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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

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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].

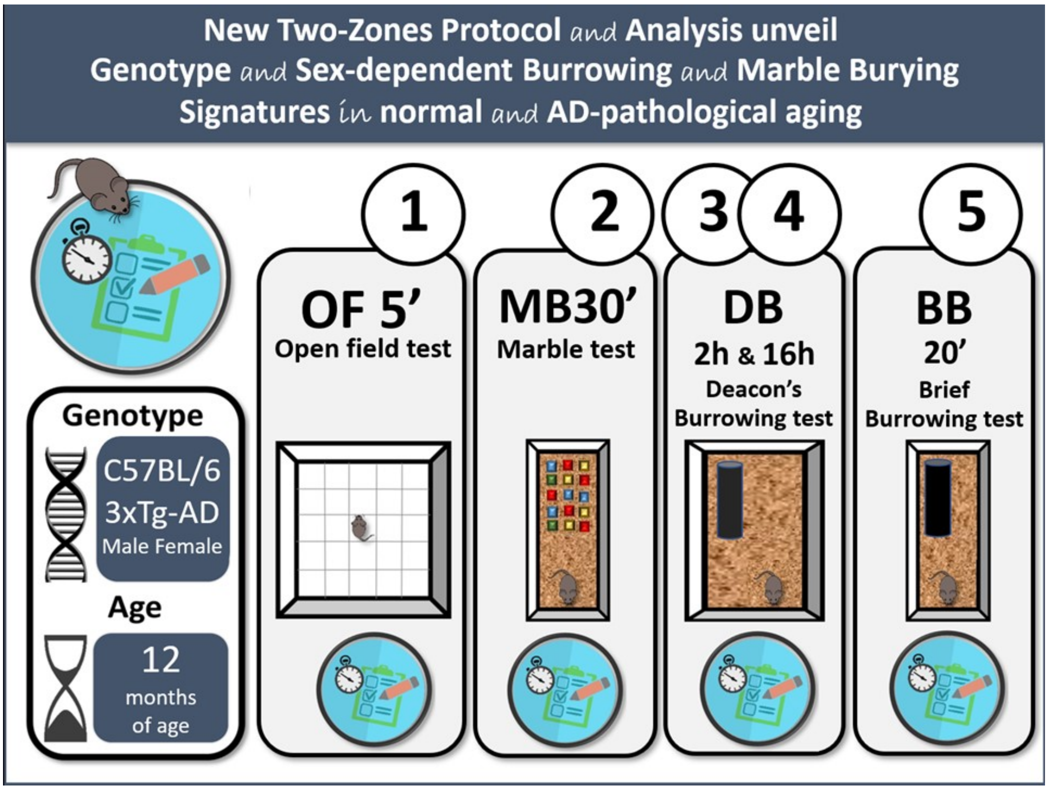

Figure 1. Graphical abstract. Experimental design: a 5-day battery of behavioral tests consisting of an open field test (OF) test on day 1, a two-zones marble test (MB) on day 2, a Deacon's burrowing test (DB) on day 3 until day 4, and a two-zones brief burrowing test (BBT) on day 5.

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].

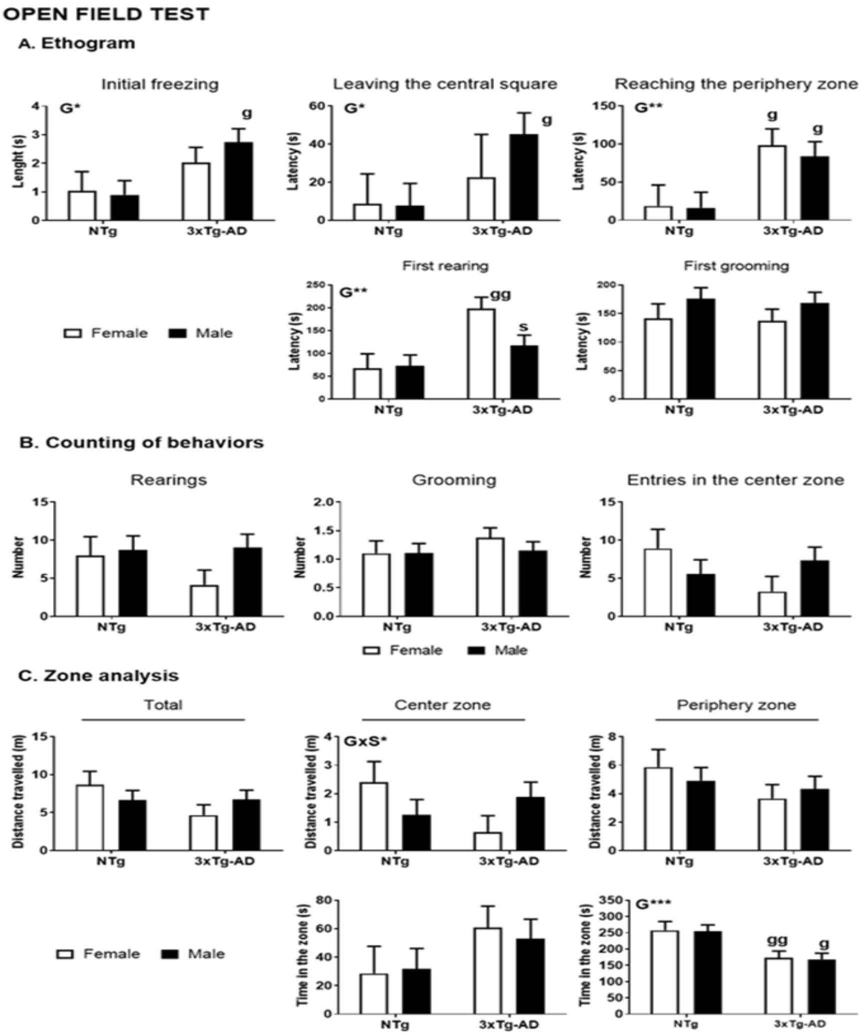

Figure 2. Open field behavioral analysis. Data are means ± SEM). Factorial analysis: Genotype (G) and sex (S) effects in mice with normal and AD-pathological aging. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Post-hoc analysis, g, genotype difference: g, $p < 0.05$; gg, $p < 0.01$.

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).

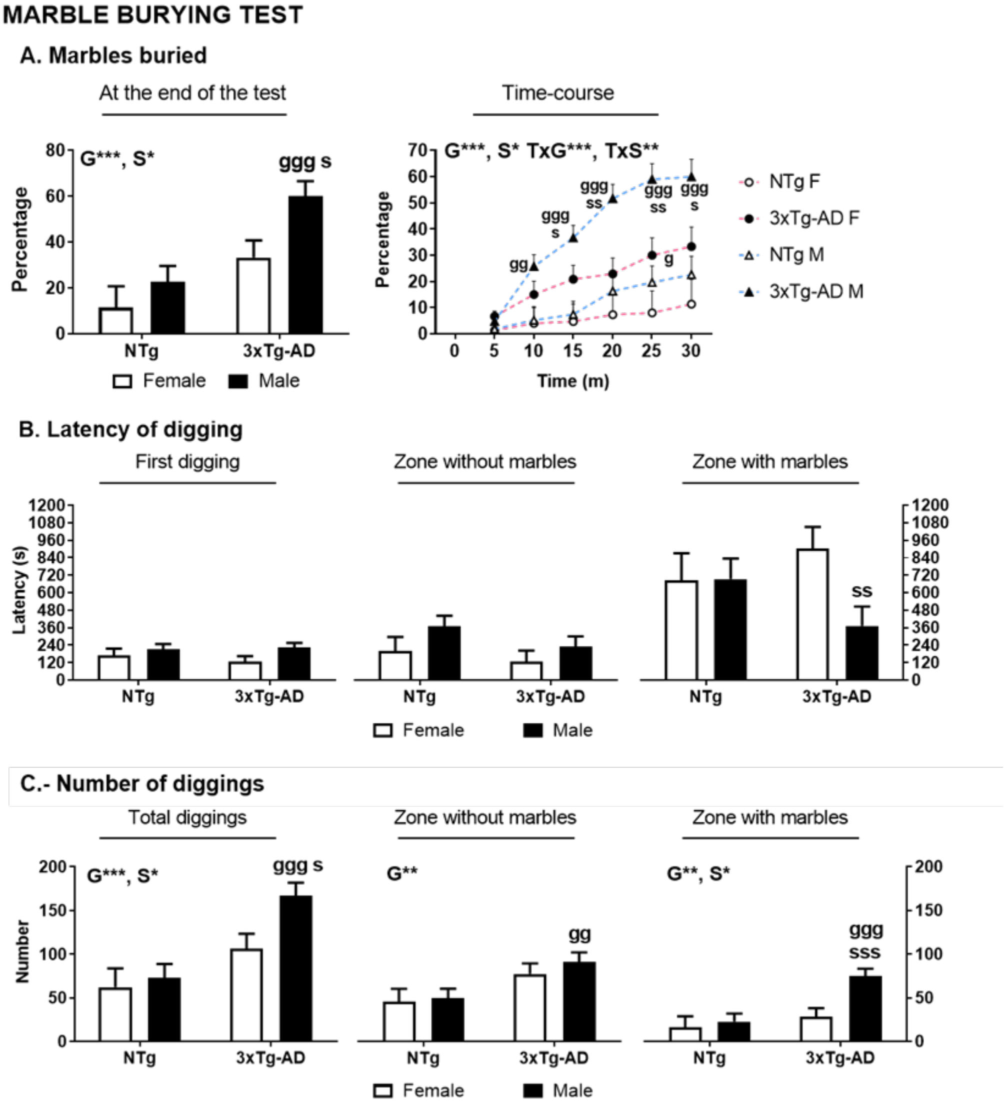

Figure 3. Marble Burying test time-course and two-zones analysis. Data are means ± SEM). Factorial analysis: Genotype (G), sex (S), time × genotype (T × G), and time × sex (T × S) in mice with normal and AD-pathological aging. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Post-hoc analysis, g, genotype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

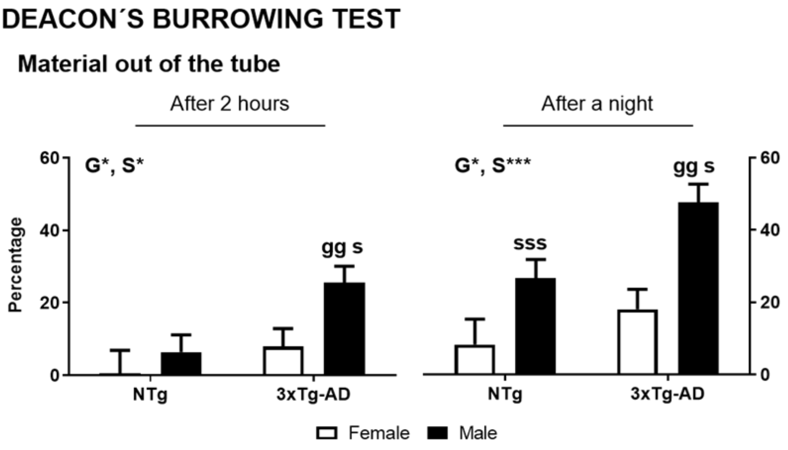

Figure 4. Deacon's burrowing test analysis. Data are means ± SEM. Factorial analysis: Genotype (G) and sex (S) effects in mice with normal and AD-pathological aging. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Post-hoc analysis, g, genotype difference; s, sex difference; gg, $p < 0.01$; s, $p < 0.05$; 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].

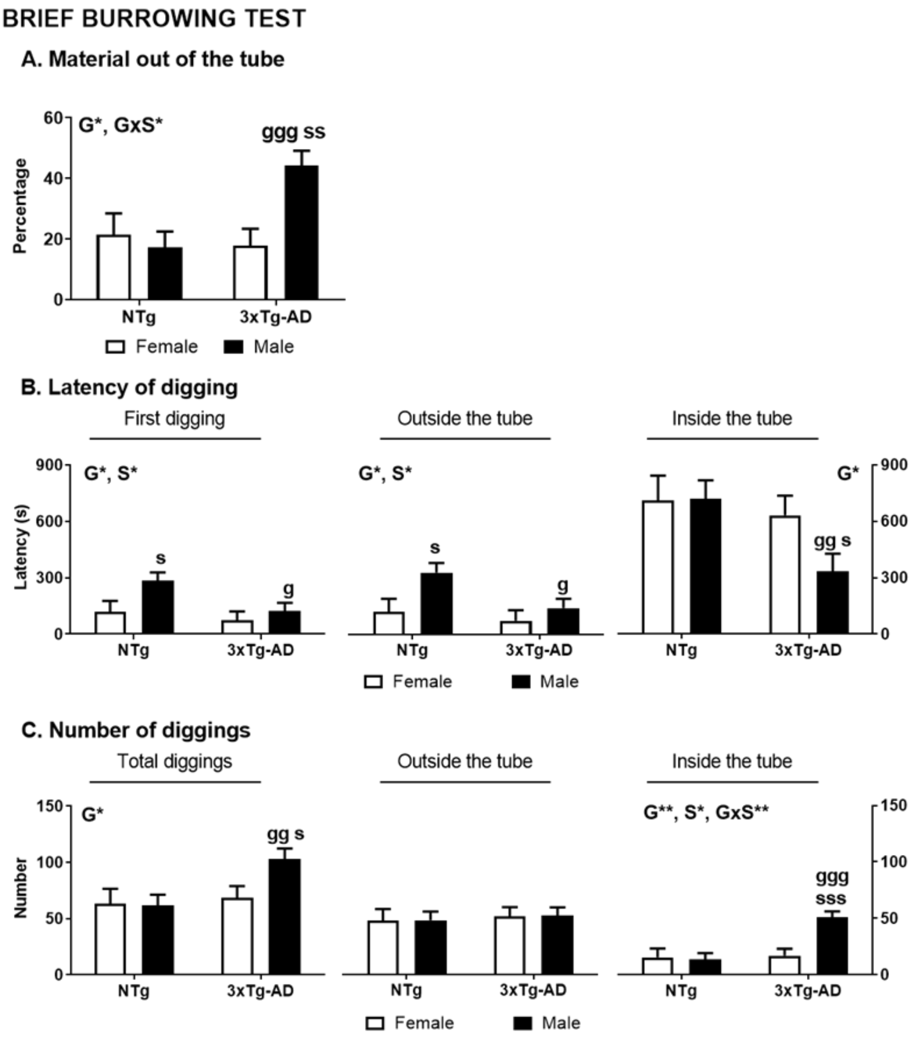

Figure 5. Dual analysis in a two-zone Brief Burrowing Test (BB). Data are means ± SEM. Factorial analysis: Genotype (G), sex (S) and genotype × sex (G × S) effects in mice with normal and AD-pathological aging. * p < 0.05, **p < 0.01 and ***p < 0.001. Post-hoc analysis, g, genotype difference; s, sex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

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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.

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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. Pharmaco*l. **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.

doi:10.20944/preprints202211.0342.v1

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.

doi:10.20944/preprints202211.0342.v1

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.