble-burying behavior via 5-HT1A receptor in mice: implications for obsessive-compulsive disorder. *Journal of pharmacological sciences, 99*(2), 154-159. https://doi.org/10.1254/jphs.fp0050144

106. Mehta, M. V., Gandal, M. J., & Siegel, S. J. (2011). mGluR5-antagonist mediated reversal of elevated stereotyped, repetitive behaviors in the VPA model of autism. *PloS one, 6*(10), e26077. https://doi.org/10.1371/journal.pone.0026077

107. Mesulam, M. M. (2000). A plasticity-based theory of the pathogenesis of Alzheimer's disease. *Annals of the New York Academy of Sciences, 924*(1), 42-52. https://doi.org/10.1111/j.1749-6632.2000.tb05559.x

108. Mielke, M. M., Vemuri, P., & Rocca, W. A. (2014). Clinical epidemiology of Alzheimer's disease: assessing sex and gender differences. *Clinical epidemiology, 6*, 37. https://doi.org/10.2147/CLEP.S37929

109. Millan, M. J., Girardon, S., Mullot, J., Brocco, M., & Dekeyne, A. (2002). Stereospecific blockade of marble-burying behaviour in mice by selective, non-peptidergic neurokinin1 (NK1) receptor antagonists. *Neuropharmacology, 42*(5), 677-684. https://doi.org/10.1016/s0028-3908(02)00021-7

110. Mioshi, E., Kipps, C. M., Dawson, K., Mitchell, J., Graham, A., & Hodges, J. R. (2007). Activities of daily living in frontotemporal dementia and Alzheimer disease. *Neurology, 68*(24), 2077-2084. https://doi.org/10.1212/01.wnl.0000264897.13722.53

111. Montine, T.J., Phelps, C.H., Beach, T.G. et al. National Institute on Aging–Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. *Acta Neuropathol 123*, 1–11 (2012). https://doi.org/10.1007/s00401-011-0910-3

112. Mora, A., Komander, D., van Aalten, D. M., & Alessi, D. R. (2004, April). PDK1, the master regulator of AGC kinase signal transduction. In *Seminars in cell & developmental biology* (Vol. 15, No. 2, pp. 161-170). Academic Press. https://doi.org/10.1016/j.semcdb.2003.12.022

113. Morley, J. E., Farr, S. A., & Nguyen, A. D. (2018). Alzheimer disease. *Clinics in geriatric medicine, 34*(4), 591-601. https://doi.org/10.1016/j.cger.2018.06.006

114. Muntsant, A. & Giménez-Llort, L. (2020). Impact of Social Isolation on the Behavioral, Functional Profiles, and Hippocampal Atrophy Asymmetry in Dementia in Times of Coronavirus Pandemic (COVID-19): A Translational Neuroscience Approach. *Frontiers in Psychiatry, 11*. https://doi.org/10.3389/fpsyt.2020.572583

115. Neistein, S., & Siegal, A. P. (1997). Agitation, wandering, pacing, restlessness, and repetitive mannerisms. *International Psychogeriatrics*, *8*(S3), 399-402. https://10.1017/s1041610297003712

116. Nicolas, L. B., Kolb, Y., & Prinssen, E. P. (2006). A combined marble burying–locomotor activity test in mice: A practical screening test with sensitivity to different classes of anxiolytics and antidepressants. *European journal of pharmacology, 547*(1-3), 106-115. https://doi.org/10.1016/j.ejphar.2006.07.015

117. Niu, H., Álvarez-Álvarez, I., Guillén-Grima, F., & Aguinaga-Ontoso, I. (2017). Prevalence and incidence of Alzheimer's disease in Europe: A meta-analysis. *Neurología (English Edition), 32*(8), 523-532. https://doi.org/10.1016/j.nrl.2016.02.016

118. Njung'e, K., & Handley, S. L. (1991a). Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. *British journal of pharmacology, 104*(1), 105-112. https://doi.org/10.1111/j.1476-5381.1991.tb12392.x

119. Njung'e, K., & Handley, S. L. (1991b). Evaluation of marble-burying behavior as a model of anxiety. *Pharmacology Biochemistry and Behavior, 38*(1), 63-67. https://doi.org/10.1016/0091-3057(91)90590-x

120. Norton, L. E., Malloy, P. F., & Salloway, S. (2001). The impact of behavioral symptoms on activities of daily living in patients with dementia. *The American Journal of Geriatric Psychiatry, 9*(1), 41-48. http://dx.doi.org/10.1097/00019442-200102000-00007

121. Nyatsanza, S., Shetty, T., Gregory, C., Lough, S., Dawson, K., & Hodges, J. R. (2003). A study of stereotypic behaviours in Alzheimer's disease and frontal and temporal variant frontotemporal dementia. *Journal of neurology, neurosurgery, and psychiatry*, *74*(10), 1398–1402. https://10.1136/jnnp.74.10.1398

122. Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B. P., & LaFerla, F. M. (2003a). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiology of aging, 24*(8), 1063-1070. https://doi.org/10.1016/j.neurobiolaging.2003.08.012

123. Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kayed, R., ... & LaFerla, F. M. (2003b). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. *Neuron, 39*(3), 409-421. https://doi.org/10.1016/s0896-6273(03)00434-3

124. Opara, J. A. (2012). Activities of daily living and quality of life in Alzheimer disease. *Journal of medicine and life, 5*(2), 162.

https://www.researchgate.net/publication/229157660_Activities_of_daily_living_and_quality_of_life_in_Alzheimer_disease

125. Ou, L. C., & Gean, P. W. (2006). Regulation of amygdala-dependent learning by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3-kinase. *Neuropsychopharmacology, 31*(2), 287-296. https://doi.org/10.1038/sj.npp.1300830

126. Pardossi-Piquard, R., Lauritzen, I., Bauer, C., Sacco, G., Robert, P., & Checler, F. (2016). Influence of genetic background on apathy-like behavior in triple transgenic AD mice. *Current Alzheimer Research, 13*(8), 942-949. https://doi.org/10.2174/1567205013666160404120106

127. Pekkala, S., Albert, M. L., Spiro, A., 3rd, & Erkinjuntti, T. (2008). Perseveration in Alzheimer's disease. *Dementia and geriatric cognitive disorders*, *25*(2), 109–114. https://doi.org/10.1159/000112476

128. Peng, Y. G., Cai, P. J., Hu, J. H., Jiang, J. X., Zhang, J. J., Liu, K. F., ... & Long, C. (2021). Altered corticostriatal synchronization associated with compulsive-like behavior in APP/PS1 mice. *Experimental Neurology, 344*, 113805. https://doi.org/10.1016/j.expneurol.2021.113805

129. Philip, S., Kaj, B., Breteler Monique, M. B., de Strooper Bart, F. G. B., & Stephen, S. (2016). Van der Flier Wiesje Maria. Alzheimer's disease. *The Lancet, 388*(10043), 505-517. https://doi.org/10.1016/S0140-6736(20)32205-4

130. Potkin, S. G. (2002). The ABC of Alzheimer's disease: ADL and improving day-to-day functioning of patients. *International Psychogeriatrics, 14*(S1), 7-26. https://doi.org/10.1017/s1041610203008640

131. Puzzo, D., Lee, L., Palmeri, A., Calabrese, G., & Arancio, O. (2014). Behavioral assays with mouse models of Alzheimer's disease: practical considerations and guidelines. *Biochemical pharmacology, 88*(4), 450-467. https://doi.org/10.1016/j.bcp.2014.01.011

132. Ritchie, K., & Kildea, D. (1995). Is senile dementia" age-related" or" ageing-related"? evidence from meta-analysis of dementia prevalence in the oldest old. *The Lancet, 346*(8980), 931-934. https://doi.org/10.1016/s0140-6736(95)91556-7

133. Deacon, R. M., & Rawlins, J. N. P. (2005). Hippocampal lesions, species-typical behaviours and anxiety in mice. *Behavioural brain research*, *156*(2), 241-249. https://doi.org/10.1016/j.bbr.2004.05.027

134. Salaberría, K., Fernández-Montalvo, J., & Echeburúa, E. (1995). Ansiedad normal, ansiedad patológica y trastornos de ansiedad: ¿un camino discontinuo?. *Boletín de Psicología, 48*, 67-81. https://academica-e.unavarra.es/xmlui/handle/2454/27969

135. Sanathara, N. M., Garau, C., Alachkar, A., Wang, L., Wang, Z., Nishimori, K., ... & Civelli, O. (2018). Melanin concentrating hormone modulates oxytocin-mediated marble burying. Neuropharmacology, 128, 22-32. https://doi.org/10.1016/j.neuropharm.2017.09.008

136. Savy, C. Y., Fitchett, A. E., McQuade, R., Gartside, S. E., Morris, C. M., Blain, P. G., & Judge, S. J. (2015). Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in adult male rats as assessed by marble burying behaviour. *Neurotoxicology, 50*, 149-156. https://doi.org/10.1016/j.neuro.2015.08.010

137. Schneider, T., & Popik, P. (2007). Attenuation of estrous cycle-dependent marble burying in female rats by acute treatment with progesterone and antidepressants. *Psychoneuroendocrinology, 32*(6), 651-659. https://doi.org/10.1016/j.psyneuen.2007.04.003

138. Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor perspectives in medicine, 1(*1), a006189. https://doi.org/10.1101/cshperspect.a006189

139. Shepherd, A., May, C., Churilov, L., Adlard, P. A., Hannan, A. J., & Burrows, E. L. (2021). Evaluation of attention in APP/PS1 mice shows impulsive and compulsive behaviours. *Genes, Brain and Behavior, 20*(1), e12594. https://doi.org/10.1111/gbb.12594

140. Shi, H.-S., Zhu, W.-L., Liu, J.-F., Luo, Y.-X., Si, J.-J., Wang, S.-J., … Lu, L. (2012). PI3K/Akt signaling pathway in the basolateral amygdala mediates the rapid antidepressant-like effects of trefoil factor 3. Neuropsychopharmacology : *Official Publication of the American College of Neuropsychopharmacology, 37*(12), 2671–83. http://doi.org/10.1038/npp.2012.131

141. Shin, I.S.; Carter, M.; Masterman, D.; Fairbanks, L.; Cummings, J.L. Neuropsychiatric symptoms and quality of life in Alzheimer disease. Am. J. Geriatr. *Psychiatry 2005*, 13, 469–474. https://doi.org/10.1176/appi.ajgp.13.6.469

142. Si, Y., Guo, C., Xiao, F., Mei, B., & Meng, B. (2022). Noncognitive species-typical and home-cage behavioral alterations in conditional presenilin 1/presenilin 2 double knockout mice. *Behavioural Brain Research, 418*, 113652. https://doi.org/10.1016/j.bbr.2021.113652

143. Slachevsky, A., Forno, G., Barraza, P., Mioshi, E., Delgado, C., Lillo, P., ... & Hornberger, M. (2019). Mapping the neuroanatomy of functional decline in Alzheimer's disease from basic to advanced activities of daily living. *Journal of Neurology, 266*(6), 1310-1322. https://doi.org/10.1007/s00415-019-09260-w

144. Slot, L. A. B., Bardin, L., Auclair, A. L., Depoortere, R., & Newman-Tancredi, A. (2008). Effects of antipsychotics and reference monoaminergic ligands on marble burying behavior in mice. *Behavioural pharmacology, 19*(2), 145-152. https://doi.org/10.1097/fbp.0b013e3282f62cb2

145. Steimer, T. (2022). Animal models of anxiety disorders in rats and mice: some conceptual issues. *Dialogues in clinical neuroscience*. https://doi.org/10.31887/DCNS.2011.13.4/tsteimer

146. Stein, D. J., & Bouwer, C. (1997). A neuro-evolutionary approach to the anxiety disorders. *Journal of Anxiety Disorders, 11*(4), 409-429. https://doi.org/10.1016/S0887-6185(97)00019-4

147. Sterniczuk, R., Antle, M. C., LaFerla, F. M., & Dyck, R. H. (2010). Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 2. Behavioral and cognitive changes. *Brain research, 1348*, 149-155. https://doi.org/10.1016/j.brainres.2010.06.011

148. Sujuan, G., Hugh, C. H., Kathleen, S., & Siu, H. (1998). The relationships between age, sex, and the incidence of dementia and Alzheimer disease. *Archives of General Psychiatry, 55*(9), 809. https://doi.org/10.1001/archpsyc.55.9.809

149. Sun, L., Cui, K., Xing, F., & Liu, X. (2020). Akt dependent adult hippocampal neurogenesis regulates the behavioral improvement of treadmill running to mice model of post-traumatic stress disorder. *Behavioural Brain Research, 379*, 112375. https://doi.org/10.1016/j.bbr.2019.112375

150. Sungur, A. Ö., Vörckel, K. J., Schwarting, R. K., & Wöhr, M. (2014). Repetitive behaviors in the Shank1 knockout mouse model for autism spectrum disorder: developmental aspects and effects of social context. *Journal of neuroscience methods, 234*, 92-100. https://dx.doi.org/10.1016/j.jneumeth.2014.05.003

151. Tan, L.L.;Wong, H.B.; Allen, H. The impact of neuropsychiatric symptoms of dementia on distress in family and professional caregivers in Singapore. *Int. Psychogeriatr. 2005*, 17, 253–263. https://doi.org/10.1017/s1041610205001523

152. Tao, W., Dong, Y., Su, Q., Wang, H., Chen, Y., Xue, W., … Chen, G. (2016). Liquiritigenin reverses depression-like behavior in unpredictable chronic mild stress-induced mice by regulating PI3K/Akt/mTOR mediated BDNF/TrkB pathway. *Behavioural Brain Research, 308*, 177–86. http://doi.org/10.1016/j.bbr.2016.04.039

153. Taylor, G. T., Lerch, S., & Chourbaji, S. (2017). Marble burying as compulsive behaviors in male and female mice. *Acta Neurobiologiae Experimentalis, 77*(3), 254-260. http://dx.doi.org/10.21307/ane-2017-059

154. Thambisetty, M., An, Y., & Tanaka, T. (2013). Alzheimer's disease risk genes and the age-at-onset phenotype. *Neurobiology of aging, 34*(11), 2696-e1. https://doi.org/10.1016/j.neurobiolaging.2013.05.028

155. Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L. A., & Paylor, R. (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology, 204*(2), 361-373. https://doi.org/10.1007/s00213-009-1466-y

156. Tian, J., Gao, X., & Yang, L. (2022). Repetitive Restricted Behaviors in Autism Spectrum Disorder: From Mechanism to Development of Therapeutics. *Frontiers in Neuroscience, 16*. https://doi.org/10.3389/fnins.2022.780407

157. Torres-Lista, V., & Giménez-Llort, L. (2013). Impairment of nesting behaviour in 3xTg-AD mice. *Behavioural brain research, 247*, 153-157. https://doi.org/10.1016/j.bbr.2013.03.021

158. Torres-Lista, V., & Giménez-Llort, L. (2019). Vibrating tail, digging, body/face interaction, and lack of barbering: sex-dependent behavioral signatures of social dysfunction in 3xTg-AD mice as compared to mice with normal aging. *Journal of Alzheimer's Disease, 69*(4). https://doi.org/10.3233%2FJAD-190253

159. Torres-Lista, V., López-Pousa, S., & Giménez-Llort, L. (2015). Marble-burying is enhanced in 3xTg-AD mice, can be reversed by risperidone and it is modulable by handling. *Behavioural processes, 116,* 69-74. https://doi.org/10.1016/j.beproc.2015.05.001

160. Torres-Lista, V., López-Pousa, S., & Giménez-Llort, L. (2019). Impact of chronic risperidone use on behavior and survival of 3xTg-AD mice model of Alzheimer's disease and mice with normal aging. *Frontiers in pharmacology, 10*, 1061. https://doi.org/10.3389/fphar.2019.01061

161. Treit, D., Pinel, J. P. J., & Fibiger, H. C. (1981). Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. *Pharmacology Biochemistry and Behavior, 15*(4), 619-626. https://doi.org/10.1016/0091-3057(81)90219-7

162. Van der Jeugd, A., Parra-Damas, A., Baeta-Corral, R., Soto-Faguás, C. M., Ahmed, T., LaFerla, F. M., Giménez-Llort, L., D'Hooge, R. & Saura, C. A. (2018). Reversal of memory and neuropsychiatric symptoms and reduced tau pathology by selenium in 3xTg-AD mice. *Scientific Reports, 8*(1). https://doi.org/10.1038/s41598-018-24741-0

163. Van der Linde, R. M., Dening, T., Matthews, F. E., & Brayne, C. (2014). Grouping of behavioural and psychological symptoms of dementia. *International journal of geriatric psychiatry, 29*(6), 562-568. https://doi.org/10.1002/gps.4037

164. Waite, K., & Eickholt, B. J. (2010). The neurodevelopmental implications of PI3K signaling. *Phosphoinositide 3-kinase in Health and Disease*, 245-265. https://doi.org/10.1007/82_2010_82

165. Webster-Cordero, F., & Giménez-Llort, L. (2022). The Challenge of Subjective Cognitive Complaints and Executive Functions in Middle-Aged Adults as a Preclinical Stage of Dementia: A Systematic Review. *Geriatrics, 7*(2), 30. http://dx.doi.org/10.3390/geriatrics7020030

166. Whitehouse, C. M., & Lewis, M. H. (2015). Repetitive behavior in neurodevelopmental disorders: clinical and translational findings. *The Behavior Analyst, 38*(2), 163-178. https://doi.org/10.1007/s40614-015-0029-2

167. Wolmarans, D. W., Stein, D. J., & Harvey, B. H. (2016). Of mice and marbles: novel perspectives on burying behavior as a screening test for psychiatric illness. *Cognitive, Affective, & Behavioral Neuroscience, 16*(3), 551-560. https://doi.org/10.3758/s13415-016-0413-8

168. Wong, H., Levenga, J., LaPlante, L., Keller, B., Cooper-Sansone, A., Borski, C., Milstead, R., Ehringer, M., & Hoeffer, C. (2020). Isoform-specific roles for AKT in affective behavior, spatial memory, and extinction related to psychiatric disorders. *eLife, 9,* e56630. https://doi.org/10.7554/eLife.56630

169. Yang, S., Pascual-Guiral, S., Ponce, R., Giménez-Llort, L., Baltrons, M. A., Arancio, O., ... & Bayascas, J. R. (2018). Reducing the levels of Akt activation by PDK1 knock-in mutation protects neuronal cultures against synthetic amyloid-beta peptides. *Frontiers in aging neuroscience, 9*, 435. https://doi.org/10.3389/fnagi.2017.00435

170. Yohn, N. L., & Blendy, J. A. (2017). Adolescent chronic unpredictable stress exposure is a sensitive window for long-term changes in adult behavior in mice. *Neuropsychopharmacology, 42*(8), 1670-1678. https://doi.org/10.1038/npp.2017.11

171. Yunusa, I., & El Helou, M. L. (2020). The use of risperidone in behavioral and psychological symptoms of dementia: a review of pharmacology, clinical evidence, regulatory approvals, and off-label use. *Frontiers in Pharmacology, 11*, 596. https://doi.org/10.3389/fphar.2020.00596

172. Zhang, J., Xue, R., Li, Y. F., Zhang, Y. Z., & Wei, H. W. (2020). Anxiolytic-like effects of treadmill exercise on an animal model of post-traumatic stress disorder and its mechanism. *The Journal of Sports Medicine and Physical Fitness, 60*(1), 172-179. https://doi.org/10.23736/S0022-4707.20.10120-8

173. Zhang, Q., Yang, C., Liu, T., Liu, L., Li, F., Cai, Y., ... & Fan, X. (2018). Citalopram restores short-term memory deficit and non-cognitive behaviors in APP/PS1 mice while halting the advance of Alzheimer's disease-like pathology. *Neuropharmacology, 131*, 475-486. https://doi.org/10.1016/j.neuropharm.2017.12.021

174. Zurashvili, T., Cordón-Barris, L., Ruiz-Babot, G., Zhou, X., Lizcano, J. M., Gómez, N., Giménez-Llort, L., & Bayascas, J. R. (2013). Interaction of PDK1 with phosphoinositides is essential for neuronal differentiation but dispensable for neuronal survival. *Molecular and cellular biology, 33*(5), 1027–1040. https://doi.org/10.1128/MCB.01052-12

Supplementary Material

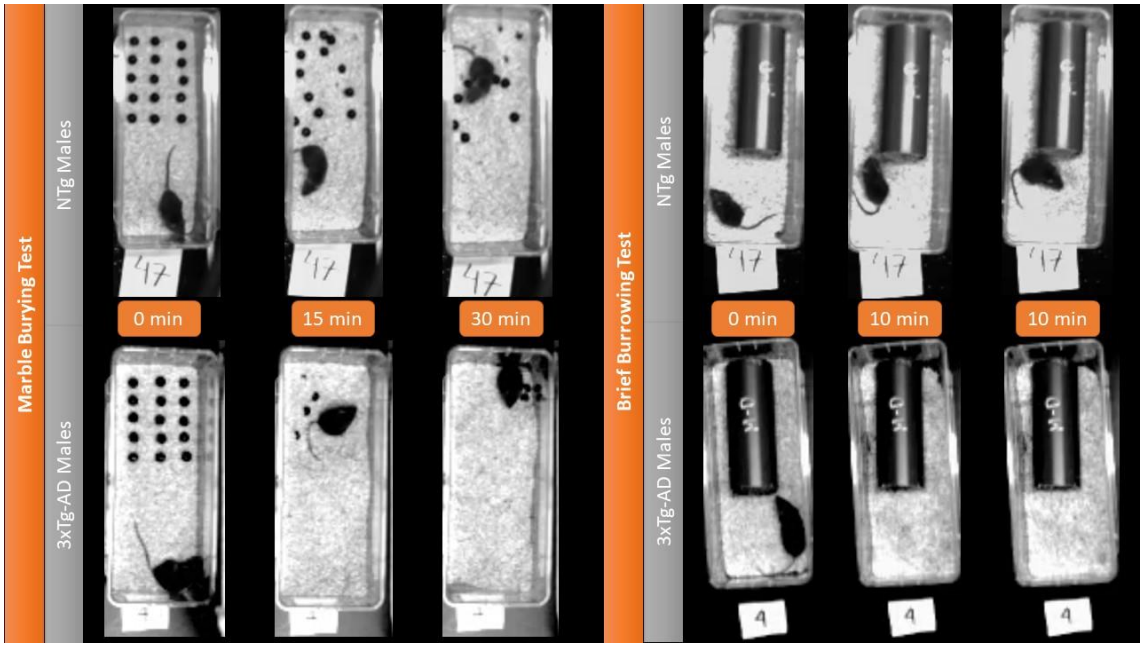

Figure 5. Temporal Progression of Marble Burying Test and Brief Burrowing Test of a 3xTg-AD male mouse and a Non-trangenic (NTg) male mouse.

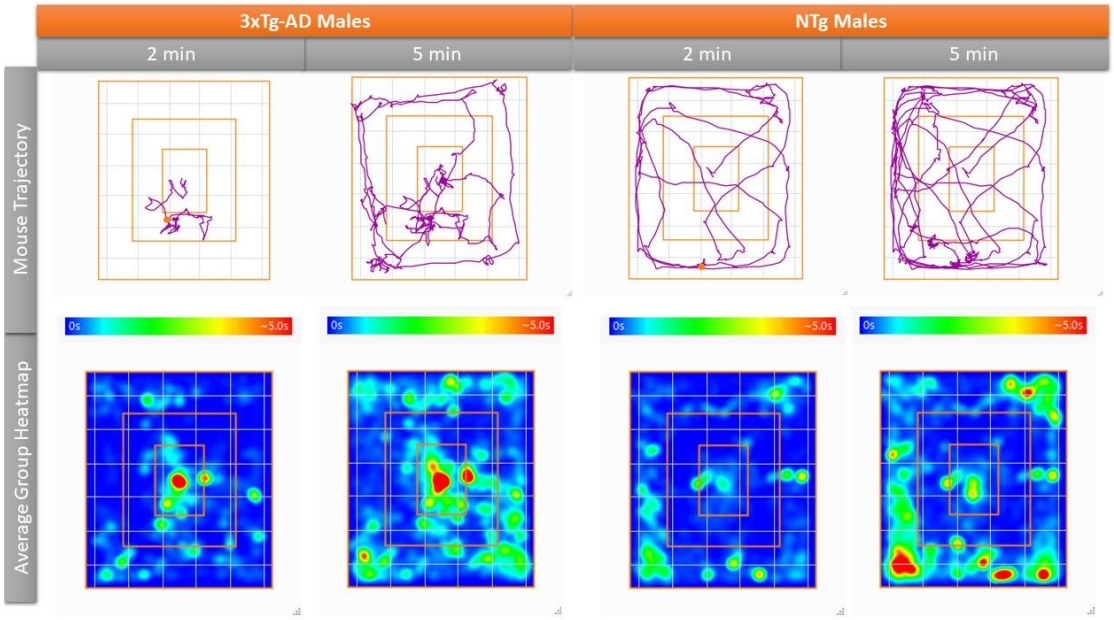

Figure 6. 3xTg-AD and Non-transgenic (NTg) male mice behavioral pattern in the Open Field Test. The figure shows an individual trajectory at both 2 minutes and 5 minutes. The average group heatmap is reported over the same period.

Publications

Study 1.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 08 May 2020
doi: 10.3389/fnbeh.2020.00061

The Impact of the PI3K/Akt Signaling Pathway in Anxiety and Working Memory in Young and Middle-Aged PDK1 K465E Knock-In Mice

Lydia Giménez-Llort[1,2]*, *Mikel Santana-Santana*[1,2] and *José Ramón Bayascas*[1,3]

[1]Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain, [2]Department of Psychiatry and Forensic Medicine, School of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain, [3]Department of Biochemistry and Molecular Biology, School of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain

OPEN ACCESS

Edited by:
Djoher Nora Abrous,
Université de Bordeaux, France

Reviewed by:
Wenqiang Chen,
Joslin Diabetes Center, Harvard Medical School, United States
Pedro Bekinschtein,
CONICET Institute of Cognitive and Translational Neuroscience (INCYT), Argentina

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

Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: 20 January 2020
Accepted: 30 March 2020
Published: 08 May 2020

Citation:
Giménez-Llort L, Santana-Santana M and Bayascas JR (2020) The Impact of the PI3K/Akt Signaling Pathway in Anxiety and Working Memory in Young and Middle-Aged PDK1 K465E Knock-In Mice. Front. Behav. Neurosci. 14:61. doi: 10.3389/fnbeh.2020.00061

Dysfunction and dysregulation at the genetic, neural, and behavioral levels point at the fine-tuning of broadly spread networks as critical for a wide array of behaviors and mental processes through the life span. This brain-based evidence, from basic to behavioral neuroscience levels, is leading to a new conceptualization of mental health and disease. Thus, the Research Domain Criteria considers phenotypic differences observed among disorders as explained by variations in the nature and degree of neural circuitry disruptions, under the modulation of several developmental, compensatory, environmental, and epigenetic factors. In this context, we aimed to describe for the first time the *in vivo* behavioral impact of tweaking the PI3K/Akt signaling pathway known to play an essential role in the regulation of cellular processes, leading to diverse physiological responses. We explored the effects in young (YA, 3–4 months of age) and mature (MA, 11–14 months of age) male and female PDK1 K465E knock-in mice in a battery of tests under different anxiogenic conditions. The results evidenced that the double mutation of the PDK1 pleckstrin homology (PH) domain resulted in an enhancement of the negative valence system shown as an increase of responses of fear- and anxiety-like behaviors in anxiogenic situations. Interestingly, this seemed to be specific of YA and found regulated at middle age. In contrast, cognitive deficits, as measured in a spatial working memory task, were found in both YA and MA mutants and independently of the level of their anxious-like profiles. These distinct age- and function-dependent impacts would be in agreement with the distinct cortical and limbic deficits in the Akt signaling in the brain we have recently described in these same animals. The elicitation of age- and neuronal-dependent specific patterns suggests that fine-tuning the intensity of the PKB/Akt signal that enables diverse physiological response has also its *in vivo* translation into the negative valence system and age is a key regulatory factor.

Keywords: RDoC, PI3K/Akt, signaling pathway, anxiety, cognition, animal model, aging, fine tuning

INTRODUCTION

The understanding of the age-dependent expression of psychiatric symptoms such as anxiety still demands important efforts to unveil and scrutinize its biological and environmental basis through the life span. In the last decade, a new consideration of psychopathology is discussed in terms of dysregulation and dysfunction in essential aspects of behavior based on basic neuroscience and behavioral science research (nimh.nih.gov/research/research-funded-by-nimh/rdoc/index.shtml). The negative valence system (NVS; primarily responsible for responses to aversive situations or context, such as fear, anxiety, and loss) and cognitive system are two of the five tentative domains convened by the National Institute of Mental Health (2020) in its Research Domain Criteria (RDoC) matrix. This matrix results from the crossword between behavioral dimensions or functional constructs and seven different levels of analysis: from genes, molecules, cells, and neuronal circuits to physiology, behavior, and self-report (Asher, 2010). In this context, while basic research on signal transduction is providing a refined close examination of the impact of cell membrane receptors and second messengers on cellular biochemistry and physiology, its downstream actions may be more difficult to characterize even more when related to age. For instance, the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which has been widely involved in controlling neuronal development and function (Waite and Eickholt, 2010). This pathway transmits the extracellular signals through the 3-phosphoinositide-dependent protein kinase-1 (PDK1), an enzyme that emerged as the major transducer of PI3K actions. PDK1 activates at least 23 AGC protein kinase family members besides Akt, the most popular effector of the pathway (Mora et al., 2004; Pearce et al., 2010). Its functional role in cellular biochemistry and physiology as well as cancer and metabolism has been largely explored; however, bottom-up approaches to elucidate the impact of dysfunctional PI3K/Akt signaling in behavior or how it differs through the aging process are still scarce.

The behavioral readout resulting from modulation of the different sites of the PDK1 enzyme is currently studied using new engineered animal models targeting key functional protein domains (Bayascas, 2008). Hypomorphic PDK1 mice, with a reduced general activity of PDK1, showed several behavioral differences related to anxiety and exploration in various tests (Leibrock et al., 2013). We previously generated neuronal-specific conditional knock-in mice in which the expression of the PDK1 L155E mutant form was targeted to neuronal tissues by means of a Nestin-Cre-driven system (Tronche et al., 1999). Disrupting the substrate docking site in PDK1 resulted in the altered activation of several AGC kinases, but intact Akt activation (Cordón-Barris et al., 2016). These animals showed altered activation of several AGC kinases, but intact Akt activation. Their phenotype was characterized by a smaller body size and showed sensorimotor problems, exacerbated disruptive behavior, and cognitive deficits. Oppositely, the PDK1-K465E pleckstrin homology (PH) domain knock-in mice (hereinafter PDK1$^{-/-}$) just have a mutation in the PH domain, only affecting the phosphorylation of PKB/Akt isoforms, but leaving intact activation of the other AGC kinase family members (Bayascas et al., 2008). These mice present a smaller body size and insulin resistance, but their behavioral phenotype and its changes through age are still unknown (Bayascas et al., 2008).

Therefore, the present study was aimed to explore the behavioral phenotype of the PDK1$^{-/-}$ PH domain knock-in mice. We recently reported that the deficits in the Akt signaling are pronounced both in the cortex and the hippocampus during young adulthood (3–4 months of age), but tend to be attenuated by middle age (11–14 months of age) in these mutant mice (Yang et al., 2018). In the present study, the behavioral and functional phenotype screening of these animals was analyzed. We used a battery of four unconditional tests differing on the levels of fear and anxiety and where the sequence of behavioral events that are successively developed in an action program (Lát, 1973) involves cognitive function. Spontaneous exploratory behavior, emotionality, bizarre and anxiety-like behaviors, habituation, as well as working memory were evaluated to address the negative valence and cognitive systems involved.

MATERIALS AND METHODS

Analysis of the PDK1 K465E Mutation

Mutation of Lys465 in PDK1, which forms key interactions with the D3 and D4 phosphates of PtdIns(3, 4, 5)P_3, to a Glu residue abolished the binding of PDK1 to phosphoinositides and localization at the plasma membrane (Komander et al., 2004). Prior to generating a knock-in mutation, the structure of the isolated PDK1(K465E) mutant PH domain was crystallized and analyzed to ensure that this mutation did not disrupt the overall PH domain fold. The mutant protein was produced in bacteria and expressed with yields similar to those for the wild-type PDK1 PH domain. The overall structure of the PDK1(K465E) PH domain was unaffected by the mutation of Lys465, except for changes observed in the side-chain conformations of residues located in the PtdIns(3,4,5)P_3-binding pocket. The K465E mutation also significantly reduced the positively charged nature of the ligand-binding interface, which accounts for its inability to bind to phosphoinositides (Komander et al., 2004).

Generation of PDK1$^{K465E/K465E}$ Mice and Genotyping Analysis

The generation and the genotyping of the PDK1$^{K465E/K465E}$ knock-in mice expressing the single-amino acid substitution of lysine 465 to glutamic acid in the PDK1 PH domain were described previously (Bayascas et al., 2008). The mice were subjected to PCR genotyping of the genomic DNA isolated from ear biopsy using primers K465E F (5′-GGG TGA AGC ATG GAA TCT GTG TCT T) and K465E R (5′-GCC AGG ATA CCT AAG AGT ACC TAG AA). PCR amplification resulted in a 196-bp product from the wild-type allele and a 236-bp product from the targeted allele.

Animals

A total of 62 mice, PDK1$^{-/-}$ ($n = 42$) and PDK1$^{+/+}$ [also referred to as wild type (WT), $n = 20$], including a pool of both sexes (50%)

and two maturation ages, YA (3- to 4-month-old young adults, n = 19) and MA (11- to 14-month-old mature adults, n = 43), were used.

Mice were maintained at the Animal House Facility of the Universitat de Lleida under standard husbandry conditions (housed three to four per cage in 35 cm × 35 cm× 25-cm Macrolon cages, with food and water *ad libitum*, 22 ± 2°C, a 12-h light/dark cycle, and relative humidity of 50–60%). Behavioral assessments and data analysis were performed blind to the experiment, in a counterbalanced manner, in the light cycle, from 09:00 to 13:00 h. All procedures were in accordance with 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 the aim to reduce the number of animals used.

Behavioral Assessments

Animals were behaviorally assessed for negative valence and cognitive systems in a battery of tests composed of a corner test, an open field test, T-maze, and marble-burying test. Somatic growth, as measured by body weight, as well as sensorimotor tasks were recorded on day 0 prior to the behavioral battery of tests in order to monitor possible confounding factors. A graphical abstract, also including the conclusions, illustrates the methodological settings and procedures (**Figure 1**).

Day 0: Reflexes and Sensorimotor Tasks

Visual reflex and posterior leg extension reflex were measured three times by holding the animals by the tail and slowly lowering them toward a black surface. Motor coordination and equilibrium were assessed twice (20-s trials) in two consecutive road tasks of increasing difficulty. The distance covered and the latency to fall off a wooden (1.3-cm width) and a metal wire (1-cm diameter) rods (both 1 m long) were recorded.

Day 1: Corner Test and Open Field Test

Corner test (CT) was used to evaluate neophobia. The animals were individually placed in the center of a clean standard home cage filled with wood shave bedding and observed for 30 s. We measured the numbers of corners visited (CTc), the latency to realize the first rearing (CTlatR), and the number of rearings (CTr). Once they completed the CT, the mice were placed in the center of an illuminated (20 lx) open field (homemade woodwork, white box, 55 cm × 55 cm × 25 cm) and observed for 5 min. First, the ethogram of action programs (sequence of behavioral events) was analyzed. Thus, the duration of freezing behavior (latM, latency of movement) and the latency of the

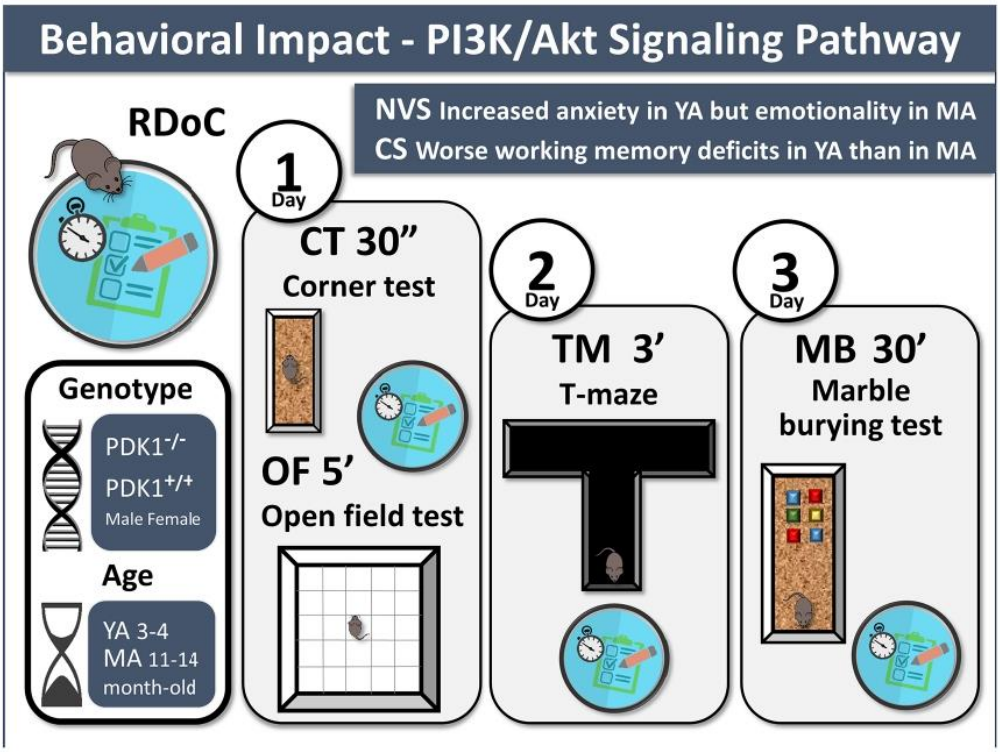

FIGURE 1 | Graphical abstract. Behavioral impact of the PI3K/Akt signaling pathway. Experimental design: 3-days battery of four behavioral tests to assess the Research Domain Criteria (*RDoC*), negative valence system (*NV*), and cognitive system (*CS*) in PDK1$^{-/-}$ and PDK1$^{+/+}$ mice at young age (*YA*) and mature age (*MA*) are illustrated. Main findings are also indicated.

behavioral events that follow to it were recorded: leaving the central square (latC), reaching the periphery (thigmotaxis, latP), performing first wall rearing (latR), and first grooming (latG). Second, the time course and total levels of exploratory activity were measured as horizontal (C, number of crossings) and vertical (Rw, rearing with wall support) locomotor activity. Third, variables of emotionality included the number of defecations (Def), the presence of urine (Ur), and the grooming behavior, through its number (nG), latency (latG), and its total time (tG). Finally, as previously described (Baeta-Corral and Giménez-Llort, 2014), we evaluated the presence of the following bizarre behaviors: stereotyped rearings without wall support (Rc), recoils (Re), scratching (Sc), turning (T), stretch attendance (Sa), jumping (Ju), and jerks (Jk). Bursting (grooming with a pattern broken in its first step, where the animal is only washing hands) was measured through its number (nB), latency (latB), and total time (tBG).

Day 2: T-Maze

Spontaneous alternation of mice was tested in a T-shaped maze (with arms 25 cm in length). The animals were placed inside the "vertical" arm of the maze with the head facing the end wall. The performance was evaluated by determining with a chronometer the time elapsed until the animal crossed (four-paw criteria) the intersection of thee three arms (latT) and the time to complete the test (latF). The task finished when the mice visited the two arms of the maze. The entry of an already visited arm in the trial before completing the test was considered an error (eT).

Day 3: Marble-Burying Test

The procedure used was as previously described (Torres-Lista et al., 2015). The mice were placed individually facing the wall in a standard home cage with six glass marbles (1 cm × 1 cm × 1 cm) on a 5-cm-thick layer of clean woodcuttings. The marbles where spaced in three rows of two marbles per row in one half of the cage. The mice were left in the cage with marbles for a 30-min period, after which the test was terminated by removing the mice. The number of marbles buried, changed of position (partially buried or turned), and left intact (I) were measured.

Statistics

Statistical analyses were performed using SPSS 15.0 software. All data are presented as the mean ± SEM and illustrated as bars (pooled data by genotype or age) or as dots that illustrate the individual values in each group segregated by sex and/or age, as indicated in the legends. To evaluate the effects of genotype (G) and age (A) group, a 2 × 2 factorial analysis design was applied. Differences were studied through multivariate general linear model analysis, followed by *post hoc* Sidak test comparisons, when possible. For categorical variables, the Fisher's exact test with 2 × 2 and 4 × 2 designs was used. Spearman's correlation analyzed the body size and behavioral correlates. Graphics were made with GraphPad Prism 6. To explore possible hints of sexual differences, males (squares) and females (circles) were represented with different symbols in the graphics. P-value < 0.05 was considered as statistically significant.

RESULTS

Somatic Growth/Body Weight

The body weights of the animals (in grams)—YA PDK1$^{-/-}$ mice, 18.67 ± 1.29; MA PDK1$^{-/-}$ mice, 22.90 ± 0.84; YA PDK1$^{+/+}$ mice, 28.14 ± 1.92; YA PDK1$^{+/+}$ mice, 32.00 ± 2.47—showed genotype and age effects (G: $F_{(1,58)} = 30.692$, $P = 0.000$; A: $F_{(1,58)} = 5.822$, $P = 0.019$), with lower body weight of PDK1$^{-/-}$ mice as compared to the age-matched WT and also for younger animals compared to mature animals. *Post hoc* comparisons showed a higher body weight in WT mice as compared to PDK1$^{-/-}$ in both ages ($P = 0.001$ and $P = 0.000$ for YA and MA, respectively). Only PDK1$^{-/-}$ MA exhibited a heavier body weight than their genotype-matched YA counterparts ($P = 0.036$).

Reflexes and Sensorimotor Tasks

No statistical differences of any type where found in the visual task. All the mice obtained the maximum score possible. In the motor tasks, no genotype differences were found in any of them. Only, an age effect was observed in the second of two trials of the metal rod, with YA mice being able to maintain the equilibrium in the road during more time than did the MA counterparts, as expected for their age-related lower weight.

Corner Test

Figures 2A,B illustrate the horizontal and vertical exploratory behaviors in the test for neophobia. The 2 × 2 GLM analysis showed genotype effects in the vertical activity, with increased latency of first rearing (G: $F_{(1,58)} = 8.350$, $P = 0.005$) and consequent reduction in the number of rearings (G: $F_{(1,58)} = 6.367$, $P = 0.014$) in the PDK1$^{-/-}$ mice vs. the WT (**Figure 2B**). Strong age effects also appeared, with higher number of corners visited (A: $F_{(1,58)} = 20.719$, $P = 0.000$) and higher latency of first rearing (A: $F_{(1,58)} = 7.481$, $P = 0.008$) in the MA vs. the YA group. Besides, a genotype × age interaction effect was found in the latency of rearing (G × A: $F_{(1,58)} = 4.072$, $P = 0.048$) since the effect was mostly due to an increase in the PDK1$^{-/-}$ YA group.

The *post hoc* comparisons analysis of the number of visited corners indicated that the performances of both YA PDK1$^{-/-}$ ($P = 0.000$) and YA PDK1$^{+/+}$ ($P = 0.017$) were lower than those of their respective older age groups. Also, YA PDK1$^{-/-}$ mice showed lower number of rearings than their older MA PDK1$^{-/-}$ group ($P = 0.009$) and as compared to the age-matched WT mice ($P = 0.009$) due to an increased latency ($P = 0.000$ and $P = 0.004$, respectively).

Open Field Test

Figures 3, **4** depict the main behavioral domains, events, and units of analysis in the open field test, showing a distinct performance on PDK1$^{-/-}$ compared to the WT groups. Although no differences were found in the latency of

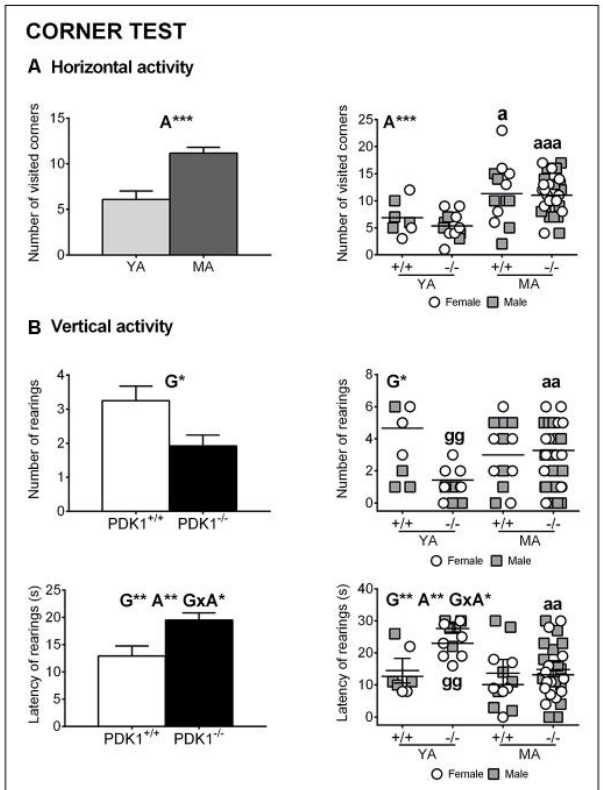

FIGURE 2 | Exploratory behaviors in the corner test in young adult (*YA*) and mature age (*MA*) PDK1$^{-/-}$ and age-matched wild-type (*WT*, PDK1$^{+/+}$) mice. Data are expressed as mean ± SEM. *Bars* illustrate the age or the genotype groups, as indicated in the *abscissae*. *Symbols* are used to illustrate individual values of females (*white circles*) and males (*gray squares*) of each genotype and age group (*YA* and *MA*), as indicated in the *abscissae*. Factorial analysis: *G*, genotype effect; *A*, age effect; *G*×*A*, genotype × age interaction effects, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *Post hoc* test: genotype (*g*), $^{gg}P < 0.01$ vs. the corresponding WT (PDK1$^{+/+}$) group; age (*a*), $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$ vs. the corresponding YA of the same genotype.

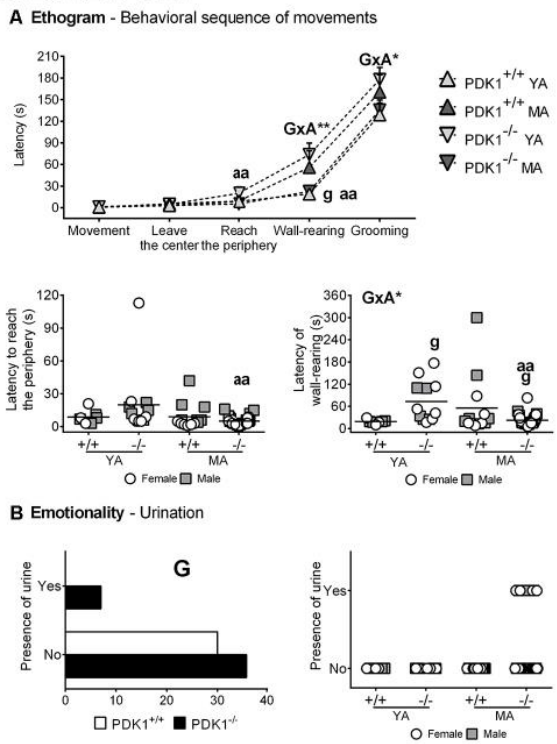

FIGURE 3 | Ethogram and emotionality in the open field test in young adult (*YA*) and mature age (*MA*) PDK1$^{-/-}$ and age-matched wild-type (*WT*; PDK1$^{+/+}$) mice. Data are expressed as the mean ± SEM or incidence. *Bars* illustrate the genotype groups, as indicated in the *Y*-axis. *Symbols* are used to illustrate the different groups or individual values, as depicted in the *legends* or *abscissae*. Factorial analysis: *G*, genotype effect; *A*, age effect, $^{*}P < 0.05$, $^{**}P < 0.01$. *Post hoc* test: genotype (*g*), $^{g}P < 0.05$ vs. the corresponding WT (PDK1$^{+/+}$) group; age (*a*), $^{aa}P < 0.01$ vs. the corresponding YA of the same genotype.

first movement or latency to leave the center (**Figure 3A**), bizarre behaviors (**Figure 4C**), scarcely elicited in the WT, were conspicuously observed in the PDK1$^{-/-}$ mice. Thus, an increased number of stretch attendance was found in the PDK1$^{-/-}$ genotype (G: $F_{(1,58)} = 5.460$, $P = 0.023$; **Figure 4C**). A genotype main effect was found in the number of wall rearings in the second minute (G: $F_{(1,58)} = 6.126$, $P = 0.016$; **Figure 4B**), with a decreased number for the PDK1$^{-/-}$ mice. Genotype × age interaction effects were shown in the latency (G × A: $F_{(1,58)} = 10.796$, $P = 0.002$; **Figure 3A**) and number of wall rearings in the first minute of the test ($F_{(1,58)} = 4.595$, $P = 0.036$; **Figure 4B**), as well as in the latency of grooming ($F_{(1,58)} = 4.139$, $P = 0.046$; **Figure 3A**).

The *post hoc* comparisons analysis showed that the ethogram and variables of analysis of YA PDK1$^{-/-}$ mice were indicative of a worse performance as compared to their older MA PDK1$^{-/-}$ counterparts. Thus, YA PDK1$^{-/-}$ exhibited more stretch attendances ($P = 0.014$), reached the periphery later ($P = 0.004$), exhibited longer latency for wall rearing ($P = 0.002$), and a consequent low number of this behavior in the first minute of the test ($P = 0.013$). As compared to the age-matched WT animals, the stretch attendance of YA PDK1$^{-/-}$ was also higher ($P = 0.023$) and the latency of wall rearing was delayed ($P = 0.016$), resulting in a lower number of rearing during the first ($P = 0.028$) and the second minute ($P = 0.020$) of the test and, consequently, a lower total number of rearings ($P = 0.031$). At mature ages, MA PDK1$^{-/-}$ exhibited faster apparition of rearing ($P = 0.034$) as compared to their age-matched WT counterparts. Finally, emotionality, as measured by the presence or not of urine and defecations, was analyzed by Fisher's exact test with 2 × 2 and 4 × 2 designs. Only for the urination were the two designs significant ($P = 0.01458$ and $P = 0.032$, respectively). The detailed representation per group hints that the enhanced urination in PDK1$^{-/-}$ mice was mostly due to MA PDK1$^{-/-}$ mice.

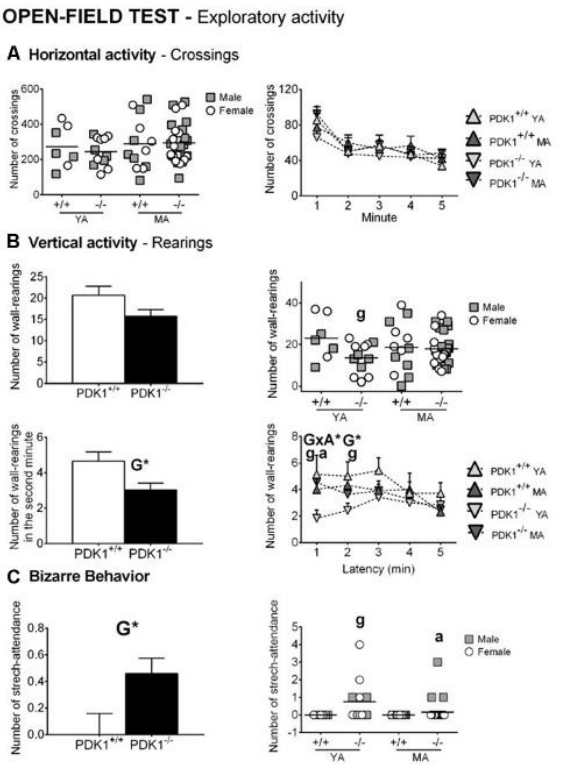

FIGURE 4 | Exploratory and bizarre behaviors in the open field test in young adult (*YA*) and mature age (*MA*) PDK1$^{-/-}$ and age-matched wild-type (*WT*; PDK1$^{+/+}$) mice. Data are expressed as the mean ± SEM. *Bars* illustrate the genotype group, as indicated in the *abscissae*. *Symbols* are used to illustrate the different groups or individual values, as depicted in the *legends* or *abscissae*. Factorial analysis: *G*, genotype effect, $^{*}P < 0.05$. *Post hoc* test: genotype (*g*), $^{g}P < 0.05$ vs. the corresponding WT (PDK1$^{+/+}$) group; age (*a*), $^{a}P < 0.05$ vs. the corresponding YA of the same genotype.

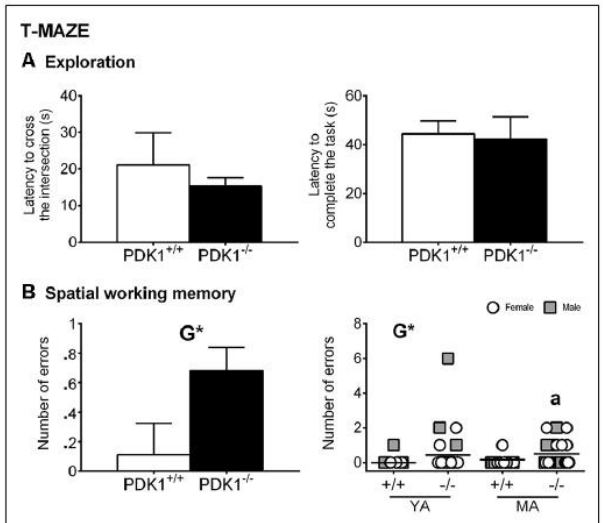

FIGURE 5 | Spontaneous alternation in the T-maze in young adult (*YA*) and mature age (*MA*) PDK1$^{-/-}$ and age-matched wild-type (*WT*; PDK1$^{+/+}$) mice. Data are expressed as the mean ± SEM. *Symbols* are used to illustrate individual values of females (*white circles*) and males (*gray squares*) for each genotype and age group. Factorial analysis: *G*, genotype effect, $^{*}P < 0.05$. *Post hoc* test: age (*a*), $^{a}P < 0.05$ vs. the corresponding YA of the same genotype.

Spontaneous Alternation in the T-Maze

Although the latency to cross the intersection of thee three arms and the latency to complete the task were similar among all groups, a trend of reduced exploratory activity was shown by PDK1$^{-/-}$ mice (**Figure 5A**). A genotype effect was found in the number of errors, with PDK1$^{-/-}$ mice exploring more visited arms (G: $F_{(1,58)} = 4.652$, $P = 0.035$; **Figure 5B**) than did the WT mice, where the incidence of errors was $n = 1$ for each age group. The *post hoc* comparisons analysis also showed an increased number of errors in the YA PDK1$^{-/-}$ as compared to its older genetic counterparts ($P = 0.048$; **Figure 5B**, right).

Marble Burying Test

Analysis of the interaction with marbles (**Figure 6**) pointed at age effects, with a reduced number of marbles buried (A: $F_{(1,58)} = 7.481$, $P = 0.009$) and an increase in those left intact (A: $F_{(1,58)} = 4.967$, $P = 0.030$) in the MA groups as compared to YA mice (**Figure 6A**). The *post hoc* comparisons analysis revealed that this age pattern was more evident in the PDK1$^{-/-}$ genotype, where MA PDK1$^{-/-}$ mice scarcely buried marbles as compared to their younger genetic counterparts ($P = 0.002$; **Figure 6B**).

Body Weight and Behavioral Correlations

Spearman's correlations between and within-test variables are detailed in **Supplementary Table S1** and summarized as follows. The body weight of the WT was correlated with the latency of rearing in the CT ($r = 0.558$, $P = 0.011$), while in the PDK1$^{-/-}$ genotype and the total sample of mice, weight was negatively correlated with the number of marbles buried ($r = -0.312$, $P = 0.044$ and $r = -0.326$, $P = 0.01$, respectively). A part of the inter-test correlations between the different variables of a test, behavioral variables of the corner and the open field test were highly correlated and confirmed the results. Neophobia in the corner test strongly predicted the subsequent behavioral ethogram (negative correlation with latencies: all $r < -0.345$, $P = 0.006$), the vertical activity in the open field test (OF; positive correlations with wall rearing in the first 2 min of the test), and the performance in the marble test ($r = -0.326$, $P = 0.01$). Similarly, neophobia in the corner and open field tests (ethogram) was correlated with the ethogram of behavioral events in the T-maze (all $r > 0.253$, $P = 0.048$). The number of errors in the T-maze was correlated with the time invested to explore the test ($r = 0.528$, $P = 0.000$) and the latency of rearing in the corner test ($r = 0.253$, $P = 0.048$). Finally, we propose an illustration of meaningful correlations as wheels (see **Figure 7**), where distinct fine-tuning of PKI3 signaling in PDK1$^{-/-}$ and WT can be visually observed in an easier manner.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

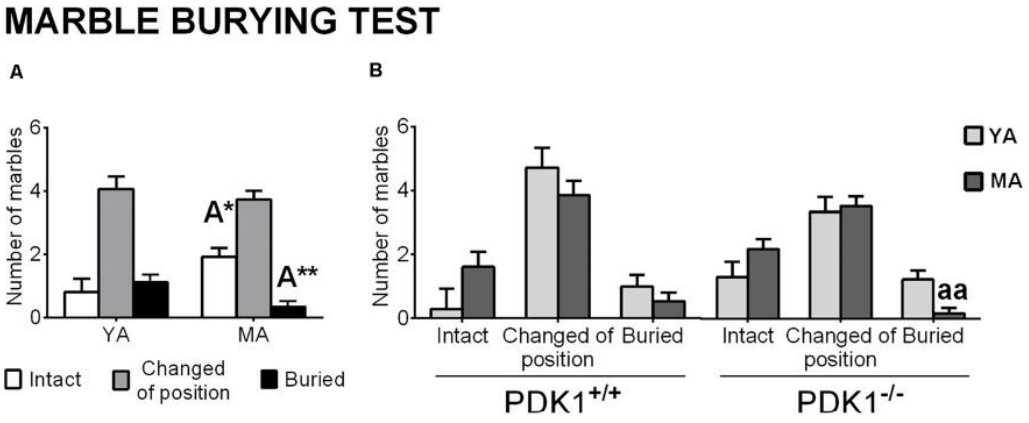

FIGURE 6 | Marble burying in young adult (*YA*) and mature age (*MA*) PDK1$^{-/-}$ and age-matched wild-type (*WT*; PDK1$^{+/+}$) mice. Data are expressed as the mean ± SEM. *Bars* are used to illustrate the different levels of marble burying, as depicted in the *abscissae*. +/+, PDK1$^{+/+}$ genotype group; −/−, PDK1$^{-/-}$ genotype group. Factorial analysis: *A*, age effect, $^{*}P < 0.05$, $^{**}P < 0.01$. *Post hoc* test: age (*a*), $^{aa}P < 0.01$, vs. the corresponding YA of the same genotype.

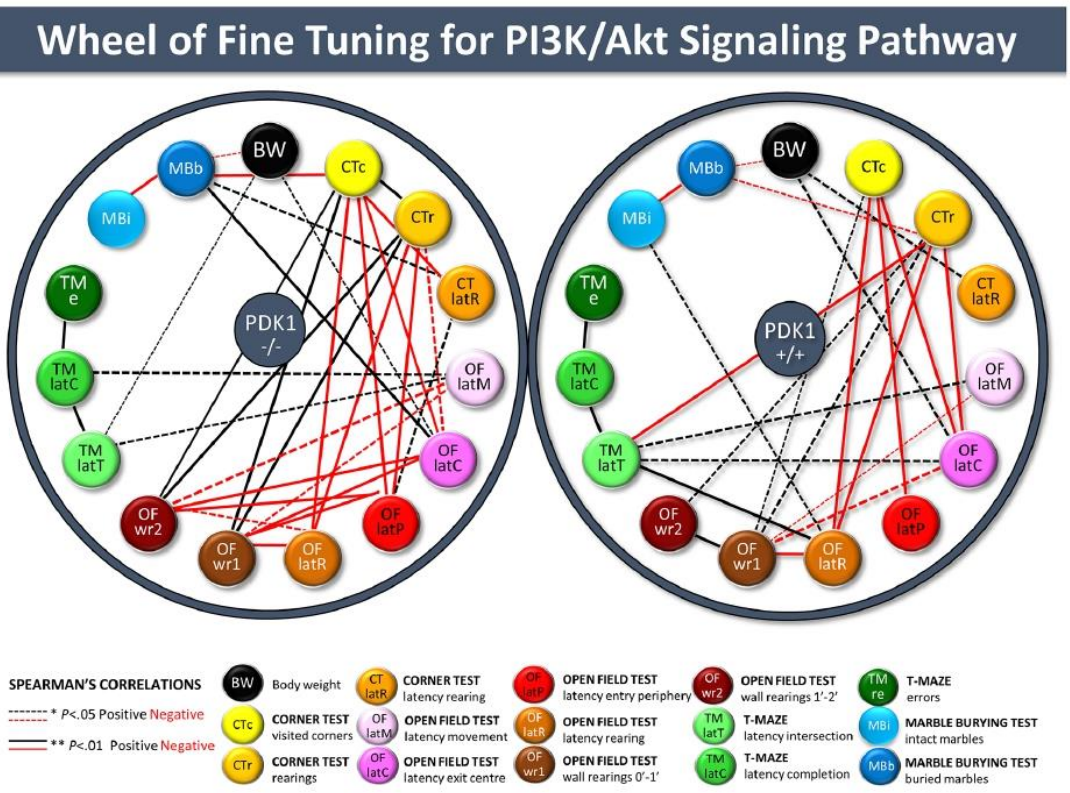

FIGURE 7 | Wheels of fine-tuning for the PI3K/Akt signaling pathway. Illustration of the meaningful behavioral correlates among variables of the different tests in the whole sample of animals, PDK1$^{-/-}$ and age-matched wild-type (*WT*; PDK1$^{+/+}$) mice. Spearman's correlation analyzed the relationship between behavioral variables of the four behavioral tests and with somatic growth (body weight). $^{*}P$ value < 0.05 was taken as statistically significant. Positive (*black*) and negative (*red*) statistically significant correlations are plotted, with *thickness of the dotted* ($^{*}P < 0.05$) and *unbroken* ($^{**}P < 0.01$) *lines* being proportional to the statistically significant value of the correlation.

DISCUSSION

Brain-based evidence on the impact of dysregulation and dysfunction, from basic neuroscience to behavioral levels, is leading to a new conceptualization of mental health and disease. Thus, the strategic plan of NIMH is "to develop new ways of classified disorders based on dimensions of observable behaviors and brain functions." Far from due to discrete etiologies, the RDoC Project considers phenotypic differences observed among disorders as explained by variations in the nature and degree of neural circuitry disruptions, under the modulation of several developmental, compensatory, environmental, and epigenetic factors (Hyman, 2007; Morris and Cuthbert, 2012). Here, we can now also add "and age/aging factors." Thus, dysfunction and dysregulation at the genetic, neural, and behavioral levels point at the fine-tuning of broadly spread networks as critical for a wide array of behaviors and mental processes (Morris and Cuthbert, 2012). In this context, tweaking of the PI3K/PDK1/Akt signaling pathway in the PDK1 K65E knock-in mice unveiled thresholds for its essential role in the regulation of cellular processes, leading to diverse physiological responses (Zhou et al., 2014).

PI3K plays fundamental roles in regulating virtually every physiological process related to cell growth, proliferation, survival, and metabolism, where Akt was meant for decades to represent the major, and perhaps unique, effector mediating these cellular responses. However, since it was first identified as the apical Akt kinase, PDK1 emerged as a major transducer of PI3K actions by regulating a number of AGC kinase family members besides Akt, including S6K, RSK, SGK, and PKC isoforms (Mora et al., 2004). The essentiality of this enzyme has been widely demonstrated by the severe phenotypes reported in different PDK1-deficient mice models (reviewed in Bayascas, 2010). Thus, the PDK1/Akt pathway has been reported as intermediating in the role of neurotropic factors, like brain-derived neurotrophic factor (BDNF), in the acquisition of fear and mood disorders like depression and anxiety (Chen et al., 2006; Duman and Monteggia, 2006; Ou and Gean, 2006; Martinowich et al., 2007). The reduced general activity of PDK1 exhibited by the hypomorphic PDK1 mice (PDK1hm) results in several behavioral differences related to anxiety and exploration assessed in various tests (Ackermann et al., 2008), while cognitively unimpaired Akt2 knockout mice ($Akt2^{-/-}$) presented an anxiety- and depressive-like phenotype (Leibrock et al., 2013). More recently, the increase of Akt phosphorylation has been implicated in the rapid antidepressant-like effects of different drugs in the basolateral amygdala, hippocampus, and the prefrontal cortex (Shi et al., 2012; Wang et al., 2015; Tao et al., 2016). Conversely, mice with high anxiety-related behaviors showed a stronger acquisition, slower extinction, and spontaneous recovery of learned fear that coincide with enhanced phosphorylation of Akt in the amygdala (Yen et al., 2012).

Biochemical and structural data permitted the rational design of two new PDK1 single-amino acid mutations within either the PH domain, namely PDK1 K465E, or the PIF-pocket domain, termed L155E, impeding the activation of Akt or substrates other than Akt, respectively. Phenotypic characterization of two knock-in mouse strains expressing each of these mutations allowed narrowing down the contribution of Akt compared to the complementary PDK1 branch in mediating PI3K actions (Bayascas et al., 2008). The PDK1 K465E mice with reduced activations were viable and exhibited no adverse phenotypes other than being smaller (Bayascas et al., 2008; Zurashvili et al., 2013). In contrast, the PDK1 L155E mice were embryonically lethal, and Cre-mediated bypass of the lethal period by targeting the expression of the mutant PDK1 L55E protein to the brain led to neurodevelopmental disorders and disrupted behavior, thereby highlighting the importance of the Akt-independent actions of this signaling toolkit regarding brain development and function (Bayascas et al., 2008). Nevertheless, in spite of the absence of overt phenotypes, the behavioral consequences of PDK1 K465E knock-in mutation and Akt activity ablation were never explored.

The present work describes, for the first time, the *in vivo* effects of PDK1 mutation in the PH domain. We studied a set of male and female $PDK1^{-/-}$ PH-domain transgenic ($PDK1^{-/-}$) mice at two stages of adult maturation—young adulthood and middle age—and as compared to age-matched WT mice with normal aging. We have recently shown, at the biochemical level, that these animals exhibited pronounced deficits in Akt signaling both in the cortex and the hippocampus during young adulthood, but tend to be attenuated at middle age (Yang et al., 2018). Here, behavioral and functional screening for negative valence systems and cognitive systems indicated that the deregulation of this signaling pathway also has a higher impact at young adulthood, as shown by their increased anxiety-like behavioral profile and working memory deficits, while changes in middle-aged animals were found restricted to emotionality. Somatic growth, as measured by body weight, was reduced, as previously described (Bayascas et al., 2008; Zurashvili et al., 2013).

The corner test for mild neophobia to a familiar environment, such as a standard home cage with new bedding, showed delayed and reduced number of rearings in the mutant mice (mostly in the YA animals), leading to genotype differences and age interaction effects. The correlation analysis showed that both horizontal and vertical activities in the corner test were intercorrelated, mostly in the $PDK1^{-/-}$ genotype. In agreement, a similar but smoother pattern was shown in the horizontal exploratory behavior of YA $PDK1^{-/-}$, albeit it did not reach statistical significance. Differences in the horizontal activity due to the age factor were notorious, indicating lower neophobia response in middle-aged animals, independently of the genotype. This could be explained by long-term habituation to husbandry routines, reducing the chances of animals to perceive a new home cage as a potential threat.

The results of increased neophobia in the corner test were confirmed in a more anxiogenic test such as the open and illuminated field. There, YA mutants showed a change in thigmotaxis, with a delay to reach the periphery and delayed latency for wall rearings. These changes in the behavioral sequence of events of the ethogram were due to the conspicuous appearance of stereotyped stretch attendance and stereotyped

rearing in mutant mice, independently of age. These risk assessment behaviors in the open field were scarcely exhibited by 6-month-old wild-type C57BL/6 × 129Sv mice, but were found enhanced in animal models with anxious-like profiles (Komander et al., 2004; Baeta-Corral and Giménez-Llort, 2014). Their presence in those mutants, considered as bizarre behaviors triggered by the extereoceptive anxiogenic stimuli of the open and illuminated arena, was related to high levels of anxiety and was reversed by early neonatal handling (Baeta-Corral and Giménez-Llort, 2014). In the case of PDK1$^{fl/fl}$ CRE^{+} mice, middle-aged females also exhibited stereotyped stretching as part of a more severe disruptive behavior that included vocalizations and hyperactivity and refusal to be handled and tested (Cordón-Barris et al., 2016). Enhanced risk assessment or vigilance is part of the pattern of responses to potential harm (anxiety), included in the behavioral dimensions or constructs within the NVS domain together with responses to acute threat (fear) and to sustained threat, frustrative non-reward, and loss (nimh.nih.gov). PDK1$^{-/-}$ mutation enhanced the anxious responses elicited in a potentially harmful environment. Among the variables of emotionality, the latency of grooming was normal, but urination was present in the PDK1$^{-/-}$ genotype due to incidence in the middle-aged mutants.

A striking parallelism in the ethogram was found in middle-aged mutants and young adult WT, both in the temporal sequence of behavioral events and the level of their expression. The action programs described by Lát (1973), which are consecutively developed by animals when confronting a new environment, were not distinguishable of those found at young adulthood, except for the first minute/s of the test when the first action, which is related to fear, was elicited. Again, the freezing behavior of YA mutant mice evidenced an increased neophobic response, here expressed in terms of a 50% reduction of rearings during the first 2 min of the test. Freezing behavior and reduced total rearings were also shown by middle-aged female PDK1$^{fl/fl}$ CRE^{+} mice, although an overall drop of activity did not allow detecting differences in thigmotaxis (Cordón-Barris et al., 2016).

In the spontaneous alternation task in the black T-maze resembling natural burrows, an innate alternation form of a win–shift behavior of foraging in the wild was elicited. In most of the WT mice, alternation behavior was performed without errors or only once in a couple of cases. In contrast, at both ages, most mutant mice explored already visited areas of the maze, which were considered errors attributed to working memory problems and prefrontal cortex dysfunction, characteristic of schizophrenia-like patterns (Goldman-Rakic, 1994). Alternation behavior has been shown to reflect short-term habituation of responding to stimuli based on their relative familiarity because of recent exposure (Sanderson and Bannerman, 2012). Importantly, similarly to the rewarded alternation and win–shift behavior on the radial arm maze, spontaneous alternation is sensitive to hippocampal lesions (Deacon et al., 2002). The role of the prefrontal cortex is under discussion and considered by these authors (Sanderson and Bannerman, 2012) as being rather involved in other more complex and demanding goal-directed behaviors where active maintenance of information is required (Miller and Cohen, 2001). Here, again, the worse performance was mostly observable in the YA mutant mice, whose brains exhibited more pronounced deficits in Akt signaling in both the cortex and the hippocampus (Yang et al., 2018), key neuroanatomical areas for these behaviors. These cognitive deficits are interesting to note since the mutations related to the PDK1 and Akt signaling pathway, such as those in Akt2 knockout mice, presented a higher anxiety-like and depressive-like behavior, but were cognitively intact (Ackermann et al., 2008). As mentioned above, middle-aged female PDK1$^{fl/fl}$ CRE^{+} mice also presented smaller size as well as sensorimotor problems, exacerbated bizarre behavior, and short-term memory deficits in the Morris water maze (Cordón-Barris et al., 2016). Therefore, while the anxious-like patterns seem to be more sensitive to be affected, a tiny modulation and Akt signal thresholds may determine distinct levels of cognitive system dysfunction.

Similarly to the elevated T-maze (Asth et al., 2012), the T-maze can be used as an animal model to simultaneously investigate memory and anxiety in mice. Thus, the latency to reach the intersection of the arms is considered a measure of copying with stress strategy, and it is used to measure anxiety in very mild conditions (Guayerbas et al., 2000). In this respect, correlations with the variables related to neophobia in the corner and open field tests were found when the whole sample was analyzed and in each genotype. However, no differences were found between genotypes, but a trend of shorter latency to reach the intersection was shown by mutants, albeit it did not reach statistical significance.

Finally, mice were assessed in the marble burying test, which has been successfully used for the pharmacological assessment of obsessive–compulsive behaviors, anxiety, and psychosis drugs (Jimenez-Gomez et al., 2011; de Brouwer et al., 2019). The pattern, with predominance in the incidence of "changed or partially buried" marbles and equal low numbers of marbles buried and left intact, shown by all YA animals, resembled that already reported by our laboratory in middle-aged males of the standard hybrid C57BL/6 × 129sv strain (Torres-Lista et al., 2015). Here, independently of the genotype, the MA animals showed a shift to the left, with increased numbers of marbles left intact in detriment of those buried as compared to the burying pattern shown by YA animals. This age effect was more clearly expressed in the PDK1$^{-/-}$ genotype, with the reduction of burying in MA *vs*. YA mice reaching statistical significance. This drop in behavior resembled the effect obtained in wild-type mice after a chronic low 1-mg/kg, s.c, non-cataleptic dose of risperidone (Bardin et al., 2006; Baeta-Corral and Giménez-Llort, 2014). A similar trend was shown in middle-aged female PDK1$^{fl/fl}$ CRE^{+} mice, whose number of buried marbles was in detriment in favor of a statistically significant increase in the number of marbles only changed of position or half buried as compared to their age-matched wild-type counterparts (Cordón-Barris et al., 2016). With respect to the integration of the results of the marble test with the other tests, meaningful correlations were found, with the number of buried marbles being directly related to neophobia in the corner test in both WT (negative correlation

with the number of rearings in the corner test) and the PDK$^{-/-}$ (negative correlation with the number of visited corners) genotypes. This was in agreement with our previous results in middle-aged standard hybrid C57BL/6 × 129sv (Baeta-Corral and Giménez-Llort, 2014), where the number of buried marbles correlated with the latency of rearing in the corner test and the incidence of urine, the variable that was found increased in MA PDK$^{-/-}$ mice.

In summary, the double mutation of the PDK1 PH domain resulted in an enhancement of NVS, shown as an increase of responses of fear- and anxiety-like behaviors in anxiogenic situations, which seemed to be specific of young adulthood and found regulated at middle age, where only an increased emotionality was noted. In contrast, cognitive deficits, as measured in a spatial working memory task, were found in both YA and MA PDK$^{-/-}$ mice and independently of the level of their anxious-like profiles. This would be in agreement with the distinct cortical and hippocampal deficits in the Akt signaling in the brain of these animals (Bayascas et al., 2008). The present results contribute to the *in vivo* characterization of the behavioral impact of Akt signaling pathway dysfunction and support the conceptualization of qualitative and quantitative variations of neural circuit disruptions underpinning phenotypic differences that are relevant to several mental disorders. The elicitation of age-dependent specific patterns suggests that fine-tuning the intensity of the Akt signal enables not only diverse physiological responses, as we have previously demonstrated in these animals, but also their readout *in vivo*. Finally, the modulation by sex factor will deserve further exploration.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by CEEAH.

AUTHOR CONTRIBUTIONS

LG-L designed and performed the behavioral tests and illustrated the graphical abstract and the proposed wheels of correlation. MS-S constructed and analyzed the matrix of data and illustrated the results. JB provided the animals and financial support. All authors participated in the scientific discussions and contributed to the writing of the manuscript.

FUNDING

This work was funded by the Ministerio de Economía y Competitividad (MINECO), Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad (SAF2014-52813-R) to JB.

ACKNOWLEDGMENTS

We thank Jessica Pairada and Núria Moix (Estabulari de Rosegadors of the Universitat de Lleida) for animal care.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00061/full#supplementary-material.

TABLE S1 | Behavioral correlates among variables of the different test in the wholes sample of animals, PDK1$^{-/-}$ and age-matched WT (PDK1$^{+/+}$) mice. Spearman's correlation analyzed the relationship between behavioral variables of the four behavioral tests and with somatic growth (body weight).
*P-value < 0.05 was taken as statistically significant; **P-value < 0.01.

REFERENCES

Ackermann, T. F., Hörtnagl, H., Wolfer, D. P., Colacicco, G., Sohr, R., Lang, F., et al. (2008). Phosphatidylinositide dependent kinase deficiency increases anxiety and decreases GABA and serotonin abundance in the amygdala. *Cell. Physiol. Biochem.* 22, 735–744. doi: 10.1159/000185557

Asher, J. (2010). *Genes and Circuitry, not Just Clinical Observation, to Guide Classification for Research (Internet) NIMH*. Available online at: https://www.nimh.nih.gov/news/science-news/2010/genes-and-circuitry-not-just-clinical-observation-to-guide-classification-for-research.shtml. Accessed 29 February, 2020.

Asth, L., Lobão-Soares, B., André, E., Soares Vde, P., and Gavioli, E. C. (2012). The elevated T-maze task as an animal model to simultaneously investigate the effects of drugs on long-term memory and anxiety in mice. *Brain Res. Bull.* 87, 526–533. doi: 10.1016/j.brainresbull.2012.02.008

Baeta-Corral, R., and Giménez-Llort, L. (2014). Bizarre behaviors and risk assessment in 3xTg-AD mice at early stages of the disease. *Behav. Brain Res.* 258, 97–105. doi: 10.1016/j.bbr.2013.10.017

Bardin, L., Kleven, M. S., Barret-Grévoz, C., Depoortère, R., and Newman-Tancredi, A. (2006). Antipsychotic-like vs. cataleptogenic actions in mice of novel antipsychotics having D2 antagonist and 5-HT1A agonist properties. *Neuropsychopharmacology* 31, 1869–1879. doi: 10.1038/sj.npp.1300940

Bayascas, J. R. (2008). Dissecting the role of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) signalling pathways. *Cell Cycle* 7, 2978–2982. doi: 10.4161/cc.7.19.6810

Bayascas, J. R. (2010). PDK1: the major transducer of PI 3-kinase actions. *Curr. Top. Microbiol. Immunol.* 346, 9–29. doi: 10.1007/82_2010_43

Bayascas, J. R., Wullschleger, S., Sakamoto, K., García-Martínez, J. M., Clacher, C., Komander, D., et al. (2008). Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. *Mol. Cell. Biol.* 28, 3258–3272. doi: 10.1128/mcb.02032-07

Chen, Z. Y., Deqiang, J., Bath, K. G., Ieraci, A., Khan, T., Siao, C. J., et al. (2006). Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* 314, 140–143. doi: 10.1126/science.1129663

Cordón-Barris, L., Pascual-Guiral, S., Yang, S., Giménez-Llort, L., Lope-Piedrafita, S., Niemeyer, C., et al. (2016). Mutation of the 3- phosphoinositide-dependent protein kinase-1 (PDK1) substrate-docking site in the developing brain causes microcephaly with abnormal brain morphogenesis independently of Akt, leading to impaired cognition and disruptive behaviors. *Mol. Cell. Biol.* 36, 2967–2982. doi: 10.1128/mcb.00230-16

de Brouwer, G., Fick, A., Harvey, B. H., and Wolmarans, W. (2019). A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: mapping the way forward. *Cogn. Affect. Behav. Neurosci.* 19, 1–39. doi: 10.3758/s13415-018-00653-4

Deacon, R. M., Bannerman, D. M., Kirby, B. P., Croucher, A., and Rawlins, J. N. (2002). Effects of cytotoxic hippocampal lesions in mice on a cognitive test battery. *Behav. Brain Res.* 133, 57–68. doi: 10.1016/s0166-4328(01)00451-x

Duman, R. S., and Monteggia, L. M. (2006). A neurotrophic model for stress-related mood disorders. *Biol. Psychiatry* 59, 1116–1127. doi: 10.1016/j.biopsych.2006.02.013

Goldman-Rakic, P. S. (1994). Working memory dysfunction in schizophrenia. *J. Neuropsychiatry Clin. Neurosci.* 6, 348–357. doi: 10.1176/jnp.6.4.348

Guayerbas, N., Sánchez, A. I., Gamallo, A., and De la Fuente, M. (2000). "Mouse performance in an exploratory activity test as a longevity biomarker," in *Animal Research Welfare: A Partnership*, eds J. A. TurMari and J. M. Orellana-Muriana (London, UK: Laboratory Animals), 159–161.

Hyman, S. E. (2007). Can neuroscience be integrated into the DSM-V? *Nat. Rev. Neurosci.* 8, 725–732. doi: 10.1038/nrn2218

Jimenez-Gomez, C., Osentoski, A., and Woods, J. H. (2011). Pharmacological evaluation of the adequacy of marble burying as an animal model of compulsion and/or anxiety. *Behav. Pharmacol.* 22, 711–713. doi: 10.1097/fbp.0b013e32834afebe

Komander, D., Fairservice, A., Deak, M., Kular, G. S., Prescott, A. R., Peter Downes, C., et al. (2004). Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. *EMBO J.* 23, 3918–3928. doi: 10.1038/sj.emboj.7600379

Lát, J. (1973). The analysis of habituation. *Acta Neurobiol. Exp.* 33, 771–789.

Leibrock, C., Ackermann, T. F., Hierlmeier, M., Lang, F., Borgwardt, S., and Lang, U. E. (2013). Akt2 deficiency is associated with anxiety and depressive behavior in mice. *Cell. Physiol. Biochem.* 32, 766–777. doi: 10.1159/000354478

Martinowich, K., Manji, H., and Lu, B. (2007). New insights into BDNF function in depression and anxiety. *Nat. Neurosci.* 10, 1089–1093. doi: 10.1038/nn1971

Miller, E. K., and Cohen, J. D. (2001). An integrative theory of prefrontal cortex function. *Annu. Rev. Neurosci.* 24, 167–202. doi: 10.1146/annurev.neuro.24.1.167

Mora, A., Komander, D., van Aalten, D. M., and Alessi, D. R. (2004). PDK1, the master regulator of AGC kinase signal transduction. *Semin. Cell Dev. Biol.* 15, 161–170. doi: 10.1016/j.semcdb.2003.12.022

Morris, S., and Cuthbert, R. N. (2012). Research Domain Criteria: cognitive systems, neural circuits, and dimensions of behaviour. *Dialogues Clin. Neurosci.* 14, 29–37. Available online at: https://www.ncbi.nlm.nih.gov/pubmed/22577302

National Institute of Mental Health. (2020). *Research Domain Criteria. (Internet) NIHM.* Available online at: https://www.nimh.nih.gov/research/research-funded-by-nimh/rdoc/index.shtml. Accessed 29 February, 2020.

Ou, L. C., and Gean, P. W. (2006). Regulation of amygdala-dependent learning by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3-kinase. *Neuropsychopharmacology* 31, 287–296. doi: 10.1038/sj.npp.1300830

Pearce, L. R., Komander, D., and Alessi, D. R. (2010). The nuts and bolts of AGC protein kinases. *Nat. Rev. Mol. Cell Biol.* 11, 9–22. doi: 10.1038/nrm2822

Sanderson, D. J., and Bannerman, D. M. (2012). The role of habituation in hippocampus-dependent spatial working memory tasks: evidence from GluA1 AMPA receptor subunit knockout mice. *Hippocampus* 22, 981–994. doi: 10.1002/hipo.20896

Shi, H. S., Zhu, W. L., Liu, J. F., Luo, Y. X., Si, J. J., Wang, S. J., et al. (2012). PI3K/Akt signaling pathway in the basolateral amygdala mediates the rapid antidepressant-like effects of trefoil factor 3. *Neuropsychopharmacology* 37, 2671–2683. doi: 10.1038/npp.2012.131

Tao, W., Dong, Y., Su, Q., Wang, H., Chen, Y., Xue, W., et al. (2016). Liquiritigenin reverses depression-like behavior in unpredictable chronic mild stress-induced mice by regulating PI3K/Akt/mTOR mediated BDNF/TrkB pathway. *Behav. Brain Res.* 308, 177–186. doi: 10.1016/j.bbr.2016.04.039

Torres-Lista, V., López-Pousa, S., and Giménez-Llort, L. (2015). Marble-burying is enhanced in 3xTg-AD mice, can be reversed by risperidone and it is modulable by handling. *Behav. Processes* 116, 69–74. doi: 10.1016/j.beproc.2015.05.001

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., et al. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat. Genet.* 23, 99–103. doi: 10.1038/12703

Waite, K., and Eickholt, J. (2010). The neurodevelopmental implications of PI3K signaling. *Curr. Top. Microbiol. Immunol.* 346, 245–265. doi: 10.1007/82_2010_82

Wang, W., Lu, Y., Xue, Z., Li, C., Wang, C., Zhao, X., et al. (2015). Rapid-acting antidepressant-like effects of acetyl-l-carnitine mediated by PI3K/AKT/BDNF/VGF signaling pathway in mice. *Neuroscience* 285, 281–291. doi: 10.1016/j.neuroscience.2014.11.025

Yang, S., Pascual-Guiral, S., Ponce, R., Giménez-Llort, L., Baltrons, M. A., Arancio, O., et al. (2018). Reducing the levels of Akt activation by PDK1 knock-in mutation protects neuronal cultures against synthetic amyloid-β peptides. *Front. Aging Neurosci.* 9:435. doi: 10.3389/fnagi.2017.00435

Yen, Y. C., Mauch, C. P., Dahlhoff, M., Micale, V., Bunck, M., Sartori, S. B., et al. (2012). Increased levels of conditioned fear and avoidance behavior coincide with changes in phosphorylation of the protein kinase B (AKT) within the amygdala in a mouse model of extremes in trait anxiety. *Neurobiol. Learn. Mem.* 98, 56–65. doi: 10.1016/j.nlm.2012.04.009

Zhou, X., Cordon-Barris, L., Zurashvili, T., and Bayascas, J. R. (2014). Fine-tuning the intensity of the PKB/Akt signal enables diverse physiological responses. *Cell Cycle* 13, 3164–3168. doi: 10.4161/15384101.2014.962954

Zurashvili, T., Cordón-Barris, L., Ruiz-Babot, G., Zhou, X., Lizcano, J. M., Gómez, N., et al. (2013). Interaction of PDK1 with phosphoinositides is essential for neuronal differentiation but dispensable for neuronal survival. *Mol. Cell. Biol.* 33, 1027–1040. doi: 10.1128/MCB.01052-12

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

Study 2.

biomedicines

MDPI

Article

Fine-Tuning the PI3K/Akt Signaling Pathway Intensity by Sex and Genotype-Load: Sex-Dependent Homozygotic Threshold for Somatic Growth but Feminization of Anxious Phenotype in Middle-Aged PDK1 K465E Knock-In and Heterozygous Mice

Mikel Santana-Santana [1,2], José-Ramón Bayascas [1,3,*] and Lydia Giménez-Llort [1,2,*]

[1] Institut de Neurociències, Universitat Autònoma de Barcelona, 08016 Barcelona, Spain; mikel.santana@e-campus.uab.cat
[2] Department of Psychiatry and Forensic Medicine, School of Medicine, Universitat Autònoma de Barcelona, 08016 Barcelona, Spain
[3] Department of Biochemistry and Molecular Biology, School of Medicine, Universitat Autònoma de Barcelona, 08016 Barcelona, Spain
* Correspondence: lidia.gimenez@uab.cat (J.-R.B.); joseramon.bayascas@uab.cat (L.G.-L.)

Abstract: According to the Research Domain Criteria (RDoC), phenotypic differences among disorders may be explained by variations in the nature and degree of neural circuitry disruptions and/or dysfunctions modulated by several biological and environmental factors. We recently demonstrated the in vivo behavioral translation of tweaking the PI3K/Akt signaling, an essential pathway for regulating cellular processes and physiology, and its modulation through aging. Here we describe, for the first time, the in vivo behavioral impact of the sex and genetic-load tweaking this pathway. The anxiety-like phenotypes of 61 mature (11–14-month-old) male and female PDK1 K465E knock-in, heterozygous, and WT mice were studied. Forced (open-field) anxiogenic environmental conditions were sensitive to detect sex and genetic-load differences at middle age. Despite similar neophobia and horizontal activity among the six groups, females exhibited faster ethograms than males, with increased thigmotaxis, increased wall and bizarre rearing. Genotype-load unveiled increased anxiety in males, resembling female performances. The performance of mutants in naturalistic conditions (marble test) was normal. Homozygotic-load was needed for reduced somatic growth only in males. Factor interactions indicated the complex interplay in the elicitation of different negative valence system's items and the fine-tuning of PI3K/Akt signaling pathway intensity by genotype-load and sex.

Keywords: RDoC; PI3K/Akt; signaling pathway; sex; genetic load; fine tuning; anxiety; aging

Citation: Santana-Santana, M.; Bayascas, J.-R.; Giménez-Llort, L. Fine-Tuning the PI3K/Akt Signaling Pathway Intensity by Sex and Genotype-Load: Sex-Dependent Homozygotic Threshold for Somatic Growth but Feminization of Anxious Phenotype in Middle-Aged PDK1 K465E Knock-In and Heterozygous Mice. *Biomedicines* **2021**, *9*, 747. https://doi.org/10.3390/biomedicines9070747

Academic Editor: Masaru Tanaka

Received: 11 May 2021
Accepted: 22 June 2021
Published: 28 June 2021

1. Introduction

The expression of psychiatric symptoms such as anxiety across lifespan still needs important research efforts to dissect and understand their modulation's biological and environmental basis [1]. Here, the new understanding of psychopathology in terms of dysregulation and dysfunction in essential behavioral features through neurobiology and behavioral neuroscience can provide a promising research scenario [2,3]. In this new conceptualization, fear, aggression, and distress are three draft constructs within the negative valence system (NVS), one of the five domains in the NIMH's Research Domain Criteria (RDoC) matrix [2,4]. This RDoC matrix comprises the interplay between behavioral dimensions or functional constructs inspected by seven different 'units of analysis', namely, genetic and molecular basis, cells and neuronal circuits, physiology of the phenotypes, behavior, and self-report [3]. The molecular genetic basis of NVS phenotypes is considered to be in its infancy, yet with few candidate genes nominated for anxiety disorders [5]. In this context, basic research on signal transduction provides an advanced close examination

of the impact of cell membrane receptors and second messengers on cellular biochemistry and physiology. However, the downstream actions unveiling how these genetic aspects translate into anxiety-related NVS constructs are challenging to characterize, mainly when related to biological factors as sex or the genetic load in aging scenarios, since female mice are underrepresented in research [6] and aging animals are scarcely studied [7].

Different PDK1/Akt mutant mice have consistently manifested a higher depressive and/or anxiety-like behavior [8–11]. The 3-phosphoinositide-dependent protein kinase-1 (PDK1) [12], an enzyme that activates Akt among other AGC kinases [13,14], is a key transmitter of extracellular signals of the phosphatidylinositol 3-kinase (PI3K) signaling, a pathway extensively involved in controlling neuronal development and function [15]. Its functional role in cellular biochemistry and physiology, as well as cancer and metabolism, has been largely explored [16], although in recent years, further evidence has been generated about its role in bipolar disorder, depression, anxiety, and schizophrenia [17–23]. In addition, antidepressants, antipsychotics, and mood stabilizers modify Aktactivity [17,24–26]. Moreover, the PDK1/Akt pathway has been also linked with suicide, alcohol drinking, and post-traumatic stress disorder [27–29]. Recently, a non-pharmacological intervention attenuated the cognitive deficit and depressive/anxiety-like behaviors induced by a stressor through the recovery of hippocampal Akt activity [30]. In addition, after postnatal maternal separation, mice treated with an early life non-pharmacological intervention showed ameliorated depressive and anxiety-like behavior through enhanced phosphorylated Akt in the hippocampus [31].

Here, mutant mice for this signaling pathway provide an experimental tool to depict the nuances of its downstream modulation. Mutation of PDK1 Lys465, a residue forms key interactions with the D3 and D4 phosphates of the PtdIns(3,4,5)P3 second messenger, to a Glu residue abolished binding of PDK1 to phosphoinositides and localization at the plasma membrane [32]. This signaling lesion selectively affected the phosphorylation and activation of PKB/Akt isoforms, but left intact the activation of other AGC kinase-family members [33]. These mice present smaller body size and insulin resistance [33]. At the brain level, they also exhibit pronounced Akt signaling deficits in both the cortex and the hippocampus during young adulthood (3–4 months of age) but tend to be attenuated by middle age (11–14 months of age) [34,35]. We recently showed that the double mutation of the PDK1 PH-domain (PDK1−/−) resulted in an enhancement of NVS shown as an increase of responses of fear and anxiety-like behaviors in anxiogenic situations [36]. Interestingly, this seemed to be specific to young adulthood and was found regulated at middle age. In contrast, as measured in a spatial working memory task, cognitive deficits were found in both young and mature mutants and independently of the level of their anxious-like profiles. These distinct age- and function-dependent impacts would agree with the distinct cortical and limbic deficits in the Akt signaling in their brains [34]. The elicitation of age- and regional-dependent specific patterns suggests that fine-tuning the PKB/Akt signal intensity that enables diverse physiological responses also has in vivo translation into the NVS, and age is a key regulatory factor.

Although women are significantly more likely than men to develop an anxiety disorder throughout the lifespan [37], fewer than 45% of animal studies into mood disorders used females [38,39]. The contribution of sex and genetic load in the anxious-like behavioral phenotype in this particular animal model is still unknown. Our previous report suggested that sex differences should be further explored [36]. Regarding genetic load, we hypothesize that heterozygous mice (PDK1+/−) may differ behaviorally from wild-type mice and/or homozygous mice due to differences in the intensity of the Akt signal under physiological conditions. On the other hand, recently, Akt deficiency in Akt isoform mutant mice altered anxiety-like behavior in an isoform- and sex-specific manner [40]. However, those sex-specific behavioral differences could not be explained by Akt expression or activation differences between the sexes.

Therefore, the present study aimed to explore further the contribution of sex and genetic load in the expression of the somatic and anxious-like behavioral phenotype of the PDK1-K465E PH-domain knock-in mice.

2. Materials and Methods

2.1. Generation of $PDK1^{K465E/K465E}$ Mice and Genotyping Analysis

The generation and genotyping of the PDK1 K465E/K465E knock-in mice expressing the single-amino-acid substitution of lysine 465 to glutamic acid in the PDK1 PH domain were described previously [17]. The mice were subjected to PCR genotyping of genomic DNA isolated from ear biopsy using primers K465E F (5′-GGG TGA AGC ATG GAA TCT GTG TCT T) and K465E R (5′-GCC AGG ATA CCT AAG AGT ACC TAG AA). PCR amplification resulted in a 196-bp product from the wild-type allele and a 236-bp product from the targeted allele.

2.2. Animals

A total of 61 mature age (MA, 11–14-month-old) mice, $PDK1^{-/-}$ (14 males, 16 females), $PDK1^{+/-}$ (8 males, 10 females) and $PDK1^{+/+}$ (also referred to as WT, 6 males, 7 females) were used.

Mice were maintained at the Animal House Facility of the Universitat de Lleida under standard husbandry conditions (housed three to four per cage in Macrolon cages, 35 × 35 × 25 cm, with food and water ad libitum, 22 ± 2 °C, a 12 h light: dark cycle and relative humidity 50–60%). Behavioral assessments and data analysis were performed blind to the experiment, in a counterbalanced manner, in the light cycle, from 9:00 to 13:00 h. All procedures were in accordance with Spanish legislation on 'Protection of Animals Used for Experimental and Other Scientific Purposes' and the EU Directive (2010/63/UE) on this subject. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used [41].

2.3. Behavioral Assessments

Animals were behaviorally assessed for NVS in the open field [42] and the marble-burying tests [43], two unconditioned tests differing in their anxiogenic conditions. A graphical abstract, also including the conclusions, illustrates the methodological setting and procedures (Figure 1).

As measured by body weight, somatic growth was recorded on Day 0 prior to the behavioral battery of tests to monitor possible confounding factors. Lack of sensorimotor problems was already described in these animals in the precedent work [36].

Day 0. Somatic Growth/Bodyweight. Bodyweight was used to measure the somatic growth and physical condition/health status of animals.

Day 1. Open field test (OF). Animals were individually placed in the center of an illuminated (20 lux) open field (homemade woodwork, white box, 55 cm × 55 cm × 25 cm) and observed for 5 min. First, the ethogram of action programs (sequence of behavioral events) was analyzed. Thus, the duration of freezing behavior (latM, latency of movement) and the latency of the behavioral events that follow it were recorded: leaving the central square (latC), reaching the periphery (thigmotaxis) (latP) and performing first wall rearing (latR). Second, the time course and total levels of exploratory activity were measured as horizontal (C, number of crossings) and vertical (Rw, rearing with wall support) locomotor activity. Finally, as previously described [44] we evaluated the presence of bizarre behaviors assessed through the number of stereotyped rearings without wall support (Rc).

Day 2. Marble-Burying Test (MB). The procedure used was as previously described [45]. The mice were placed individually facing the wall in a standard home cage with six glass marbles (1 cm × 1 cm × 1 cm) on a 5-cm-thick layer of clean wood cuttings. The marbles were spaced in three rows of two marbles per row in one half of the cage. The mice were left in the cage with marbles for a 30-min period. The test was terminated by removing

per sex and genotype (Figure 2C, G × S, $F_{(1,55)} = 22.035$, $p = 0.000$), post hoc comparisons showed that the higher body weight in males is preserved in all the genotypes (Figure 2C, $PDK1^{+/+}$, s, $p = 0.024$; $PDK1^{+/-}$, sss, $p = 0.001$; $PDK1^{-/-}$, s, $p = 0.030$). Moreover, in females, somatic growth followed a progressive decrease with genotype load (Figure 2C, $PDK1^{+/+}$, g, $p = 0.018$), but heterozygous males had normal weight (Figure 2C, $PDK1^{+/+}$, ggg, $p = 0.000$; $PDK1^{+/-}$, ggg, $p = 0.000$).

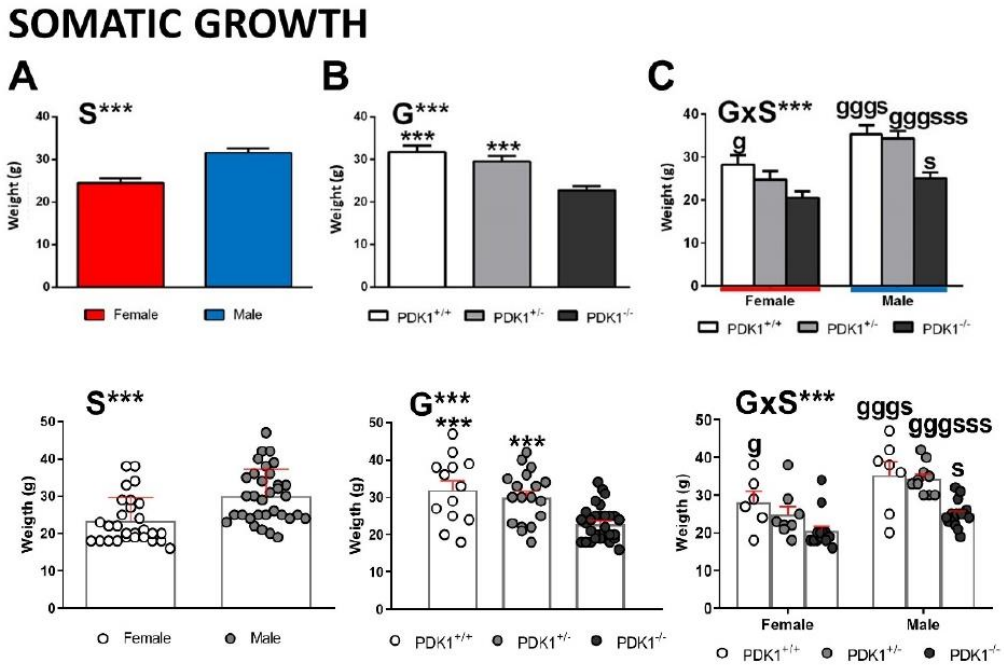

Figure 2. Somatic growth/ body weight in female and male mature $PDK1^{-/-}$, heterozygous $PDK1^{+/-}$ and homozygous WT ($PDK1^{+/+}$) mice. Top panel: Data are expressed as mean ± SEM. Bottom panel: Individual data are depicted. Bars illustrate the genotype or sex groups. Factorial analysis: (**A**) S, sex effect; (**B**) G, genotype effect; (**C**) G × S, genotype × sex interaction effects; *** $p < 0.001$. *Post-hoc* test: genotype: ^{g} $p < 0.05$, ^{ggg} $p < 0.01$ vs. the corresponding KO ($PDK1^{-/-}$, black bar) group; s (sex), ^{s} $p < 0.05$, ^{sss} $p < 0.001$ vs. the corresponding male of the same genotype.

3.2. Open Field Test

Figures 3 and 4 depict the main behavioral domains, events, and units of analysis in the open-field test, showing the distinct sex-dependent performances of homozygous and heterozygous PDK1 mutants compared to WT groups.

Fear and Thigmotaxis—Immediate response to exposure to the open-field was similar among groups, with no differences in the latency of first movement (not shown). Sex differences were found in the latency to leave the center (Figure 3A, S, $F_{(1,55)} = 5.538$; $p = 0.022$) and to reach the periphery (Figure 3B, S, $F_{(1,55)} = 11.057$; $p = 0.002$) with females being faster than males. Post hoc multicomparison analysis showed that this difference was due to sex dimorphism in the behavior of WT mice (Figure 3A, lat center, ss, $p = 0.006$), since PDK+/− and PDK1−/− males also left the center faster than WT males, albeit this difference only reached the statistical significance in the heterozygous group (Figure 3A, lat center, g, $p = 0.018$). This genotype × sex interaction reached statistical significance in the latency to reach the periphery (Figure 3B, G × S, $F_{(2,55)} = 4.028$; $p = 0.023$). Post hoc multicomparison analysis showed that WT females arrived faster than males (Figure 3B, lat periphery, sss, $p = 0.000$) and that both PDK1+/− and PDK1−/− males also reached the periphery sooner than WT (Figure 3B, gg, $p = 0.003$ and gg, $p = 0.008$, respectively).

Vertical behavior—Latency of rearing showed a genotype main effect (Figure 3C, G, $F_{(1,55)} = 3.675$; $p = 0.032$), where both PDK1+/− and PDK1 −/− genotypes performed rearing earlier than WT (Figure 3C, *, $p = 0.035$ and *, $p = 0.040$, respectively). Post hoc multicomparison analysis also showed that latency of rearing was shorter in female WT as compared to males (Figure 3C, s, $p = 0.031$) and that both PDK1+/− and PDK1−/− males performed rearing earlier than WT males (Figure 3C, g, $p = 0.016$ and gg, $p = 0.008$, respectively). As shown in Figure 4, the min-by-min analysis of the temporal course of horizontal locomotor activity indicated similar habituation curves in the three genotypes. In the last minute of the test, the female sex performed less activity than males (Figure 4A, S, $F_{(1,55)} = 5.763$; $p = 0.020$). Post hoc multiple comparison analysis indicated that this sex effect was mostly due to the sexual dimorphism of WT mice in minute 5 (Figure 4A, s, $p = 0.043$).

Vertical activity (Wall Rearing): No main but interaction effects were found (Figure 4B, G × S, $F_{(2,55)} = 3.340$; $p = 0.043$). Post hoc multiple comparison analysis showed differences in the heterozygotes where males outperformed more than heterozygote females in minutes 1 (s, $p = 0.036$), 2 (ss, $p = 0.006$), and 3 (s, $p = 0.025$), resulting in a total higher total vertical activity (Figure 4B, s, $F_{(1,55)} = 6.631$, $p = 0.013$). WT females showed a higher rearing behavior in minute 4 (s, $p = 0.047$).

Bizarre behavior (Rearings in the center): No main but interaction effects were also found in the rearings performed in the center of the apparatus (Figure 4C, G × S, $F_{(2,55)} = 3.360$; $p = 0.043$). Post hoc multiple comparison analysis showed homozygote mutant females performed less than homozygote mutant males in minutes 2 (s, $p = 0.034$), 3 (s, $p = 0.048$), 5 (s, $p = 0.018$), resulting in a total lower vertical rearing activity in the center (Figure 4C, s, $F_{(1,55)} = 4.700$, $p = 0.035$). Moreover, in minute 5, homozygote mutant males exhibited more than wild-type mice (Figure 4C, g, $p = 0.039$).

3.3. Marble Burying Test

The qualitative (three levels of interaction) and quantitative (number) analysis of the marble-burying test did not show any statistically significant effect and/or differences between groups (see Figure 5). However, in contrast to the standard quantitative evaluation protocol, the consideration of several levels of interaction with small objects enabled us to uncover the predominant behaviors. That is, in the three strains, the most common behavioral interaction did not result in the complete burying of the marbles but their change of position (turned or partially buried).

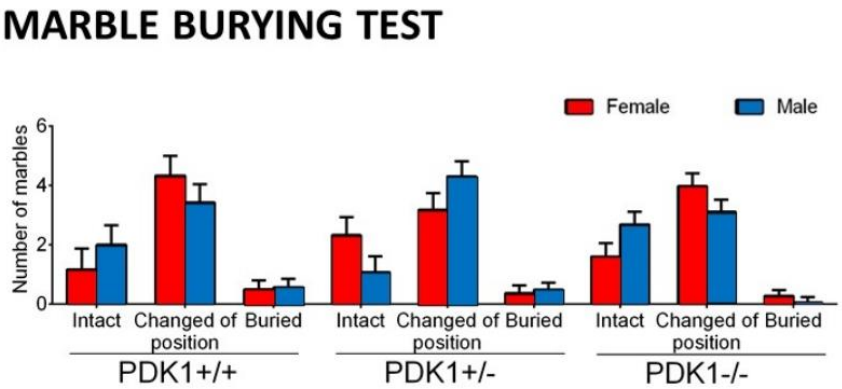

Figure 5. Marble burying test in female and male mature PDK1$^{-/-}$, heterozygous PDK1$^{+/-}$ and homozygous WT (PDK1$^{+/+}$) mice. Top panel: Data are expressed as mean ± SEM or incidence. Bottom panel: Individual data are depicted. Bars illustrate the genotype groups, as indicated in the Y-axis. Symbols illustrate the different groups or individual values, as depicted in the legends or abscissae. Factorial analysis: G, genotype effect; S, sex effect; all $p > 0.05$, *n.s.*

4. Discussion

In the present work, we describe for the first time the in vivo effects of the PDK1 mutation in the PH domain depending on age and genetic load. Mature (11–14 months of age) male and female PDK1$^{-/-}$ and PDK1$^{+/-}$ PH-domain knock-in mice were studied and compared to age- and sex-matched WT mice with normal aging. The results unveil the fine-tuning of the signaling pathways by sex and genetic load, with a feminization of the behavioral profiles.

4.1. Homozygous-Load of the Mutant Gene Is Needed for the Reduced Somatic Growth Effects Only in Mutant Males

As previously described [33,35,36], somatic growth, measured through bodyweight, was found reduced in both male and female PDK1$^{-/-}$ mice. It is known that homozygous male and female PDK1$^{-/-}$ are −35% smaller from birth than WT littermates [33]. In that precedent work, magnetic resonance imaging-obtained images or physical sections of fixed organs using the Cavalieri method described reduced brain volume (−20%) but also of metabolic (liver), immune (spleen, −20%), male gonadal (testis, −50%) organs and a slight reduction in kidney size (albeit did not reach statistical significance) in PDK1$^{-/-}$ mice compared to littermates [33]. Disector principle, a quantitative and unbiased stereological approach to estimate cell volume, also indicated that a reduction in organ volume translated into a reduction of cell size. Thus, in the case of adrenal glands of PDK1$^{-/-}$ animals, a −40% reduction in the relative cell size of zona fasciculata cells was found compared to littermates. Interestingly, the present report shows that heterozygosis was enough to sustain normal weight. However, further analysis segregated by sex unveiled that the half genetic load of wild type PDK1 could guarantee normal somatic growth only in males since heterozygous females were already sensitive to this somatic effect of PDK1/Akt signaling pathway. We hypothesize that this sex-dependent modulation of size translates into the previously reported organ sizes, glucose resistance, hyperinsulinemia, and insulin resistance [33], and future experiments are needed to study it further.

4.2. Similar Neophobia and Locomotion, but Increased Anxiety-Like Phenotype in Mutant Females and Feminized Anxious-Like Phenotype of Mutant Males

In the PDK1$^{-/-}$ mice, the cortical and hippocampal deficits in the PDK1/Akt signaling [34] and the anxious-like phenotype [36] are found attenuated at 11–14 months of age as compared to young adulthood. In the present work, we provide further evidence of a fine-tuning modulation of this signaling pathway by sex and genetic load. Neophobia, an amygdala-dependent immediate fear response to novelty, was similar among the six groups of animals. However, after that, mutant females exhibited a coping with stress strategy characterized by shorter latencies to develop the ethogram, thigmotaxis, increased wall rearing, and presence of bizarre rearings. As previously described, according to their temporal (repetitive/stereotyped or not) and spacial (horizontal/vertical) features, behaviors apparently without a purpose but considered coping-with-stress strategies can be classified as stereotyped stretching, stereotyped rearing, backward movement, and jumping [44]. These disrupted behaviors are scarcely observed in young animals and still difficult to record at middle age, as shown here by the low number in male WT. However, in C57bL/6 mice, we described bizarre behavior that could be conspicuous at 6 months of age when confronting the anxiogenic environments such as the open-field test, mainly in females due to their increased anxious-like profiles as compared to males, or when these responses are found exacerbated by neuropathological conditions [10,44,46]. Here, the bizarre emergent behavior was vertical rearing, which resembles escape behavior in the behavioral despair test. More importantly, in the present work, this female pattern was emulated by homozygous PDK1$^{-/-}$ and heterozygous PDK1$^{+/-}$ male mice, to the extent that the male WT profile was dissonant with the one exhibited by all the other groups. We have shown that bizarre behaviors delay the exploratory activity in adults [44] and aged mice [46], and can be modulated by early-life interventions [44]. Therefore, the selective

effects of sex and PDK/Akt signaling genotype-load on the vertical but not the horizontal activity, as shown by normal habituation curves, is noticeable and suggests that underlying mechanisms are mediated by anxiety but not by hyperactivity [47,48].

4.3. Performance of Mutants Can Resemble Normal under Naturalistic Anxiogenic Conditions

In the precedent work [36], we demonstrated that in the PDK1 homozygous mice, anxiety but not working memory was modulated by age with a reduction in its expression at 11–13 months of age. This would also explain that here, similarly to previous work [44], the anxiogenic environment of the open and illuminated field test was found to be the best to observe the elicitation of bizarre behaviors, as well the fine-tuning of genotype and sex modulation, but when the behavior of animals was assessed in the marble burying test, behaviors did not differ. Thus, in the current work, similar signatures were shown by the different groups and the 'moved or semi-buried marbles' was found the dominant behavioral readout at the end of the 30 min test. Compared to an anxiogenic open-field test, marble burying is a neuroethological paradigm eliciting spontaneous responses of vigorous and deep digging of beddings to bury the pieces (food pellets or small objects) the animal finds in its environment [49,50]. The interpretation of this test is in constant debate, since it is sensitive to anxiolytic but also antipsychotic drugs [45,51], and it is proposed for modeling compulsive-like characteristics of OCD or autism spectrum disorders [51,52]. Here, as in other research, its use was aimed to in vivo identification of biological impacts in mice [53,54], in our case of genetic load and sex. Furthermore, digging can also be understood as a measure of general activity rather than a measure of repetitive or anxiety-related behavior [51,55–57] and conversely, the animal's general activity can be a confounding factor. Due to this controversy, the open-field or other anxiety tests that also monitor the general activity are a must for interpretations and discard confounding factors. In all cases, in the present work, the impact of sex and genetic load on specific vertical but not on locomotor activity may also agree with the similar signatures observed in this paradigm. This would also agree with reports on drug-induced dose-dependent reduction in marble-burying independently of its locomotor effects [58] or our most recent report in a model for neuropathological aging [47].

5. Conclusions

Of the two unconditioned tests used, the forced (open-field) but not naturalistic (marble arena) anxiogenic environmental conditions were sensitive to detect sex and genetic-load differences at middle age in the PDK1 mutant mice. Thus, despite similar initial fear response (freezing indicating increased neophobia) and horizontal locomotion among the six groups of animals, females exhibited faster ethograms than males, with increased thigmotaxis with shorter latencies to reach the periphery and perform wall rearings, and increased wall and bizarre rearing. Genotype-load unveiled increased anxiety in males in elicitation of male ethograms and profiles resembling the performances characteristic of the female phenotype. While a heterozygous genotype-load was enough to elicit reduced somatic growth (bodyweight) in females, an homozygotic load of PDK1 was needed to exert this somatic effect in males. In summary, factor interactions indicated the complex interplay in the involvement of PI3K/Akt signaling pathway in the elicitation of different NVS's construct items and somatic growth and the relevance of genotype-load and sex in the fine-tuning of its intensity.

Author Contributions: Conceptualization, L.G.-L.; animal model, J.-R.B.; behavioral performance, L.G.-L.; behavioral analysis, statistics and illustrations, M.S.-S.; writing—original draft preparation, M.S.-S. and L.G.-L.; writing—review and editing, M.S.-S., L.G.-L. and J.-R.B.; funding acquisition, J.-R.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministerio de Economía y Competitividad (MINECO), Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad (SAF2014-52813-R) to J.-R.B.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Departament de Medi Ambient i Habitatge, Generalitat de Catalunya (CEEAH 2291/DMAH 7493) the 17 March 2014.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Jessica Pairada and Núria Moix, Estabulari de Rosegadors of the Universitat de Lleida, for animal care.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Lenze, E.J.; Wetherell, J.L. A lifespan view of anxiety disorders. *Dialogues Clin. Neurosci.* **2011**, *13*, 381–399. [CrossRef]
2. National Institute of Mental Health. Research Domain Criteria. (Internet) NIHM. Available online: https://www.nimh.nih.gov/research/research-funded-by-nimh/rdoc/index.shtml (accessed on 29 February 2020).
3. Asher, J. Genes and Circuitry, Not Just Clinical Observation, to Guide Classification for Research (Internet). NIMH; 2010. Available online: https://www.nimh.nih.gov/news/science-news/2010/genes-and-circuitry-not-just-clinical-observation-to-guide-classification-for-research.shtml (accessed on 29 February 2020).
4. Cuthbert, B.N.; Insel, T.R. Toward the future of psychiatric diagnosis: The seven pillars of RDoC. *BMC Med.* **2013**, *11*, 126. [CrossRef]
5. Savage, J.E.; Sawyers, C.; Roberson-Nay, R.; Hettema, J.M. The genetics of anxiety-related negative valence system traits. *Am. J. Med. Genet. Part B Neuropsychiatr. Genet.* **2017**, *174*, 156–177. [CrossRef]
6. Prendergast, B.J.; Onishi, K.G.; Zucker, I. Female mice liberated for inclusion in neuroscience and biomedical research. *Neurosci. Biobehav. Rev.* **2014**, *40*, 1–5. [CrossRef] [PubMed]
7. Mitchell, S.J.; Scheibye-Knudsen, M.; Longo, D.L.; de Cabo, R. Animal Models of Aging Research: Implications for Human Aging and Age-Related Diseases. *Annu. Rev. Anim. Biosci.* **2015**, *3*, 283–303. [CrossRef]
8. Ackermann, T.F.; Hörtnagl, H.; Wolfer, D.P.; Colacicco, G.; Sohr, R.; Lang, F.; Hellweg, R.; Lang, U.E. Phosphatidylinositide Dependent Kinase Deficiency Increases Anxiety and Decreases GABA and Serotonin Abundance in the Amygdala. *Cell. Physiol. Biochem.* **2008**, *22*, 735–744. [CrossRef] [PubMed]
9. Leibrock, C.; Ackermann, T.F.; Hierlmeier, M.; Lang, F.; Borgwardt, S.; Lang, U.E. Akt2 Deficiency is Associated with Anxiety and Depressive Behavior in Mice. *Cell. Physiol. Biochem.* **2013**, *32*, 766–777. [CrossRef] [PubMed]
10. Cordón-Barris, L.; Pascual-Guiral, S.; Yang, S.; Giménez-Llort, L.; Lope-Piedrafita, S.; Niemeyer, C.; Claro, E.; Lizcano, J.M.; Bayascas, J.R. Mutation of the 3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1) Substrate-Docking Site in the Developing Brain Causes Microcephaly with Abnormal Brain Morphogenesis Independently of Akt, Leading to Impaired Cognition and Disruptive Behaviors. *Mol. Cell. Biol.* **2016**, *36*, 2967–2982. [CrossRef]
11. Bergeron, Y.; Bureau, G.; Laurier-Laurin, M.-É.; Asselin, E.; Massicotte, G.; Cyr, M. Genetic Deletion of Akt3 Induces an Endophenotype Reminiscent of Psychiatric Manifestations in Mice. *Front. Mol. Neurosci.* **2017**, *10*, 102. [CrossRef] [PubMed]
12. Alessi, D.R.; James, S.R.; Downes, C.; Holmes, A.B.; Gaffney, P.R.; Reese, C.B.; Cohen, P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Curr. Biol.* **1997**, *7*, 261–269. [CrossRef]
13. Mora, A.; Komander, D.; van Aalten, D.M.; Alessi, D.R. PDK1, the master regulator of AGC kinase signal transduction. *Semin. Cell Dev. Biol.* **2004**, *15*, 161–170. [CrossRef]
14. Pearce, L.R.; Komander, D.; Alessi, D. The nuts and bolts of AGC protein kinases. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 9–22. [CrossRef] [PubMed]
15. Waite, K.; Eickholt, B.J. The Neurodevelopmental Implications of PI3K Signaling. *Curr. Top. Microbiol. Immunol.* **2010**, *346*, 245–265. [CrossRef]
16. Bayascas, J.R. PDK1: The Major Transducer of PI 3-Kinase Actions. *Curr. Top. Microbiol. Immunol.* **2010**, *346*, 9–29. [CrossRef] [PubMed]
17. Jope, R.S.; Roh, R.S. Glycogen Synthase Kinase-3 (GSK3) in Psychiatric Diseases and Therapeutic Interventions. *Curr. Drug Targets* **2006**, *7*, 1421–1434. [CrossRef]
18. Beaulieu, J.-M.; Gainetdinov, R.; Caron, M.G. Akt/GSK3 Signaling in the Action of Psychotropic Drugs. *Annu. Rev. Pharmacol. Toxicol.* **2009**, *49*, 327–347. [CrossRef]
19. Freyberg, Z.; Ferrando, S.J.; Javitch, J.A. Roles of the Akt/GSK-3 and Wnt Signaling Pathways in Schizophrenia and Antipsychotic Drug Action. *Am. J. Psychiatry* **2010**, *167*, 388–396. [CrossRef]
20. Jope, R.S. Glycogen Synthase Kinase-3 in the Etiology and Treatment of Mood Disorders. *Front. Mol. Neurosci.* **2011**, *4*, 16. [CrossRef] [PubMed]

21. Sachs, B.D.; Rodriguiz, R.M.; Siesser, W.B.; Kenan, A.; Royer, E.L.; Jacobsen, J.P.R.; Wetsel, W.C.; Caron, M.G. The effects of brain serotonin deficiency on behavioural disinhibition and anxiety-like behaviour following mild early life stress. *Int. J. Neuropsychopharmacol.* **2013**, *16*, 2081–2094. [CrossRef] [PubMed]
22. Singh, K. An emerging role for Wnt and GSK3 signaling pathways in schizophrenia. *Clin. Genet.* **2013**, *83*, 511–517. [CrossRef]
23. Beurel, E.; Grieco, S.F.; Jope, R.S. Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacol. Ther.* **2015**, *148*, 114–131. [CrossRef] [PubMed]
24. Shi, H.-S.; Zhu, W.-L.; Liu, J.; Luo, Y.-X.; Si, J.-J.; Wang, S.-J.; Xue, Y.-X.; Ding, Z.-B.; Shi, J.; Lu, L. PI3K/Akt Signaling Pathway in the Basolateral Amygdala Mediates the Rapid Antidepressant-like Effects of Trefoil Factor 3. *Neuropsychopharmacology* **2012**, *37*, 2671–2683. [CrossRef]
25. Wang, W.; Lu, Y.; Xue, Z.; Li, C.; Wang, C.; Zhao, X.; Zhang, J.; Wei, X.; Chen, X.; Cui, W.; et al. Rapid-acting antidepressant-like effects of acetyl-l-carnitine mediated by PI3K/AKT/BDNF/VGF signaling pathway in mice. *Neuroscience* **2015**, *285*, 281–291. [CrossRef] [PubMed]
26. Tao, W.; Dong, Y.; Su, Q.; Wang, H.; Chen, Y.; Xue, W.; Chen, C.; Xia, B.; Duan, J.; Chen, G. Liquiritigenin reverses depression-like behavior in unpredictable chronic mild stress-induced mice by regulating PI3K/Akt/mTOR mediated BDNF/TrkB pathway. *Behav. Brain Res.* **2016**, *308*, 177–186. [CrossRef] [PubMed]
27. Dwivedi, Y.; Rizavi, H.S.; Zhang, H.; Roberts, R.C.; Conley, R.R.; Pandey, G.N. Modulation in Activation and Expression of Phosphatase and Tensin Homolog on Chromosome Ten, Akt1, and 3-Phosphoinositide-Dependent Kinase 1: Further Evidence Demonstrating Altered Phosphoinositide 3-Kinase Signaling in Postmortem Brain of Suicide Subjects. *Biol. Psychiatry* **2010**, *67*, 1017–1025. [CrossRef]
28. Neasta, J.; Ben Hamida, S.; Yowell, Q.V.; Carnicella, S.; Ron, D. AKT Signaling Pathway in the Nucleus Accumbens Mediates Excessive Alcohol Drinking Behaviors. *Biol. Psychiatry* **2011**, *70*, 575–582. [CrossRef]
29. Dahlhoff, M.; Siegmund, A.; Golub, Y.; Wolf, E.; Holsboer, F.; Wotjak, C. AKT/GSK-3β/β-catenin signalling within hippocampus and amygdala reflects genetically determined differences in posttraumatic stress disorder like symptoms. *Neuroscience* **2010**, *169*, 1216–1226. [CrossRef] [PubMed]
30. Sun, L.; Cui, K.; Xing, F.; Liu, X. Akt dependent adult hippocampal neurogenesis regulates the behavioral improvement of treadmill running to mice model of post-traumatic stress disorder. *Behav. Brain Res.* **2020**, *379*, 112375. [CrossRef]
31. Huang, H.; Wang, Q.; Guan, X.; Zhang, X.; Zhang, Y.; Cao, J.; Li, X. Effects of enriched environment on depression and anxiety-like behavior induced by early life stress: A comparison between different periods. *Behav. Brain Res.* **2021**, *411*, 113389. [CrossRef]
32. Komander, D.; Fairservice, A.; Deak, M.; Kular, G.S.; Prescott, A.; Downes, C.P.; Safrany, S.; Alessi, D.; Van Aalten, D.M.F. Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. *EMBO J.* **2004**, *23*, 3918–3928. [CrossRef]
33. Bayascas, J.R.; Wullschleger, S.; Sakamoto, K.; García-Martínez, J.M.; Clacher, C.; Komander, D.; van Aalten, D.M.F.; Boini, K.M.; Lang, F.; Lipina, C.; et al. Mutation of the PDK1 PH Domain Inhibits Protein Kinase B/Akt, Leading to Small Size and Insulin Resistance. *Mol. Cell. Biol.* **2008**, *28*, 3258–3272. [CrossRef]
34. Yang, S.; Pascual-Guiral, S.; Ponce, R.; Giménez-Llort, L.; Baltrons, M.A.; Arancio, O.; Palacio, J.R.; Clos, V.M.; Yuste, V.J.; Bayascas, J.R. Reducing the Levels of Akt Activation by PDK1 Knock-in Mutation Protects Neuronal Cultures against Synthetic Amyloid-Beta Peptides. *Front. Aging Neurosci.* **2018**, *9*, 435. [CrossRef] [PubMed]
35. Zurashvili, T.; Cordón-Barris, L.; Ruiz-Babot, G.; Zhou, X.; Lizcano, J.M.; Gómez, N.; Giménez-Llort, L.; Bayascas, J.R. Interaction of PDK1 with Phosphoinositides Is Essential for Neuronal Differentiation but Dispensable for Neuronal Survival. *Mol. Cell. Biol.* **2013**, *33*, 1027–1040. [CrossRef] [PubMed]
36. Giménez-Llort, L.; Santana-Santana, M.; Bayascas, J.R. The Impact of the PI3K/Akt Signaling Pathway in Anxiety and Working Memory in Young and Middle-Aged PDK1 K465E Knock-In Mice. *Front. Behav. Neurosci.* **2020**, *14*, 61. [CrossRef] [PubMed]
37. McLean, C.P.; Asnaani, A.; Litz, B.T.; Hofmann, S.G. Gender differences in anxiety disorders: Prevalence, course of illness, comorbidity and burden of illness. *J. Psychiatr. Res.* **2011**, *45*, 1027–1035. [CrossRef]
38. Zucker, I.; Beery, A. Males still dominate animal studies. *Nat. Cell Biol.* **2010**, *465*, 690. [CrossRef]
39. Beery, A.; Zucker, I. Sex bias in neuroscience and biomedical research. *Neurosci. Biobehav. Rev.* **2011**, *35*, 565–572. [CrossRef]
40. Wong, H.; Levenga, J.; Laplante, L.; Keller, B.; Cooper-Sansone, A.; Borski, C.; Milstead, R.; Ehringer, M.; Hoeffer, C. Isoform-specific roles for AKT in affective behavior, spatial memory, and extinction related to psychiatric disorders. *eLife* **2020**, *9*, 56630. [CrossRef]
41. Kilkenny, C.; Browne, W.J.; Cuthill, I.C.; Emerson, M.; Altman, D.G. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol.* **2010**, *8*, e1000412. [CrossRef]
42. Hall, C.S.; Ballachey, E.L. A study of the rat's behavior in a field: A contribution to method in comparative psychology. *Univ. Calif. Publ. Psychol.* **1932**, *6*, 1–12.
43. Deacon, R.M.J. Digging and marble burying in mice: Simple methods for in vivo identification of biological impacts. *Nat. Protoc.* **2006**, *1*, 122–124. [CrossRef]
44. Baeta-Corral, R.; Giménez-Llort, L. Bizarre behaviors and risk assessment in 3xTg-AD mice at early stages of the disease. *Behav. Brain Res.* **2014**, *258*, 97–105. [CrossRef] [PubMed]
45. Torres-Lista, V.; López-Pousa, S.; Giménez-Llort, L. Marble-burying is enhanced in 3xTg-AD mice, can be reversed by risperidone and it is modulable by handling. *Behav. Process.* **2015**, *116*, 69–74. [CrossRef] [PubMed]

46. Castillo-Mariqueo, L.; Giménez-Llort, L. Translational Modeling of Psychomotor Function in Normal and AD-Pathological Aging With Special Concerns on the Effects of Social Isolation. *Front. Aging* **2021**, *2*, 5. [CrossRef]
47. Gimenez-Llort, L.; Alveal-Mellado, D. Digging Signatures in 13-Month-Old 3xTg-AD Mice for Alzheimer's Disease and Its Disruption by Isolation Despite Social Life Since They Were Born. *Front. Behav. Neurosci.* **2021**, *14*, 611384. [CrossRef]
48. Muntsant, A.; Giménez-Llort, L. Impact of Social Isolation on the Behavioral, Functional Profiles, and Hippocampal Atrophy Asymmetry in Dementia in Times of Coronavirus Pandemic (COVID-19): A Translational Neuroscience Approach. *Front. Psychiatry* **2020**, *11*, 572583. [CrossRef]
49. Pinel, J.P.; Treit, D. Burying as a defensive response in rats. *J. Comp. Physiol. Psychol.* **1978**, *92*, 708–712. [CrossRef]
50. Gyertyán, I. Analysis of the marble burying response: Marbles serve to measure digging rather than evoke burying. *Behav. Pharmacol.* **1995**, *6*, 24–31.
51. De Brouwer, G.; Fick, A.; Harvey, B.H.; Wolmarans, D.W. A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive–compulsive disorder: Mapping the way forward. *Cogn. Affect. Behav. Neurosci.* **2019**, *19*, 1–39. [CrossRef]
52. Mahmood, H.M.; Aldhalaan, H.M.; Alshammari, T.K.; Alqasem, M.A.; Alshammari, M.A.; Albekairi, N.A.; AlSharari, S.D. The Role of Nicotinic Receptors in the Attenuation of Autism-Related Behaviors in a Murine BTBR T + tf/J Autistic Model. *Autism Res.* **2020**, *13*, 1311–1334. [CrossRef]
53. Broekkamp, C.L.; Rijk, H.W.; Joly-Gelouin, D.; Lloyd, K.L. Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. *Eur. J. Pharmacol.* **1986**, *126*, 223–229. [CrossRef]
54. Deacon, R.M.; Bannerman, D.; Kirby, B.; Croucher, A.; Rawlins, J.P. Effects of cytotoxic hippocampal lesions in mice on a cognitive test battery. *Behav. Brain Res.* **2002**, *133*, 57–68. [CrossRef]
55. Greene-Schloesser, D.M.; Van Der Zee, E.A.; Sheppard, D.K.; Castillo, M.R.; Gregg, K.A.; Burrow, T.; Foltz, H.; Slater, M.; Bult-Ito, A. Predictive validity of a non-induced mouse model of compulsive-like behavior. *Behav. Brain Res.* **2011**, *221*, 55–62. [CrossRef] [PubMed]
56. Mitra, S.; Bastos, C.; Chesworth, S.; Frye, C.; Bult-Ito, A. Strain and sex based characterization of behavioral expressions in non-induced compulsive-like mice. *Physiol. Behav.* **2017**, *168*, 103–111. [CrossRef] [PubMed]
57. Dixit, P.V.; Sahu, R.; Mishra, D.K. Marble-burying behavior test as a murine model of compulsive-like behavior. *J. Pharmacol. Toxicol. Methods* **2020**, *102*, 106676. [CrossRef] [PubMed]
58. Jimenez-Gomez, C.; Osentoski, A.; Woods, J.H. Pharmacological evaluation of the adequacy of marble burying as an animal model of compulsion and/or anxiety. *Behav. Pharmacol.* **2011**, *22*, 711–713. [CrossRef] [PubMed]

biomedicines

MDPI

Article

Sex-Dependent Signatures, Time Frames and Longitudinal Fine-Tuning of the Marble Burying Test in Normal and AD-Pathological Aging Mice

Mikel Santana-Santana [1,2], José-Ramón Bayascas [1,3] and Lydia Giménez-Llort [1,2,*]

[1] Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain; mikel.santana@e-campus.uab.cat (M.S.-S.); joseramon.bayascas@uab.cat (J.-R.B.)
[2] Department of Psychiatry and Forensic Medicine, School of Medicine, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
[3] Department of Biochemistry and Molecular Biology, School of Medicine, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
* Correspondence: lidia.gimenez@uab.cat

Abstract: The marble burying (MB) test, a classical test based on the natural tendency of rodents to dig in diverse substrates and to bury small objects, is sensitive to some intrinsic and extrinsic factors. Here, under emerging neuroethological quantitative and qualitative analysis, the MB performance of 12-month-old male and female 3xTg-AD mice for Alzheimer's disease and age-matched counterparts of gold-standard C57BL6 strain with normal aging unveiled sex-dependent signatures. In addition, three temporal analyses, through the (1) time course of the performance, and (2) a repeated test schedule, identified the optimal time frames and schedules to detect sex- and genotype-dependent differences. Besides, a (3) longitudinal design from 12 to 16 months of age monitored the changes in the performance with aging, worsening in AD-mice, and modulation through the repeated test. In summary, the present results allow us to conclude that (1) the marble burying test is responsive to genotype, sex, aging, and its interactions; (2) the male sex was more sensitive to showing the AD-phenotype; (3) longitudinal assessment shows a reduction in females with AD pathology; (4) burying remains stable in repeated testing; (5) the time-course of marbles burying is useful; and (6) burying behavior most likely represents perseverative and/or stereotyped-like behavior rather than anxiety-like behavior in 3xTg-AD mice.

Keywords: neuroethology; behavioral neuroscience methodology; sexual differences; aging; Alzheimer's disease; comorbidities; phobia; anxiety; OCD (obsessive-compulsive symptoms)

Citation: Santana-Santana, M.; Bayascas, J.-R.; Giménez-Llort, L. Sex-Dependent Signatures, Time Frames and Longitudinal Fine-Tuning of the Marble Burying Test in Normal and AD-Pathological Aging Mice. *Biomedicines* **2021**, *9*, 994. https://doi.org/10.3390/biomedicines9080994

Academic Editor: Masaru Tanaka

Received: 9 July 2021
Accepted: 6 August 2021
Published: 11 August 2021

1. Introduction

The behavioral and psychological symptoms associated with dementia (BPSD), including neuropsychiatric symptoms (NPS) such as anxiety and phobias, paranoia and delusion, hallucinations, stereotypes, and other disturbances, are comorbidities manifested in 50–90% of people with Alzheimer's disease (AD) [1]. These non-cognitive problems affect their quality of life [2], are an important source of distress for patients and caregivers [3], and frequently lead to premature institutionalization [4]. Furthermore, recent studies suggest a distinct distribution of NPS comorbidities among sexes, and therefore there is a need to characterize these differences, elucidate the underlying pathophysiology, and identify better treatment targets with a gender perspective [5]. At the translational level, the modeling of BPSD/NPS in basic and preclinical research of AD under the sex perspective is also needed to develop better pharmacological and non-pharmacological preventive/therapeutical interventions that could be effectively translated into clinical scenarios. In this context, natural species-typical behaviors representing active interaction with the environment are excellent ethological scenes to reflect the interplay of cognitive

and non-cognitive disturbances induced by normal and AD-pathological aging. In agreement with this, we have proven the validity of the 3xTg-AD mice to assess the impact of the disease on naturally occurring executive functions and daily life activities based on species-typical behaviors when interacting with the environment, such as burying behavior [6], and nest-building [7,8].

Burying behavior is commonly measured with the marble burying (MB) test [9], a classical behavioral test employed in rodents that exploit the tendency of these animals to dig in diverse substrates and to bury small objects, such as glass marbles, in a test cage with beddings [10]. Initially, this test was pharmacologically validated for its use to measure anxiety-related behaviors and screening for anxiolytic pharmacological drugs [11,12]. However, controversy exists regarding its specificity as it is also proposed as modeling meaningless repetitive and perseverative behaviors mimicking psychotic and obsessive-compulsive (OCD) symptoms [13]. Actually, some authors consider that for MB to be regarded as a reliable screening test for a specific assessment of a neuropsychological construct, the introduction of methodological changes or better experimental designs is needed [13–15]. Thus, two-zone configuration, repeated trials, and limitations inherent to MB score and ceiling/floor effects are among the experimental considerations discussed. Additionally, emerging neuroethological analysis of behavior, which integrates the sequence of behavioral events in an ethogram, may provide a better understanding of the functional role, its modulation, and underlying mechanisms than classical behavioral analysis.

Marble burying behavior is altered in the 3xTg-AD mice. Specifically, it is enhanced in 12-month-old 3xTg-AD male mice, an age mimicking advanced stages of the disease [16,17], can be reversed by risperidone, and be modulated by handling [6,18]. In addition, we have recently proven that at 15-months of age, just 2–3 months of naturalistic isolation, which occurs when congeners die, is enough to exacerbate this behavior despite social lives since they were born, modeling the worsening of OCD described in the current COVID-19 scenario [19]. However, there are still various unresolved questions regarding the effect of sex and age factors on marble burying behavior in normal and AD-pathological aging. First, as in the case in other fields with rodent experimentation [20], the inclusion of female mice in MB testing is not the most common choice [21]. The inclusion of females in animal studies of AD is relevant, even if similar incidence between sexes is found, since risk factors may differentially affect multiple pathways and evolve into different manifestations of NPS and comorbidities [5]. Second, how aging and AD-pathological processes affect burying behavior and the MB profile evolves in a long-term perspective. This is a significant concern due to the intrinsic nature of AD, in which cognitive and psychiatric symptoms are present in early stages and worsen over time as the disease progresses [22]. Hence, to increase the translational value of experimental designs, rather than a transversal comparison of the performance at different age stages, longitudinal studies allow monitoring oof the progression of cognitive and non-cognitive deficits through an AD-pathological life-span.

Therefore, the present study aimed to explore further the contribution of sex and aging in the normal and AD-pathological brain in marble burying behavior. We used middle-aged 3xTg-AD mice through a longitudinal study including methodological modifications (two-zone configuration, repeated trials, and time-course counting of marbles buried) to have a better approach to the possible neuropsychiatric constructs involved in their alteration, and we compared them with those presented in their non-transgenic (NTg) counterparts with the gold-standard C57BL/6 strain genetic background.

2. Materials and Methods

2.1. Animals

A total number of forty-six 12-month-old male and female mice, homozygous 3xTg-AD (males $n = 15$, females $n = 8$) and non-transgenic (NTg, males $n = 10$, females $n = 13$) mice on a C57BL/6 background (after embryonic transfer and backcrossing at least 10 generations), established in the Universitat Autònoma de Barcelona [23] were used. The 3xTg-AD mice harboring transgenes were genetically engineered at the University of Cali-

fornia Irvine, as previously described [16]. Animals were maintained in groups of 3–4 mice per cage (Macrolon, 35 × 35 × 25 cm) filled with 5 cm thick layer of clean woodchips that were the same used for behavioral testing (Ecopure, Chips6, DateSand, UK; Uniform cross-cut wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 × 20 cm, White). All animals were maintained under standard laboratory conditions of food and water ad libitum, 22 ± 2°C, 12 h light: dark cycle with lights on at 8:00 a.m., and relative humidity 50–60%.

2.2. Experimental Design

As illustred in Figure 1, animals were behaviorally assessed at middle-age (12 months of age) and re-tested four months later when they reached old age (16 months of age). In the AD-genotype, these time points correspond to two different advanced stages of the disease with the progressive development of βA and tau pathologies [17].

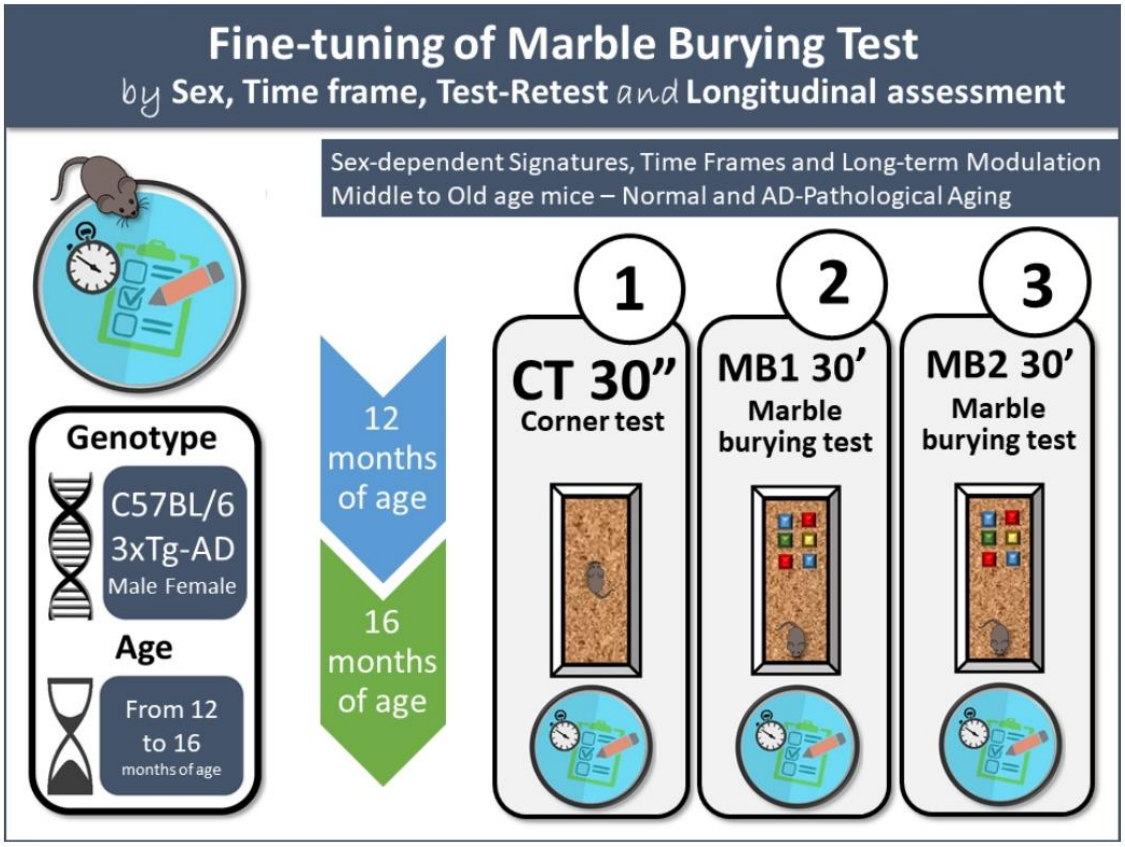

Figure 1. Fine-tuning of the marble burying test by sex, time frame, test–re-test, and longitudinal assessment. Experimental design: a 3-day battery of behavioral tests consisting of a corner test on day 1, a marble burying test on day 2 (MB1), and a repeated test on day 3 (MB2).

2.3. Behavioral Assessments

Behavioral assessments in the corner test and marble burying test under dim white light (20 lx) were conducted during the light phase of the light: dark cycle (from 10 a.m. to 1 p.m.). The tests were performed in a counterbalanced manner, by direct observation by a trained observer, blind to the genotype, and a camera's support. All procedures

were in accordance with 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 protocol CEEAH 3588/DMAH 9452 was approved the 8th of March 2019 by the 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 [24].

Day 1—Corner test (CT) was used to evaluate neophobia. The animal was placed in the center of a clean standard home cage filled with woodchip shave bedding and observed for 30 s. We measured the numbers of corners visited (CTc), the latency to perform the first rearing (CTlatR), and the number of rearings (CTr). The ratio of the number of visited corners and rearings variables (Ratio CTc/r) was calculated.

Days 2 and 3—Marble burying test (MB1) and re-test (MB2): Nine ceramic marbles were put in a standard home cage (Macrolon, 35 × 35 × 25 cm^3) with a 5 cm thick layer of clean woodchips. The marbles were placed evenly spaced (three rows of three) in one-quarter of the cage and allowing the mice to avoid interaction with the marbles. Then, the mouse was introduced in the zone without marbles facing the wall and left to interact with the cage freely. A picture of the cage was taken every 5 min to assess the buried marbles' progress. After 30 min the mice were gently removed from the cage, and the buried marbles were counted. Marbles were counted as buried when their surface was covered at least 90% with bedding material. The number of marbles buried was transformed in a percentage (MBx.y; x, day, y, time of measurement) for further statistical analysis. Twenty-four hours later, animals repeated the test under the same conditions.

2.4. Statistics

Statistical analyses were performed using SPSS 23.0 software. In the corner test, the variables recorded were analyzed by a split-plot design with the factors genotype (G), aging (A), sex (S), according to the experimental design G(2)×A(2)×S(2). ANOVA split-plot designs analyzed the number of marbles buried with the factors time (T), genotype (G), aging (A), sex (S), and day (D), according to the experimental design T(7)×G(2)×S(2)×A(2)×D(2). Post-hoc comparisons were run with Bonferroni corrections. Both the F and the degrees of freedom values were reported when it was possible. Spearman correlations were made to analyze behavioral correlates between the CT and the MB. Correlation coefficients (r) are indicated. A p-value < 0.05 was considered as statistically significant. Graphics were made with GraphPad Prism 6. Abbreviation: sexAgeMBday·minute (i.e, m12MB2.30, male at 12 months of age, re-test, 30 min).

3. Results

3.1. Corner Test for Neophobia

In the corner test (Figure 2), all the variables were sensitive to the aging factor, as the longitudinal analysis showed the reduction of the number of crossings (A, $F_{(1,42)} = 80.104$; $p < 0.001$), the number of rearings (A, $F_{(1,42)} = 24.564$; $p < 0.001$), the crossings/rearings ratio (A, $F_{(1,42)} = 23.903$; $p < 0.001$), and, conversely, the enhancement of the latency of rearing (A, $F_{(1,42)} = 18.085$; $p < 0.001$). Moreover, the crossings/rearings ratio was also sensitive to the genotype and aging interaction (G×A, $F_{(1,42)} = 18.085$; $p < 0.001$).

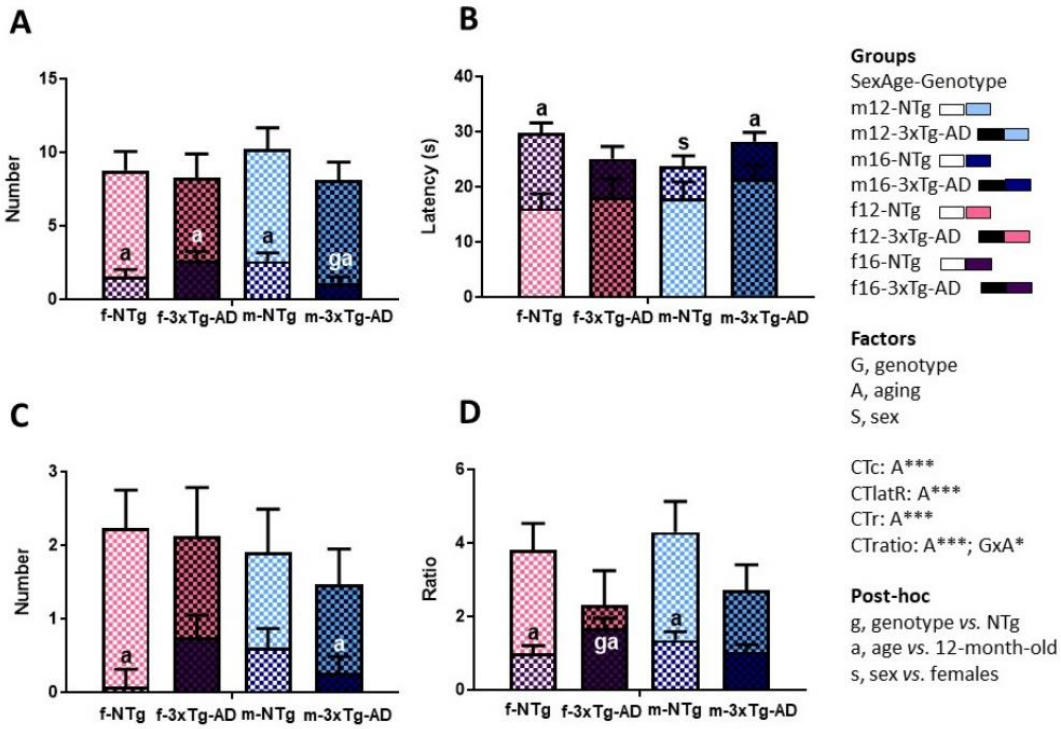

Figure 2. Sex and age effects in the longitudinal assessment in the corner test in mice with normal and AD-pathological aging. (**A**) Number of crossings (CTc); (**B**) Latency of rearing (CTlatR); (**C**) Number of rearings (CTr); (**D**) Ratio CTc/CTr (CTratio); Factorial analysis: G, genotype (NTg, 3xTg-AD mice); A, aging (12, 16 months of age); S, sex (male, female). * $p < 0.05$; *** $p < 0.001$.

The post-hoc analysis indicated meaningful differences as described hereinafter (see g, s, a at each variable in Figure 2). Thus, at 16 months, AD-males and AD-females exhibited a lower number of crossings (g, $F_{(1, 42)} = 4.474$; $p = 0.040$) and higher crossings/rearings ratio (g, $F_{(1, 42)} = 4.335$; $p = 0.043$), respectively, than their NTg counterparts. Regarding sex, NTg-males showed a lower latency of rearing than NTg-females at 16 months of age. With aging, all the groups manifested a reduction in the number of crossings (NTg-females: $p < 0.001$; AD-females: $p = 0.003$; NTg-males: $p < 0.001$; AD-males: $p < 0.001$). However, the rearing behavior only was affected in NTg-females (CTlatR: $p = 0.0001$; CTr: $p = 0.001$) and AD-males (CTlatR: $p = 0.048$; CTr: $p = 0.035$). At 16 months, these mice showed both a delayed elicitation of rearing and a lower number of total rearings. Finally, a reduction with aging in the ratio of crossings/rearings was also presented in NTg-females ($p = 0.001$), AD-males ($p = 0.021$) and WT-males ($p = 0.001$). The post-hoc analysis of these results is also depicted in Table 1.

Table 1. Post-hoc analysis of genotype, sex, and aging differences in the corner test.

Post-Hoc Analysis CORNER TEST	Genotype (vs. NTg Mice)		Sex (vs. Female Mice)		Aging (vs. 12-Month-Old Mice)	
Behavioral variable	12 mo	16 mo	12 mo	16 mo	Each group	
CTc	All n.s.	Males $p = 0.040$	All n.s.	All n.s.	fNTg f3xTg-AD mNTg m3xTg-AD	$p < 0.001$ $p = 0.003$ $p < 0.001$ $p < 0.001$
CTlatR	All n.s.	All n.s.	All n.s.	NTg $p = 0.024$	fNTg f3xTg-AD mNTg m3xTg-AD	$p < 0.001$ n.s. n.s. $p = 0.048$
CTr	All n.s.	All n.s.	All n.s.	All n.s.	fNTg f3xTg-AD mNTg m3xTg-AD	$p = 0.001$ n.s. n.s. $p = 0.035$
CTratio	All n.s.	Females $p = 0.043$	All n.s.	All n.s.	fNTg f3xTg-AD mNTg m3xTgAD	$p = 0.001$ n.s. $p = 0.001$ $p = 0.021$

Abbreviations: mo, months-old; CTc, number of crossings; CTlatR, latency of rearing; CTr, number of rearings; CTratio, CTc/CTr ratio; fNTg, female NTg mice; f3xTg-AD, female 3xTg-AD mice; mNTg, male NTg mice; m3xTg-AD, male 3xTg-AD mice; n.s., *p*-value value is not statistically significant.

3.2. Longitudinal Assessment of Marble Burying Test and Repeated Test

The marble burying test (Figure 3) was sensitive to the main factors genotype (G, $F_{(1, 42)} = 4.212$; $p = 0.046$) and aging (A, $F_{(1, 42)} = 4.325$; $p = 0.044$), while sex effects depended on the genotype (G×S, $F_{(1, 42)} = 12.768$; $p = 0.001$). The time-course analysis indicated that time (minute) (T, $F_{(2.373, 99.681)} = 68.644$; $p < 0.001$) was determinant to detect genotype, sex, and age effects and interactions (T×G×A×S, $F_{(3.234, 135.830)} = 3.442$, $p = 0.016$; TxA, $F_{(3.234, 135.830)} = 3.385$; $p = 0.017$, T×G×S, $F_{(2.373, 99.681)} = 5.589$; $p = 0.003$), while re-test 24 h later reduced the performances of 12-month-old animals in a lower/higher intensity manner depending on the genotype and sex (G×A×S×D, $F_{(1,42)} = 5.598$; $p = 0.023$). In general, T×G×A×S×D interactions effects were not statistically significant ($F_{(3.499, 146.951)} = 5.400$; $p = 0.061$).The post-hoc analysis indicated meaningful differences as described hereinafter, providing evidence that the observation windows are critical (see g, a, s, d at each time point). The post-hoc analysis of these results is summarized in Table 2.

In the first MB testing, several meaningful differences between genotypes in the test performance were exhibited. At 12 months of age, post-hoc comparisons showed increased marble burying in AD-males compared to NTg-males (mMB5: $p = 0.010$; mMB10: $p = 0.013$; mMB15: $p = 0.009$; mMB20: $p = 0.014$), but no differences were found between females at this age.

However, when animals reached 16 months of age, the AD-phenotype was found up-regulated in males and down-regulated in females compared to their NTg counterparts. Thus, AD-males showed increased marble burying compared to NTg-males (m16MB1·10: $p = 0.016$; m16MB1·15: $p < 0.001$; m16MB1·20: $p < 0.001$; m16MB·25: $p = 0.001$), whereas AD-females buried less marbles than their NTg counterparts (f16MB1·15: $p = 0.046$; f16MB1·20: $p = 0.040$; f16MB1·25: $p = 0.011$; f16MB1·30: $p = 0.002$).

Table 2. Post-hoc analysis of genotype, sex, and aging differences in the marble test.

Post-Hoc Analysis MARBLE TEST	**Genotype (vs. NTg Mice)**		**Sex (vs. Female Mice)**		**Aging (vs. 12 mo)**	**Repeated Test(vs. Day 1)**
Behavioral variable	12 mo	16 mo	12 mo	16 mo	Each group	Each group
Day 1 (MB1)						
MB1.5	Females $p = 0.010$	All n.s.	3xTg-AD $p = 0.036$	NTg $p = 0.025$	All n.s.	All n.s.
MB1.10	Females $p = 0.013$	Males $p = 0.016$	All n.s.	NTg $p = 0.043$	All n.s.	All n.s.
MB1.15	Females $p = 0.009$	Females $p = 0.046$ Males $p = 0.000$	All n.s.	NTg $p = 0.002$ 3xTg-AD $p = 0.011$	All n.s.	All n.s.
MB1.20	Females $p = 0.014$	Females $p = 0.040$ Males $p = 0.000$	All n.s.	NTg $p = 0.003$ 3xTg-AD $p = 0.003$	f3xTg-AD $p = 0.043$	All n.s.
MB1.25	All n.s.	Females $p = 0.011$ Males $p = 0.001$	All n.s.	NTg $p = 0.004$ 3xTg-AD $p = 0.003$	f3xTg-AD $p = 0.010$	All n.s.
MB1.30	All n.s.	Females $p = 0.002$	All n.s.	NTg $p = 0.012$ 3xTg-AD $p = 0.007$	f3xTg-AD $p = 0.010$	All n.s.
Day 2 (MB2)						
MB2.5	Males $p = 0.005$	Males $p = 0.021$	3xTg-AD $p = 0.009$	All n.s.	All n.s.	All n.s.
MB2.10	All n.s.	Males $p = 0.024$	3xTg-AD $p = 0.045$	All n.s.	All n.s.	All n.s.
MB2.15	Males $p = 0.006$	Males $p = 0.008$	All n.s.	All n.s.	All n.s.	All n.s.
MB2.20	Males $p = 0.007$	Males $p = 0.004$	All n.s.	3xTg-AD $p = 0.012$	All n.s.	All n.s.
MB2.25	Males $p = 0.024$	Males $p = 0.020$	Alln.s.	3xTg-AD $p = 0.039$	All n.s.	All n.s.
MB2.30	All n.s.	Males $p = 0.038$	All n.s.	3xTg-AD $p = 0.025$	All n.s.	fNTg $p = 0.005$

Abbreviations: mo, months-old; (MBx.y), MB, Marble test; x, day; y, time (accumulated counts); fNTg, females NTg; n.s., *p*-value value is not statistically significant.

Besides, only the female sex exhibited longitudinal differences in the performance of the test. At 16 months of age, AD-female mice showed a lower percentage of marbles buried in the test's final minutes (f12-16MB1·20: $p = 0.043$; f12-16MB1·25: $p = 0.010$; f12-16MB1·30: $p = 0.010$) compared to their scores at 12 months of age.

Besides, several significant sex differences were found. At 12 months of age, significant post-hoc differences only appeared at the first five minutes of the test, where AD-males buried more marbles than AD-females (mf12MB1·5, $p = 0.036$). No differences were found between NTg mice at 12 months. Nevertheless, several meaningful differences were manifested by both AD-mice and NTg-mice at 16 months old. At this age, NTg-

males buried less marbles than NTg-females early in the test (mf16MB1·5: $p = 0.025$; mf16MB1·10: $p = 0.043$; mf16MB1·15: $p = 0.002$; mf16MB1·20: $p = 0.003$; mf16MB1·25: $p = 0.004$; mf16MB1·30: $p = 0.012$). Conversely, AD-males buried a higher percentage than AD-females (mf16MB1·15: $p = 0.011$; mf16MB1·20: $p = 0.003$; mf16MB1·25: $p = 0.003$; mf16MB1·30: $p = 0.007$).

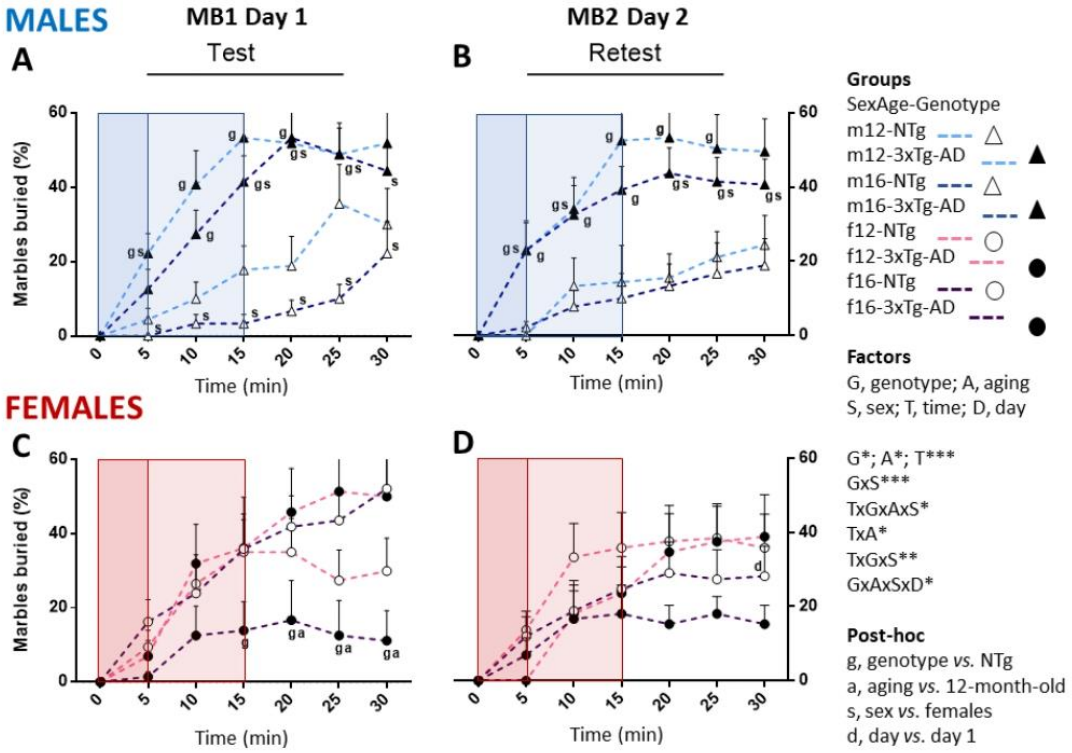

Figure 3. Sex, age, time, and day effects in the longitudinal assessment in the marble burying test and repeated test in mice with normal and AD-pathological aging. (**A**) Males marble burying test on day 1 (MB1 Day 1) and (**B**) re-test 24 h later (MB2 Day 2); (**C**) Females marble burying test on day 1 (MB1 Day 1) and (**D**) re-test 24 h later (MB2 Day 2); Factorial analysis: G, genotype (NTg, 3xTg-AD mice); A, aging (12, 16 months of age); S, sex (male, female); T, time (0–30 min); D, day (Day 1, Day 2). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In the re-test, 24 h later, 12-month-old AD-males buried more marbles than NTg-males at several time points of the test (m12MB2·5: $p = 0.005$; m12MB2·15: $p = 0.006$; m12MB2·20: $p = 0.007$; m12MB2·25: $p = 0.0024$). Conversely, there was an absence of differences between 12-month-old AD-females and NTg-females. When the animals reached 16 months of age, genotype differences still persisted between AD and NTg males (m16MB2·5: $p = 0.021$; m16MB2·10: $p = 0.024$; m16MB2·15: $p = 0.008$; m16MB2·20: $p = 0.004$; m16MB2·25: $p = 0.020$; m16MB2·30: $p = 0.038$), but those observed between females disappeared.

Regarding sex post-hoc differences in the re-test, they were clearly shown in the group of AD mice at both ages studied. Thus, 12-month-old AD-males buried a higher percentage of marbles than NTg-males at the beginning of the test (m12MB2·5: $p = 0.009$; m12MB2·10: $p = 0.0045$). Four months later, when they reached 16 months of age, AD-males showed very similar test and re-test patterns (m16MB2·20: $p = 0.012$; m16MB2·25: $p = 0.0039$; m16MB2·30: $p = 0.025$). However, at 16 months, differences between NTg-males and NTg-females were not found.

Finally, the percentage of marbles buried between the test and the re-test on the first and the second day of testing in all the time measures for all the groups were compared. At 12 months of age, no differences were found, while the performance of these animals at 16 months showed one difference: the NTg-females buried fewer marbles at the end time point of the second test (f16MB2·30: $p = 0.005$). Therefore, the time-course analysis is essential to unveil sex, aging, and re-test differences otherwise under-detected.

3.3. Corner Test and Marble Burying Test Correlations

In calculating these correlations, the variables genotype and sex were taken into account to generate the tables. The relationship between these tests was analyzed for the two ages studied. To simplify the analysis, we paid attention mainly to both the percentage of marbles buried at five and thirty minutes for the first marble test testing (MB1·5 and MB1·30, respectively).

At 12 months of age, correlations between the CT and the MB tests were only exhibited by female mice. For NTg-female mice, the number of rearings in the CT was positively correlated with the percentage of marbles buried at both five and thirty minutes (CTr © f12MB1·5, r = 0.716; $p = 0.006$; CTr © f12MB1·30, r = 0.620; $p = 0.024$). While for AD-female mice, the number of rearings in the CT were positively correlated with the percentage of marbles buried at five minutes (CTr © f12MB1·5, r = 0.835; $p = 0.010$), the latency of rearing was inversely correlated also with the percentage of marbles buried at five minutes (CTlatR © f12MB1·5, r = −0.845; $p = 0.008$). At 16 months of age, none of the groups exhibited correlations. Among others, all these results are summarized in Table 3.

Table 3. Corner test and marble burying test Spearman correlation analysis.

		CTc	CTlatR	CTr	CTratio
fNTg ($n = 13$) at 12 moa					
MB1.5	Spearman correlation Sig. (2-tailed)	n.s.	n.s.	0.716 ** 0.006	n.s.
MB1.10	Spearman correlation Sig. (2-tailed)	n.s.	n.s.	0.617 * 0.025	n.s.
MB1.15	Spearman correlation Sig. (2-tailed)	n.s.	n.s.	0.697 ** 0.008	n.s.
MB1.20	Spearman correlation Sig. (2-tailed)	n.s.	n.s.	0.631 * 0.021	n.s.
MB1.25	Spearman correlation Sig. (2-tailed)	n.s.	n.s.	n.s.	n.s.
MB1.30	Spearman correlation Sig. (2-tailed)	n.s.	n.s.	0.620 * 0.024	n.s.
fNTg at 16 moa					
MB1.25	Spearman correlation Sig. (2-tailed)	0.568 * 0.043	n.s.	n.s.	n.s.
f3xTg-AD ($n = 8$) at 12 moa					
MB1.5	Spearman correlation Sig. (2-tailed)	n.s.	−0.845 ** 0.008	0.0835 ** 0.010	n.s.
All the other groups					
MB1.all	Spearman correlation Sig. (2-tailed)	n.s.	n.s.	n.s.	n.s.

Only statistically significant correlations are indicated. Abbreviations: fNTg, females NTg; f3xTg-AD, females 3xTg-AD mice; moa, months of age; (MBx.y) MB, marble test; x, day; y, time (accumulated counts); fNTg Sig., significant; **, correlation significant at the 0.01 level (2-tailed), *, correlation significant at the 0.05 level (2-tailed).

4. Discussion

In the present work, we corroborate the previously described higher burying of marbles in 3xTg-AD male mice at 12 months of age [6,18], and we demonstrate the complexity of factor interplay in the performance of the MB test. For the first time, we show that the higher performance in 3xT-AD male mice is also observed in the female sex, albeit statistically significant genotype differences in females are only reached at 16 months. Moreover, the longitudinal design allows the monitoring of changes in the performance with worsening of the disease only in AD-female mice, and normal aging in NTg mice, from 12 to 16 months of age, and its modulation through the repeated test. Most importantly, the time-course analysis provides a tool to discriminate the best temporal windows of observation depending on these factors.

4.1. New Insight of Burying Behavior in 3xTg-AD Mice

We provide evidence that the higher burying of the AD-male mice than NTg mice over 30 min [6,19] is a phenomenon sustained throughout the test and could also be scored since the beginning. For the first time, the inclusion of 3xTg-AD female mice in MB testing is reported. In contrast, despite AD-females showing higher percentages than their NTg counterparts at the end of the test, these differences did not reach statistical significance. Sexual differences were present in the AD-phenotype at the beginning of the test at 12 months old. However, with aging, these differences were exhibited in the second half of the test. In both stances, AD-female mice presented the lower percentage of marbles buried. Although it is known that the estrous cycle can affect burying activity [25–27], comparative studies between sexes are scarce. Those available do not find differences [28], and if they do, they are dependent on the menstrual cycle [28]. Therefore, this finding represents a step forward in the exploration of sex differences in burying behavior.

In addition, for the first time, the longitudinal design showed that pathological aging influenced MB performance. At 16 months of age, males did not show any significant difference in performance compared to their assessment at 12 months. In contrast, the performance of AD-females was lower at 16 months through all the tests, although statistical significance was reached from minute 20, and the performance of NTg-females was relatively similar at both ages. In normal aging, NTg-males showed significantly lower MB compared to NTg-females of the same age. Also, the genotype differences between male mice persisted at this age through the MB test. Therefore, we found a differential influence of how normal and AD-pathological aging affects MB performance. Firstly, aging has a differentiated response, whether accompanied by pathology or not, as shown by the fact that NTg animals do not undergo percentage changes. However, in addition, the aging of 3xTg-AD mice produced a sex-dependent differential response in their behavior, with significant differences between 16-month-old AD-females and AD-males in the second half of the MB test.

Several authors advocate for repeated trials as necessary for using MB as a model of neophobia/anxiety or OCD [13]. Following these recommendations, at both ages, we applied two consecutive days of MB testing. When the time-course of the test and re-test of all groups was compared, the standard pattern was the absence of differences in their performance at both ages, although NTg-females showed significantly lower performance at min 30 in the re-test performed at 16 months of age. As can be seen, these variations are not large enough to generate significant changes concerning their performance on the previous day. At 16 months of age, the lower performance of NTg-females eliminates both the genotype and sexual differences with AD-females and NTg-males, respectively. Moreover, the reduction of the performance of marbles buried in AD-females at 12 months suggests that aging differences with respect to AD-females at 16 months did not show up, and caused the temporal amplification of sexual differences at the beginning of the test regarding AD-males. For the rest, genetic and sexual differences manifested by AD-males at both ages were still conserved. Although slight variations are exhibited in some measures, they do not change their manifested behavioral phenotype interpretation.

4.2. Corner Test and Its Relationship with Marble Burying Test in 3xTg-AD Mice

In the present work, we did not find genotype differences in neophobia, contrary to what occurred on other occasions [6,19]. However, we did observe a reduction in mouse activity and a slowdown of latency due to aging. This occurs in all variables, although it does not always occur in all groups in a statistically significant way, although a tendency can be appreciated.

The relationship between the burrowing percentage in the MB and the CT variables could be described as inconsistent and poor. To simplify its interpretation, we focused our attention on the first measure, which should be more sensitive to neophobia, and the last one, for comparative purposes. If we hypothesize that the higher percentage of buried marbles is due to anxiety, a clear relationship should be visible between the two tests, especially for AD-males. However, correlations only appeared in both NTg and AD female mice. To summarize, these results would be in line with the poor relationship showed between the MB and other tests for assessing anxiety-like behavior in other studies [29–31].

4.3. Marble Test as a Model of Anxiety-Like or OCD-Like Behavior in 3xTg-AD Mice?

With all of the previously discussed, the modeling of anxiety-like behavior in the burying behavior of this animal model is certainly questionable. First of all, due to the utilization of the two-zone configuration, the animals can avoid the marbles, so we can assume that the interaction with the marbles is, to some extent, voluntary. Then, it would be expected that AD-mice would show passive avoidance of marbles, which is not the case. These results would be consistent with those shown by other studies using a two-zone configuration [11,14,31–34]. Furthermore, in the re-testing, no change would indicate that habituation to a stressful situation is produced, either assuming two different fight-to-flight scenarios: a case where the animals were so frightened of the marbles that they buried as a defensive strategy (AD-mice), or they would avoid their interaction with them (NTg mice). However, they interact as well, but to a lesser extent. Moreover, although there are no clear genotype differences in neophobia, measured through CT, differences still appeared in burying behavior in MB. Finally, correlations between the burying percentages and the CT variables are scarce and inconsistent. A possible hypothesis to support the anxiety-like modeling of burying behavior could be that the inherited anxiety trait of these mice could make their response to marbles resistant to habituation [35,36] and thereby invoking either active burying or passive avoidance behavior as coping strategies [36–41]. Since 3xTg-AD mice present higher baseline anxiety [23], which produces differentiated anxious responses depending on the test [18], the previous hypothesis is still possible. Interestingly, we already reported that other animal models for anxiety, such as the A1 receptor knock out mice, also show reduced habituation [42]. Moreover, in our precedent Gimenez-Llort and Alveal-Mellado's work [19], 3xTg-AD mice showed a higher freezing behavior in the open-field test accompanied with higher amounts of marble burying, contrary to NTg-mice. Therefore, although the modeling of anxious-like behavior is questionable, with the methodology employed it is not completely discardable.

It seems pretty clear that regardless of whether they bury more or fewer marbles, their performance in this test is persistent and stable over time, in concordance with other studies with repeated MB application [28,31,32,43–45]. This event would support the current practice of using burying behavior as an indication of OCD-like behavior, although this approach also has certain validity concerns [13,45,46]. While OCD may be a risk factor for developing AD [47,48], it is unlikely to be the construct modeled in the 3xTg-AD mice. Due to the repetitive and perseverative nature of burying activity, this behavior could represent NPS such as perseverative behavior and/or stereotyped behavior. Both are NPS usually present in patients with Alzheimer's and other dementias [49–51]. In addition, 3xTg-AD mice have been shown to present more significant errors due to preserverative behavioral hopelessness paradigms [52] and attentional tasks [53], and greater presence of stereotyped behaviors at early stages of the disease [23]. Therefore, it is quite possible that burying behavior in 3xTg-AD mice reflects perseverative and/or stereotyped-like

behavior rather than anxiety-like behavior. However, this is not necessarily denying that anxiety may influence the performance on the marble test, mainly through differentiated coping strategies.

Far from discussions about which pathology models the test in our animal model, what remains clear is that the burying behavior is stable and resistant to repetition, regardless of the group or whether the animals show high or low percentages. Therefore another way of interpreting the results would be to look at them with a neuroethological perspective, in which burying is an inherent behavior of the animal [13], in this case, sensitive to AD-pathology and with differentiated response depending on sex, aging, and their interactions. Considering that burying represents the application of digging [13], defined as the displacement of a substrate mainly using forepaws [54], to a more complex task, it would be expected that digging was the sensitive behavior to the AD pathology. Thus, 3xTg-AD mice should show a similar profile in other tests involving digging behavior shown in the MB. Therefore, further research is needed to confirm this hypothesis.

4.4. Integrating Our New Findings into Our Previous Knowledge of The Burying Behavior of 3xTg-AD Mice

The inter-test stability in the burying behavior also has important implications for supporting the conclusions developed in previous works of our research group with the 3xTg-AD mice. In Torres-Lista's previous studies [6,18], chronic administration of risperidone reduced the number of marbles buried compared to their pre-treatment testing for both AD-male and NTg-male mice. Moreover, this number was modulated in saline groups, albeit presenting a different pattern depending on the genotype. In AD-mice, as happened with risperidone, the number of marbles buried was reduced, whereas for NTg-males, this number was higher. These changes in the marble activity were attributed to an effect of the repetitive handling for the administration of the saline compounds. The results obtained in the present study would support that conclusion since, without the handling, the animal's burying behavior should have been unaltered in the resetting. We attributed the reduction of marble activity in the AD saline group to the anxiolytic effect of handling [55–57], and the increase in marble activity in NTg-males with an increase in their emotional state. However, this claim remains unclear partly for our present results and partly because burying behavior can be enhanced in mice by stressing the animal through non-pharmacological intervention [58–60]. Thus, it may have been possible that the repetitive subcutaneous administration of the saline compound acted as a stressor affecting the inherited burying behavior of each group and thereby either increasing or decreasing it depending on their inherited pattern.

4.5. Benefits of Implementing the Time-Course of Marble Buried

This is the first time that time-course of marbles buried by 3xTg-AD mice in MB was studied as a methodological novelty. As we previously explained in the method, it consisted of counting the number of marbles buried at five minute intervals until the end of the test. Although we have already commented on their results in the previous section, we would like to make some remarks to promote its use. First of all, its application is easy and affordable. It can be done through photography or video, not interfering in the normal development of the test. Moreover, the intervals can be easily adapted to the needs of the study, although it would be advisable to include at least one measurement in the middle of the test. The reason for this is that, at least in our model, differences usually appear at 10–15 min, and the score at that time does not differ significantly from the score obtained at 30 min (analysis not shown). To us, the most important reason for its use is that it gives us valuable information about the behavioral pattern of the animal throughout the test, helping us to establish a more accurate profile of the animal and the possible differences between them. This may be especially important in pharmacological and non-pharmacological interventions as well, as such interventions could modify the pattern of the animal and not just the final test score. Concerning the latter, time-course could save many nightmares to researchers using the MB. If we consider only the final

measurement, we could erroneously conclude that there are significant differences (false positive) or not (false negative) between the two groups when in fact, throughout the test, this was not the case. In our experiment, we can see an example of each. As a false positive in the final measurement, we have that the 16-month-old AD-females burying percentage is significantly different regarding their score on the previous testing, but only in that score. While as a false negative, we see that in the last measurement of the re-test, the significant difference between AD-males and NTg-males at 12 months disappears when there have been significant differences in all the previous scores. In addition, although the counting of marbles through the test has existed for a long time [43], its use is not widespread. It is difficult to find examples of its use in the literature, although they certainly exist [59,61]. As in our experiment, the differences found at the end of the test are usually manifested from the first measurements and are relatively stable over the time course. Considering the foregoing, we consider that the advantages of its application far outweigh the costs of its implementation.

4.6. Methodological Limitations of the Study

Although, in our view, the data support the above discussions, certain methodological limitations may influence the degree of certainty of our conclusions. Here, we'll discuss them to warn the reader regarding the possible impact of these limitations on interpretability and, consequently, the conclusions drawn.

First, it is necessary to discuss the statistical analysis employed. MB is commonly analyzed using linear models, such as the *t*-test and ANOVA. In our case, we have also employed a split-plot ANOVA. However, as Lazic [15] points out, this type of analysis may be inadequate because, as the number of marbles is a counted data, it does not meet the requirements of this type of analysis, leading to 95% confidence intervals that include impossible values (less than zero or greater than the number of marbles present), misleading p-values, and impossible predictions. There are other more appropriate (non-parametric) types of analysis. However, in our case, we decided to use the analysis for several reasons: 1) to be in line with our previous studies, 2) to use the most commonly used method in the literature, and 3) the complexity of the experimental design made it very difficult to use non-parametric analysis techniques.

On the other hand, there are several limitations in ascertaining which construct the MB test relates to or models. In the case of anxiety, there are more classical tests than the CT for measuring anxiety-like behavior (e.g., open field, elevated plus maze). We used the CT because in our previous studies [6], there were correlations concerning the MB. However, as mentioned above, other studies have explored the relationship between anxiety tests and the MB and obtained relatively poor results [29–31], so it is unlikely that this would be any different in our case. In perseverative behavior, we also do not have an alternative behavior (e.g., grooming) in the MB or another test to validate this hypothesis. However, in this case, the design relies on the resistance to habituation of the marble test, as is the case in other experimental tests e.g., [31]. The main intention of this paper is not to conclude what behavior models the MB in the 3xTg-AD model, although we do hypothesize, based on the data obtained, that it could be due to the previous points of the discussion.

All in all, it is clear that more research is needed to explore these questions further and overcome the limitations present in this study.

5. Conclusions

In summary, the present results allow us to conclude that (1) the marble test is responsive to genotype, sex, aging, and its interactions; (2) the male sex was more sensitive to showing the AD-phenotype; (3) longitudinal study shows a reduction of burying in females with AD pathology; (4) burying remains stable in repeated testing; (5) the time-course of marbles buried is a useful methodological modification; and (6) burying behavior in the MB test most likely represents perseverative and/or stereotyped-like behavior rather than anxiety-like behavior in 3xTg-AD mice. More research is needed in the 3xTg-AD mice to

approach further the modeling of perseverative and stereotyped-like behavior in MB and to be able to verify if the profile shown in the MB test is transferable to other tests that imply digging behavior.

Author Contributions: Conceptualization, L.G.-L. and M.S.-S.; Behavioral performance, analysis, statistics, M.S.-S.; Illustrations: M.S.-S. and L.G.-L. Writing—original draft preparation, M.S.-S.; writing—review and editing, M.S.-S., L.G.-L. and J.-R.B.; Funding acquisition, L.G.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by UAB-GE-260408 to L.G.-L. The colony of 3xTg-AD mice is sustained by ArrestAD H2020 Fet-OPEN-1-2016-2017-737390, European Union's Horizon 2020 research and innovation program under grant agreement No 737390 to L.G.-L. It also received financial support from Memorial Mercedes Llort Sender 2021/80/09241941.6.

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

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Frank M. LaFerla, Institute for Memory Impairments and Neurological Disorders, University of California Irvine, CA, USA, for kindly providing the progenitors of the Spanish colonies of homozygous 3xTg-AD and NTg mice.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Ballard, C.; Corbett, A. Management of neuropsychiatric symptoms in people with dementia. *CNS Drugs* **2010**, *24*, 729–739. [CrossRef] [PubMed]
2. Shin, I.S.; Carter, M.; Masterman, D.; Fairbanks, L.; Cummings, J.L. Neuropsychiatric symptoms and quality of life in Alzheimer disease. *Am. J. Geriatr. Psychiatry* **2005**, *13*, 469–474. [CrossRef] [PubMed]
3. Tan, L.L.; Wong, H.B.; Allen, H. The impact of neuropsychiatric symptoms of dementia on distress in family and professional caregivers in Singapore. *Int. Psychogeriatr.* **2005**, *17*, 253–263. [CrossRef]
4. Hope, T.; Keene, J.; Gedling, k.; Fairburn, C.G.; Jacoby, R. Predictors of institutionalization for people with dementia living at home with a carer. *Int. J. Geriatr. Psychiatry* **1998**, *13*, 682–690. [CrossRef]
5. Mielke, M.M. Sex and Gender Differences in Alzheimer's Disease Dementia. *Psychiatr. Times* **2018**, *35*, 14–17. [PubMed]
6. Torres-Lista, V.; López-Pousa, S.; Giménez-Llort, L. Marble-burying is enhanced in 3xTg-AD mice, can be reversed by risperidone and it is modulable by handling. *Behav. Process.* **2015**, *116*, 69–74. [CrossRef]
7. Torres-Lista, V.; Giménez-Llort, L. Impairment of nesting behaviour in 3xTg-AD mice. *Behav. Brain Res.* **2013**, *247*, 153–157. [CrossRef] [PubMed]
8. Giménez-Llort, L.; Torres-Lista, V. Social Nesting, Animal Welfare, and Disease Monitoring. *Animals* **2021**, *11*, 1079. [CrossRef]
9. Deacon, R.M. Digging and marble burying in mice: Simple methods for in vivo identification of biological impacts. *Nat. Protoc.* **2006**, *1*, 122–124. [CrossRef] [PubMed]
10. Pinel, J.P.J.; Treit, D. Burying as a defensive response in rats. *J. Comp. Physiol. Psychol.* **1978**, *92*, 708–712. [CrossRef]
11. Broekkamp, C.L.; Rijk, H.W.; Joly-Gelouin, D.; Lloyd, K.L. Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. *Eur. J. Pharmacol.* **1986**, *126*, 223–229. [CrossRef]
12. Njung'e, K.; Handley, S.L. Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. *Br. J. Pharmacol.* **1991**, *104*, 105–112. [CrossRef] [PubMed]
13. de Brouwer, G.; Fick, A.; Harvey, B.H.; Wolmarans, W. A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward. *Cogn. Affect. Behav. Neurosci.* **2019**, *19*, 1–39. [CrossRef] [PubMed]
14. de Brouwer, G.; Wolmarans, W. Back to basics: A methodological perspective on marble-burying behavior as a screening test for psychiatric illness. *Behav. Process.* **2018**, *157*, 590–600. [CrossRef]
15. Lazic, S.E. Analytical strategies for the marble burying test: Avoiding impossible predictions and invalid *p*-values. *BMC Res. Notes* **2015**, *8*, 1–10. [CrossRef] [PubMed]

16. Oddo, S.; Caccamo, A.; Shepherd, J.D.; Murphy, M.P.; Golde, T.E.; Kayed, R.; Metherate, R.; Mattson, M.P.; Akbari, Y.; LaFerla, F.M. Triple-transgenic model of Alzheimer's disease with plaques and tangles: Intracellular Abeta and synaptic dysfunction. *Neuron* **2003**, *39*, 409–421. [CrossRef]
17. Belfiore, R.; Rodin, A.; Ferreira, E.; Velazquez, R.; Branca, C.; Caccamo, A.; Oddo, S. Temporal and regional progression of Alzheimer's disease-like pathology in 3xTg-AD mice. *Aging Cell* **2019**, *18*, e12873. [CrossRef]
18. Torres-Lista, V.; López-Pousa, S.; Giménez-Llort, L. Impact of Chronic Risperidone Use on Behavior and Survival of 3xTg-AD Mice Model of Alzheimer's Disease and Mice With Normal Aging. *Front. Pharmacol.* **2019**, *10*, 1061. [CrossRef]
19. Gimenez-Llort, L.; Alveal-Mellado, D. Digging Signatures in 13-Month-Old 3xTg-AD Mice for Alzheimer's Disease and Its Disruption by Isolation Despite Social Life Since They Were Born. *Front. Behav. Neurosci.* **2021**, *19*. [CrossRef]
20. Zucker, I.; Beery, A.K. Males still dominate animal studies. *Nature* **2010**, *465*, 690. [CrossRef]
21. Çalışkan, H.; Şentunali, B.; Özden, F.M.; Cihan, K.H.; Uzunkulaoğlu, M.; Çakan, O.; Kankal, S.; Zaloğlu, N. Marble burying test analysis in terms of biological and non-biological factors. *J. Appl. Biol. Sci.* **2017**, *11*, 54–57.
22. Giménez-Llort, L.; Blázquez, G.; Cañete, T.; ohansson, B.; Oddo, S.; Tobeña, A.; LaFerla, F.M.; Fernández-Teruel, A. Modeling behavioral and neuronal symptoms of Alzheimer's disease in mice: A role for intraneuronal amyloid. *Neurosci. Biobehav. Rev.* **2007**, *31*, 125–147. [CrossRef]
23. Baeta-Corral, R.; Giménez-Llort, L. Bizarre behaviors and risk assessment in 3xTg-AD mice at early stages of the disease. *Behav. Brain Res.* **2014**, *258*, 97–105. [CrossRef]
24. Kilkenny, C.; Browne, W.J.; Cuthill, I.C.; Emerson, M.; Altman, D.G. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol.* **2010**, *24*. [CrossRef]
25. Schneider, T.; Popik, P. Attenuation of estrous cycle-dependent marble burying in female rats by acute treatment with progesterone and antidepressants. *Psychoneuroendocrinology* **2007**, *32*, 651–659. [CrossRef] [PubMed]
26. Llaneza, D.C.; Frye, C.A. Progestogens and estrogen influence impulsive burying and avoidant freezing behavior of naturally cycling and ovariectomized rats. *Pharmacol. Biochem. Behav.* **2009**, *93*, 337–342. [CrossRef] [PubMed]
27. Mitra, S.; Bastos, C.P.; Chesworth, S.; Frye, C.; Bult-Ito, A. Strain and sex based characterization of behavioral expressions in non-induced compulsive-like mice. *Physiol. Behav.* **2017**, *168*, 103–111. [CrossRef] [PubMed]
28. Taylor, G.T.; Lerch, S.; Chourbaji, S. Marble burying as compulsive behaviors in male and female mice. *Acta Neurobiol. Exp. (Wars)* **2017**, *77*, 254–260. [CrossRef] [PubMed]
29. Sanathara, N.M.; Garau, C.; Alachkar, A.; Wang, L.; Wang, Z.; Nishimori, K.; Xu, X.; Civelli, O. Melanin concentrating hormone modulates oxytocin-mediated marble burying. *Neuropharmacology* **2018**, *128*, 22–32. [CrossRef] [PubMed]
30. Savy, C.Y.; Fitchett, A.E.; McQuade, R.; Gartside, S.E.; Morris, C.M.; Blain, P.G.; Judge, S.J. Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in adult male rats as assessed by marble burying behaviour. *Neurotoxicology* **2015**, *50*, 149–156. [CrossRef]
31. Thomas, A.; Burant, A.; Bui, N.; Graham, D.; Yuva-Paylor, L.A.; Paylor, R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology* **2009**, *204*, 361–373. [CrossRef]
32. Njung'e, K.; Handley, S.L. Evaluation of marble-burying behavior as a model of anxiety. *Pharmacol. Biochem. Behav.* **1991**, *38*, 63–67. [CrossRef]
33. Kaehler, S.T.; Singewald, N.; Sinner, C.; Philippu, A. Nitric oxide modulates the release of serotonin in the rat hypothalamus. *Brain Res.* **1999**, *835*, 346–349. [CrossRef]
34. Nicolas, L.B.; Kolb, Y.; Prinssen, E.P. A combined marble burying-locomotor activity test in mice: A practical screening test with sensitivity to different classes of anxiolytics and antidepressants. *Eur. J. Pharmacol.* **2006**, *547*, 106–115. [CrossRef] [PubMed]
35. Stein, D.J.; Bouwer, C. A neuro-evolutionary approach to the anxiety disorders. *J. Anxiety Disord.* **1997**, *11*, 409–429. [CrossRef]
36. Steimer, T. Animal models of anxiety disorders in rats and mice: Some conceptual issues. *Dialogues Clin. Neurosci.* **2011**, *13*, 495–506. [CrossRef]
37. Koolhaas, J.M.; Everts, H.; de Ruiter, A.J.; de Boer, S.F.; Bohus, B. Coping with stress in rats and mice: Differential peptidergic modulation of the amygdala-lateral septum complex. *Prog. Brain Res.* **1998**, *119*, 437–448. [CrossRef]
38. Koolhaas, J.M.; de Boer, S.F.; Buwalda, B.; van Reenen, K. Individual variation in coping with stress: A multidimensional approach of ultimate and proximate mechanisms. *Brain Behav. Evol.* **2007**, *70*, 218–226. [CrossRef] [PubMed]
39. Bruins Slot, L.A.; Bardin, L.; Auclair, A.L.; Depoortere, R.; Newman-Tancredi, A. Effects of antipsychotics and reference monoaminergic ligands on marble burying behavior in mice. *Behav. Pharmacol.* **2008**, *19*, 145–152. [CrossRef] [PubMed]
40. Coppens, C.M.; de Boer, S.F.; Koolhaas, J.M. Coping styles and behavioural flexibility: Towards underlying mechanisms. *Philos. Trans. R. Soc. B Biol. Sci.* **2010**, *365*, 4021–4028. [CrossRef]
41. Kinsey, S.G.; O'Neal, S.T.; Long, J.Z.; Cravatt, B.F.; Lichtman, A.H. Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay. *Pharmacol. Biochem. Behav.* **2011**, *98*, 21–27. [CrossRef] [PubMed]
42. Giménez-Llort, L.; Masino, S.A.; Diao, L.; Fernández-Teruel, A.; Tobeña, A.; Halldner, L.; Fredholm, B.B. Mice lacking the adenosine A1 receptor have normal spatial learning and plasticity in the CA1 region of the hippocampus, but they habituate more slowly. *Synapse* **2005**, *57*, 8–16. [CrossRef]
43. Gyertyán, I. Analysis of the marble burying response: Marbles serve to measure digging rather than evoke burying. *Behav. Pharmacol.* **1995**, *6*, 24–31.

44. Poling, A.; Cleary, J.; Monaghan, M. Burying by rats in response to aversive and nonaversive stimuli. *J. Exp. Anal. Behav.* **1981**, *35*, 31–44. [CrossRef] [PubMed]
45. Wolmarans de, W.; Stein, D.J.; Harvey, B.H. Of mice and marbles: Novel perspectives on burying behavior as a screening test for psychiatric illness. *Cogn. Affect. Behav. Neurosci.* **2016**, *16*, 551–560. [CrossRef]
46. de Boer, S.F.; Koolhaas, J.M. Defensive burying in rodents: Ethology, neurobiology and psychopharmacology. *Eur. J. Pharmacol.* **2003**, *463*, 145–161. [CrossRef]
47. Mrabet Khiari, H.; Achouri, A.; Ben Ali, N.; Cherif, A.; Batti, H.; Messaoud, T.; Mrabet, A. Obsessive-compulsive disorder: A new risk factor for Alzheimer disease? *Neurol Sci.* **2011**, *32*, 959–962. [CrossRef]
48. Dondu, A.; Sevincoka, L.; Akyol, A.; Tataroglu, C. Is obsessive-compulsive symptomatology a risk factor for Alzheimer-type dementia? *Psychiatry Res.* **2015**, *225*, 381–386. [CrossRef]
49. Pekkala, S.; Albert, M.L.; Spiro, A., 3rd; Erkinjuntti, T. Perseveration in Alzheimer's disease. *Dement. Geriatr. Cogn. Disord.* **2008**, *25*, 109–114. [CrossRef]
50. Deardorff, W.J.; Grossberg, G.T. Behavioral and psychological symptoms in Alzheimer's dementia and vascular dementia. *Handb. Clin. Neurol.* **2019**, *165*, 5–32. [CrossRef]
51. Cipriani, G.; Vedovello, M.; Ulivi, M.; Nuti, A.; Lucetti, C. Repetitive and stereotypic phenomena and dementia. *Am. J. Alzheimers Dis Other Demen.* **2013**, *28*, 223–227. [CrossRef]
52. Torres-Lista, V.; Giménez-Llort, L. Persistence of behaviours in the Forced Swim Test in 3xTg-AD mice at advanced stages of disease. *Behav. Processes* **2014**, *106*, 118–121. [CrossRef] [PubMed]
53. Romberg, C.; Mattson, M.P.; Mughal, M.R.; Bussey, T.J.; Saksida, L.M. Impaired attention in the 3xTgAD mouse model of Alzheimer's disease: Rescue by donepezil (Aricept). *J. Neurosci.* **2011**, *31*, 3500–3507. [CrossRef]
54. Layne, J.N.; Ehrhart, L.M. Digging behavior of four species of deer mice (Peromyscus). In *American Museum Novitates*; American Museum of Natural History: Washington, DC, USA, 1970; p. 2429.
55. Boix, F.; Fernandez-Terula, A.; Tobeña, A. The anxiolytic action of benzodiazepines is not present in handling-habituated rats. *Pharmacol. Biochem. Behav.* **1988**, *31*, 541–546. [CrossRef]
56. Cañete, T.; Blázquez, G.; Tobeña, A.; Giménez-Llort, L.; Fernández-Teruel, A. Cognitive and emotional alterations in young Alzheimer's disease (3xTgAD) mice: Effects of neonatal handling stimulation and sexual dimorphism. *Behav. Brain Res.* **2015**, *281*, 156–171. [CrossRef]
57. Gouveia, K.; Hurst, J.L. Improving the practicality of using non-aversive handling methods to reduce background stress and anxiety in laboratory mice. *Sci. Rep.* **2019**, *9*, 20305. [CrossRef] [PubMed]
58. Barnum, C.J.; Pace, T.W.; Hu, F.; Neigh, G.N.; Tansey, M.G. Psychological stress in adolescent and adult mice increases neuroinflammation and attenuates the response to LPS challenge. *J. Neuroinflamm.* **2012**, *9*, 9. [CrossRef]
59. Kedia, S.; Chattarji, S. Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice. *J. Neurosci Methods.* **2014**, *233*, 150–154. [CrossRef] [PubMed]
60. Yohn, N.L.; Blendy, J.A. Adolescent Chronic Unpredictable Stress Exposure Is a Sensitive Window for Long-Term Changes in Adult Behavior in Mice. *Neuropsychopharmacology* **2017**, *42*, 1670–1678. [CrossRef]
61. Sugimoto, Y.; Tagawa, N.; Kobayashi, Y.; Hotta, Y.; Yamada, J. Effects of the serotonin and noradrenaline reuptake inhibitor (SNRI) milnacipran on marble burying behavior in mice. *Biol. Pharm. Bull.* **2007**, *30*, 2399–2401. [CrossRef]

Anexes

biomedicines

MDPI

Article

Burying and Burrowing Behavior in Male and Female Normal and 3xTg-AD Mice: A New Comprehensive Study Based on the Two-Zone Configuration.

Mikel Santana-Santana [1,2], José-Ramón Bayascas[1,3] and Lydia Giménez-Llort [1,2,*]

[1] Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain
[2] Department of Psychiatry and Forensic Medicine, School of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain
[3] Department of Biochemistry and Molecular Biology, School of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain
* Correspondence: lidia.gimenez@uab.cat

Abstract: Burying and burrowing are promising rodent-typical behaviors to model neuropsychiatric symptoms (NPS). However, the original tests could be insufficient to conclude which NPS are modeled. Here, we propose methodological modifications such as the two-zone configuration and dual analysis in the Marble Burying Test (MB). Also, a new Brief Burrowing Test (BB), a 20 min brief version of the Deacon's Burrowing Test (DB). We comprehensively studied these behaviors in 12-month-old male and female mice with normal and Alzheimer's disease (AD)-pathological aging. The results: 1) confirm our precedent report of sexual dimorphism, with enhanced burying in male 3xTg-AD mice; 2) describe for the first time burrowing behavior in 3xTg-AD mice and its sex dependence; 3) regardless of the pattern, MB and BB reflected a goal-directed rather than an indiscriminate digging; 4) using the MB and BB to model anxiety-like behavior it's not recommended; 5) burying and burrowing represent a repetitive rather than a stereotyped-like or perseverative behavior. In addition: 1) burying and burrowing behavioral patterns are alike, connected by several correlations; 2) the two-zones configuration is a useful tool to assess the intentionality of the burying and burrowing behaviors and to perform a more accurate screening of the NPS modeled by them.

Keywords: neuroethology; methodology; sexual differences; aging; Alzheimer's disease; marble burying test; burrowing test; repetitive behavior

Citation: Santana-Santana, M.; Bayascas, J.-R.; Giménez-Llort, L. Burying and burrowing behavior in Male and Female Normal and 3xTg-AD mice: A Comprehensive Study Based on the Two-Zone Configuration. *Biomedicines* **2022**, *9*, x. https://doi.org/10.3390/xxxxx
Academic Editor(s):
Received: date
Accepted: date
Published: date
Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Natural species-typical behaviors involve the animal's active use of cognitive and non-cognitive functions to interact with its environment. These can be excellent ethological scenarios to reflect the interplay of cognitive and non-cognitive disturbances induced by Alzheimer's Disease (AD). From a translational perspective, these behaviors also represent an important opportunity to model neuropsychiatric symptoms (NPS), also called "Behavioral and Psychological Symptoms associated with Dementia" (BPSD) [1], as well as alterations to perform "daily living activities" (DLA) [2] presented in most of the patients, that increase the disease and caregiver burden [3-5]. In this context, the neuroethological features of these models are important as pre-clinical tools for drug design, development and assessment, but also to investigate non-pharmacological strategies before they can be effectively translated into clinical scenarios. Our research in 3xTg-AD mice is

committed to such a multidimensional approach, investigating the impact of the AD genotype not only on the classical cognitive hallmarks of the disease [6] but also on the ethological repertoire of the animal through the analysis of rodent-typical behaviors [7-9].

The burying behavior is among the rodent-typical behaviors usually considered that model anxiety-related disorders. Burying can be defined as the concerted effort to either cover a particular object with a substrate or displace an object beneath any available substrate [10]. This is commonly measured using the Marble Burying test (MB) [11]. This test was initially pharmacologically validated for its use to measure anxiety-related behaviors and screen for anxiolytic drugs [i.e., 12,13]. Currently, controversy exists regarding its specificity, as it is also proposed as modeling meaningless repetitive and perseverative behaviors mimicking psychotic and obsessive-compulsive (OCD) symptoms [10]. In this scenario, several authors [10,14-18] argue the importance of introducing methodological changes and better experimental designs to consider the MB as a reliable screening test for any specific assessment of a neuropsychological construct. We agree with this statement, so over the years, we have investigated this behavior by carrying out various experimental designs, adding methodological modifications to the test, and analyzing new variables.

Initially, we have proven enhanced burying behavior assessed in the MB in 12-month-old 3xTg-AD male mice that can be reversed by risperidone and be modulated by handling [8,19]. We also showed that at more advanced stages of disease (15 months of age), a 2-3 months of naturalistic isolation, which occurs when congeners die, exacerbates this digging behavior despite the animal having had social lives since birth [20]. Our previous work [21] demonstrated that MB is sensitive to AD-genotype, sex, aging, and these biological factors' interactions. There, the male sex was more sensitive to show enhanced burying, whereas the female sex was affected by AD-pathological aging showing a reduction of burying at 16 months. The results also showed, for the first time, that burying remains stable in repeated testing; that the time-course of buried marbles is a useful methodological improvement to prevent false-negative and false-positive results and identify early signatures in burying behavior. Also, we concluded that in the 3xTg-AD mice, burying behavior most likely represents perseverative and/or stereotyped-like behavior rather than anxiety-like behavior.

However, these findings raise new questions. The first question is whether high/low burying is due only to an increase or decrease in global digging behavior or whether it is due to an increase or decrease in goal-directed behavior, revealing that the animal has the intention to perform or not to perform such behavior. As de Brower et al.[10] pointed out, burying represents the application of digging to a more complex task. Therefore, to answer this question, we considered it necessary to perform protocols with two zones (with and without marbles), which allow the animal to interact or not with the marbles. Here, we further propose and demonstrate the relevance of analyzing specific variables related to the ethogram of behavioral performances in these two zones.

A second question is whether the pattern shown in MB is transferable to other behaviors involving digging, such as burrowing behavior. Burrowing is a rodent-typical behavior that consists of digging with the intention of tunneling for habituation [10]. One way to test it is using the Burrowing test (DB) [2]. In recent years, the use of this test has increased, and it has been shown to be a valuable test for measuring well-being and motor function, testing pain and stress, and modeling neurological and psychiatric conditions [2,10]. Since the burrowing behavioral pattern of the 3xTg-AD mice is still unknown, one of the aims of the present work was to describe it for the first time.

We also conceived the Brief Burrowing test (BB), to implement the two-zone approach and achieve better comparability with the data obtained in the MB. In this way, we could also assume or discard intentionality for burrowing behavior, preventing the same classical confounding factors observed in the MB.

Therefore, the present study in males and females with normal and AD-pathological aging aimed 1) to describe for the first time the burrowing behavior in male and female

3xTg-AD mice as compared to NTg counterparts; 2) to investigate the animal's intentionality to perform burying and burrowing behaviors in both genotypes and sexes using a two-zones protocol and dual analysis; 3) to explore the relationship between burying and burrowing behaviors; 4) to examine the possible NPS-like constructs related to AD modeled by theses tests ; 5) to promote the use of the two-zone approach as a necessary methodological tool for a better evaluation of any proposed neuropsychiatric construct to be modeled by burying and burrowing behaviors. For this purpose, we tested 12-month-old male and female mice through the MB, the DB, and the BB. The two-zone approach was implemented to consider better the possible NPS-like constructs involved in their alterations. In the AD-genotype, this middle-age time point corresponds to an advanced stage of the disease with the development of βA and tau pathologies [22]. The sex- and age-matched non-transgenic (NTg) counterparts of the gold-standard C57bl/6J strain genetic background were used for comparison.

2. Materials and Methods

2.1. Animals

A total number of sixty-four 12-month-old male and female mice, homozygous 3xTg-AD (males n=20, "*AD males*"; females n=16, "*AD females*") and NTg (males n=18, *"NTg males"*; females n=10, *"NTg females"*) mice on a C57BL/6J background after embryonic transfer and backcrossing at least 10 generations, established in the Universitat Autònoma de Barcelona [23] were used. The 3xTg-AD mice harboring transgenes were genetically engineered at the University of California Irvine, as previously described [24]. Animals were maintained in groups of 3-4 mice per cage (Macrolon, 35 × 15 × 15 cm) filled with a 5 cm thick layer of clean woodchips that were the same used for behavioral testing (Ecopure, Chips6, DateSand, UK; Uniform cross-cut wood granules with 2.8–1.0 mm chip size) and nesting materials (Kleenex, Art: 08834060, 21 × 20 cm, White). All animals were maintained under standard laboratory conditions of food and water ad libitum, 22 ± 2°C, 12 h light: dark cycle with lights on at 8:00 am, and relative humidity 50–60%.

2.2. Experimental Design

As illustrated in figure 1, animals were behaviorally assessed for four consecutive days in a counterbalanced manner using a factorial design genotype (G) x sex (S).

2.3. Behavioral Assessment

Behavioral assessments in the different tests were conducted under dim white light (20 lx) and during the light phase of the light: dark cycle, in the morning (from 10 am to 1 pm) except for the Deacon's burrowing test that started at 3 pm and ended on the next day at 9 am, as detailed below. A trained observer performed direct observation assessments, blind to the genotype and with a camera's support. All procedures were in accordance with 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 protocol CEEAH 3588/DMAH 9452 was approved on the 8th of March 2019 by the 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 [25].

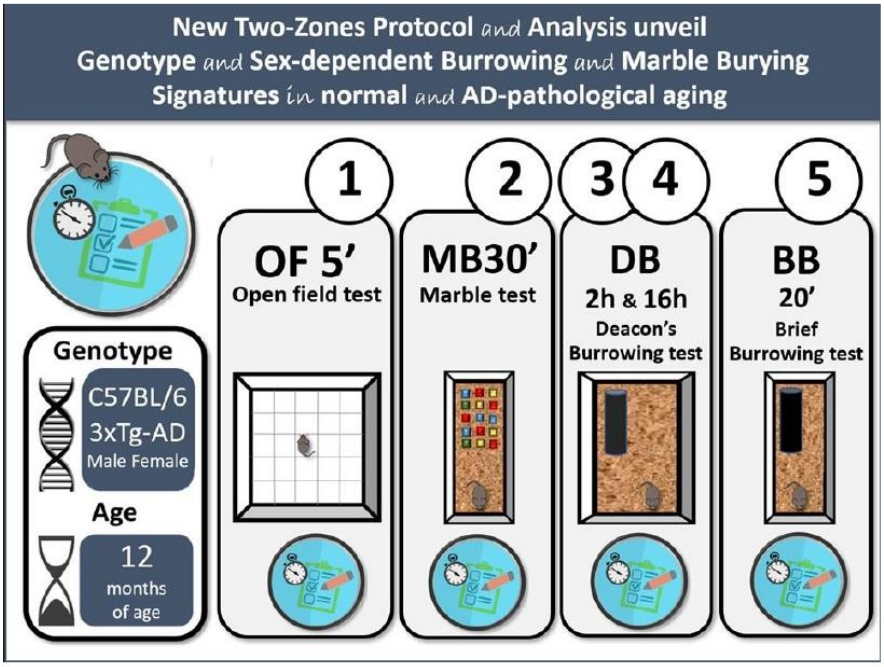

Figure 1. Graphical abstract. Experimental design: a 5-day battery of behavioral tests consisting of an open field test (OF) test on day 1, a two-zones marble test (MB) on day 2, a Deacon's burrowing test (DB) on day 3 until day 4, and a two-zones brief burrowing test (BBT) on day 5.

Day 1— Open field test (OF)

This classical anxiety test was used to evaluate the ethogram of anxiety-like behaviors and exploratory activity. The animal was placed in the center of an open and illuminated field (homemade woodwork, white box, 5 × 5 squares distribution, 55 × 55 × 25 cm) and observed for 5 minutes. First, the ethogram of action programs (sequence of behavioral events) was recorded. Thus, the duration of freezing behavior (OFlatM) and the latency of the behavioral events that follow it were recorded: leaving the central square (OFlatC), reaching the periphery zone (OFlatP), performing the first rearing (OFlatR) and the first grooming (OFlatG). Additionally, the number of rearings (OFnR), the number of grooming episodes (OFnG), the distance traveled (OFd), the number of entries in the center zone (OFeC), the time spent in the center zone (OFtC), the distance traveled in the center zone (OFdC), the time spent in the periphery zone (OFtP) and the distance traveled in the periphery zone (OFdP) were also recorded.

Day 2— Dual analysis in the two-zones Marble Burying test (MB)

The Marble Burying test (marbles equally spaced in a cage) is usually used to evaluate burying behavior. In the present work, we propose using our two-zone configuration [8] and a dual analysis, evaluating marble burying and digging behaviors.

The two-zones protocol consisted of virtually dividing a standard home cage (Macrolon, 35 × 15 × 15 cm), with a 5 cm thick layer of clean woodchips, into two zones: with marbles (w/MB) and without marbles (w/oMB). In this way, we allow the animals to avoid interacting with the marbles if they do not want to. In this work, fifteen glass marbles were placed evenly spaced (five rows of three) in one-half of the cage (zone w/MB), and the test was video recorded. Then, the mouse was introduced in the zone w/oMB facing the wall and left to interact with the cage freely. After 30 minutes, the mouse was gently removed from the cage, and the buried marbles were counted (MB30). Later, to assess the buried

marbles' time-course [21], the number of buried marbles was counted every 5 minutes in the video recording (MBx, x=minute). In all the measures, the number of marbles buried was transformed into a percentage for further statistical analysis. The burying criteria was strict: marbles were counted as buried when their surface was covered at least 90% with bedding material.

Additionally, for a better understanding of the animal's intention (goal-directed behavior) to dig, the latency of digging appearance and the number of diggings episodes were registered, taking into account the area in which it was made (MBlatDw/ and MBnDw/, in the zone with marbles)(MBlatDw/o and MBnDw/o in the zone without marbles). Subsequently, regardless of the zone, the latency of digging appearance in the test was established (MBlatD), and the number of total digging episodes was calculated (MBnD). All these variables were counted through the video recording. Digging was defined as using front legs and/or hind legs to displace the substrate of the cage.

Day 3 and 4— Deacon's Burrowing Test (DB)

Burrowing behavior was measured using this test [2]. A burrowing tube (PVC plastic, 20 cm) filled with 200 grams of food pellets was introduced into a big home cage (Macrolon, 50 × 22 × 14 cm) with a 3 cm thick layer of woodchips. At 3 pm, mice were placed in the cage facing the wall opposite the tube and left to explore freely. After two hours, the tube was retired to be weighed and refilled. Then, the tube was reintroduced and left until the following day. Sixteen hours later, at 9 am, the tube was retired and weighed again. Finally, the animals were returned to their home cage until the following day. The amount of food out of the tube was calculated and converted into a percentage in both the 2 hours measure (short, DB%s) and the overnight measure (overnight, DB%o).

Day 5— Dual analysis in a two-zones Brief Burrowing Test (BB)

To assess burrowing behavior in a format easily comparable to the data obtained in the MB, here we propose a two-zone approach of the protocol proposed by Deacon and a dual analysis, that is, evaluating burrowing and digging behaviors. This test was performed the day after completing the Deacon's test.

A burrowing tube (PVC plastic, 20 cm) filled with 80 g of woodchip bedding material was weighed and introduced into a standard home cage (Macrolon, 35 × 15 × 15 cm) with a 5 cm thick layer of woodchips. Then, the mouse was placed in the cage facing the wall opposite the tube and left to explore freely. After 20 minutes, the mouse was gently removed from the cage, and the tube was weighed. Thus, the amount of wood chips out of the tube was calculated and converted into a percentage (BB%).

Digging was defined as using front legs and/or hind legs to displace the substrate of the cage. The latency of digging appearance and the number of diggings episodes were recorded for each zone: outside the tube (BBlatDout and BBnDout) and inside the tube (BBlatDin and BBnDin). Afterward, regardless of the zone, the latency of digging appearance in the test was established (BBlatD), and the number of total digging episodes was calculated (BBnD). All these variables, except for diggings inside the tube, were counted through the video recording.

2.4. Statistics

Statistical analyses were performed using SPSS 23.0 software. In all the tests, variables were analyzed by ANOVA split-plot analysis, with (G) genotype and (S) sex as the main factors, in a G(2)×S(2) design. In the case of the percentage of marbles buried, the time (T) was included as a within factor according to the experimental design G(2)×S(2)×T(7). Post-hoc comparisons were run with Bonferroni corrections. Spearman correlations were made to analyze behavioral correlates. Correlation coefficients (r) are indicated. A p-value < 0.05 was considered statistically significant. Graphics were made with GraphPad Prism 6.

3. Results

3.1. Open Field Test (OF)

In the open field test (Figure 2) the temporal ethogram was sensitive to the genotype as shown in the increased latencies of movement [G, F(1, 60) = 6.646; p = 0.012], to leave the center [G, F(1, 60) = 4.02; p = 0.049], to reach the periphery [G, F(1, 60) = 10.562; p = 0.002] and to perform the first rearing [G, F(1, 60) = 11.557; p = 0.001]. Thus, the 3xTg-AD mice exhibited a 3-fold significant delay in the development of the ethogram compared to NTg mice. This temporal delay also resulted in an AD-dependent reduction of the time spent in the periphery [G, F(1, 60) = 14.832; p = 0.000]. Besides, a significant genotype and sex interaction effect for distance traveled in the center [G×S, F(1, 60) = 4.024; p = 0.049] was found.

To further depict this aspect, the post-hoc analysis indicated meaningful genotype (g) and sex (g) differences as described hereinafter. Thus, AD males exhibited higher latency of movement [g, F(1, 60) = 7.130; p = 0.010], to leave the center [g, F = (1, 60) = 5.449; p = 0.023] and to reach periphery [g, F(1, 60) = 5.641; p = 0.021] than their NTg counterparts. In the case of -females, the delay was observed as a higher latency to reach the periphery [g, F(1, 60) = 5.107; p = 0.027] and in the appearance of the first rearing [g, F(1, 60) = 10.594; p = 0.002] than NTg females. In addition, both, AD males [g, F(1, 60) = 9.478; p = 0.003] and AD females [g, F(1, 60) = 6.076; p = 0.017] spent less time in the periphery zone than their corresponding NTg groups. However, the delayed rearing was higher in AD females than in AD males [s, F(1, 60) = 5.803; p = 0.019].

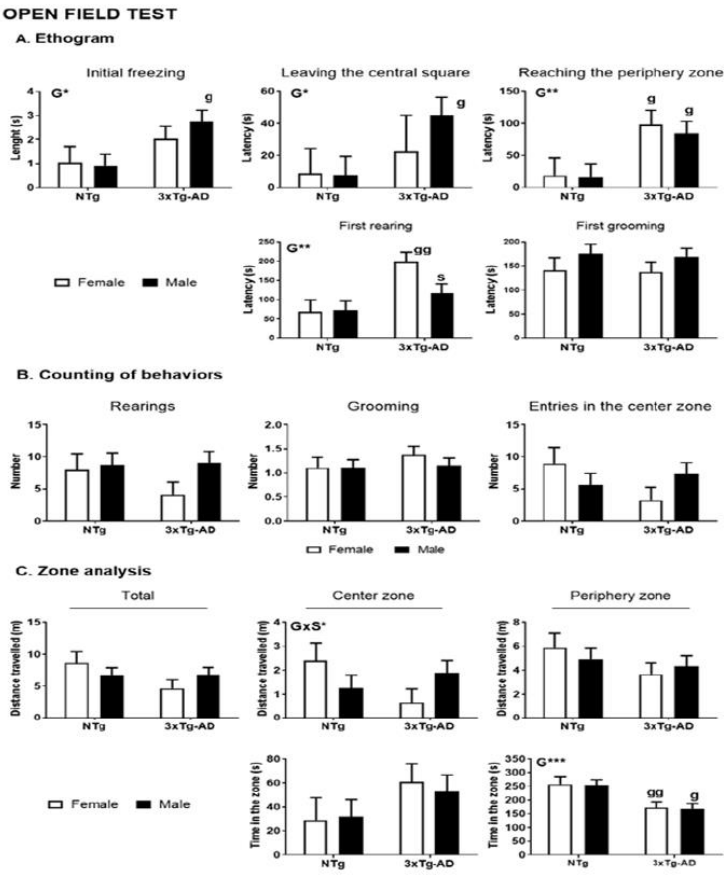

Figure 2. Open field behavioral analysis. Data are means ± SEM). Factorial analysis: Genotype (G) and sex (S) effects in mice with normal and AD-pathological aging. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Post-hoc analysis, g, genotype difference: g, $p < 0.05$; gg, $p < 0.01$.

3.2. Dual Analysis in the Two-zones Marble Burying Test (MB)

In the time-course analysis of the percentage of marbles buried (Figure 3A), the main effects were significant for genotype [G, F(1, 60) = 18.239; p = 0.000] and sex [S, F(1, 60) = 5.833; p = 0.019]. Time factor (T) showed interaction effects: time × genotype [T×G, F(2.343, 140.583) = 111.291; p = 0.000] and time × sex [T×S, F(2.343; 140.583) = 6.244; p = 0.001]. Thus, post-hoc analysis evidenced specific differences along the time course of the different groups. First, AD females showed a higher percentage of marbles buried than NTg females, but only at 25 minutes (MB25, p = 0.045), a time point close to the end of the test. However, in the male sex, 3xTg-AD mice exhibited a higher percentage than NTg males all along the test (MB10, p = 0.003; MB15, p = 0.000; MB20, p = 0.000; MB25, p = 0.000; MB30, p = 0.000). Additionally, AD males also exhibited a higher activity (MA) than AD females along the task (MB15, p = 0.031; MB20, p = 0.001; MB25, p = 0.002; MB30, p = 0.010).

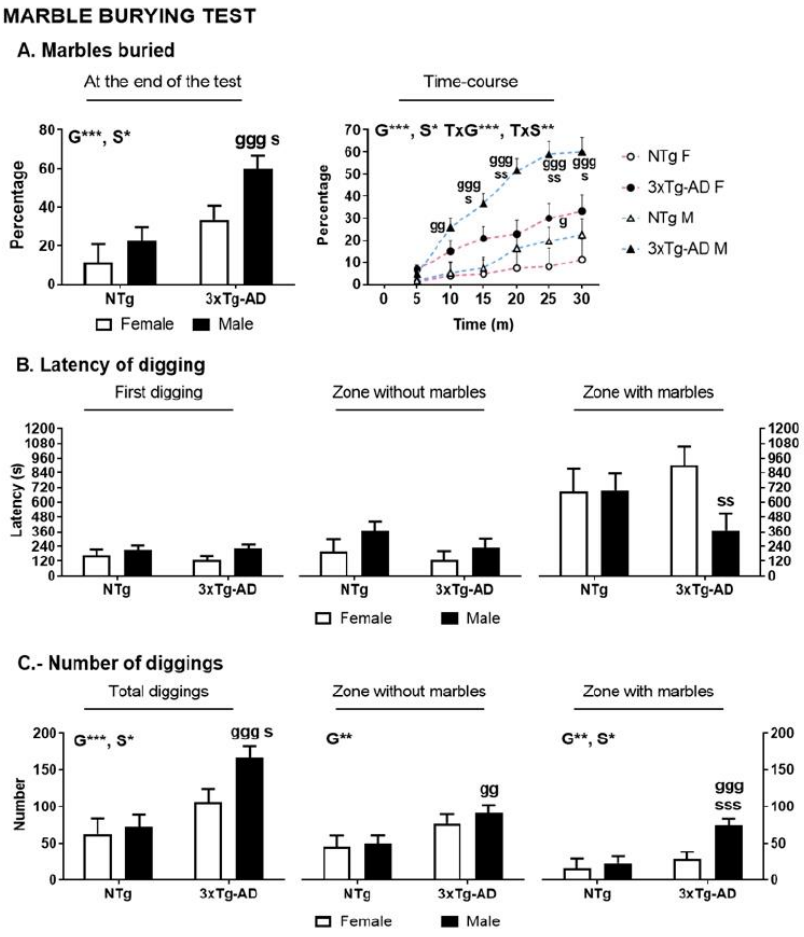

Figure 3. Marble Burying test time-course and two-zones analysis. Data are means ± SEM). Factorial analysis: Genotype (G), sex (S), time × genotype (T × G), and time × sex (T × S) in mice with normal and AD-pathological aging. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Post-hoc analysis, g, genotype difference; s, sex difference; gg, $p < 0.01$; ggg , $p < 0.001$; s, $p < 0.05$, ss, $p < 0.01$; sss, $p < 0.001$.

The digging ethogram in the two zones MB analysis is depicted in Figure 3B. The latency of digging in each zone was analyzed. The results show that the first digging occurred in the zone without marbles, in a time window of 2-5 minutes, thus indicating that the onset of the response was performed after the animal explored most of the cage and was elicited in the bedding area free of unknown objects. In the zone with marbles, the temporal appearance was over the 10th minute of the test. Latencies showed no genotype nor sex effects, although trends of increased latencies in the males and AD-genotype could be observed in the zone without marbles. However, the latency of digging in the zone with marbles was significantly shorter in AD males than in AD females [post-hoc, s, $F(1, 60) = 7.019$; $p = 0.01$].

The total number of diggings (Figure 3C) showed significant genotype [G, $F(1, 60) = 15.364$; $p = 0.000$] and sex [S, $F(1, 60) = 4.017$; $p = 0.05$] effects, being higher in males and the AD-genotype. Post-hoc analysis also indicated that AD males performed a total number of diggings higher than NTg males [g, $F(1, 60)=18.039$; $p=0.000$] and AD females [s, $F(1, 60) = 6.947$; $p = 0.011$]. Considering each zone, a significant genotype effect was found in the number of diggings in the zone without marbles [G, $F(1, 60) = 9.098$; $p = 0.004$], where AD mice performed more diggings than NTg mice, an effect mostly due to results in the male sex [g, $F(1, 60) = 7.410$; $p = 0.008$]. In the zone with marbles, both genotype [G, $F(1, 60) = 10.018$; $p = 0.002$] and sex [S, $F(1, 60) = 6.826$; $p = 0.011$] effects were shown. In this case, AD-genotype and male sex performed more diggings than NTg mice and female sex, respectively. In the post-hoc analysis, AD males performed a higher number of diggings than NTg males [g, $F(1, 60) = 16.818$; $p = 0.000$] and their female counterparts [s, $F(1, 60) = 12.574$; $p = 0.001$].

3.3. Deacon's Burrowing Test (DB)

Differences in the burrowed food after two hours were found to be significant for genotype [G, $F(1, 60) = 6.557$; $p = 0.013$] and sex [S, $F(1, 60) = 5.179$; $p = 0.026$], were AD mice and male mice showed a higher percentage of food outside of the tube. The post-hoc comparisons only showed a higher burrowed food by AD males to both, NTg males [$F(1, 60) = 8.717$; $p = 0.004$] and AD females [$F(1, 60) = 6.970$; $p = 0.011$]. In the overnight measurement, a similar pattern was shown. Genotype [G, $F(1, 60) = 7.152$; $p = 0.010$] and sex [S, $F(1, 60) = 17.313$; $p = 0.000$] were significant, so again AD mice and male mice burrowed more food outside of the tube. Post-hoc differences again showed that AD males burrowed more than NTg males [$F(1, 60) = 8.499$; $p = 0.005$] and AD females [$F(1, 60) = 4.347$; $p = 0.041$] but we also found that NTg males burrowed more food than its female counterparts [$F(1, 60) = 15.770$; $p = 0.000$] (see Figure 4).

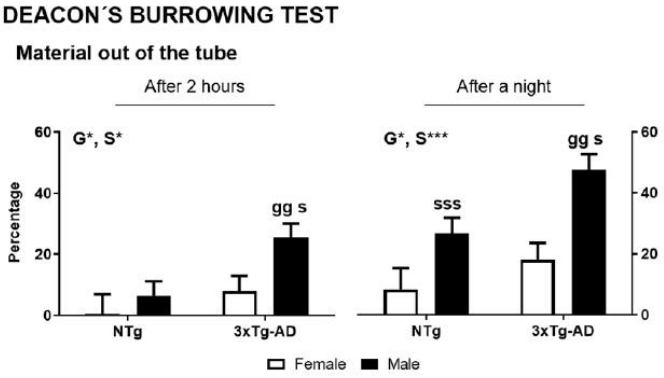

Figure 4. Deacon's burrowing test analysis. Data are means ± SEM. Factorial analysis: Genotype (G) and sex (S) effects in mice with normal and AD-pathological aging. * $p < 0.05$, ** $p < 0.01$ and *** p

< 0.001. Post-hoc analysis, g, genotype difference; s, sex difference; gg, $p < 0.01$; s, $p < 0.05$; sss, $p < 0.001$.

3.3. Dual Analysis in the Brief Burrowing Test (BB)

Genotype [G, $F(1, 60) = 4.092$; $p = 0.048$] and genotype × sex interaction [GxS, $F(1, 60) = 6.973$; $p = 0.011$] effects were found in the percentage of bedding material out of the tube (Figure 5A). Despite the amount of material being higher in the AD genotype than in NTg mice, the post-hoc comparisons only showed a higher percentage in AD males than both, NTg males [$F(1, 60) = 13.807$; $p = 0.000$] and AD females [$F(1, 60) = 12.424$; $p = 0.001$].

Similar to MB, we also analyzed the latency of digging (Figure 5B). A genotype [G, $F(1, 60) = 4.215$; $p = 0.044$] and sex [S, $F(1, 60) = 4.814$; $p = 0.032$] effect was manifested. The occurrence of the first digging was sooner for AD mice and female mice than for their counterparts. Post-hoc analysis showed that AD males exhibited this behavior sooner than NTg males [$F(1, 60) = 6.544$; $p=0.013$]. Additionally, NTg males displayed this behavior later than NTg females [$F(1, 60)=4.839$; $p=0.032$].

Furthermore, when the zone where the dig was done was considered, genotype [G, $F(1, 60) = 4.211$; $p = 0.045$] and sex [S, $F(1, 60) = 5.763$; $p = 0.019$] effects were found for the latency of digging outside the tube. Again, AD mice and female mice showed a lower latency than NTg mice and male mice, respectively. Post-hoc comparisons showed that AD males did the digging earlier than NTg males [$F(1, 60) = 6.844$; $p = 0.011$] and NTg males did it later than their female counterparts [$F(1, 60)=5.683$; $p=0.020$]. However, only a difference between genotypes [G, $F(1, 60)=4.559$; $p=0.037$] was manifested in the latency to dig inside the tube. As happened outside the tube, AD mice did it earlier than NTg mice. But in this variable, post-hoc analysis evidenced that AD-male did it earlier than both NTg-male and AD-female.

The analysis of the number of diggings also manifested meaningful results (Figure 5C). When the total number of diggings was analyzed, the genotype main effect [G, $F(1, 60) = 4.782$; $p = 0.044$] was meaningful. This implies that AD mice did more diggings than NTg mice, although the post-hoc analysis only manifested a higher number of diggings in AD males than NTg males [$F(1, 60) = 9.538$; $p = 0.003$]. Also, AD males did it more diggings than AD females [$F(1,60) = 6.179$; $p = 0.016$]. Then, we considered the area in which the diggings were made. There were no differences between groups in the number of diggings outside the tube. However, differences occurred in the diggings inside the tube. Genotype [G, $F(1, 60) = 9.399$; $p = 0.003$], sex [S, $F(1, 60) = 6.343$; $p = 0.014$] and genotype × sex interaction [G × S, $F(1, 60) = 8.084$; $p = 0.006$] were showed significant. Similar to the pattern of other variables, AD mice and male mice did more diggings than NTg mice and female mice, respectively. Post-hoc comparisons exhibited that AD males did more diggings inside the tube than NTg males [$F(1, 60) = 22.167$; $p = 0.000$] and AD females [$F(1, 60) = 17.125$; $p = 0.000$].

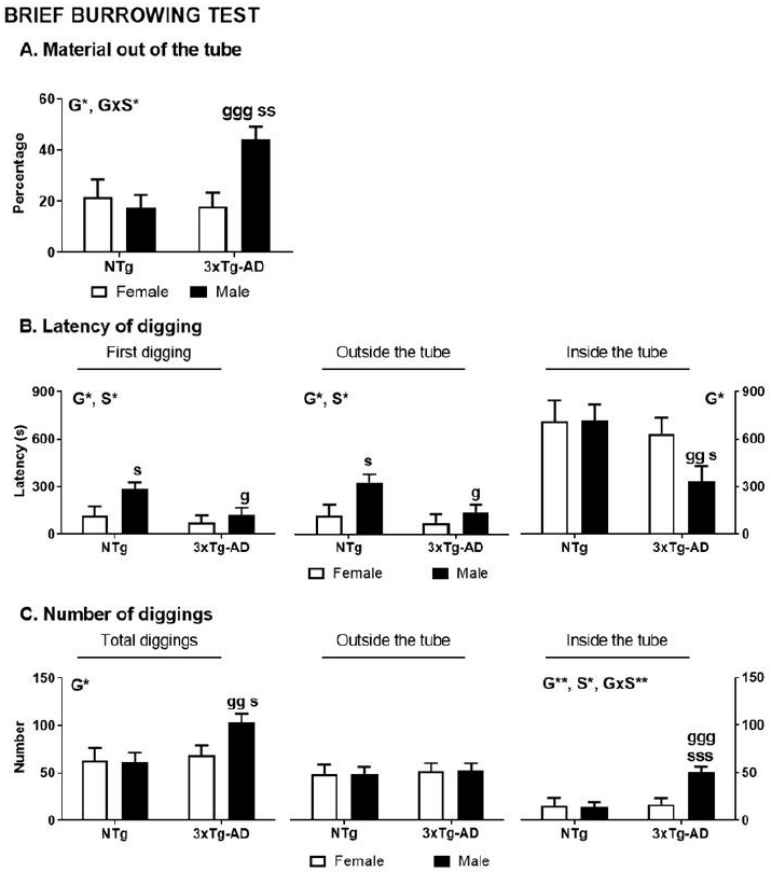

Figure 5. Dual analysis in a two-zone Brief Burrowing Test (BB). Data are means ± SEM. Factorial analysis: Genotype (G), sex (S) and genotype × sex (G × S) effects in mice with normal and AD-pathological aging. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Post-hoc analysis, g, genotype difference; s, sex difference; g, $p < 0.05$; gg, $p < 0.01$; ggg , $p < 0.001$; s, $p < 0.05$; sss, $p < 0.001$.

3.4. Meaningful Behavioral Correlations

In this section we will mention the most relevant correlations found between the different tests. Correlacions tables are included in the supplementary material.

3.4.1.Marble Burying Test and Open Field Test correlations

Correlations were calculated by genotype and sex (Table S1). First, NTg females only showed a meaningful correlation between the latency of digging and the time spent in the periphery in the OF ($r = 0.673$; $p = 0.033$). In NTg males, both the latency of digging and the latency of digging in the zone with marbles were negatively correlated with the number of rearings in the OF ($r = -0.488$; $p = 0.004$) ($r= -0.492$; $p = 0.038$). In AD females, both the total number of diggings and the number of diggings in the zone without marbles positively correlated with the latency of movement in the OF ($r = 0.511$; $p = 0.043$ and $r = 0.560$; $p = 0.024$, respectively). AD males were the group with more meaningful correlations. We highlight the inverse relationship between the percentage of marbles buried at 5 minutes (MB5) and the latency to leave the central zone in the OF ($r = -0.490$; $p = 0.028$). Furthermore, the latency of digging in the zone with marbles also positively correlated with the latency of leaving the central zone ($r = 0.540$; $p = 0.014$).

3.4.2. Burrowing Tests and Open Field Test Correlations

Due to genotype and sex being significant factors in almost every variable in both burrowing tests, correlation analyses were performed accordingly (Table S2). First, NTg females only exhibited a meaningful correlation between the latency of digging in the BB and the time spent in the peripheral zone of the OF. However, NTg males exhibited a higher number of significant correlations. Among others, we found that the overnight measure of the DB was negatively correlated to the latency to entry in the periphery in the OF ($r = -0.643$, $p = 0.004$) and positively to the time spent in this zone. Furthermore, the number of diggings inside the tube in the BB showed a negative correlation with the latency of rearing in the OF ($r = -0.475$, $p = 0.047$).

AD females also showed several meaningful correlations. Especially relevant are the negative correlations between the latency of digging inside the tube in the BB and both, the latency to reach the periphery ($r = -0.608$; $p = 0.012$) and the latency to do the first rearing ($r = -0.498$; $p = 0.05$) in the OF. Finally, in AD males, only a correlation was manifested, an inverse relationship between the latency of digging in the BB and the time spent in the periphery of the OF ($r = -0.457$; $p = 0.043$).

3.4.3. Marble Burying, Deacon's Burrowing Test and Brief Burrowing Test Correlations

The correlations tables were generated without any categorical division (see Table S3). The most relevant correlations are discussed to see how the test variables were related at an intra- and inter-test level.

The intra-test analysis showed interesting results. In the MB, the percentage of marbles buried at 30 minutes (MB30) positively correlated for both the number of digging in the zone without marbles ($r = 0.557$; $p = 0.000$) and the number of diggings in the zone with marbles ($r = 0.870$; $p = 0.000$), being stronger the relationship with this last one. Also, the final percentage negatively correlated with the latency of digging in the zone with marbles ($r = -0.612$; $p = 0.000$), but did not correlate with the latency of digging in the zone without marbles. This pattern was similar to all the percentage measures along the task. For the DB, a positive correlation was found between the two burrowed food measures ($r = 0.550$; $p = 0.000$).

Finally, in the BB, the percentage of bedding material out of the tube negatively correlated with the latency of digging inside the tube ($r = -0.750$; $p = 0.000$) and positively with the number of diggings inside the tube ($r = 0.931$; $p = 0.000$). Nevertheless, both the latency of digging outside the tube and the number of diggings outside the tube did not correlate with the percentage of material outside the tube.

Furthermore, at an inter-test level, the measures of the percentage of the three tests were positively correlated: the percentage of marbles buried at 30 minutes (MB30), the two percentages of burrowed food in the DB, and the percentage of borrowed material in our new brief version of the test. Moreover, only the number of diggings in the zone with marbles correlates with both the 2 hours measure ($r = 0.420$; $p = 0.001$) and the overnight measure of the DB ($r = 0.467$; $p = 0.000$). However, both the number of diggings in the zone with marbles and the zone without marbles correlated with the percentage of burrowed material ($r = 0.502$; $p = 0.000$) ($r = 0.302$; $p = 0.015$) in our adaptation of the burrowing test.

Regarding the variables of the BB, the number of diggings inside the tube correlated in the MB with the last percentage of marbles buried ($r = 0.373$; $p= 0.002$), the number of diggings in both the zone with marbles ($r=0.474$; $p=0.000$) and without its ($r=0.348$; $p=0.001$). In addition, this measure correlated with the 2 hours measure ($r=0.441$; $p=0.000$) and the overnight measure ($r=0.499$; $p=0.000$) of the DB. However, none of these variables correlated with the number of diggings outside the tube.

4. Discussion

4.1. New Insights from the Two-zones Configuration in the Marble Burying Test.

Our two-zones configuration protocol [8,24] allows the animal to voluntarily avoid or interact with marbles as goal-directed behaviors [10]. This configuration is especially relevant to characterizing anxiety-like responses in the MB and is sensitive to antipsychotic and anxiolytic-like interventions [8]. However, the marble-burying behavior may be insufficient for a neuroethological understanding of animal behavior when mimicking advanced AD scenarios where the array of BPSD-like symptoms of dementia is present. Therefore, here we propose a neuroethological behavioral analysis in the two zones. Other behavioral variables should accompany the classical measure to help us understand the ethogram, how the animal behaves in the two zones, and monitor competing behaviors that may work as confounding factors. Thus, this is the first time we have recorded specific behavioral measures relative to the zones. In particular, since digging behavior is a primary action necessary for more complex or goal-directed tasks, like burying or burrowing, the latency and number of diggings in each zone were measured. Therefore, these variables should exhibit an explicit and robust relationship with the percentage of buried marbles. Hence, their use would be more appropriate than other measures, such as the time the animal spends in each zone. In the following paragraphs, these measures are discussed.

The time course analysis of marbles buried replicates our previous works [21]. Higher marble burying was shown by AD males but not by AD females. This variable's differences between AD males and their NTg counterparts are manifested again early in the test. However, it's important to mention that in the present work, both AD and NTg females exhibited lower percentages of buried marbles (MB30: AD, $\bar{X}$ = 33.33; NTg, $\bar{X}$ = 11.33) than in our last work (MB30: AD, $\bar{X}$ = 50; NTg, $\bar{X}$ = 29,92) [21]. In addition, AD females showed higher percentages along the tests. This phenomenon led to a statistically significant difference between them at 25 minutes.

The latency of their first digging and that in the zone w/oMB were similar in the four groups of animals. This is explained by the fact that the first digging performed by the animals is usually in the area w/oMB. Although the absence of differences was the norm in the zone w/MB, the graphical representation allowed us to appreciate valuable information for interpreting results. In this zone, the latencies are notably increased for all the groups except the AD males, with differences with AD females reaching statistical significance. In addition, it is noteworthy that AD males had a small gap in the latency of digging in both zones.

The analysis of the number of diggings reported numerous statistically significant differences. The total diggings were higher for AD mice, especially in males, who also differed from AD females. Although AD mice manifested a higher number of diggings in the zone w/oMB, genotype differences reached statistical significance only in males. Moreover, in the marble zone, all the groups performed a similar number of diggings except for the AD males. In contrast to the other three groups, their digging behavior was enhanced compared to their peers, reaching quantitative values similar to those recorded in the area without marbles.

The intra-test correlations of the MB provide valuable insights into how variables are related to each other (Table S3). First of all, the absence of a relationship between the latencies of diggings of the two different zones could indicate that the occurrence of digging in each zone, w/MB and w/oMB, were independent events subjected to the mouse will. Of these two latencies, only the one done in the marble zone was related to the percentage of marbles buried at the test's beginning and end. This suggests that it does not matter whether the animals start digging earlier or later in the zone w/oMB because the buried marbles only depend on how long it takes to start digging in the marble zone.

Furthermore, the idea that the animal's behavior in each zone is voluntary and independent of each other is suggested when we observe that the latency in each one of the zones correlates only with the number of diggings of their respective zone and not with de diggings of the other zone.

Finally, it is important to consider that both the number of diggings in the zone w/oMB and the marble zone correlated with the percentage of marbles buried at the beginning and the final of the test, albeit being bigger for the marbles-zone digging. This implies that the burying percentage does not depend solely on the diggings in the marbles-zone, indicating a certain "contamination" from the non-marble-zone activity to the other. This could be caused by the absence of physical separation between the two areas in two different ways: throwing woodchips over the marbles and covering them from the zone w/oMB when they dig, and/or shifting the marbles to the zone w/oMB and then burying there.

In conjunction with the analysis of the percentages, the insight from these new variables allows us to elaborate more complete behavioral profiles or ethological signatures of our mice and thus increase and improve our understanding of how they behaved in the MB. First, both NTg males and females showed lower percentages of buried marbles, which would agree with the lower number of diggings in the marble zone. Since they started later to dig in the marble zone and their digging episodes in the zone w/oMB were clearly higher than their diggings in the marble zone, this would suggest that these animals show a preference for digging in the zone without marbles and avoiding, to a certain extent, digging in the area with marbles.

On the other hand, the behavioral pattern exhibited by 3xTg-AD mice would be sex-dependent. As well as NTg mice, AD females would also present a preference for digging in the zone w/oMB and avoiding burying in the area with marbles, but even so, their burying percentage is slightly higher than NTg females. This could be due to a more efficient burying in the marble zone and/or "contamination" from the activity in the zone w/oMB. Meanwhile, AD males exhibited an earlier and higher activity in the marble zone. However, this increased activity was not detrimental to the activity in the zone without marbles since they showed similar latencies and activity in both zones and similar to the shown by the other groups.

4.2. Burrowing Behavior in the 3xTg-AD Mice

The application of the DB yielded novel and interesting results about the burrowing behavior in the 3xTg-AD mice. After two hours of testing, AD males manifested a higher borrowing than their NTg counterparts and AD females. In addition, AD females showed a similar burrowing percentage to both NTg sexes. It is important to highlight that the burrowing percentage in NTg females was nearly zero. The overnight measure resulted in higher burrowed material than at two hours for all the groups. AD males exhibited a higher burrowing than the other three groups on this occasion. Surprisingly, NTg males manifested a higher burrowing than NTg females. This phenomenon could be due to the sum of the higher activity of NTg male at night and the extremely low burrowing percentages of NTg females.

Additionally, the BB and the incorporation of the two-zone analysis provided richer information about the burrowing behavior in the 3xTg-AD mice. As in the MB, this approach can provide valuable information about its ethogram, the animal's intentionality, and potentially BPSD modeling symptoms. First, the burrowing percentage depended on the interaction between genotype and sex. This implied a higher burrowing behavior exhibited in AD males but not in AD females. Both NTg sexes showed a similar percentage, comparable to the exhibited by AD females and lower than the expressed by AD males.

The four groups of animals showed similar latencies in their first digging and the latency recorded outside the tube. This is explained by the fact that the first digging performed by the animals was usually performed in this area. However, male mice exhibited higher latencies to dig outside the tube regardless of genotype. Inside the tube, the latencies were high in all the groups except the AD males, reaching statistical significance regarding NTg males and AD females. In addition, the small gap between the latency of digging in both zones was highlighted in AD males.

The analysis of the number of diggings reported numerous statistically significant differences. The total diggings were higher for AD mice, especially in males, who also differed from AD females. Outside the tube, all the groups presented a similar number of diggings events. In contrast, inside the tube, all the groups displayed a similar number of diggings except for the AD males. Contrary to the other three groups, their digging behavior was enhanced compared to their peers.

Valuable insights into how variables are related are provided by intra-test correlations of the BB (Table S3). First, the absence of a relationship between the latencies of diggings of the two zones, inside and outside the tube, could indicate that digging in each zone was an independent event subjected to the mouse's will. Of these two latencies, only the one done inside the tube is related to the percentage of woodchip outside the tube. This suggests that it does not matter whether the animals start digging earlier or later outside the tube because the material only depends on how long it takes to start digging inside. Furthermore, the latency in each one of the zones correlates only with the number of diggings of their respective zone and not with de diggings of the other zone. This supports the idea that the animal's behavior in each zone is voluntary and independent of each other. Finally, it's important to note that only the number of diggings inside the tube correlated with the burrowing percentage. This implies that the burrowing percentage did not depend on the diggings done outside the tube. Hence there is no contamination between the activity done in each zone. This is probably due to the physical separation between the two zones caused by the tube walls.

In conjunction with the DB analysis, the insight obtained for the two-zone analysis from the BB allows us to elaborate a comprehensive behavioral profile or ethological signature of burrowing behavior in AD mice and that of NTg counterparts. First, both NTg males and females showed lower percentages of burrowed material, which would agree with the lower number of diggings done inside the tube. Since they started later to dig inside the tube and their digging episodes outside were higher than their diggings inside, this would suggest that these animals prefer digging outside the tube. Therefore, they manifest some hesitation to dig inside the tube. On the other hand, the burrowing pattern exhibited by 3xTg-AD mice would be sex-dependent. As well as NTg mice, AD females prefer digging outside the tube and show similar burrowing percentages in both tests. Meanwhile, AD males exhibited an earlier and higher activity inside the tube, which translates into a higher percentage of burrowing. However, this increased activity was only manifested inside the tube.

This is the first time burrowing behavior has been assessed in the 3xTg-AD mice. Contrary to our results, other AD models tested in the DB or similar protocols have shown impaired burrowing [26-31]. In contrast, Wistar rats injected with amyloid-beta peptides in the hippocampus manifested enhanced burrowing behavior [32]. The differences in the onset and progression of AD brain pathology in the different AD transgenic mouse models is a topic well documented [i.e., 30, 33-35]. However, differences in behavioral phenotypes do not receive the same depth of study. Some examples in the bibliography show how these behavioral phenotypes do or do not manifest themselves or do so distinctly depending on the mouse model employed [31, 36-38]. In addition, it is important to keep in mind that burrowing behavior is sensitive to strain differences [i.e., 39]. Therefore, the strain selected for both NTg mice and Tg mice could influence the appearance or not of differences. Furthermore, the strain could influence how we interpret such burrowing if such differences emerge. For instance, the same burrowing manifested by some Tg mouse models could be interpreted as excessive or impaired depending on whether we employ an NTg strain with lower or higher burrowing. In our opinion, the results obtained in this study do not invalidate and cannot be invalidated by the results of other AD mouse models. We advocate for the careful study of each mouse model of which AD BPSD-like symptoms are susceptible to be modeled in their behavioral alterations. Since an impaired or excessive burrowing does not have the same theoretical implications. Consequently, the

possible constructs modeled by burrowing behavior in the 3xTg-AD mice will be addressed later.

4.3. – The Relationship Between Burying and Burrowing Behavior in the 3xTg-AD Mice

The burying and burrowing behavioral patterns exhibited by the 3xTg-AD mice were remarkably similar. AD males showed increased percentages of burying and burrowing, shortened latencies to initiate the digging in the zone with marbles/inside the tube, and increased episodes of diggings in such zones. However, there were also some performance differences in the AD males. Perhaps the most relevant one was that AD males displayed increased diggings in the area without marbles, in contrast to diggings outside the tube in the BB. This could be because there is no physical separation between the two MB zones, and the MB and BB have different durations. Besides this, AD females had no sign of altered burying or burrowing. Even so, this absence of alterations was consistent in all tests. The different behavioral patterns warn about the presence of sexual dimorphism. Recently, Dennison and colleagues [40] reviewed the differential expression of behavioral phenotypes depending on sex in the 3xTg-AD model.

In addition, the correlations reported in this work provide valuable information on how burying and burrowing behavior are related. First, the burying percentage and all the burrowing percentages from both DB and BB, are positively correlated. This supports the idea that the performance shown in one test is, to a certain extent, transferable to other tests. This finding has important implications. Thus, an animal that performs a low burying is likely also to present a similar level of burrowing and vice versa.

Furthermore, implementing the two-zone analysis in the MB and BB allows a deeper understanding of the relationship between these tests. Firstly, the latencies of digging in the zone with marbles and inside the tube are positively related. This relationship is exclusive, as they do not correlate with other latencies. Surprisingly, the latencies performed in the zone without marbles and outside the tube do not correlate. In addition, we can observe how the latencies of digging in the zone with marbles and inside the tube are negatively related to the percentages of the other tests. However, each latency is uniquely related to a different percentage in the DB. Then, the initiation of both burying and burrowing is intentional, exclusive, and closely related. Moreover, the number of diggings in both zones of the MB is positively correlated with diggings done inside the tube in the BB. However, diggings done outside the tube are unrelated to both digging measures in MB. This pattern is also observed when the burying percentage is compared to diggings measures in the BB, and conversely. This pattern mirrors the contamination effect previously described in the MB and absents in the BB. Therefore, burying and burrowing behavior is the manifestation of goal-directed digging, which in turn are related to each other. Finally, only the number of diggings in the zone with marbles and inside the tube are correlated with both DB burrowing percentages.

Contrary to the BB, the contamination effect of the MB is not transferred to the DB percentages. This may be due to the methodological differences between MB and DB, as MB and BB present a similar methodological design. Altogether, burying and burrowing are two goal-directed digging behaviors coherently interconnected to each other through correlations of direct and indirect behavioral variables, but they are not entirely alike.

This is the first time, to our knowledge, that the relationship between burying and burrowing behaviors has been studied through correlations. However, other authors have explored, in the same study, how both behaviors are manifested. However, the number of studies is scarce since, most often, only one of these behaviors is tested. Below, we will examine those studies in which both burying and burrowing have been included. Burying and burrowing were investigated in both the 5-HTT overexpressing mice (5-HTT OEs) and the 5-HTT knockout mice (5-HTT KOs) [41]. Each of them was compared with their respective wild-type mice. 5-HTT OEs mice manifested an enhanced burrowing behavior while the burying was unaffected. However, the unaffected burying could be caused by a ceiling effect, as both the 5-HTT OEs mice and their wild types bury almost all the marbles

(approximately 9 out of 10). Besides this, reduced burying and burrowing behavior is exhibited in the 5-HTT KOs mice. In other research, Konsolaki and colleagues [42] studied the burying and burrowing in mice lacking high-affinity nicotinic receptors (β2-/-) and their wild-type mice at two different ages, adult (4-6 months) and old (22-24 months). Older β2-/- showed reduced burrowing. The other groups did not present any differences in both behaviors. Finally, a double knockout model of AD displayed decreased burying and burrowing behavior [31]. The review of these studies yields the following conclusions. Examples of burying and burrowing showing reversed patterns (i.e., increased burying and decreased burrowing) do not exist. Usually, either both behaviors are altered, or only one of them does. Therefore, the similar burying and burrowing behavioral pattern displayed by the 3xTg-AD mice is not an exclusive event of this transgenic mice model.

Overall, it is clear that burying and burrowing behaviors were closely related. This is not only based on the mere correlation of percentages but also the interconnection of intertest latencies and diggings variables. Furthermore, it is confirmed that burying and burrowing percentages result from goal-directed diggings. This means that the indiscriminate use of digging did not cause them. There were differences in the burying and burrowing patterns and correlations that could be influenced by some methodological limitations. These will be further developed in a later section.

4.4. Is Anxiety Modeled in the Burying and Burrowing Behavior in 3xTg-AD Mice?

The 3xTg-AD mice showed anxiety-like behavior in the OF, as shown by the delay in the temporal development of the ethogram compared to their NTg counterparts. However, sex-dependent nuances were observed. Thus, in both sexes, thigmotaxis and increased time spent in the periphery are considered indicators of increased anxious response in fight-to-flight coping strategies. In behavioral paradigms where the animals are introduced in the center of the arena, if a freezing response is used instead of the dichotomic strategy, the indicators appear inversed (reduced). The elicitation of freezing has been linked to an overload increase of amygdala activation in situations with no capacity to make a choice. Nevertheless, the correlations between the OF variables and the other tests were scarce and inconsistent between groups (Table S1 and Table S2). Not only to the percentages of burying and burrowing but also to the new variables incorporated in our research. Suppose we hypothesize that animals with less burying and burrowing (NTg male and female, AD female) avoid it because of anxiety. In that case, we should expect negative correlations between open field latencies and direct and indirect measures of burying/burrowing (percentages, number of diggings in the zone with marbles/inside the tube) and positive correlations with the latency of digging in the zone with marbles and inside the tube. However, this is not reflected in our correlations, only the NTg males presented this relationship between OFlatC/OflatP with DB%O. In AD males, the hypothesis would be the opposite. If increased burying and burrowing are related to anxiety, there should be positive correlations between OF latencies and direct and indirect measures of burying/burrowing and negative correlations with latency. The results were not like that but were incongruous. MB percentages in the first 15 minutes were negatively related to OFC and OFM, and MBlatDw/ was positively related to OFlatC. There was some congruence in the negative relationship between OFlatC and the number of diggings in the zone w/oMB. Although it is not a direct mean, due to contamination, it influenced the final burying percentage, although it had less relevance than other variables. In the burrowing test in this group, there were no correlations. In view of the results, it is quite questionable to relate the presence of anxiety to performance on this test, even hypothesizing different responses.

This phenomenon is similar to what occurred when, in our previous works, we evaluated the relationship between MB and neophobia using the corner test [21]. Moreover, other researchers have obtained relatively poor results exploring the relationship between anxiety tests and the MB [43-45]. Given the common behavioral substrate of burrowing and burying, it is not surprising that in the present work, it also occurs with the burrowing

test and the OF. In addition, the two-zone configuration of the MB and BB allows no interaction with the marbles or the tube, but all the groups show some interaction with them. These results would be consistent with other studies using a two-zone configuration in the MB [12,15,43,46-48].

Furthermore, we have proven that burying behavior is resistant to habituation in 3xTg-AD and their NTg counterparts [21]. Given the evidence, a possible hypothesis to support the anxiety-like modeling of burying behavior could be that the inherited anxiety trait of these mice [23] invokes either active burying/burrowing or passive avoidance behavior as coping strategies [19,54-59] and make their response to marbles resistant to habituation [59,60]. This hypothesis is not supported by the data obtained from the two-zone analyses of the MB. First, the correlation analysis did not support this hypothesis. Moreover, the digging done by the AD males is higher in both zones of the test, which discards that they only seek to bury the marbles because they are aversive. In addition, AD females present a slight increase in digging outside the marbles zone and a higher burying percentage than their NTg counterparts, although they are not statistically different. These facts show that they do not avoid marbles. If this "avoidance" behavior were caused by anxiety, it would not be "clinically" different from the anxiety shown by the NTg mice. If we transfer this hypothesis to the BB we would get a similar response. Although the AD males show only increased digging inside the tube, this could be due to the physical separation of the two zones (the tube walls). The AD females show a profile similar to that of the NTg females and males, with normal burrowing that is far from being avoidant. Given this and above, both MB and BB do not seem appropriate tests for modeling anxiety-like behavior in the 3xTg-AD mice.

We do not deny that anxiety could mediate or may have some influence on the behavioral outcome of these tests. Both the marbles and the tube make the digging appear much later than in the zone w/oMB or outside the tube. This tells us that there is some initial hesitation in all groups to burying or burrowing. This could indicate some level of neophobia. But, even so, the 3xTg-AD mice do not show higher latencies but even lower latencies, especially in males. In summary, it seems that anxiety would also not have a major role in the behavior manifested by the 3xTg-AD mice in the MB and BB.

4.5. Burying and Burrowing as a Model of Repetitive, Stereotyped or Perseverative Behavior in AD?

In our previous work [21], we concluded that the higher burying exhibited by the 3xTg-AD mice is more likely to reflect a repetitive/stereotyped-like and/or perseverative behavior. The results obtained from the two-zone analysis and the new tests incorporated in this work provide valuable information for further exploration of this issue.

However, before discussing them, we must define what we mean by repetitive, stereotypical and perseverative. This is not an easy task, even though they are terms that we usually handle in the field of psychology, psychiatry, and neuroscience. Depending on the source consulted, we can find definitions for the same term with notable differences, belonging to different classifications and using different terms as synonyms [i.e., 61-65]. All this confuses and makes the interpretation of the results difficult. Our intention is not to redefine these terms or to create a theoretical framework but to specify, as far as possible, what these constructs mean to us. In this way, we intend to give clarity to the conclusions, avoiding confusion and misunderstanding of our results so that other researchers can transfer the conclusions obtained to their field. These behaviors have been extensively studied in autism spectrum disorders (ASD) [i.e., 64, 66], but they are not exclusive to this pathology. Repetitive behavior, stereotypy and perseveration are manifested in AD patients [65, 67-70], and our research group has also described them in the 3xTg-AD mouse [23,71,72]. In the following, we will define these constructs and discuss how our results conform to these.

First, we will define repetitive behavior as that behavior or response that occurs in an excessive repeated manner. This behavior may be functional in the situation in which

it appears, but it occurs in individuals in greater quantities than under normal conditions. This definition would align with what Ridley [61] refers to as productive stereotypy. The higher number of diggings and the higher percentage of burying shown by the AD males with respect to their NTg and female counterparts in the MB confer an excessive character to such behavior. Moreover, this phenomenon not only occurs in the MB but is also present in the two tests used to measure burrowing. Digging being the primary behavior behind burying and burrowing, we can say that this is a repetitive and persistent behavior, not only resistant to habituation but also consistently manifested in the different contexts that facilitate its occurrence.

Stereotyped-like behavior can be defined as abnormal repetitive movements or behaviors. They are considered maladaptive and/or malfunctional [62]. They are usually present in captive animals and can even lead to self-injurious behavior. It would be the equivalent of what Ridley [58] defined as deprivation-stereotypies and confinement-stereotypy. In mice, these behaviors have been widely studied and include behaviors such as grooming, jumping, barbering, or circling [i.e., 23,73]. First, digging is not a maladaptive or malfunctioning behavior per se in our context because even if excessive, a test that needs digging to be performed is not abnormal, nor is it unrelated to the context. And this argument is transferable to all the tests used.

Second, there is no correlation of any kind between the grooming observed in the OF with any of the variables of the other tests. This is important, as grooming is a deeply studied and well-documented rodent-typical behavior for studying stereotypic behaviors [74]. It is important to note, however, that in the OF there were no differences between genotypes in this behavior, and it occurred in very low numbers. However, on other occasions, we have documented the presence of this type of behavior in the 3xTg-AD model [23]. Therefore, attributing this construct to excessive digging by the animals in these tests is neither theoretically nor empirically supported.

Finally, we would define perseverance as the performance of a behavior or strategy several times that, although it may make sense in a given situation, is not adapted to the current demand. It is demonstrated by the inability to shift, change or cease a behavior pattern once started [75]. In our opinion, this construct is difficult to test with the tests used, or at least with the methodology employed. First, we observed that AD-male mice present a greater number of diggings both in the zone without and in the zone with marbles, while in the BB this phenomenon does not occur since they only present greater digging inside the tube. We could make parallelism and say that the digging done in the zone without marbles would be synonymous with this perseverance. A reflection of the animal's insistence to continue burying or make a burrow when it is impossible to do so. However, it has been proven that the mere presence of bedding material in the cage is sufficient to elicit this behavior in mice [43]. Therefore, performing the digging behavior makes sense from a neuroethological perspective, whether the marbles are there or not, whether it is done inside the tube or outside. Moreover, we should not forget that digging in the area without marbles or outside the tube is the most common behavior in the other groups. These show a certain reluctance to diggings in the area with marbles and inside the tube, as corroborated by the greater latency to bury the marbles or empty the donkey-wing tube. Therefore, it is difficult to prove with the current protocol that the digging behavior present is perseverative in nature.

Due to the repetitive nature of digging behavior in the 3xTg-AD male mice, this could be a consequence of the presence of impulsivity. Garner [63] defines impulsive behavior as repetitive behavior that usually varies in form and motor pattern and is goal-directed. It is a BPSD manifested in patients with AD [76] and animal models of the disease [i.e., 77-79]. Some authors have argued and employed the MB as a model of impulsive behavior [49, 52, 80-82]. This approach could be very interesting and promising. Recently we have proven impairment in gait and exploratory activity accompanied by muscular pathology in 3xTg-AD male mice [83]. How are animals in this physical state motivated to dig? Is this impulsivity making this animal bury or burrow in high quantities even when NTg

mice show some type of hesitation to do it? Could impulsivity mediate to do such activity even in their poor physical state? These are interesting hypotheses to approach in further investigations.

In summary, burying and burrowing behaviors in 3xTg-AD mice represent a repetitive behavior, understood as excessive in quantity but with functionality and directed to a goal. They imply performing excessive digging towards a specific task. However, it cannot be attributed to stereotypical properties. It is a persistent behavioral pattern that does not change in repeated trials [21] and manifests itself in different tests involving such behavior. In order to prove the presence of perseverance, it would be necessary to devise methodological modifications or other experimental protocols. This pattern may be related to impulsivity. In the future, it would be relevant to continue exploring this line of work and find the neuroanatomical substrates and functional correlates involved in the increased burying and burrowing behaviors exhibited by 3xTg-AD males.

4.5. Benefits and Future Directions of Implementing the two-zone analysis

The results obtained on burying and burrowing behavior in our work have important implications, not only in the 3xTg-AD model but also in future studies carried out in other strains or rodent species. The two-zone configuration could be a helpful tool for modeling OCD or ASD through burying and burrowing behaviors.

First, the similarity between the behavioral patterns and the strong correlations, not only in burrowing and burrowing percentages but also in the other behavioral variables, seems to indicate that, to some extent, they are highly overlapping tests. They could be homologous and interchangeable tests. For practical purposes, our recommendation would always be to apply both tests since, although there is some overlap, there are likely to be differences between them. Both tests are simple to perform, inexpensive, and do not cause any harm to the animal. If this is not possible, the ideal would be to perform a screening study in the model or species to be studied to verify that the phenomenon of similar profiles and correlations is present. Finally, if neither of the two previous options is possible, the researcher would have to choose which test best suits the objectives, taking into account the needs of his research. In this regard, it is important to consider several aspects.

First, the MB allows a simple way to record the time-course of buried marbles, while in the BB it is not possible to record the time-course of the material outside the tube without interrupting the test, or at least to do it in a simple way. As we presented in our previous work [21], the time-course provides valuable information on the burying behavioral pattern of the animal and provides robustness to the results obtained since it protects us from conclusions based on differences that could be considered "false positives" and "false negatives". Another important aspect is that, although both tests are of low economic cost, the BBT involves the purchase or the use of self-made tubes, while in the MB, it is only necessary to buy marbles. On the other hand, in both tests, it is important to consider the substrate on which the digging behavior is performed since they can alter the percentage of burying or burrowing in both tests [2,15,84]. In addition, in the MB, it is also necessary to consider the density of the material to be buried [43], since this can also influence whether or not it is easier to bury the object. Another difference is that in the tests that measure burrowing, an objective measure is used since it is calculated based on the differential of the weight of the tube before and after the test. In contrast, in the MB it depends on the criteria established by the researcher to count a marble as buried, which can alter the results [15].

Another point to consider would be the presence of "contamination". If there is no natural separator between the MB zones, the activity of the zone without marbles may affect, to a certain extent, the percentage of buried marbles, which does not occur in the BB. This would imply that to prevent this phenomenon, it would be necessary to put some physical separator to avoid this effect. Another aspect is the resistance to habituation present in MB, which allows its application on consecutive days [13,43,49-53]. This may have

important advantages when designing experimental protocols. This phenomenon has not been tested in any burrowing test, or we are unaware of it. Finally, a differential factor may be evidence accumulated behind each test. The MB is a test with a greater volume of research, while the DT is a more recent test whose use has been booming relatively recently. Moreover, both have been used in different paradigms, models, constructs, or drugs, so depending on the research scope, it may be more convenient to use one or the other.

However, we must analyze the two zones in-depth regardless of which test we choose. And this is another of the implications of our work for other researchers. As stated above, this is already recommended in the MB [10], but we also encourage implementing tests to measure burrowing. Without this approach, it is impossible to capture the intentionality of the animal correctly, so the interpretations we make of the results obtained may not be entirely robust. For example, concerning burrowing behavior, in our study, we can observe that there is only a significant increase in digging inside the tube and not outside it. Therefore, we can affirm that the 3xTg-AD males show an increase in burrowing behavior and not digging. Applying this protocol to studies focused on neuropathic pain (for example), by counting the number of diggings, we could see if the differences in the percentage of burrowing are due to an absence of this behavior (they omit the digging both outside and inside the tube) or a greater difficulty to perform the burrowing correctly (they perform diggins inside the tube but do not empty it correctly or perform digging outside the tube). This is just an example of possible uncertainties that could be answered by obtaining information derived from the two-zone analysis. We consider this approach especially relevant in the study of burrowing since it is a behavior increasingly implemented in behavioral evaluations, and we would avoid making the historical mistakes made with the MB.

Regarding which variable to use for this analysis, we consider the number of diggins and the latency of diggings in each zone as the minimum indispensable measures. From there onwards, any extra measure used is welcome and will add richness to the interpretation of the results, but these cannot be substituted by others, such as the time spent by the animal in each zone. Both measures have strong correlations with the percentage of burying or burrowing. The number of diggings within each zone is particularly relevant since it represents a direct measure of the behavior to be captured. Calculating times in each zone or digging latencies are indirect measures, as is counting the number of marbles or weighing the material outside the tube. The inclusion of the latency record is justified, in our opinion, by the ease of obtaining this measure and provides information on the onset of burrowing behavior, which we believe is a more direct variable than the time in each zone. Counting the number of diggings can be laborious, especially in investigations with very large samples or very long protocols. However, we believe that the gains in interpreting the results far outweigh the costs.

4.6. Limitations of the Study

Although, in our opinion, the data support the conclusions drawn, some methodological limitations may influence the degree of certainty of such conclusions. Some of them have been addressed in previous sections. In order to warn the reader regarding the possible impact of these limitations on the interpretability of the results, we will enumerate and discuss them below.

First, we analyzed our data using ANOVAS. This type of analysis may be inadequate in count-type data [14]. We decided to use this analysis because we employed it in our previous studies; its commonly used in the literature, and the analyses proposed by Lazic [14] are complicated to implement in our experimental design.

Second, the empirical justification to discard the use of burying and burrowing as models of perseverative or stereotypic behavior may be limited (see section 4.4 for further development). However, it would be desirable in future studies to use other variables, tests or experimental designs to explore this hypothesis further.

Third, the level of familiarity with the tests used could affect the results. This may be especially relevant in DB, as it was the first one performed in our protocol. The animals were not previously exposed to the burrowing tube. In addition, in the DB it was necessary to isolate the animals during that day to perform the test. In the BB these problems would not occur since the previous day they had been exposed to the tube in the DB, and the isolation of the animals was not necessary. However, that previous exposure to the tube may be an issue when comparing the results with the MB, since the mice are not previously exposed to the marbles. From our previous study, repeating the MB does not alter the percentage of buried marbles, so it would not be a major concern.

Fourth, the material inside the tube in DB and BB was different. It is possible that employing food may have made diggings inside the DB tube more challenging or not as attractive as woodchip may be in BB. In Deacon's work [2], it can be observed that the material used inside the tube can influence the performance in the test, with the percentages being lower when food is used. This could explain why burrowing percentages are too low in NTg mice and AD females after 2 hours of testing. Despite the different materials, the digging behavioral patterns are quite similar in our work.

Fifth, MB and BB have different duration periods. We chose 20 minutes for the BB to avoid a ceiling effect (animals emptying the tube), since, in this test, we could not record the time-course of the material out of the tube. Because in the 3xTg-AD mice, 20 minutes was more than enough time for differences in MB to appear, we chose that duration for the BB. This could partially explain why AD males exhibit higher episodes of diggings in the zone without marbles while they do not appear outside the tube.

5. Conclusions

In summary, the present results allow us to conclude that 1) 3xTg-AD burying sexual dimorphism is replicated; 2) AD-male mice show increased burrowing behavior; 3) burying and burrowing are closely related, their behavioral patterns are alike and several correlations connect such behaviors; 4) regardless of the behavioral pattern, the outcome of the MB and BB is the results of a goal-directed digging, rather than an indiscriminate use of digging; 5) there is no evidence to recommend the use of the MB and BB as a test to model behavioral anxiety in the 3xTg-AD mice; 6) 3xTg-AD burying and burrowing behaviors represents a repetitive behavior rather than a stereotyped-like or perseverative behavior; 7) the two-zone analysis is a useful tool to assess the intentionality of the burying and burrowing behaviors and to perform a more accurate screening of the neuropsychiatric symptoms modeled by them.

It would be relevant to explore the neuroanatomical substrates and functional correlates involved in the increased burying and burrowing behaviors exhibited by 3xTg-AD males.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1,Table S1: Marble Burying Tests and Open Field Test correlations; Table S2: Burrowing Tests and Open Field Test correlations; Table S2: Marble Burying, Deacon's Burrowing Tests and Brief Burrowing Test correlations.

Author Contributions: Conceptualization, L.G.-L. and M.S.-S.; Behavioral performance, analysis, statistics, M.S.-S.; Illustrations: M.S.-S and L.G.-L. Writing—original draft preparation, M.S.-S.; writing—review and editing, M.S.-S., L.G.-L and J.R.B.; Funding acquisition, L.G.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by UAB-GE-260408 to L.G.-L. The colony of 3xTg-AD mice is sustained by ArrestAD H2020 Fet-OPEN-1-2016-2017-737390, European Union's Horizon 2020 research and innovation program under grant agreement No 737390 to L.G.-L. It also received financial support from Memorial Mercedes Llort Sender 2021/80/09241941.6.

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

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Prof. Frank M. LaFerla, Institute for Memory Impairments and Neurological Disorders, University of California Irvine, CA, USA, for kindly providing the progenitors of the Spanish colonies of homozygous 3xTg-AD and NTg mice.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Ballard, C.; Corbett, A. Management of neuropsychiatric symptoms in people with dementia. *CNS drugs*. **2010,** 24, 729–739; doi:10.2165/11319240-000000000-00000.
2. Deacon, R.M.J. Burrowing: a sensitive behavioural assay, tested in five species of laboratory rodents. *Behav. Brain. Res.* **2009**, 200(1), 128-133; DOI: 10.1016/j.bbr.2009.01.007
3. Hope, T.; Keene, J.; Gedling; K., Fairburn, C.G.; Jacoby, R. Predictors of institutionalization for people with dementia living at home with a carer. *Int. J. Geriatr. Psychiatry*. **1998**, 13, 682–690; DOI:10.1002/(sici)1099-1166(1998100)13:10<682::aid-gps847>3.0.co;2-y.
4. Shin, I.S.; Carter, M.; Masterman, D.; Fairbanks, L.; Cummings, J.L. Neuropsychiatric symptoms and quality of life in Alzheimer disease. *Am. J. Geriatr. Psychiatry*. **2005**, 13, 469–474; DOI:10.1176/appi.ajgp.13.6.469.
5. Tan, L.L.; Wong, H.B.; Allen, H. The impact of neuropsychiatric symptoms of dementia on distress in family and professional caregivers in Singapore. *Int. Psychogeriatr*. **2005**, 17, 253–263; DOI:10.1017/s1041610205001523.
6. Giménez-Llort, L. Blázquez, G.; Cañete, T.; ohansson, B.; Oddo, S.; Tobeña, A.; LaFerla, F.M.; Fernández-Teruel, A. Modeling behavioral and neuronal symptoms of Alzheimer's disease in mice: A role for intraneuronal amyloid. *Neurosci. Biobehav. Rev.* **2007**, 31, 125–147; DOI:10.1016/j.neubiorev.2006.07.007.
7. Torres-Lista, V.; Giménez-Llort, L. Impairment of nesting behaviour in 3xTg-AD mice. *Behav. Brain Res.* **2013**, *247*, 153–157; DOI:10.1016/j.bbr.2013.03.021.
8. Torres-Lista, V., López-Pousa; S., Giménez-Llort; L. Marble-burying is enhanced in 3xTg-AD mice, can be reversed by risperidone and it is modulable by handling. *Behav. Processes*. **2015**, *116*, 69–74; DOI:10.1016/j.beproc.2015.05.001.
9. Giménez-Llort, L.; Torres-Lista; V. Social Nesting, Animal Welfare, and Disease Monitoring. *Animals (Basel)*. **2021**, 11, 1079; DOI:10.3390/ani11041079.
10. de Brouwer, G.M; Fick, A.; Harvey; B.H.; Wolmarans, W. A critical inquiry into marble-burying as a pre-clinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward. *Cogn Affect. Behav Neurosci*. **2019**, 19, 1–39; DOI:10.3758/s13415-018-00653-4.
11. Deacon, R.M. Digging and marble burying in mice: Simple methods for in vivo identification of biological impacts. *Nat. Protoc.* **2006**, 1, 122–124; DOI:10.1038/nprot.2006.20.
12. Broekkamp, C.L.; Rijk, H.W.; Joly-Gelouin, D.; Lloyd, K.L. Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. *Eur. J. Pharmacol.* **1986**, *126*, 223–229; DOI:10.1016/0014-2999(86)90051-8.
13. Njung'e, K.; Handley, S.L. Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. *Br. J. Pharmacol*. **1991,** 104, 105–112; DOI:10.1111/j.1476-5381.1991.tb12392.x.
14. Lazic, S.E. Analytical strategies for the marble burying test: Avoiding impossible predictions and invalid p-values. *BMC Res. Notes*. **2015**, *8*, 141; DOI:10.1186/s13104-015-1062-7.
15. de Brouwer, G.; Wolmarans, W. Back to basics: A methodological perspective on marble-burying behavior as a screening test for psychiatric illness. *Behav. Processes*. **2018**, *157*, 590–600; DOI:10.1016/j.beproc.2018.04.011.
16. Jimenez-Gomez C, Osentoski A, Woods JH. Pharmacological evaluation of the adequacy of marble burying as an animal model of compulsion and/or anxiety. Behav Pharmacol. 2011 Oct;22(7):711-3. doi: 10.1097/FBP.0b013e32834afebe.
17. Nicolas LB, Kolb Y, Prinssen EP. A combined marble burying-locomotor activity test in mice: a practical screening test with sensitivity to different classes of anxiolytics and antidepressants. Eur J Pharmacol. 2006 Oct 10;547(1-3):106-15. doi: 10.1016/j.ejphar.2006.07.015
18. Dixit PV, Sahu R, Mishra DK. Marble-burying behavior test as a murine model of compulsive-like behavior. J Pharmacol Toxicol Methods. 2020 Mar-Apr;102:106676. doi: 10.1016/j.vascn.2020.106676.

19. Torres-Lista, V., López-Pousa, S., Giménez-Llort, L. Impact of Chronic Risperidone Use on Behavior and Survival of 3xTg-AD Mice Model of Alzheimer's Disease and Mice With Normal Aging. *Front. Pharmacol.* **2019**, *10*, 1061; DOI:10.3389/fphar.2019.01061.
20. Gimenez-Llort, L.; Alveal-Mellado, D. Digging Signatures in 13-Month-Old 3xTg-AD Mice for Alzheimer's Disease and Its Disruption by Isolation Despite Social Life Since They Were Born. *Front. Behav. Neurosci.* **2021**, 14; DOI:10.3389/fnbeh.2020.611384.
21. Santana-Santana, M.; Bayascas, J.R.; Giménez-Llort, L. Sex-Dependent Signatures, Time Frames and Longitudinal Fine-Tuning of the Marble Burying Test in Normal and AD-Pathological Aging Mice. *Biomedicines*. **2021,** 9(8), 994; DOI: 10.3390/biomedicines9080994.
22. Oddo, S.; Caccamo, A.; Shepherd, J.D., Murphy, M.P.; Golde, T.E.; Kayed, R.; Metherate, R.; Mattson, M.P.; Akbari, Y.; LaFerla, F.M. Triple-transgenic model of Alzheimer's disease with plaques and tangles: Intracellular Abeta and synaptic dysfunction. *Neuron*. **2003**, 39, 409–421; DOI:10.1016/s0896-6273(03)00434-3.
23. Baeta-Corral, R.; Giménez-Llort, L. Bizarre behaviors and risk assessment in 3xTg-AD mice at early stages of the disease. *Behav. Brain. Res*. **2014**, *258*, 97–105; DOI:10.1016/j.bbr.2013.10.017.
24. Belfiore, R.; Rodin, A.; Ferreira, E.; Velazquez; R., Branca, C.; Caccamo, A.; Oddo, S. Temporal and regional progression of Alzheimer's disease-like pathology in 3xTg-AD mice. *Aging Cell*. **2019**, 18; DOI:10.1111/acel.12873.
25. Kilkenny, C.; Browne, W.J.; Cuthill, I.C.; Emerson, M.; Altman, D.G. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol.* **2010**, 8; DOI:10.1371/journal.pbio.1000412.
26. Deacon, R.M.J.; Cholerton, L.L.; Talbot, K.; Nair-Roberts, R.G.; Sanderson, D.J.; Romberg, C.; Koros, E.; Bornemann, K.D.; Rawlins, J.N.P. Age-dependent and-independent behavioral deficits in Tg2576 mice. *Behav. Brain. Res*. **2008**, 189(1), 126-138; DOI: 10.1016/j.bbr.2007.12.024.
27. Deacon, R.M.J.; Koros, E.; Bornemann, K.D.; Rawlins, J.N.P. Aged Tg2576 mice are impaired on social memory and open field habituation tests. *Behav. Brain. Res*. **2009**, 197(2), 466-468; DOI: 10.1016/j.bbr.2008.09.042.
28. Sagare, A.P.; Bell, R.D.; Zhao, Z.; Ma, Q.; Winkler, E A.; Ramanathan, A.; Zlokovic, B.V. Pericyte loss influences Alzheimer-like neurodegeneration in mice. *Nat. Commun.* **2013**, 4(1), 1-14; DOI: 10.1038/ncomms3932.
29. Janus, C.; Flores, A.Y.; Xu, G.; Borchelt, D.R. Behavioral abnormalities in APPSwe/PS1dE9 mouse model of AD-like pathology: comparative analysis across multiple behavioral domains. *Neurobiol Aging*. **2015**, 36(9), 2519-2532. DOI: 10.1016/j.neurobiolaging.2015.05.010.
30. Lippi, S.L.; Smith, M.L.; Flinn, J.M. A novel hAPP/htau mouse model of Alzheimer's disease: inclusion of APP with Tau exacerbates behavioral deficits and zinc administration heightens tangle pathology. *Front. Aging Neurosci*. **2018**, 10, 382; DOI: 10.3389/fnagi.2018.00382.
31. Si, Y.; Guo, C.; Xiao, F.; Mei, B.; Meng, B. Noncognitive species-typical and home-cage behavioral alterations in conditional presenilin 1/presenilin 2 double knockout mice. *Behav. Brain Res*. **2022**, 418, 113652; DOI: 10.1016/j.bbr.2021.113652.
32. Salgado-Puga, K., Prado-Alcalá, R.A; Peña-Ortega, F. Amyloid β enhances typical rodent behavior while it impairs contextual memory consolidation. *Behav. Neurol*. **2015**, 526912; DOI: 10.1155/2015/526912.
33. Janus, C.; Westaway, D. Transgenic mouse models of Alzheimer's disease. *Physiol. & Behav*. **2001**, 73(5), 873-886; DOI: 10.1016/s0031-9384(01)00524-8.
34. LaFerla, F.M.; Green, K.N. Animal models of Alzheimer disease. *Cold Spring Harb. Perspect. Med*. **2012**, 2(11), a006320; DOI: 10.1101/cshperspect.a006320.
35. Götz, J.; Bodea, L.G.; Goedert, M. Rodent models for Alzheimer disease. Nature Reviews Neuroscience. **2018**, 19(10), 583-598; DOI: 10.1038/s41583-018-0054-8.
36. Kobayashi, D.T.; Chen, K.S. Behavioral phenotypes of amyloid-based genetically modified mouse models of Alzheimer's disease. Genes, Brain and Behavior. **2005**, 4(3), 173-196; DOI: 10.1111/j.1601-183X.2005.00124.x.
37. Bryan, K. J., Lee, H. G., Perry, G., Smith, M. A., & Casadesus, G. Transgenic mouse models of Alzheimer's disease: behavioral testing and considerations. In *Methods of Behavior Analysis in Neuroscience*, 2nd ed.; Bucafusso, J.J.; CRC Press: Boca Raton, Florida, 2009, ISBN: 1420052349.
38. Puzzo, D;, Lee, L;, Palmeri, A; Calabrese, G.; Arancio, O. Behavioral assays with mouse models of Alzheimer's disease: practical considerations and guidelines. *Biochem. Pharmacol*. **2014**, 88(4), 450-467; DOI: 10.1016/j.bcp.2014.01.011.
39. Contet, C.;+8 Rawlins, J.N.P.; Deacon, R.M. A comparison of 129S2/SvHsd and C57BL/6JOlaHsd mice on a test battery assessing sensorimotor, affective and cognitive behaviours: implications for the study of genetically modified mice. *Behav. Brain Res*. **2001**, 124(1), 33-46; DOI:10.1016/s0166-4328(01)00231-5.
40. Dennison, J. L. Ricciardi, N.R.; Lohse, I.; Volmar, C.H.; Wahlestedt, C. Sexual dimorphism in the 3xTg-AD mouse model and its impact on pre-clinical research. *J. Alzheimers Dis.* **2021**, 80(1), 41-52 DOI: 10.3233/JAD-201014.
41. Line, S. J.; Barkus, C.; Coyle, C.; Jennings, K.A.; Deacon, R.M.; Lesch, K.P.; Sharp, T.; Bannerman, D. M. Opposing alterations in anxiety and species-typical behaviours in serotonin transporter overexpressor and knockout mice. *Euro. Neuropsychopharmacol*. **2011**, 21(1), 108-116; DOI: 10.1016/j.euroneuro.2010.08.005.

42. Konsolaki, E.; Tsakanikas, P.;0 Polissidis, A.V.; Stamatakis, A.; Skaliora, I. (2016). Early signs of pathological cognitive aging in mice lacking high-affinity nicotinic receptors. *Front. Aging Neurosci*. **2016**, 8, 91; DOI: 10.3389/fnagi.2016.00091.
43. Thomas, A.; Burant, A.; Bui, N., Graham, D., Yuva-Paylor, L.A.; Paylor, R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl)*. **2009**, 204, 361–373; DOI:10.1007/s00213-009-1466.
44. Savy, C.Y.; Fitchett, A.E.; McQuade, R.; Gartside, S.E.; Morris, C.M.; Blain, P.G.; Judge, S.J. Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in adult male rats as assessed by marble burying behaviour. *Neurotoxicology*. **2015**, *50*, 149–156; DOI:10.1016/j.neuro.2015.08.010.
45. Sanathara, N.M.; Garau, C.; Alachkar, A.; Wang, L.; Wang, Z.; Nishimori, K.; Xu, X.; Civelli, O. Melanin concentrating hormone modulates oxytocin-mediated marble burying. *Neuropharmacology*. **2018**, *128*, 22–32; DOI:10.1016/j.neuropharm.2017.09.008. 29
46. Njung'e, K.; Handley, S.L. Evaluation of marble-burying behavior as a model of anxiety. Pharmacol. *Biochem. Behav*. **1991**, 38, 63–67; DOI:10.1016/0091-3057(91)90590-x.
47. Kaehler, S.T.; Singewald, N.; Sinner, C.; Philippu, A. Nitric oxide modulates the release of serotonin in the rat hypothalamus. *Brain Res*. **1999**, 835, 346–349; DOI:10.1016/s0006-8993(99)01599-1.
48. Nicolas, L.B.; Kolb, Y.; Prinssen, E.P. A combined marble burying-locomotor activity test in mice: A practical screening test with sensitivity to different classes of anxiolytics and antidepressants. *Eur, J. Pharmacol*. **2006**, 547, 106–115; DOI:10.1016/j.ejphar.2006.07.015.
49. Gyertyán, I. Analysis of the marble burying response: Marbles serve to measure digging rather than evoke burying. *Behav Pharmacol*. **1995**, 6, 24–31.
50. Poling, A.; Cleary, J.; Monaghan, M. Burying by rats in response to aversive and nonaversive stimuli. J Exp Anal Behav. **1981**, 35, 31–44; DOI:10.1901/jeab.1981.35-31.
51. Wolmarans de, W.; Stein, D.J.; Harvey, B.H. Of mice and marbles: Novel perspectives on burying behavior as a screening test for psychiatric illness. *Cogn Affect. Behav Neurosci*. **2016**, 16, 551–560; DOI:10.3758/s13415-016-0413-. 45
52. Taylor, G.T.; Lerch, S.; Chourbaji, S. Marble burying as compulsive behaviors in male and female mice. *Acta Neurobiol Exp. (Wars)*. **2017**, 77, 254–260. PMID: 29182616.
53. Menashe, N.; Salama, Y.; Steinauer, M.L.; Spaan, J.M. Do behavioral test scores represent repeatable phenotypes of female mice?. *J. Pharmacol. Toxicol. Methods*. **2022** , 115, 107170; DOI: 10.1016/j.vascn.2022.107170.
54. Koolhaas, J.M.; Everts, H.; de Ruiter, A.J.; de Boer, S.F.; Bohus, B. Coping with stress in rats and mice: Differential peptidergic modulation of the amygdala-lateral septum complex. *Prog Brain Res*. **1998**, *119*, 437–448; DOI:10.1016/s0079-6123(08)61586-1. 37
55. Koolhaas, J.M.,; de Boer, S.F.; Buwalda, B.; van Reenen, K. Individual variation in coping with stress: A multidimensional approach of ultimate and proximate mechanisms. *Brain Behav Evol*. **2007**, 70, 218–226; DOI:10.1159/000105485.
56. Bruins Slot, L.A.; Bardin, L.; Auclair, A.L.; Depoortere, R.; Newman-Tancredi, A. Effects of antipsychotics and reference monoaminergic ligands on marble burying behavior in mice. *Behav Pharmacol.* **2008**, 19, 145–152; DOI:10.1097/FBP.0b013e3282f62cb2.
57. Coppens, C.M.; de Boer, S.F., Koolhaas, J.M. Coping styles and behavioural flexibility: Towards underlying mechanisms. *Philos Trans. R Soc. Lond B Biol Sci*. **2010**, 365, 4021–4028; DOI:10.1098/rstb.2010.0217.
58. Kinsey, S.G.; O'Neal, S.T.; Long, J.Z.; Cravatt, B.F.; Lichtman, A.H. Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay. *Pharmacol Biochem Behav*. **2011**, 98, 21–27; DOI:10.1016/j.pbb.2010.12.002. 41
59. Steimer, T. Animal models of anxiety disorders in rats and mice: Some conceptual issues. *Dialogues Clin. Neurosci*. **2011**, 13, 495–506, DOI:10.31887/DCNS.2011.13.4/tsteimer.
60. Stein, D.J.; Bouwer, C. A neuro-evolutionary approach to the anxiety disorders. *J. Anxiety Disord*. **1997**, 11, 409–429; DOI:10.1016/s0887-6185(97)00019-.
61. Ridley, R.M. The psychology of perseverative and stereotyped behaviour. *Prog. Neurobiol*. **1994**, 44(2), 221-231; DOI: 10.1016/0301-0082(94)90039-6.
62. Garner, J.P. Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of scientific outcomes. ILAR J. **2005**, 46(2), 106-117; DOI: 10.1093/ilar.46.2.106.
63. Garner, J.P. (2006). Perseveration and stereotypy: systems-level insights from clinical psychology. In *Stereotypic animal behaviour: fundamentals and applications to welfare*, 2nd ed; Mason, G., Rushen, J.; CABI: Cambridge, USA, 2006; 121, 142, ISBN: 1-84593-042-8.
64. Lewis, M., Kim, S.J. (2009). The pathophysiology of restricted repetitive behavior. *J. Neurodev. Disord*. **2009**, 1(2), 114-132; DOI: 10.1007/s11689-009-9019-6.
65. Cipriani, G.; Vedovello, M.; Ulivi, M.; Nuti, A.; Lucetti, C. Repetitive and stereotypic phenomena and dementia. *Am. J. Alzheimers Dis Other Demen*. **2013**, 28, 223–227; DOI:10.1177/1533317513481094.
66. Tian, J.; Gao, X.; Yang, L. Repetitive Restricted Behaviors in Autism Spectrum Disorder: From Mechanism to Development of Therapeutics. *Front. Neurosci.* **2022**, 16, 780407; DOI: 10.3389/fnins.2022.780407.
67. Neistein, S.; Siegal, A.P. Agitation, wandering, pacing, restlessness, and repetitive mannerisms. *International Psychogeriatrics*. **1997**, 8(S3), 399-402; DOI: 10.1017/S1041610297003712.

68. Nyatsanza, S.; Shetty, T.; Gregory, C.; Lough, S.; Dawson, K; Hodges, J.R. A study of stereotypic behaviours in Alzheimer's disease and frontal and temporal variant frontotemporal dementia. *J. Neurol. Neurosurg. Psychiatry*. **2003**, 74(10), 1398-1402. DOI: 10.1136/jnnp.74.10.1398.
69. Pekkala, S.; Albert, M.L.; Spiro, A., 3rd; Erkinjuntti, T. Perseveration in Alzheimer's disease. *Dement. Geriatr Cogn Disord.* **2008,** 25, 109–114; DOI:10.1159/000112476.
70. Deardorff, W.J.; Grossberg, G.T. Behavioral and psychological symptoms in Alzheimer's dementia and vascular dementia. *Handb Clin. Neurol*. **2019**, *165*, 5–32; DOI:10.1016/B978-0-444-64012-3.00002-2.
71. Torres-Lista, V.; Giménez-Llort, L. Persistence of behaviours in the Forced Swim Test in 3xTg-AD mice at advanced stages of disease. *Behav Processes*. **2014**, *106*, 118–121; DOI:10.1016/j.beproc.2014.05.001.
72. Baeta-Corral, R.; Giménez-Llort, L. Persistent hyperactivity and distinctive strategy features in the Morris water maze in 3xTg-AD mice at advanced stages of disease. *Behav. Neurosci.* **2015**, 129(2), 129; DOI: 10.1037/bne0000027.
73. Kim, H.; Lim, C.S.; Kaang, B.K. Neuronal mechanisms and circuits underlying repetitive behaviors in mouse models of autism spectrum disorder. *Behav. Brain Funct.* **2016**, 12(1), 1-13; DOI: DOI: 10.1186/s12993-016-0087-y.
74. Kalueff, A.V.; Stewart, A.M.; Song, C.; Berridge, K.C.; Graybiel, A.M.; Fentress, J.C. (2016). Neurobiology of rodent self-grooming and its value for translational neuroscience. *Nat. Rev. Neurosci*. **2016**, 17(1), 45-59; DOI: 10.1038/nrn.2015.8.
75. Gillen, G.; Rubio, K.B. Treatment of Cognitive-Perceptual Deficits: A Function-Based Approach. In *Stroke rehabilitation: a function-based approach*, 4th ed; Gillen. G.; Mosby: Riverport Lane, USA, 2015, ISBN: 978-0-323-17281-3.
76. Keszycki, R.M.; Fisher, D.W.; Dong, H. The hyperactivity–impulsivity–irritiability–disinhibition–aggression–agitation domain in Alzheimer's disease: current management and future directions. *Front. Pharmacol.* **2019**, 10, 1109; DOI: 10.3389/fphar.2019.01109.
77. Adriani, W.; Ognibene, E.; Heuland, E., Ghirardi, O.; Caprioli, A.; Laviola, G. Motor impulsivity in APP-SWE mice: a model of Alzheimer's disease. *Behav. Pharmacol.* **2006**, 17(5-6), 525-533; DOI: 10.1097/00008877-200609000-00019.
78. Masuda, A.; Kobayashi, Y.; Kogo, N.; Saito, T.; Saido, T.C., Itohara, S. (2016). Cognitive deficits in single App knock-in mouse models. *Neurobiol. Learn. Mem.* **2016**, 135, 73-82; DOI: 10.1016/j.nlm.2016.07.001.
79. Shepherd, A.; May, C.; Churilov, L.; Adlard, P.A.; Hannan, A.J.; Burrows, E.L. Evaluation of attention in APP/PS1 mice shows impulsive and compulsive behaviours. *Genes Brain Behav*. **2021**, 20(1), e12594; 10.1111/gbb.12594.
80. Millan, M.J.; Girardon, S.; Mullot, J.; Brocco, M.; Dekeyne, A. Stereospecific blockade of marble-burying behaviour in mice by selective, non-peptidergic neurokinin1 (NK1) receptor antagonists. *Neuropharmacology*. **2002**, 42(5), 677-684; 10.1016/s0028-3908(02)00021-7.
81. Schneider, T.; Popik, P. Attenuation of estrous cycle-dependent marble burying in female rats by acute treatment with progesterone and antidepressants. *Psychoneuroendocrinology*. **2007**, 32(6), 651-659; DOI: 10.1016/j.psyneuen.2007.04.003.
82. Llaneza, D.C.; Frye, C.A. Progestogens and estrogen influence impulsive burying and avoidant freezing behavior of naturally cycling and ovariectomized rats. *Pharmacol. Biochem. Behav.* **2009**, 93(3), 337-342; DOI: 10.1016/j.pbb.2009.05.003.
83. Castillo-Mariqueo, L.; Pérez-García, M.J.; Giménez-Llort, L. Modeling functional limitations, gait impairments, and muscle pathology in Alzheimer's disease: studies in the 3xTg-AD mice. *Biomedicines*. **2021**, 9(10), 1365; DOI: 10.3390/biomedicines9101365.
84. Homma, C.; Yamada, K. (2009). Physical properties of bedding materials determine the marble burying behavior of mice (C57BL/6J). *Open Behav. Sci.* **2009**, 3(1); DOI: 10.2174/1874230000903010034.

Study 4. Sumplementary Material

TABLE S1: Marble Burying Tests and Open Field Test correlations.

NTg FEMALE N=10

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
MB5	Spearman Correlation	-,349	-,609	-,348	,000	,348	,000	-,108	,087	,044	,261	,174	-,087	-,087
	Sig. (2-tailed)	,323	,062	,324	1,000	,324	1	1	,811	,904	,466	,631	,811	,811
MB10	Spearman Correlation	-,045	-,440	-,112	,052	,157	,060	-,138	,052	-,091	,022	-,037	-,246	-,097
	Sig. (2-tailed)	,902	,203	,758	,886	,666	,869	,703	,886	,803	,951	,919	,493	,790
MB15	Spearman Correlation	-,214	-,405	-,337	-,472	,315	,373	-,139	,247	,080	,112	,180	-,292	,157
	Sig. (2-tailed)	,552	,246	,341	,168	,376	,288	,702	,491	,826	,757	,619	,413	,664
MB20	Spearman Correlation	-,072	-,347	,020	,046	,229	-,086	-,203	-,216	-,060	,334	,098	-,479	-,361
	Sig. (2-tailed)	,843	,325	,957	,900	,524	,814	,575	,548	,869	,345	,787	,162	,306
MB25	Spearman Correlation	-,088	-,376	,019	,110	,234	-,140	-,200	-,240	-,076	,344	,091	-,448	-,396
	Sig. (2-tailed)	,809	,284	,957	,762	,516	,699	,579	,504	,835	,331	,803	,195	,258
MB30	Spearman Correlation	,048	-,374	-,241	,032	-,063	-,229	-,235	-,336	-,080	,380	,114	-,101	-,488
	Sig. (2-tailed)	,896	,287	,503	,931	,862	,524	,514	,343	,825	,279	,754	,781	,153
MBlatD	Spearman Correlation	-,243	,127	-,406	-,588	-,442	,201	,674	,030	-,062	-,491	-,285	,673(*)	,091
	Sig. (2-tailed)	,498	,726	,244	,074	,200	,577	,033	,934	,866	,150	,425	,033	,803
MBlatDw/o	Spearman Correlation	-,383	-,212	-,479	-,479	-,248	,134	,539	,091	,092	-,188	-,127	,467	-,006
	Sig. (2-tailed)	,275	,556	,162	,162	,489	,712	,108	,803	,800	,603	,726	,174	,987
MBlatDw/	Spearman Correlation	-,146	,432	-,085	-,456	-,231	,046	,199	,049	-,068	-,407	-,274	,231	,122
	Sig. (2-tailed)	,687	,213	,815	,185	,521	,900	,581	,894	,852	,243	,444	,521	,738
MBnD	Spearman Correlation	,049	,018	-,164	-,006	-,236	-,213	,135	-,539	-,474	-,164	-,212	,321	-,442
	Sig. (2-tailed)	,894	,960	,651	,987	,511	,554	,710	,108	,166	,651	,556	,365	,200
MBnDw/o	Spearman Correlation	-,012	,164	-,176	-,006	-,370	-,372	,270	-,588	-,585	-,382	-,370	,612	-,467
	Sig. (2-tailed)	,973	,651	,627	,987	,293	,290	,451	,074	,076	,276	,293	,060	,174
MBnDw/	Spearman Correlation	,315	-,240	,148	,283	-,160	-,019	,042	-,302	-,184	,068	-,080	-,191	-,345
	Sig. (2-tailed)	,376	,504	,684	,428	,659	,959	,909	,397	,610	,853	,826	,598	,329

NTg MALE N=18

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
MB5	Spearman Correlation	,043	-,191	,020	-,321	-,363	,322	,396	,070	-,220	-,023	-,188	,053	,105
	Sig. (2-tailed)	,866	,449	,937	,193	,139	,192	,104	,783	,380	,929	,456	,836	,678
MB10	Spearman Correlation	-,019	-,184	-,086	-,395	-,160	,319	,073	,270	,073	,134	,083	-,017	,223
	Sig. (2-tailed)	,940	,466	,733	,105	,527	,197	,775	,279	,774	,595	,744	,946	,373
MB15	Spearman Correlation	-,108	-,198	-,137	-,108	-,144	,114	,231	,107	-,037	,079	-,057	-,049	,040
	Sig. (2-tailed)	,670	,432	,587	,669	,568	,651	,357	,672	,885	,755	,824	,847	,874
MB20	Spearman Correlation	-,006	-,122	-,162	-,163	-,088	,274	,038	,321	,230	,188	,221	-,089	,251
	Sig. (2-tailed)	,980	,630	,520	,517	,729	,272	,879	,195	,358	,455	,379	,726	,315
MB25	Spearman Correlation	,096	-,256	-,246	-,425	-,282	,502(*)	,244	,432	,230	,196	,212	-,018	,418
	Sig. (2-tailed)	,706	,305	,326	,079	,256	,034	,328	,073	,358	,435	,399	,944	,085
MB30	Spearman Correlation	,138	-,224	-,199	-,329	-,201	,378	,213	,406	,252	,256	,259	-,108	,350
	Sig. (2-tailed)	,584	,370	,428	,183	,423	,122	,397	,095	,314	,305	,299	,671	,155
MBlatD	Spearman Correlation	,067	-,005	-,018	,214	,142	-,488(*)	,109	-,150	,172	,292	,130	-,302	-,185
	Sig. (2-tailed)	,791	,984	,945	,395	,573	,040	,667	,553	,494	,240	,606	,223	,463
MBlatDw/o	Spearman Correlation	-,009	-,194	-,127	,027	,067	-,368	,138	-,176	,090	,232	,059	-,187	-,179
	Sig. (2-tailed)	,972	,440	,616	,916	,793	,133	,586	,486	,721	,354	,818	,458	,478
MBlatDw/	Spearman Correlation	-,125	,086	,024	,340	-,131	-,492(*)	,052	-,109	,104	-,024	,004	-,013	-,046
	Sig. (2-tailed)	,621	,735	,925	,168	,604	,038	,837	,666	,680	,925	,987	,958	,855
MBnD	Spearman Correlation	,027	-,108	-,158	-,193	-,272	,153	,166	,179	-,112	-,255	-,204	,286	,271
	Sig. (2-tailed)	,916	,669	,531	,443	,276	,543	,510	,478	,659	,307	,417	,250	,276
MBnDw/o	Spearman Correlation	,118	,066	,006	-,015	-,319	-,002	,103	,072	-,171	-,321	-,266	,300	,202
	Sig. (2-tailed)	,640	,794	,981	,951	,197	,993	,684	,776	,498	,194	,286	,227	,420
MBnDw/	Spearman Correlation	,054	-,172	-,159	-,288	-,010	,414	,128	,353	,205	,220	,215	-,135	,253
	Sig. (2-tailed)	,832	,496	,528	,246	,969	,088	,613	,150	,413	,380	,392	,592	,311

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).

3xTg-AD FEMALE N=16

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
MB5	Spearman Correlation	-,228	-,127	,091	-,166	-,310	,232	,488	-,007	,158	,245	-,008	-,269	-,068
	Sig. (2-tailed)	,396	,638	,739	,538	,243	,387	,055	,980	,559	,360	,977	,314	,801
MB10	Spearman Correlation	-,029	-,068	,063	-,213	-,278	,247	,430	,104	,165	,160	-,007	-,195	,060
	Sig. (2-tailed)	,914	,802	,817	,429	,297	,357	,097	,702	,541	,554	,981	,469	,826
MB15	Spearman Correlation	,053	-,131	,116	-,249	,015	,314	,123	,063	,055	,029	,038	-,248	,023
	Sig. (2-tailed)	,845	,629	,668	,352	,955	,237	,649	,817	,841	,916	,889	,354	,932
MB20	Spearman Correlation	,069	-,115	,119	-,237	,026	,315	,116	,023	,047	,003	,025	-,287	-,008
	Sig. (2-tailed)	,799	,670	,660	,376	,923	,235	,669	,932	,861	,991	,927	,281	,977
MB25	Spearman Correlation	,245	,050	,053	-,133	,171	,214	-,047	-,021	-,094	-,130	-,118	,011	-,029
	Sig. (2-tailed)	,360	,854	,845	,623	,526	,425	,864	,938	,729	,631	,663	,969	,915
MB30	Spearman Correlation	,353	,143	,103	-,060	,256	,138	-,147	-,113	-,182	-,225	-,217	-,014	-,109
	Sig. (2-tailed)	,180	,597	,705	,825	,338	,611	,588	,676	,499	,403	,419	,960	,688
MBlatD	Spearman Correlation	-,083	,272	-,083	,185	,035	-,120	,078	,078	-,093	-,081	,011	,227	,083
	Sig. (2-tailed)	,761	,307	,760	,493	,896	,658	,774	,774	,732	,766	,967	,397	,760
MBlatDw/o	Spearman Correlation	-,083	,272	-,083	,185	,035	-,120	,078	,078	-,093	-,081	,011	,227	,083
	Sig. (2-tailed)	,761	,307	,760	,493	,896	,658	,774	,774	,732	,766	,967	,397	,760
MBlatDw/	Spearman Correlation	,013	-,032	-,284	-,225	-,326	,122	,174	,325	,252	-,180	,246	,333	,358
	Sig. (2-tailed)	,962	,905	,287	,403	,218	,652	,519	,220	,347	,504	,359	,207	,173
MBnD	Spearman Correlation	,511(*)	,233	,055	-,100	,209	,229	-,034	,006	-,098	-,336	-,076	,038	,016
	Sig. (2-tailed)	,043	,386	,840	,711	,436	,394	,901	,983	,717	,203	,781	,888	,952
MBnDw/o	Spearman Correlation	,560(*)	,197	-,006	-,135	,275	,244	-,130	,059	-,068	-,424	,033	,109	,086
	Sig. (2-tailed)	,024	,464	,983	,619	,303	,362	,631	,829	,803	,102	,902	,687	,752
MBnDw/	Spearman Correlation	,157	,082	,109	,098	,220	-,006	-,095	-,131	-,167	,068	-,221	-,058	-,150
	Sig. (2-tailed)	,563	,762	,688	,718	,414	,983	,727	,628	,535	,803	,410	,832	,580

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).

3xTg-AD MALE N=20

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
MB5	Spearman Correlation	-,299	-,490(*)	-,282	,006	,078	,078	,032	,062	,012	-,037	,079	,243	,200
	Sig. (2-tailed)	,200	,028	,228	,981	,744	,744	,894	,795	,961	,876	,740	,301	,399
MB10	Spearman Correlation	-,478(*)	-,310	-,112	-,046	,092	,145	-,053	-,071	-,247	-,111	-,182	,215	,083
	Sig. (2-tailed)	,033	,184	,637	,847	,700	,542	,826	,766	,294	,642	,442	,363	,727
MB15	Spearman Correlation	-,400	-,533(*)	-,370	-,114	,091	,355	,022	,227	,122	-,034	,132	,384	,394
	Sig. (2-tailed)	,080	,016	,109	,631	,704	,124	,928	,335	,610	,886	,579	,095	,086
MB20	Spearman Correlation	-,192	-,418	-,283	-,096	,244	,294	,073	,210	,079	-,125	,099	,330	,384
	Sig. (2-tailed)	,417	,067	,226	,688	,300	,209	,760	,375	,741	,598	,679	,156	,094
MB25	Spearman Correlation	-,257	-,287	-,240	,026	,288	,104	-,101	,038	-,056	-,264	,011	,243	,244
	Sig. (2-tailed)	,273	,220	,307	,914	,219	,663	,673	,874	,814	,261	,965	,301	,300
MB30	Spearman Correlation	-,222	-,300	-,246	,167	,221	-,004	-,020	,186	,102	-,193	,151	,201	,337
	Sig. (2-tailed)	,348	,198	,297	,481	,350	,986	,935	,433	,667	,416	,526	,396	,146
MBlatD	Spearman Correlation	,427	,527(*)	,278	-,103	-,030	-,119	-,094	-,290	-,119	-,021	-,132	-,245	-,399
	Sig. (2-tailed)	,060	,017	,236	,665	,900	,616	,694	,214	,618	,930	,579	,297	,082
MBlatDw/o	Spearman Correlation	,393	,507(*)	,234	-,121	-,110	-,011	-,095	-,202	-,054	-,023	-,078	-,188	-,301
	Sig. (2-tailed)	,087	,023	,321	,611	,645	,965	,691	,394	,821	,924	,745	,427	,197
MBlatDw/	Spearman Correlation	,326	,540(*)	,259	-,212	-,026	-,060	,017	-,281	-,224	-,026	-,185	-,192	-,369
	Sig. (2-tailed)	,161	,014	,269	,369	,915	,800	,943	,230	,343	,912	,435	,418	,109
MBnD	Spearman Correlation	-,241	-,557(*)	-,363	,018	,217	,167	,054	,357	,287	-,109	,281	,311	,510(*)
	Sig. (2-tailed)	,306	,011	,116	,940	,357	,481	,822	,123	,221	,647	,230	,182	,022
MBnDw/o	Spearman Correlation	-,366	-,607(**)	-,312	-,017	,427	,075	-,062	,328	,213	-,111	,207	,215	,437
	Sig. (2-tailed)	,112	,005	,181	,942	,061	,753	,795	,158	,367	,640	,380	,363	,054
MBnDw/	Spearman Correlation	,005	-,205	-,268	,029	-,038	,321	,211	,253	,357	,152	,361	,238	,381
	Sig. (2-tailed)	,985	,386	,253	,905	,875	,168	,371	,282	,122	,523	,118	,312	,097

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).

Table S2: Burrowing Tests and Open Field Test correlations.

NTg FEMALE N=10

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
DB%S	Spearman Correlation	,093	-,425	-,074	-,062	,000	,433	,426	,289	,059	-,178	,074	,178	,289
	Sig. (2-tailed)	,799	,221	,839	,866	1,000	,211	,220	,418	,871	,622	,839	,622	,418
DB%O	Spearman Correlation	,365	,188	,212	,115	-,576	,104	,614	-,152	-,178	-,479	-,345	,406	-,042
	Sig. (2-tailed)	,300	,603	,556	,751	,082	,776	,059	,676	,622	,162	,328	,244	,907
BB%	Spearman Correlation	-,109	-,612	-,224	-,091	,164	,409	,412	,212	,105	,055	,212	,127	,176
	Sig. (2-tailed)	,763	,060	,533	,803	,651	,241	,237	,556	,774	,881	,556	,726	,627
BBlatD	Spearman Correlation	,353	-,127	-,321	,115	-,588	-,091	,150	-,127	-,400	-,527	-,370	,733(*)	-,103
	Sig. (2-tailed)	,318	,726	,365	,751	,074	,802	,680	,726	,252	,117	,293	,016	,777
BBlatDout	Spearman Correlation	,353	-,127	-,321	,115	-,588	-,091	,150	-,127	-,400	-,527	-,370	,733(*)	-,103
	Sig. (2-tailed)	,318	,726	,365	,751	,074	,802	,680	,726	,252	,117	,293	,016	,777
BBlatDin	Spearman Correlation	-,308	,411	,018	-,092	,276	-,123	-,212	-,043	,069	,153	,104	-,092	,080
	Sig. (2-tailed)	,387	,238	,960	,800	,440	,734	,556	,906	,851	,672	,774	,800	,827
BBnD	Spearman Correlation	-,529	-,127	,127	,079	,152	-,140	,607	-,224	-,111	-,103	-,212	,006	-,285
	Sig. (2-tailed)	,116	,726	,726	,829	,676	,699	,063	,533	,761	,777	,556	,987	,425
BBnDout	Spearman Correlation	-,552	,170	,274	,116	,109	-,419	,346	-,419	-,182	-,067	-,365	-,237	-,517
	Sig. (2-tailed)	,098	,638	,444	,751	,763	,228	,328	,228	,615	,854	,300	,510	,126
BBnDin	Spearman Correlation	-,163	-,550	-,281	-,069	,100	,340	,464	,238	,006	-,150	,075	,338	,219
	Sig. (2-tailed)	,653	,099	,431	,850	,783	,337	,177	,509	,986	,679	,837	,340	,544

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).

NTg MALE N=18

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
DB%S	Spearman Correlation	,003	,032	-,003	-,333	-,277	,481(*)	,119	,457	,331	,286	,311	-,139	,395
	Sig. (2-tailed)	,990	,900	,990	,176	,266	,043	,638	,056	,180	,250	,210	,581	,104
DB%O	Spearman Correlation	-,461	-,600(**)	-,643(**)	-,451	,149	,153	-,079	,110	-,098	-,309	-,208	,536(*)	,253
	Sig. (2-tailed)	,054	,009	,004	,060	,556	,543	,754	,663	,698	,213	,407	,022	,311
BB%	Spearman Correlation	-,118	-,245	-,300	-,273	-,245	,342	,369	,174	-,058	-,119	-,196	,265	,389
	Sig. (2-tailed)	,642	,328	,226	,272	,328	,165	,132	,489	,818	,639	,437	,287	,111
BBlatD	Spearman Correlation	,009	,018	,133	-,366	,060	,200	,164	-,067	,020	,191	,139	-,150	-,112
	Sig. (2-tailed)	,971	,945	,598	,135	,813	,426	,517	,791	,938	,448	,583	,553	,657
BBlatDout	Spearman Correlation	-,032	-,011	,096	-,346	,098	,165	,107	-,117	-,046	,115	,083	-,092	-,158
	Sig. (2-tailed)	,900	,964	,705	,160	,699	,513	,674	,645	,856	,651	,744	,717	,531
BBlatDin	Spearman Correlation	,209	,309	,363	,414	,073	-,468	,045	-,205	,083	,326	,174	-,409	-,325
	Sig. (2-tailed)	,404	,212	,139	,088	,775	,050	,858	,415	,744	,187	,490	,092	,188
BBnD	Spearman Correlation	,020	,138	,064	-,018	-,172	,073	-,012	,255	,055	-,108	-,080	,047	,282
	Sig. (2-tailed)	,937	,586	,800	,945	,495	,774	,961	,308	,827	,671	,753	,854	,256
BBnDout	Spearman Correlation	,040	,130	,035	,032	-,176	-,069	-,066	,352	,222	,022	,084	-,045	,304
	Sig. (2-tailed)	,875	,607	,890	,900	,484	,787	,795	,151	,376	,932	,739	,858	,220
BBnDin	Spearman Correlation	-,041	-,202	-,226	-,475(*)	-,213	,578(*)	,220	,196	-,124	-,247	-,199	,311	,385
	Sig. (2-tailed)	,873	,420	,368	,047	,396	,012	,380	,435	,623	,324	,428	,209	,115

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).

3xTg-AD FEMALE N=16

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
DB%S	Spearman Correlation	-,137	-,476	-,472	-,240	-,225	,212	,211	,174	,300	-,013	,204	,314	,170
	Sig. (2-tailed)	,614	,062	,065	,370	,403	,431	,434	,520	,259	,963	,448	,236	,528
DB%O	Spearman Correlation	-,327	-,271	-,188	-,058	,021	,135	,094	-,079	-,049	,150	-,126	,124	-,102
	Sig. (2-tailed)	,216	,310	,485	,830	,939	,619	,729	,770	,857	,580	,642	,648	,706
BB%	Spearman Correlation	,181	,266	,431	,324	,251	-,217	-,103	-,394	-,518(*)	-,141	-,391	-,195	-,431
	Sig. (2-tailed)	,502	,319	,095	,221	,349	,419	,704	,131	,040	,603	,134	,470	,095
BBlatD	Spearman Correlation	,149	,056	,034	,052	,239	-,018	-,365	,038	-,021	,283	,160	-,077	,084
	Sig. (2-tailed)	,582	,837	,900	,849	,373	,948	,164	,888	,939	,288	,554	,778	,756
BBlatDout	Spearman Correlation	,149	,056	,034	,052	,239	-,018	-,365	,038	-,021	,283	,160	-,077	,084
	Sig. (2-tailed)	,582	,837	,900	,849	,373	,948	,164	,888	,939	,288	,554	,778	,756
BBlatDin	Spearman Correlation	-,315	-,416	-,608(*)	-,498(*)	-,494	,388	,312	,631(**)	,752(**)	,437	,619(*)	,323	,656(**)
	Sig. (2-tailed)	,235	,109	,012	,050	,052	,138	,240	,009	,001	,091	,011	,223	,006
BBnD	Spearman Correlation	-,267	-,353	,024	-,183	-,190	,253	-,046	,000	,113	,390	,266	-,182	-,024
	Sig. (2-tailed)	,318	,179	,931	,497	,480	,344	,865	1,000	,678	,135	,319	,501	,931
BBnDout	Spearman Correlation	-,365	-,524(*)	-,306	-,538(*)	-,475	,544(*)	,108	,312	,436	,360	,440	-,022	,321
	Sig. (2-tailed)	,165	,037	,249	,031	,063	,029	,691	,239	,091	,171	,088	,935	,225
BBnDin	Spearman Correlation	,233	,350	,493	,430	,380	-,282	-,252	-,517(*)	-,531(*)	-,123	-,363	-,327	-,525(*)
	Sig. (2-tailed)	,385	,184	,052	,097	,146	,291	,347	,040	,034	,649	,167	,216	,037

3xTg-AD MALE N=20

		OFlatM	OFlatC	OFlatP	OFlatR	OFlatG	OFnR	OFnG	OFd	OFeC	OFtC	OFdC	OFtP	OFdP
DB%S	Spearman Correlation	-,182	-,035	,029	,194	,203	,200	-,171	,170	,240	,189	,205	-,041	,187
	Sig. (2-tailed)	,442	,885	,902	,412	,391	,397	,471	,474	,309	,424	,387	,863	,429
DB%O	Spearman Correlation	,020	-,123	-,139	-,232	-,132	,250	,122	,063	,253	,117	,087	,387	,244
	Sig. (2-tailed)	,932	,605	,558	,325	,578	,288	,609	,791	,281	,623	,716	,092	,299
BB%	Spearman Correlation	-,279	-,362	-,278	-,197	,072	,154	-,226	,126	,174	,062	,092	,202	,154
	Sig. (2-tailed)	,233	,116	,236	,404	,762	,516	,338	,596	,463	,795	,699	,392	,516
BBlatD	Spearman Correlation	-,048	,252	,367	,200	,408	-,080	-,343	,016	-,003	-,037	,026	-,457(*)	-,164
	Sig. (2-tailed)	,841	,284	,111	,399	,074	,738	,138	,947	,991	,876	,913	,043	,490
BBlatDout	Spearman Correlation	-,177	,226	,302	,270	,377	-,147	-,422	,074	,011	-,104	,041	-,388	-,090
	Sig. (2-tailed)	,456	,339	,195	,250	,101	,535	,064	,758	,962	,662	,865	,091	,705
BBlatDin	Spearman Correlation	,207	,178	,268	,323	,268	-,150	,104	-,076	-,005	,184	,067	-,421	-,160
	Sig. (2-tailed)	,381	,454	,253	,165	,254	,527	,661	,750	,983	,438	,780	,065	,501
BBnD	Spearman Correlation	,002	,014	,242	,154	,068	,009	-,070	-,182	,089	,162	-,031	-,133	-,119
	Sig. (2-tailed)	,995	,955	,304	,517	,774	,971	,771	,442	,709	,495	,898	,576	,618
BBnDout	Spearman Correlation	,032	,100	,196	-,037	-,059	,119	-,003	-,209	-,005	,107	-,058	-,037	-,126
	Sig. (2-tailed)	,892	,676	,408	,877	,805	,617	,991	,377	,983	,654	,807	,876	,598
BBnDin	Spearman Correlation	-,164	-,138	,048	,205	,100	-,115	-,198	-,066	,113	,124	,000	-,064	-,046
	Sig. (2-tailed)	,489	,562	,841	,387	,674	,628	,402	,784	,636	,603	,999	,787	,849

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).

Table S3: Marble Burying, Deacon´s Burrowing Tests and Brief Burrowing Test correlations.

N=64

		MB5	MB10	MB15	MB20	MB25	MB30	MBlatD	MBlatDw/o	MBlatDw/	MBnD	MBnDw/o	MBnDw/	DB%s	DB%o	BB%	BBlatD	BBlatDout	BBlatDin	BBnD	BBnDout
MB5	Spearman Correlation	1																			
	Sig. (2-tailed)	.																			
MB10	Spearman Correlation	,683(**)	1																		
	Sig. (2-tailed)	,000	.																		
MB15	Spearman Correlation	,570(**)	,801(**)	1																	
	Sig. (2-tailed)	,000	,000	.																	
MB20	Spearman Correlation	,477(**)	,788(**)	,903(**)	1																
	Sig. (2-tailed)	,000	,000	,000																	
MB25	Spearman Correlation	,497(**)	,760(**)	,853(**)	,923(**)	1															
	Sig. (2-tailed)	,000	,000	,000	,000	.															
MB30	Spearman Correlation	,444(**)	,664(**)	,779(**)	,867(**)	,959(**)	1														
	Sig. (2-tailed)	,000	,000	,000	,000	,000	.														
MBlatD	Spearman Correlation	-,335(**)	-,374(**)	-,263(*)	-,293(*)	-,247(*)	-,262(*)	1													
	Sig. (2-tailed)	,007	,002	,035	,019	,049	,036	.													
MBlatDw/o	Spearman Correlation	-,186	-,303(*)	-,238	-,258(*)	-,208	-,238	,901(**)	1												
	Sig. (2-tailed)	,142	,015	,058	,040	,099	,058	,000	.												
MBlatDw/	Spearman Correlation	-,490(**)	-,522(**)	-,524(**)	-,613(**)	-,598(**)	-,612(**)	,449(**)	,275(*)	1											
	Sig. (2-tailed)	,000	,000	,000	,000	,000	,000	,000	,028	.											
MBnD	Spearman Correlation	,361(**)	,580(**)	,701(**)	,756(**)	,800(**)	,825(**)	-,323(**)	-,373(**)	-,428(**)	1										
	Sig. (2-tailed)	,003	,000	,000	,000	,000	,000	,009	,002	,000	.										
MBnDw/o	Spearman Correlation	,205	,370(**)	,458(**)	,484(**)	,516(**)	,557(**)	-,338(**)	-,445(**)	-,129	,877(**)	1									
	Sig. (2-tailed)	,105	,003	,000	,000	,000	,000	,006	,000	,311	,000	.									
MBnDw/	Spearman Correlation	,415(**)	,639(**)	,737(**)	,819(**)	,875(**)	,870(**)	-,158	-,125	-,722(**)	,692(**)	,298(*)	1								
	Sig. (2-tailed)	,001	,000	,000	,000	,000	,000	,211	,325	,000	,000	,017	.								
DB%s	Spearman Correlation	,163	,418(**)	,333(**)	,365(**)	,421(**)	,374(**)	-,155	-,168	-,263(*)	,378(**)	,238	,420(**)	1							
	Sig. (2-tailed)	,200	,001	,007	,003	,001	,002	,223	,186	,036	,002	,058	,001	.							
DB%o	Spearman Correlation	,118	,299(*)	,335(**)	,370(**)	,392(**)	,344(**)	,094	,062	-,226	,409(**)	,228	,467(**)	,550(**)	1						
	Sig. (2-tailed)	,353	,016	,007	,003	,001	,005	,458	,627	,073	,001	,070	,000	,000	.						
BB%	Spearman Correlation	,275(*)	,395(**)	,430(**)	,395(**)	,438(**)	,372(**)	-,015	,009	-,391(**)	,458(**)	,302(*)	,502(**)	,411(**)	,462(**)	1					
	Sig. (2-tailed)	,028	,001	,000	,001	,000	,002	,904	,945	,001	,000	,015	,000	,001	,000	.					
BBlatD	Spearman Correlation	-,040	-,046	-,219	-,209	-,176	-,168	,180	,257(*)	-,010	-,247(*)	-,203	-,178	-,050	-,042	-,157	1				
	Sig. (2-tailed)	,754	,719	,082	,098	,164	,184	,154	,041	,935	,049	,107	,160	,697	,742	,214	.				
BBlatDout	Spearman Correlation	-,075	-,051	-,190	-,175	-,135	-,108	,162	,235	-,049	-,202	-,163	-,138	-,004	-,003	-,112	,968(**)	1			
	Sig. (2-tailed)	,555	,688	,134	,167	,286	,394	,200	,061	,699	,109	,198	,279	,977	,983	,379	,000	.			
BBlatDin	Spearman Correlation	-,114	-,289(*)	-,350(**)	-,367(**)	-,405(**)	-,357(**)	,034	,071	,280(*)	-,366(**)	-,240	-,439(**)	-,225	-,405(**)	-,750(**)	,235	,145	1		
	Sig. (2-tailed)	,370	,021	,005	,003	,001	,004	,787	,579	,025	,003	,056	,000	,073	,001	,000	,062	,251	.		
BBnD	Spearman Correlation	,251(*)	,401(**)	,420(**)	,438(**)	,375(**)	,312(*)	-,156	-,162	-,295(*)	,480(**)	,371(**)	,407(**)	,453(**)	,421(**)	,568(**)	-,300(*)	-,278(*)	-,429(**)	1	
	Sig. (2-tailed)	,045	,001	,001	,000	,002	,012	,218	,200	,018	,000	,003	,001	,000	,001	,000	,016	,026	,000	.	
BBnDout	Spearman Correlation	,169	,224	,194	,211	,109	,056	-,087	-,068	-,022	,186	,148	,127	,218	,151	,031	-,259(*)	-,303(*)	,010	,759(**)	1
	Sig. (2-tailed)	,181	,075	,125	,093	,393	,661	,493	,592	,866	,140	,242	,315	,083	,234	,808	,039	,015	,940	,000	
BBnDin	Spearman Correlation	,277(*)	,387(**)	,411(**)	,385(**)	,435(**)	,373(**)	-,072	-,070	-,383(**)	,484(**)	,348(**)	,474(**)	,441(**)	,499(**)	,931(**)	-,120	-,060	-,788(**)	,639(**)	,055
	Sig. (2-tailed)	,026	,002	,001	,002	,000	,002	,570	,583	,002	,000	,005	,000	,000	,000	,000	,346	,637	,000	,000	,668

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).