Episodic-Like Memory for What-Where-Which Occasion is Selectively Impaired in the 3xTgAD Mouse Model of Alzheimer's Disease

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Running title: Episodic-like memory deficit in 3xTgAD mouse

Abstract

Episodic memory loss is a defining feature of early-stage Alzheimer's disease (AD). A test of episodic-like memory for the rat, the What-Where-Which occasion task (WWWhich; Eacott and Norman, 2004; [1]), requires the association of object, location and contextual information to form an integrated memory for an event. The WWWhich task cannot be solved by use of non-episodic information such as object familiarity and is dependent on hippocampal integrity. Thus, it provides an ideal tool with which to test capacity for episodic-like memory in the 3xTg murine model for AD. As this model captures much of the human AD phenotype, we hypothesised that these mice would show a deficit in the WWWhich episodic-like memory task. To test the specificity of any episodic-like deficit, we also analysed whether mice could perform components of the WWWhich task that do not require episodic-like memory. These included object (Novel Object Recognition), location (Object Location Task, What-Where task) and contextual (What-Which) memory, as well as another three-component task that can be solved without reliance on episodic recall (What-Where-When; WWWhen). The results demonstrate for the first time that control 129sv/c57bl6 mice could form WWWhich episodic-like memories, whereas, 3xTgAD mice at 6 months of age were impaired. Importantly, even though 3xTgAD mice showed some deficit on spatial component tasks, they were unimpaired in the more complex WWWhen combination task (which includes a spatial component and is open to a non-episodic solution). These results strongly suggest that AD pathology centred on the hippocampal formation mediates a specific deficit for WWWhich episodic-like memory in the 3xTgAD model.

Introduction

Alzheimer's Disease (AD) is defined by early episodic memory loss and the progressive accumulation of amyloid-beta (Aβ), hyperphosphorylated tau and cholinergic cell loss [2, 3]. The 3xTgAD mouse carries the familial AD transgenes for APP$_{SWE}$ (KM670/671NL) and Presenilin 1 (PS1$_{M146V}$), and an additional tauopathy mutation in human tau Tau$_{P301L}$ [4] and mimics human AD progression, developing Aβ pathology throughout the medial temporal lobe (MTL) and neocortex in a manner that is temporally and spatially matched to human AD [4, 5]. The impact of 3xTgAD pathology on cognitive performance has been investigated through several behavioural paradigms, including water maze, inhibitory avoidance and contextual fear [6-8]. However, there are no reports specifically examining episodic memory in the 3xTgAD mouse.

Although episodic memory has been considered an uniquely human trait [9], recent work in birds [10, 11] and rats [1, 12-14] has demonstrated episodic-like memories in non-human species. In particular, two tasks, termed What-Where-When (WWWhen) or What-Where-Which-occasion (WWWhich) have been developed as tests of episodic-like memory for rodents. These tasks allow animals to demonstrate memory for an object (What), its location (Where) and the occasion (temporal or otherwise) on which it was experienced (When or Which occasion). Forming integrated and flexible associations between these three components provides enough information to define separate experiences and has been claimed to be crucial to demonstrating episodic-like memory in WWWhen [11] and WWWhich [1] memory tasks. Crucially, although both WWWhen and WWWhich initially appear to be open to familiarity-based solutions, both have been argued to rely on episodic-like processes. For example, Eacott and colleagues [15, 16] have argued that the pattern of deficits and spared performance on the WWWhich (and related tasks involving its spatial or contextual task components) are not compatible with the deficit on the WWWhich task being one of familiarity. Thus, it is claimed that

performance in these tasks that require integration of object (what), location (where) and occasion (temporal or otherwise) require episodic-like memory. However, the term 'episodic-like' is used, as in non-human animals we cannot demonstrate that it includes the sense of self or autonoetic consciousness required in Tulving's definition of episodic memory [9].

In support of the view that performance in the WWWhich task relies on episodic-like memory, previous studies in the rat have shown that WWWhich memories are dependent on the hippocampus [1, 17, 18] and dissociable from familiarity-based component spatial or contextual deficits [1]. Moreover, a recent study [19] examined the subjective experience of human participants given a WWWhich task modelled closely on the WWWhich task given to rats. The results suggested that above chance accuracy in this task was associated with a sense of "remembering", while responses associated with a sense of "knowing" were at chance levels. As human episodic memory is accompanied by a feeling of remembering, while familiarity processes are associated with a feeling of knowing [20], this provides strong support for the view that above chance performance in the WWWhich task is reliant on episodic memory processes. Therefore, the WWWhich task is a good candidate to examine episodic-like memory processes in transgenic disease models with symptomatic hippocampal pathology. WWWhen memory tasks have also been claimed to be reliant on episodic memory processes [14, 21]. However, this task has been criticised on the basis that the temporal identifier 'When' is typically defined in terms of "how long ago" the event took place rather than memory for the specific temporal occasion [16, 22, 23]. This can allow animals to use memory for object location (what-where) in combination with trace strength to guide accurate performance. If this strategy is adopted, the task no longer meets the requirements of episodic-like memory in being reliant on an integrated representation of the three components (what, where and occasion). For this reason, the

WWWhich task is a preferable measure of episodic-like memory in non-human animals, as it is less susceptible to such confounds. However, the WWWhich task has to date only been used with rats. In the present study, therefore, we adapted the WWWhich task for use in mice. The aim of this study was to determine whether both control and 3xTgAD mice could form episodic-like memories in the WWWhich task. For comparison, their ability to process single (What and Where) and dual (What-Where and What-Which) sub-components of the WWWhich task was also examined. In addition, performance on the alternative three-component WWWhen task (which can be solved either by episodic-like recall or non-episodic memory trace strength) was determined. These further tasks were included to explore the impact of task difficulty on performance and to gauge performance in hippocampus-independent tasks.

Methods

Animals

Behavioural experiments were performed using 12 female 3xTgAD mice carrying the AD transgenes for APP$_{SWE}$, PS1$_{M146V}$ and the additional human tau mutation for fronto-temporal dementia Tau$_{P301L}$ [4] and 10 age-matched female control mice. A further subset of mice not included in the behavioural tested were used for immunohistochemistry to determine AD-like pathology (see below). All mice in this study were bred in-house at the University of Manchester from homozygous pairing of mice donated from the original 3xTgAD and control lines [4]. Thus, control mice were bred from the 129sv/c57bl6 founder strain for the 3xTgAD line. Mice were housed in groups of 5 or 6 individuals on a 12:12 light/dark cycle with access to food and water *ad libitum*. All experiments were carried out using a longitudinal within-subjects design over a period of 8 months as follows (task/age): (1) WWWhich task (6 months old); (2) What-Where task (9 months); (3) novel object recognition (NOR; 'What') and object location task (OLT; 'Where') tasks (11 months); (4) What-Which task (12 months); and (5) WWWhen task

(14 months). All mice were ear punched for identification and genotyping (confirmed in a random sample including one control and four 3xTgAD mice). All procedures conformed to the European Communities Council Directive of 24 November 1986 (86/609/EEC), were licensed by the UK Home Office and approved by the University of Manchester research ethics committee.

The development of Aβ patholgy in female 3xTgAD mice is accelerated compared to males, possibly due to increased β-secreatase activity, decreased neprilysin levels and the involvement of oestrogen and progesterone on Aβ regulation [24, 25]. Therefore, females were used in this study due to the expectation of an earlier cognitive decline compared to males. Previous reports found intracellular Aβ to be the earliest detectable pathology in the 3xTgAD mouse, developing from 3 months of age in hippocampus and neocortex and in layers II and III of entorhinal cortex [4, 5]. Extracellular Aβ plaque deposits initiate in CA1 and subiculum from 12 months of age [4, 5, 26] and paired-helical filament tau pathology is detectable in hippocampus from 12 months [4, 5]. It was expected, therefore, that our 3xTgAD mice would have intracellular Aβ pathology in hippocampus at the time of WWWhich testing and that pathology would become increasingly widespread and severe over the period of testing for subsequent tasks. We confirmed the presence of Aβ pathology at 5, 8, 11 and 15 months of age (i.e., at an age point prior to the start of behavioural testing, at 2 age-points used in component tasks and at the end of the longitudinal testing period) through immunohistochemical analysis of a small subset of female mice with the 6E10 APP/Aβ antibody. The number of mice used for Aβ immunohistochemistry were as follows: 5 months (n = 4 3xTgAD and n = 2 control), 8 months (n = 6 3xTgAD and n = 2 control), 11 months (n = 4 3xTgAD and n = 1 control) and 15 months (n = 2 3xTgAD and n = 2 control).

Apparatus

Two open field arenas were constructed from 5mm white Perspex (Gilbert Curry, white 050) with floor dimensions of 30 x 30cm and a wall height of 25cm. These were modified further into two different contexts by attaching a tactile floor made from a LEGO® base plate and a stripe around the wall at mouse rearing height in one arena (context 1) and adding alternating vertical strips using black tape in the second (context 2; see Fig 1 A and B). Objects for testing were constructed from LEGO® and a combination of plastic alphabet letters glued to a single LEGO® block (Fig 1 C-G). Objects were adhered to the base of the arena in context 1 by the LEGO® base plate and in context 2 via Blu-Tack® hidden from view under the block. All objects were made from plastic to prevent material preference and to ease cleaning to prevent odour cues. Four identical copies of each object were constructed and different copies were used in the acquisition and test phases to control for olfactory cues.

Distal visual cues to the testing area included the camera mounted on a tripod to the south wall, A4-sized images of geometric shapes attached to the east wall of the testing room, and a 3D cylindrical striped can outside the arena on the North-West corner. Ambient lighting was provided from four ceiling lights and a low level spotlight attached to the video camera. All experimental phases were videotaped and object exploration was scored offline using a stopwatch.

Habituation

Following a 10-minute cage group habituation session to each context on day 1, mice were exposed singly to each context for 5 minutes per day over 5 days prior to testing. One object was placed in the centre of the area during habituation to ensure animals became used to the presence of an object and to prevent future object neophobia (this object was then excluded from future test sessions). Mice were run in a consistent order

in cage mate sessions during habituation and testing to maintain olfactory cues for the following mouse. During the test and habituation sessions, the arenas were left to saturate with scent, as it was previously observed that thoroughly cleaning the arena for each mouse elicited anxious behaviour. As all experimental phases were thoroughly counterbalanced, any build-up of scent could not be used as a cue for either the position or novelty of objects. This is because any scent marking by an animal could only have provided a cue when in combination with memory for what object was where on which occasion when the marking was made. Urine and faeces were removed from the arena between groups, however, as an aseptic environment affected the exploration of the first mouse in each cage, it was only at the end of the daily session that the arenas were cleaned thoroughly using 70% ethanol and/or soapy water. This could have affected the first mouse on a daily testing session, however, due to the counterbalancing of cage start order, any effect would have been spread evenly across genotypes.

Experiment 1: What-Where-Which task for episodic-like memory

The WWWhich task [1] was the first task experienced when mice were 6 months of age. Each test session was composed of two separate 3-minute acquisition phases and one 3-minute test phase, separated by one of five Inter-Trial-Intervals (ITIs). At test, mice were presented with a familiar and a novel object-location-context combination (Fig 2 A).

*** FIG 2 HERE ***

During the acquisition phases two objects (one 'Letter' type and one made from LEGO®) were placed in the top half of the context arena. The left/right position of the objects was reversed between acquisition phases 1 and 2, (e.g., object type Letter appeared on the left in context 1 and object type Lego® on the right, and vice versa for context 2). Mice were given 3 minutes to explore the context arena in acquisition phase 1, and were then removed to a holding cage briefly (for less than 20 seconds) so that the arena for acquisition stage 2 could be substituted in the location previously holding the first arena.

The mouse was then placed in this second context arena for a further 3 minutes. Mice were observed at these acquisition stages to ensure that they had approached the objects; however, no set exclusion criteria were applied. Upon completion of acquisition phase 2, mice were returned to a holding cage for one of 5 ITIs (either 2, 5, 10, 15 or 30 minutes) before testing. Thereafter, the mouse was returned to one of the two context arenas for the test phase, where it experienced two copies of one of the objects presented at acquisition. Direct object exploration was recorded when the mouse's nose was within 1cm and orientated towards or touching the object. Periods where the mouse was either rearing or sitting on the object, or facing but looking past the object, were excluded.

Mice were tested on one trial per day for 5 or 6 days per week, with each ITI being repeated 4 times over a counterbalanced period of 20 testing days. Mice were housed singly during the ITI delay in this and all subsequent tasks. The order in which contexts were experienced as phase 1 or 2, the choice of context for the test session, the choice of Letter or Lego® objects at test and the left/right position of the novel configuration were all counterbalanced in this and subsequent tests. Upon entry at each stage, the mouse was placed into the arena orientated to face the south wall away from the objects (which always appeared in the middle of the top half of the box). This south facing orientation, referred to here as egocentric, was also used in all other subsequent behavioural tests, except the What-Where allocentric test. The following behavioural tasks are presented in order of task complexity (number of components) rather than chronologically (see above), as we provide these results for examination of component features which could have influenced performance on the WWWhich task at 6 months of age, rather than to comment on the progression of AD pathology.

Experiment 2: Novel Object Recognition (What) and Object Location task (Where)

To examine whether memory capacities for 'What' and 'Where' were intact, mice were tested in a Novel Object Recognition task (NOR) and Object Location Task (OLT) at an ITI of 2 minutes. Each task comprised a 3-minute acquisition phase, followed by an ITI delay of 2 minutes and a final 3-minute test phase. Both tasks took place in Context 1 and objects from the WWWhich task were re-used in novel configurations (with the addition of extra Lego® blocks to the original object) and in novel pairings.

For the NOR acquisition phase, mice were presented with two identical copies of an object in the middle of the arena equidistant from the walls. They were then removed to a holding cage for the 2-minute interval. At test one novel object and a copy of the original object replaced the two acquisition objects. Increased exploration of the novel object was taken as an indication of intact object memory [27].

For the OLT acquisition phase, two identical copies of an object were placed on the left and right of the bottom half of the box. At test, the object on either the left or the right was spatially displaced to the upper half of the box (copies of the objects were used at test). This acquisition phase procedure is reversed to the other tests in order to prevent any effect of location neophobia, as the latter was found previously to influence the performance of both 3xTgAD and control mice in the OLT (Fig 2C). Novelty at test appearing on the left or right was counterbalanced for each mouse, so that they experienced it twice on the left and twice on the right. Testing took place over 8 consecutive days in a block of 4 days of OLT followed by 4 days of NOR.

Experiment 3: What-Where task of Object-Location spatial memory: Allocentric and Egocentric

To examine whether mice could form an association between an object and location, they were tested in the What-Where paradigm. Mice were tested at an ITI of 5 minutes first with an egocentric test starting orientation and then with an allocentric novel starting

location. The latter was included to examine; (a) whether a novel starting location at test would engage an allocentric strategy; and (b) to determine whether an egocentric start point was sufficient to make spatial judgements in these spontaneous tasks. The What-Where task consisted of a single acquisition phase of 5 minutes and an ITI of 5 minutes before a 3-minute test phase (see Fig 2 D and E). In the acquisition phase, mice were presented with two different objects, located to the upper half of the arena. Following the ITI delay in a holding cage, mice were returned to the arena, now with two copies of one of the previously presented objects. Thus, at test one object appeared in a location that was novel and one appeared in its previously encountered location. Increased exploration of the novel object-location combination would indicate memory for the test object's previous location. The left/right position of the novel object-location combination was counterbalanced for each mouse.

For the egocentric phase of this task, mice were placed into the arena at test facing the south wall, as per all other experiments described here (Fig 2D). In the allocentric test phase, mice were placed into the box in a novel location that was to the side of the static familiar object, facing the north wall (Fig 2E). This alternated left and right depending on the location of novelty at test and was chosen to emphasise that increased exploration of the novel combination (i.e., away from the allocentric starting location) demonstrated the motivation to move toward and explore novelty. Trials were run as follows; four allocentric and four egocentric tests at an ITI of 5 minutes, alternating task type over 8 consecutive days. Due to the alternation of egocentric and allocentric tasks daily (and inherent random allocation of novel combination to left or right side of the arena), it was assumed that mice would have no pattern of experience with which to predict the side containing novelty at test. In total, data from 8 trials (4 repeats x 2 task start points) were collected. As in the NOR and OLT tasks, only context 1 was used as the background setting and novel object configurations were used for all repeats.

Experiment 4: What-Which task for Object-Context memory

Two-component memory performance was tested further in an object-context (What-Which) memory task. Two acquisition phases of 3 minutes (with minimal delay to move the mouse between them) were followed by an ITI of 2 minutes prior to a 3-minute test phase. Acquisition phase 1 consisted of two copies of the same object presented in one context, followed by acquisition phase 2 containing two copies of a different object in the other context. At test, a copy of each object was presented in either context 1 or context 2, thus, one of these objects was now in a novel context (Fig 2F). Memory for the What-Which association was demonstrated as increased exploration of the novel object-context combination. The order of experiencing either context 1 or 2 first, and which context was used at test, was counterbalanced such that data from four trials were collected, with two from each context at test.

Experiment 5: What-Where-When task of episodic-like memory

In order to compare the performance of mice on the WWWhich task with that of an alternative three-component task, mice were run in the episodic-like WWWhen task. The protocol for WWWhen testing was adapted from that of Good et al. [21]. Combinations of both Letter and Lego® objects were used and all phases took place in context 1 only. Mice were run on four occasions over four subsequent testing days.

Each test session comprised two acquisition phases separated by an ITI of 2 minutes prior to one test phase, also separated from the acquisition phases by a 2 minute ITI (Fig 2G). All phases were 5 minutes in length. During acquisition phases 1 and 2, there were two objects placed in the arena, either in the top left and right corners, or bottom left and right corners. The order in which phase (top or bottom) occurred was alternated over the four trials. At test, all four items were returned into the corners of the arena, creating combinations as follows: one item from the first acquisition phase remained in a static position (Static-Old), one item from the first acquisition stage was spatially displaced

(Displaced-Old), one item from the second acquisition phase remained in a static position (Static-Recent) and finally one item from the second acquisition phase was spatially displaced (Displaced-Recent; Figure 2G). For each trial the location of the four object-location-temporal appearance combinations were moved, such that no combination (e.g. Static-Old) appeared in the same location at test more than once. Increased exploration of the less recent, spatially displaced object (Displaced-Old) would suggest that the mouse has combined object-location and temporal information to create a What-Where-When memory.

Data Analysis

Proportional differences in exploration between a novel and familiar object/location/contextual combination (the displacement value D2) was calculated as described previously [27]. Specifically, D2 equates to the time of exploration for the novel combination, minus the time exploring the familiar combination, with the result divided by the total exploration time. On this measure, a score of zero indicates no difference in exploration of the two objects and values above zero (maximum of 1) indicate greater exploration of the novel object or configuration. D2 values were analysed with a mixed ANOVA for WWWhich (or time in seconds for WWWhen task) to detect main effects. Group differences were compared using t-tests with Bonferroni correction for WWWhich performance across the 5 ITIs and in WWWhen for the four object combinations, and in the single and dual component tasks with single two-tailed, unpaired t-tests. One-sample t-tests (1-tailed) were also used in each task for comparing the performance of each group to chance (a ratio of 0) for each test. For the WWWhen task, time in seconds was used as the measure to allow comparison to those reported previously [21]. However, analysing WWWhen data as a proportion of time spent exploring each item (compared to chance at 0.25), and examining preferences for the Displaced-Old object combination versus the other combinations revealed the same

result (data not shown). Locomotor activity (LMA) in the arena at test was measured offline by counting the quadrant crosses made by the mouse during the 3-minute period. A quadrant cross was counted every time the mouse moved its entire body across one of the four imaginary lines separating the four quadrants of the test arena. The total exploration time for both objects at test was also analysed to assess motivation to approach and examine the objects (and to preclude the possibility that a D2 value of zero simply represented no object exploration). For each animal, the D2 value was calculated for each task repeat and later combined to produce an overall mean. When scoring videos offline, it was impossible to blind the experimenter to genotype at test as control and 3xTgAD mice often appeared identifiable as control mice had a tendency to wear out the fur on their nose through over-grooming. However, all videos were re-coded prior to analysis to blind the experimenter to both time delay and novelty position.

Staining for Aβ in the 3xTgAD mouse using the 6E10 anti- APP/Aβ antibody

Aβ pathology was determined using the 6E10 antibody to label the N-terminal 1-16 amino acids of Aβ and also the non-proteolytically processed isoform of human APP [4, 5]. While the 6E10 anti-body also labels non-processed APP, there is a strong correlation between the progression of 6E10 staining and that of specific Aβ 1-42 antibodies [5]. 30 micron thick sections were incubated with primary antibody (6E10, Signet 1:3000) in 0.1M KPB/ 0.1% Triton-x + 1% normal horse serum overnight. Slices were then incubated with secondary antibody (biotinylated anti-mouse, Vector Labs, UK, 1:200) in 0.1M KPB /0.1% Triton-x 100 + 1% normal horse serum for 1 hour and, thereafter, with Vectorstain Horseradish Peroxidase ABC kit (Vector Labs, UK) for 30 minutes. Staining was visualised using a DAB peroxidise kit (Vector Labs, UK) with nickel enhancement for a period up to 3 minutes. Sections were mounted in dH_2O onto 2.5 % gelatine coated slides and left to dry overnight, prior to cover-slipping using DPEX

solution. Slices were visualised under a light microscope (Olympus BX41) and pictures were taken using a camera (Olympus DP11).

Results

What-Where-Which task of episodic-like memory at 6 months of age

D2 performance was analysed in a 2 (genotype) by 5 (ITI) mixed ANOVA revealing an effect of genotype ($F_{(1,80)}=7.95$, $P<0.05$), but no effect of delay ($F_{(4,80)}=1.35$, $P>0.05$) or an interaction ($F_{(4,80)}=1.33$, $P>0.05$). The performance of control mice was significantly above chance at ITI delays up to 10 minutes (all delays: $t_{(9)}=>2.93$, $P<0.01$) but thereafter fell to chance. In contrast, the performance of 3xTgAD mice did not significantly differ from chance at any delay (Fig 3 Top). To test whether differences in motivation might explain these results a 2 (genotype) x 5 (ITI) mixed ANOVA was conducted on both LMA activity and on total object observation at test (Fig 3 Bottom right and left). For LMA there was a significant effect of ITI ($F_{(4,60)}=2.99$, $P<0.05$), but not of genotype ($F_{(1,80)}=4$, $P>0.05$). For total observation there was no effect of ITI ($F_{(4,80)}=0.73$, $P>0.05$) and there were no significant differences in observation between genotypes ($F_{(1,80)}=0.09$, $P>0.05$). Due to a main effect of genotype for performance, and the lack of significant performance versus chance in the 3xTgAD mice, we conclude that 3xTgAD mice are impaired in WWWhich episodic-like memory.

Novel Object recognition (What) and Object location task (Where) at 11 months of age

In NOR, both 3xTgAD and control mice performed significantly above chance levels ($t_{(11)}= 6.40$, $P<0.0001$ and $t_{(9)}=2.60$, $P<0.05$ respectively; Fig 4 Top panel). Furthermore, there were no significant differences between the performance of the two groups ($t_{(20)}=0.50$, $P>0.05$), nor between their total object observation ($t_{(20)}=1.39$, $P>0.05$) or LMA ($t_{(20)}=0.57$, $P>0.05$).

In OLT, there were no significant differences between the performance of the two groups (t(20)=1.193,P>0.05) see Fig 4 Bottom panel). Control mice performed significantly above chance (t(9)=3.63, P<0.01). 3xTgAD performance approached but did not quite reach significance (t(11)= 1.74, P= 0.0545). There were no significant differences between groups for either total observation (t(20)=1.102, P>0.05) or LMA (t(20)=0.359, P>0.05).

In summary, we conclude that object ('what') memory is unimpaired in the 3xTgAD model. While the performance of 3xTgAD mice did not differ significantly from controls in the OLT, their performance did not quite reach significance. Thus, we conclude from this that 3xTgAD mice have a mild memory deficit for spatial locations.

What-Where task for Object-Location associative memory at 9 months of age

The D2 results of the What-Where egocentric verses allocentric task with an ITI of 5 minutes were analysed in a 2 (genotype) by 2 (task type) mixed ANOVA. There was a significant effect of task type (F(1,20)= 6.10, P<0.05), but not genotype (F(1,20) = 0.10, >0.05) with no interaction (Fig 5 Top panel). It was clear that neither group was above chance in the allocentric version of the task (control t(9)=0.58, P= 0.29; 3xTgAD t(11)=0.7, P= 0.25), whereas, control mouse performance were above chance for the egocentric task (t(9)=2.52,P<0.05). As with the OLT, the performance of 3xTgAD mice approached, but did not reach, significance (t(11) = 1.694, P = 0.059). Due to the lack of genotype performance differences in the allocentric and egocentric mixed ANOVA, and the poor performance of both genotypes on the allocentric task, D2 values for the egocentric task alone were compared to identify any genotype differences in a 2-way un-related paired t-test. Although 3xTgAD mice were slightly worse than controls on this egocentric task, with a lower performance mean, there were no significant genotype differences (t(20) = 1.27, P>0.05).

To explore the results of the ITI 5 minute What-Where task further, both LMA and total observation results were analysed in a 2 (genotype) by 2 (task type) mixed ANOVA (Fig 5 Bottom panel). For LMA, there was a significant effect of task type ($F_{(1,20)} = 4.36$, $P<0.05$) but not genotype ($F_{(1,20)} = 2.99$, $P> 0.05$). For total observation, there was a significant effect of task type ($F_{(1,20)} = 4.96$, $P<0.05$), genotype ($F_{(1,20)} = 5.93$, $P<0.05$) and an interaction ($F_{(1,20)} = 4.47$, $P<0.05$). Post-hoc Bonferroni t-tests found this difference to lie in the egocentric task ($t_{(20)} = 3.12$, $P<0.01$) with 3xTgAD animals showing increased total observation.

As per the OLT task, no significant genotype differences were seen in What-Where task performance. However, as in the OLT task, 3xTgAD animals did not show performance levels that were significantly different from chance levels. Thus, we find a slight impairment in object-location associative memory in the 3xTgAD model (but see WWWhen memory section and later Discussion).

What-Which task for object-context associative memory at 12 months of age

D2 values for the What-Which task were compared with t-tests between each genotype and versus chance. Both 3xTgAD and controls performed significantly above chance at the delay of 2 minutes (3xTgAD $t_{(11)}=3.40$, $P<0.01$, 129sv $t_{(9)}=3.82$, $P<0.01$) and a further unpaired t-test revealed no significant differences in performance between genotypes ($t_{(20)}=0.78$, $P>0.05$: see Fig 6). There were also no significant genotype differences for total object observation ($t_{(20)}= 0.45$, $P>0.05$) or LMA ($t_{(20)} = 0.08$, $P>0.05$) at test. Thus, we conclude that object-contextual memory is intact in the 3xTgAD mouse.

What-Where-When episodic-like memory at 14 months of age

In order to control for multiple-component difficulty, all mice were tested in a 3-component WWWhen task. For this, we used exploration duration as the measure of performance. A 4 (object combination) by 2 (genotype) mixed ANOVA revealed an effect of object combination ($F_{(3,60)}=31.23$, $P<0.0001$) and an interaction ($F_{(3,60)}=3.72$, $P<0.05$), but no effect of genotype ($F_{(1,60)}=1.42$, $P>0.05$: see Fig 7 Top panel). However, Bonferroni post hoc t-tests revealed a significant genotype difference for the Displaced-Old object ($t(20) = 3.28$, $P< 0.01$). This was due to increased exploration of the Displaced-Old object by 3xTgAD mice. Further Bonferroni post-hoc t-tests revealed the Displaced-Old object to have significantly higher exploration than Static-Old for both groups ($t(9/11)= >5.0$, $P<0.0001$) and also higher than Static-Recent ($t(9/11)= >2.33$, $P<0.05$). Displaced-Old also had significantly more exploration than Displaced-Recent for 3xTgAD mice ($t(11)=6.08$, $P<0.0001$), thus, both 3xTgAD and control mice preferentially explored the Displaced-Old object combination, suggesting that three-component memory (what-where-when) was intact. This result, in contrast to the results of OLT and What-Where tasks suggest that under different circumstances, 3xTgAD mice are capable of demonstrating intact object-location memory, observable in their preferential exploration of the more remote What-Where combination. There were no significant genotype differences between either total observation ($t(20) = 0.73$, $P>0.05$) or LMA ($t(20)=0.69$, $P>0.05$).

Amyloid-Beta pathology in the 3xTgAD mouse, localised with 6E10 anti APP/Aβ antibody

To confirm the presence and progression of Aβ intracellular pathology, small cohorts of 5, 8, 11 and 15 month old female mice were screened with the 6E10 APP/Aβ antibody (Figure 8). Multiple sections in the horizontal and sagittal planes were examined to

estimate pathology in neocortex, hippocampus, medial and lateral entorhinal cortex and also in postrhinal and perirhinal cortices (extra-hippocampal structures associated with some of the component tasks). Intracellular staining for Aβ was widespread throughout the pyramidal cell layer of hippocampus proper (CA1 and CA3) and subiculum at 5 months of age (Figure 8 A and B top row) while dentate gyrus remained unstained at all ages examined (Figure 8 B). At 5 months of age, deep, layer-specific staining could be seen throughout cortical structures (Figure 8 A), with heavy labelling in LEC and MEC (layer V) and sparse cell labelling in perirhinal and postrhinal cortices (layer V) and neocortex (IV and V). Clear labelling with 6E10 could be seen in amygdala from 5 months onwards (data not shown). By 11 months of age onwards, there were the beginnings of extracellular Aβ pathology within subiculum and this spread to CA1 by 15 months of age (Figure 8 B bottom). Non-transgenic control mice displayed no specific staining for 6E10 at any age, consistent with their lack of human AD transgenes (See figure 8 B at 5 and 15 months of age).

Discussion

The results of this study show that control mice can demonstrate memory for objects (What), locations (Where), configurations of object and location (What-Where), object and context (What-Which), object, location and recency (What-Where-When) and object, location and context (What-Where-Which occasion). This demonstrates, for the first time, episodic-like memory in control mice in the robust WWWhich-occasion task. Similarly, 3xTgAD mice, carrying Alzheimer's disease transgenes for APP_{SWE}, $PS1_{M146V}$ and Tau_{P301L} have intact object memory (What), object and context memory (What-Which), and intact memory for object, location and recency (What-Where-When). However, in stark contrast to controls, they are impaired at identifying a novel configuration of What, Where and Which occasion and demonstrate slight impairment in

memory for locations (Where) and combinations of object and location (What-Where). As the pattern of pathology seen in the Manchester 3xTgAD colony is qualitatively similar to that demonstrated previously [4, 5, 26] it is likely that our 3xTgAD mouse cohort had widespread intracellular hippocampal Aβ pathology prior to the onset of WWWhich testing at 6 months of age. Due to the longitudinal design of the study, we can only compare the progression of Aβ pathology during the subsequent months of testing with non-behavioural animals sacrificed for the purpose of histology. However, it is extremely unlikely that the progression of pathological hallmarks of AD in these mice would not be identical to the presented immunohistological data, which are entirely consistent with other published accounts of 6E10 pathology in the 3xTgAD model [4, 5]. We argue that this impairment in identifying a novel configuration of What, Where and Which occasion represents a selective impairment in episodic-like memory in the 3xTgAD mouse, without any major influence from an underlying spatial deficit. While the theoretical basis for the claim that performance on this task represents episodic-like memory has been previously discussed [16, 28], there are specific issues to be addressed with this demonstration in mice. First, control mice performed above chance in this task only at delays of up to 10 minutes, while rats show above chance performance with delays of up to one hour [1] and human episodic memory also typically lasts much longer than this. Nonetheless, the limit of performance in the WWWhich task does not necessarily reflect the absolute limit of episodic-like memory: success here relies on maintaining distinct details of two highly similar events. Mice (or rats) may still retain significant information about aspects of the event even at the delay at which they can no longer perform the task. It is clear that without specific motivation to remember, the ability to retain the details (and maintain separate representation of) two highly similar events may not last long, even in humans. Indeed, recent research suggests that human patients with hippocampal damage struggle to maintain object-in-scene

information when memory load is high (i.e., when multiple representations are required to be held and/or for a period of time longer than working memory [29]). Therefore, the present mouse performance may be more comparable to human episodic memory than it at first would appear. Thus, we claim that the present control mice are demonstrating episodic-like memory.

The episodic-like impairment in the 3xTgAD mice could to be due to the presence of early AD pathology within the hippocampal formation, as no motivational differences were found for any of the present tasks. Indeed, the presence of intracellular Aβ has been shown to disrupt the performance of the 3xTgAD mouse during spatial reference memory water-maze testing, where performance was improved following the hippocampal clearance of Aβ using anti- Aβ antibodies [6]. It is highly unlikely that the deficit in WWWhich task would have been influenced by tau pathology, as this does not occur to any great extent in the 3xTgAD model until at least 12 months of age [4, 5].

An issue that does arise concerns the specificity of the impairment in the 3xTgAD mice. While the 3xTgAD mice were impaired in the WWWhich episodic-like memory task and were unimpaired in some component tasks, these mice were tested longitudinally at different ages and, therefore, would have expressed advancing levels of pathology during the period of testing encompassing the component tasks. However, a benefit of this design is that mice acted as their own controls throughout the testing period and the impairment in episodic-like memory was demonstrated at the earliest age point and the unimpaired tasks were tested at later points, when pathology would be more severe (or at least similar). While 3xTgAD mice did not demonstrate unambiguously intact spatial performance (i.e., significantly above chance) in the spatial OLT and What-Where tasks in the months after WWWhich testing, they were not significantly different from control animals and their performance approached significance. For the What-Where task, there is a potential confound in that a longer acquisition phase (5 minutes versus 3 minutes)

was given; thus, there is the possibility that more object encoding during acquisition could facilitate performance during test. Whilst this additional encoding time resulted in no obvious differences between the level of performance of either control or 3xTgAD mice in the spatial component tasks, it is possible that this additional time could have masked a clear spatial impairment in the 3xTgAD model. However, 3xTgAD mice were able to identify spatially displaced objects in the WWWhen task to exactly the same level as control mice (albeit with a different task arrangement than in OLT and What-Where tasks) suggesting that it is unlikely that such a mild impairment in spatial (where) or object-location (what-where) memory could account for the level of poor performance seen in the WWWhich task. Therefore, the pattern of WWWhich results is consistent with a robust deficit in episodic-like memory rather than an interpretation of an underlying deficit in the spatial task components.

Our conclusion that a hippocampal deficit in 3xTgAD mice could underlie their WWWhich performance deficit in the current study is supported by selective hippocampal lesion data [1, 18]. Furthermore, our results are consistent with data suggesting that lesions within the hippocampal system leave performance on the component tasks intact [1, 30]. The current results also support accumulating evidence that the WWWhich-occasion task is a measure of episodic-like memory that is dissociable from memory for its components [16, 28, 31]. One possible criticism of this view is that the three-component WWWhich task may simply be more difficult than the single or dual component tasks: that is, increased difficulty, rather than a specific reliance on episodic processing mechanisms, may be the cause of the dissociations observed here and in previous reports. The current results argue against this view: the 3xTgAD mice were severely impaired on the three-component WWWhich occasion task yet were unimpaired on the three-component WWWhen task. Thus, the crucial parameter is not the number of task components; it is rather the specific combination of

component factors that meets the requirement for episodic-like memory processes (What, Where and Which occasion). The current results are entirely consistent with this view and supported by lesion data [1, 18]. Thus, performance on component tasks (supported by non-hippocampal cortical association areas) remained largely intact here, whereas, pervasive pathology in the hippocampus could have affected the association of the three WWWhich components into an integrated episodic-like memory.

The use of spontaneous recognition tasks, rather than stressful behavioural paradigms (e.g. water maze), avoids adding further factors to the interpretation of the present results, such as an exaggerated stress response in female 3xTgAD in water maze [32]. While the current study represents the first time that 3xTgAD mice have been tested for episodic-like memory in a spontaneous recognition task, mice carrying the single APP Swedish gene mutation (Tg2576) have been tested on similar tasks. Good and colleagues [21, 33] reported that Tg2576 mice have similar impairments to those shown here; however, some crucial differences are apparent. Firstly, Tg2576 mice are reported to be impaired on a variant of the What-Where task [33] while in our spatial component tasks 3xTgAD mice were only mildly impaired at the ages we tested. Moreover, the 3xTgAD mice were able to detect a novel object-location combination on the WWWhen task. This result is seemingly paradoxical, as the single gene mutation appears to have produced robust impairment whereas the more inclusive triple gene mutation has not. However, methodological differences might account for these discrepancies: In the current study in our component tasks, we used a constant starting point, rendering the spatial tasks egocentric in nature. Good and Hale's [33] What-Where task used a much bigger arena and had four objects spaced further apart than in the current study, perhaps encouraging a stronger allocentric spatial strategy. Specifically, mice in an arena with widely spaced objects would have a further distance to travel (and may have to view each object in isolation), whereas in the current study, pairs of objects could be

seen simultaneously, facilitating egocentric processing. In addition, in the protocol of Good and Hale [33], re-located objects swapped positions, therefore, different mechanisms could be required when recognising this type of change (object swap versus object relocation to a novel location). It is possible that 3xTgAD mice express a mild deficit in spatial processing that was not strongly revealed in our egocentric What-Where task. In support of this argument, 3xTgAD demonstrated a preference for object spatial relocation on the 'harder' three component (WWWhen) spatial task. Thus, the What-Where task used by Good and Hale [33] may be more spatially taxing, eliciting allocentric processes and, therefore, revealing a spatial impairment in the Tg2576 mice. However, in our hands, neither controls nor 3xTgAD mice were able to perform the allocentric version of the What-Where task, perhaps because it represented a shift of strategy for the mice, which have been using constant starting points for the other tasks tested.

There is independent evidence that this difference between egocentric and allocentric starting points may be important in some spatial tasks. For example, Langston and Wood [18] demonstrated that rats with hippocampal lesions were unable to perform an allocentric version of the What-Where task, whereas they could perform the egocentric version. They could not, however, form WWWhich episodic-like memories from an egocentric viewpoint, as used here. In the current study, 3xTgAD mice with hippocampal pathology could form an egocentric What-Where memory to some extent (as a component of the WWWhich task) but failed to show an egocentric WWWhich-occasion memory, suggesting that further demands on the hippocampus are required to make an episodic-like judgement than for a What-Where combination, regardless of starting orientation. Further, the 3xTgAD pathology appears to elicit a similar effect to hippocampal lesions in rats. We suggest that despite the finding of a spatial processing deficit in similar AD mice in the literature, our use of an egocentric starting position

allowed the mice to overcome this potential impairment, at least in the WWWhen task.

This suggestion is supported by water maze data, where an enforced allocentric strategy reveals spatial deficits in young 3xTgAD mice [6, 32]. In conclusion, while 3xTgAD mice may show subtle deficits in spatial processing at the ages tested here, the poorer performance of 3xTgAD mice in these component tasks does not account for the level of impairment seen in the WWWhich task. Specifically, as object and contextual information remained intact, we suggest that deficits in WWWhich performance were not simply an additive impairment derived from an underlying spatial impairment.

Another discrepancy between the results of the 3xTgAD and Tg2576 mice is the performance on the WWWhen task [21]. This task is based on the seminal work of Clayton and colleagues with birds [10, 11] and has been developed as a model of episodic-like memory for rodents. It is thought that WWWhen task performance, like that of WWWhich, depends on the hippocampus functioning to associate object and place information from the association cortices with contextual or temporal information [28, 34].

However, the WWWhen task has been criticised for being open to non-episodic solutions [16, 19, 22, 35] such that the differential trace strength of What-Where memory can give results that may appear as an integrated episodic memory for What-Where-When. Indeed, Tg2576 mice showed an awareness of object recency (What-When), exploring recently seen objects less than objects presented earlier in the sequence [21].

However, their deficit in What-Where was again apparent in that they did not preferentially explore the novel object-location configuration; thus, the failure to demonstrate WWWhen memory in Tg2576 mice [21] may be entirely dependent on the impaired spatial component. In contrast, in the current study, 3xTgAD mice were not impaired in WWWhen memory (including the What-Where component of this task). This supports the suggestion that the WWWhen task is open to trace strength confounds (i.e., a non-episodic solution) rather than assuming that different neural processes are

required for the two tasks, as hippocampal lesions are known to impair both WWWhich and WWWhen types of memory [1, 13, 17, 18, 36]. It is possible rather than having intact WWWhen episodic-like memory, that 3xTgAD mice were able to use recency cues (i.e., trace strength) to guide performance.

However, another potential explanation of the apparently intact WWWhen memory in the 3xTgAD mice is that, as a result of their memory impairment, the mice could have forgotten (or have relatively weak memory for) the first presentation phase of the WWWhen task. This would result in the remote objects appearing to be (relatively) novel at test, inducing increased exploratory behaviour. Increased exploration of the old-displaced object based on object familiarity differences resulting from the forgotten first phase could be misinterpreted as evidence for WWWhen episodic memory. However, such forgetting would result in both the objects which appeared in the first presentation phase appearing to be (relatively) novel and thus would result not only in high levels of exploration of the old-displaced object (as seen) but also equally high exploration of the old-static object, which was not seen in the present results. Therefore this potential explanation does not bear detailed scrutiny. Thus, due to the potential of such non-episodic solutions being employed in tasks with a 'When' component, we suggest that the WWWhich occasion task is a stronger paradigm for assessing episodic-like memory in both mice and rats.

In contrast to the WWWhen task, 3xTgAD mice were severely impaired on the WWWhich episodic-like task despite being unimpaired at some component tasks at short time delays: What and What-Which. It has been argued that the WWWhich task tests episodic-like memory, and is hippocampus-dependent [16, 18]. Moreover, the task is dissociable from at least some of its component tasks (What-Which [30]) as, despite being a recognition task, it specifically tests episodic (recollected) memory over

familiarity-based processes. Our behavioural evidence suggests, therefore, that the neural circuits responsible for object recognition may be functionally intact at short time delays in the 3xTgAD model. In support of this argument, the 3xTgAD mouse is impaired from 9 months of age in the NOR task at long time delays of 1.5 and 24 hours [32], however, these are delays which are likely to recruit the hippocampus, not just perirhinal cortex [37], and from our WWWhich results, we show there is likely to be a hippocampal impairment in the model. Interestingly, although there was evidence for Aβ pathology within the association cortices, we did not see impairment in the object and contextual memory tasks at the short delays tested. Thus, it seems that the hippocampus is more susceptible to impairment due to AD-related pathology.

In contrast to our findings in the 3xTgAD mouse, the previously reported deficit in WWWhen memory in the single mutation Tg2576 mice can be attributed directly to their impairment in the What-Where task [33]. Therefore, the present results are the first report of a selective deficit in a spontaneous recognition test of episodic-like memory in a transgenic model of AD. The results further suggest that there could be a heightened susceptibility of the hippocampal formation to early AD pathology in the 3xTgAD mouse and parallels the progression of early human AD, where episodic memory is often lost and patients become more reliant on familiarity based processes [38, 39]. In the current study design, determining the pathological state of 3xTgAD mice tested for behaviour was not possible due to the longitudinal design of the study; however, in the months following WWWhich testing we saw relatively intact component performance. In our separate sample of mice sacrificed for immunohistochemistry at a later date, we saw worsening of Aβ pathology from 5 months onwards, thus, it appears that the observed specific and early impairment in episodic-like memory was caused by the earliest stage AD pathology. For future work, it would be beneficial to quantify AD pathology

specifically from 3xTgAD animals sacrificed from the behavioural sample and to investigate whether episodic-like memory is intact in younger 3xTgAD mice, which carry a lower Aβ load. It would also be useful in order to concretely implicate one type of pathology over another to use multiple strains to separate out the relative contribution or Aβ or tau pathology on performance.

In summary, the current results demonstrate a selective impairment in episodic-like memory in mice carrying the Alzheimer's disease transgenes for APP$_{SWE}$, PS1$_{M146V}$ and Tau$_{P301L}$. These results mirror the early stages of Alzheimer's disease in human patients, showing separation between the level of impairment seen in different forms of memory (episodic versus familiarity-based). Finally, the results also demonstrate dissociation between performance on the WWWhich-occasion task of episodic-like memory and the WWWhen task, suggesting that the former is a more robust task for episodic memory.

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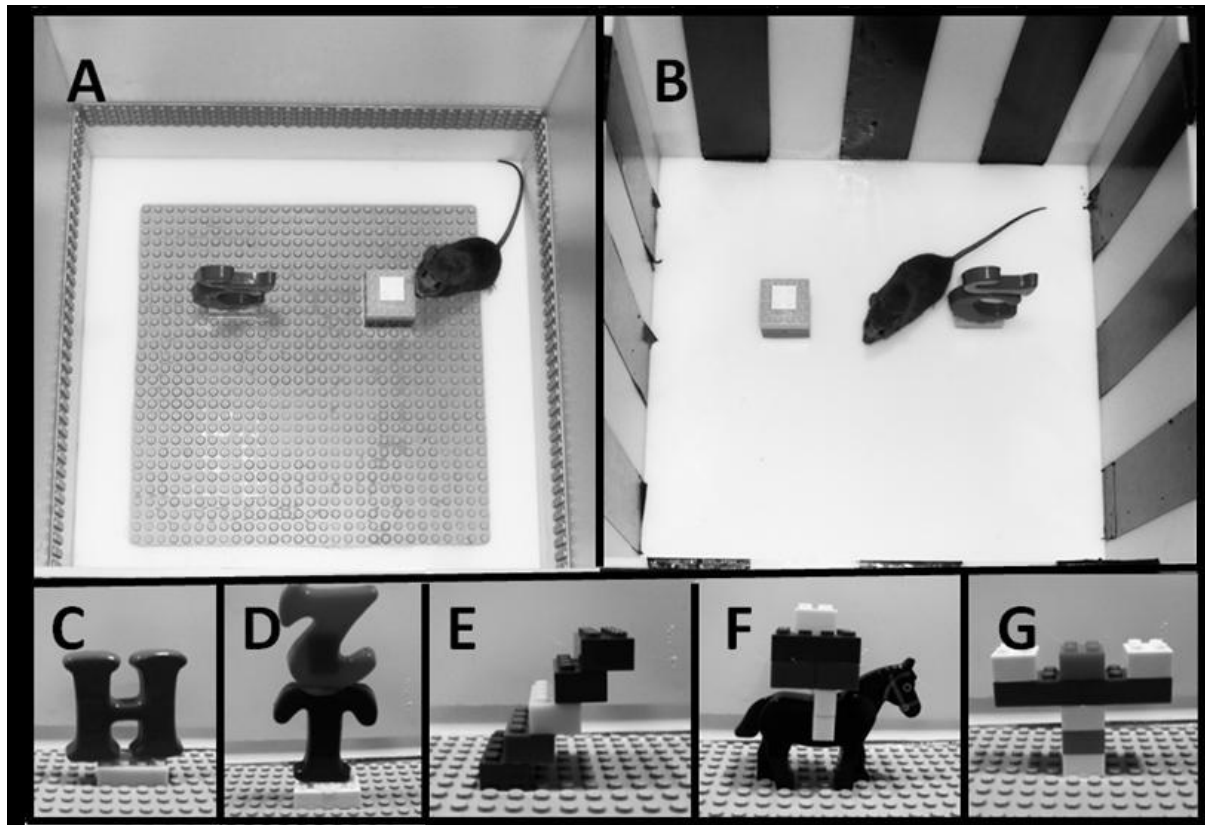
Figures

Figure 1: Apparatus used in the study. The top two panels show examples of Context 1 [A] and Context 2 [B] for the What-Where-Which task. Typical stimuli are displayed on the lower row. Examples of 'letter' [C,D] and LEGO® [E,F,G] objects. Combinations of object type were used in all tasks.
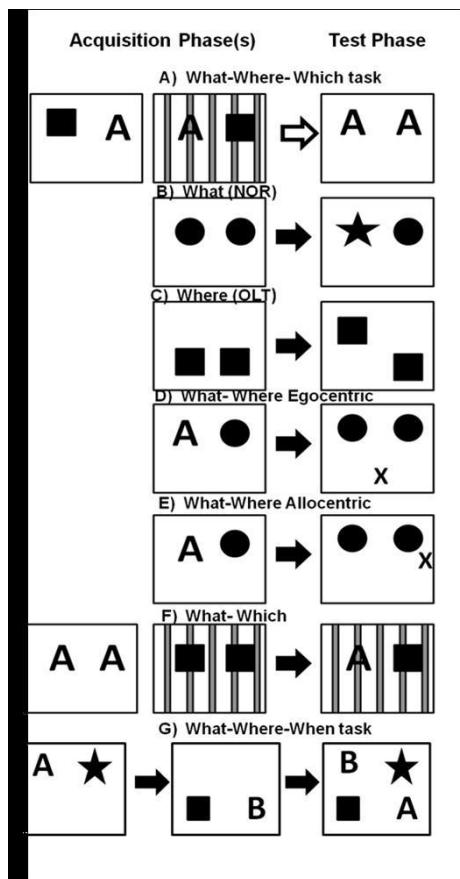
Figure 2: Behavioural protocols. Tasks consisted of either one (NOR, OLT, What-Where), or two acquisition phases (What-Which, What-Where-Which, What-Where-When) followed by a test. The black arrow depicts ITI. In tasks A-F, novelty is shown occurring at test on the left. Protocols: [A] What-Where-Which task; [B] What (NOR) task; [C] Where (OLT) task; [D] What-Where egocentric position at test. Black cross depicts starting position facing the south wall; [E] What-Where allocentric task in which mice enter the box on the opposite side to novelty at test, facing the north wall (black cross); [F] What-Which task; [G] What-Where-When task. At test A= Displaced-Old, B= Displaced-Recent, Star= Static-Old, Square= Static-Recent.
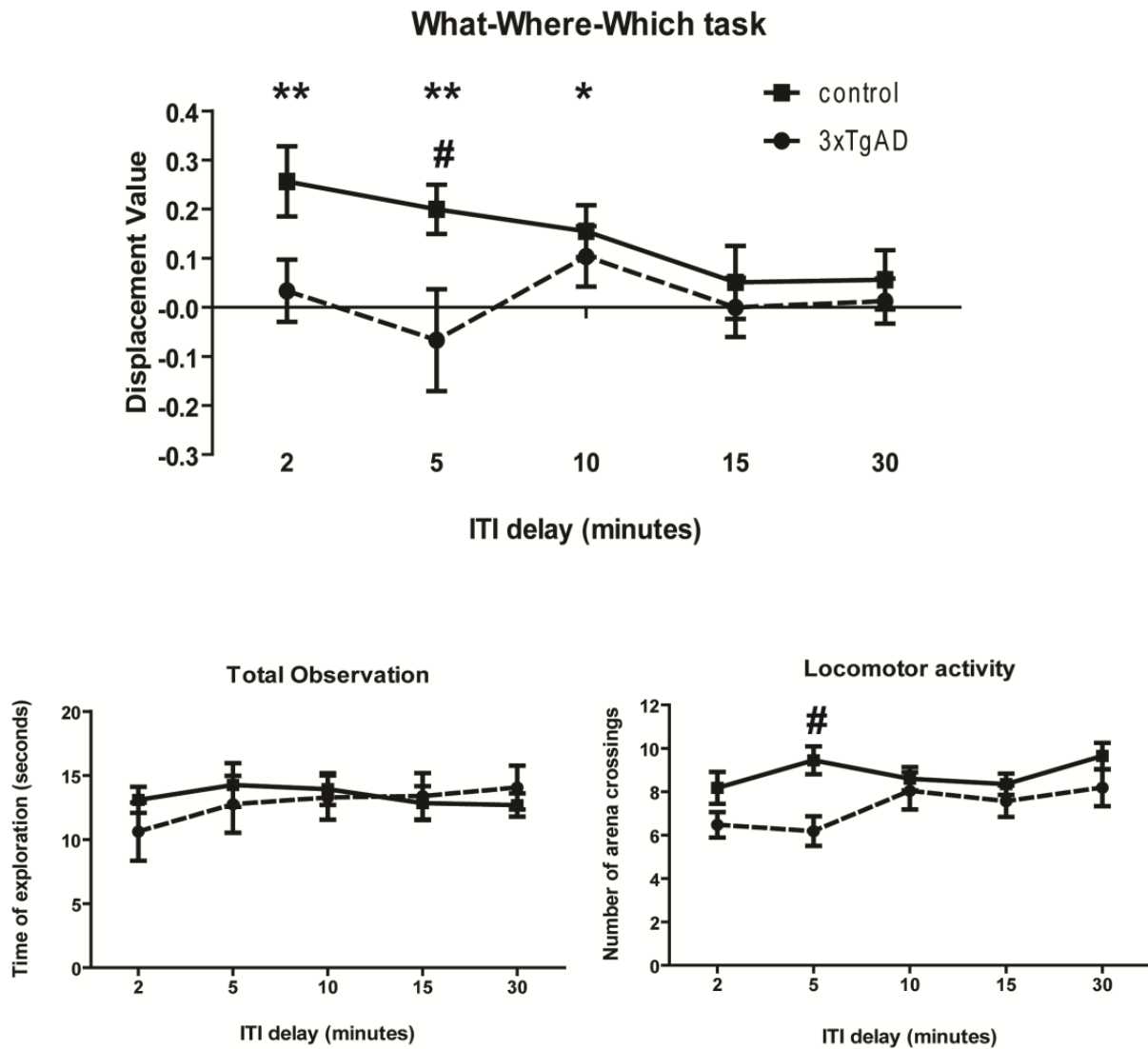
Figure 3: Episodic-like memory performance in the What-Where-Which task in 3xTgAD versus control mice. 3xTgAD mice display an episodic memory deficit at 6 months of age. Top panel: D2 values for 3xTgAD mice (n=12) and controls (n=10) at each delay. Bottom left: Total observation. Bottom Right: Locomotor activity at test. Asterisks denote significantly better performance of control mice versus chance (P<0.01 **, P<0.05 *). Hash denotes pair-wise genotype difference in this and all other figures (P<0.05 #). All data in this and subsequent Figures are represented as mean (±SEM).
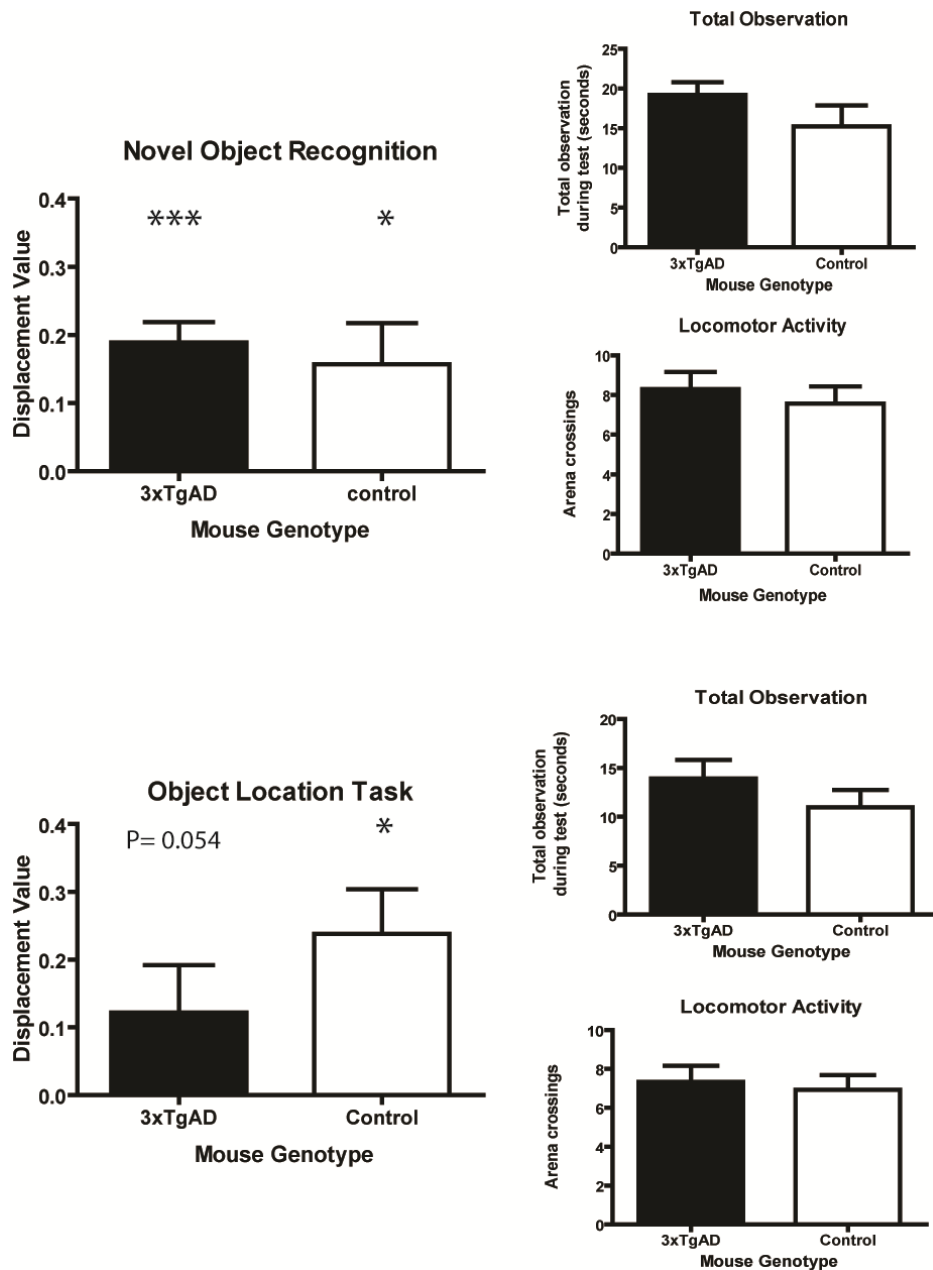
Figure 4: Memory for What and Where at a delay of 2 minutes. Top panel: Novel Object Recognition task D2 for 3xTgAD and control mice suggests intact object memory. Top right: Exploratory measures. Bottom panel: Object Location task D2 for control mice suggests normal object-location memory. 3xTgAD performance approached but did not reach significance (P= 0.054). Bottom right: Exploratory measures. Asterisks denote the significantly better performance of mice versus chance at P<0.05 *, P<0.01 ** and P<0.0001 ***.
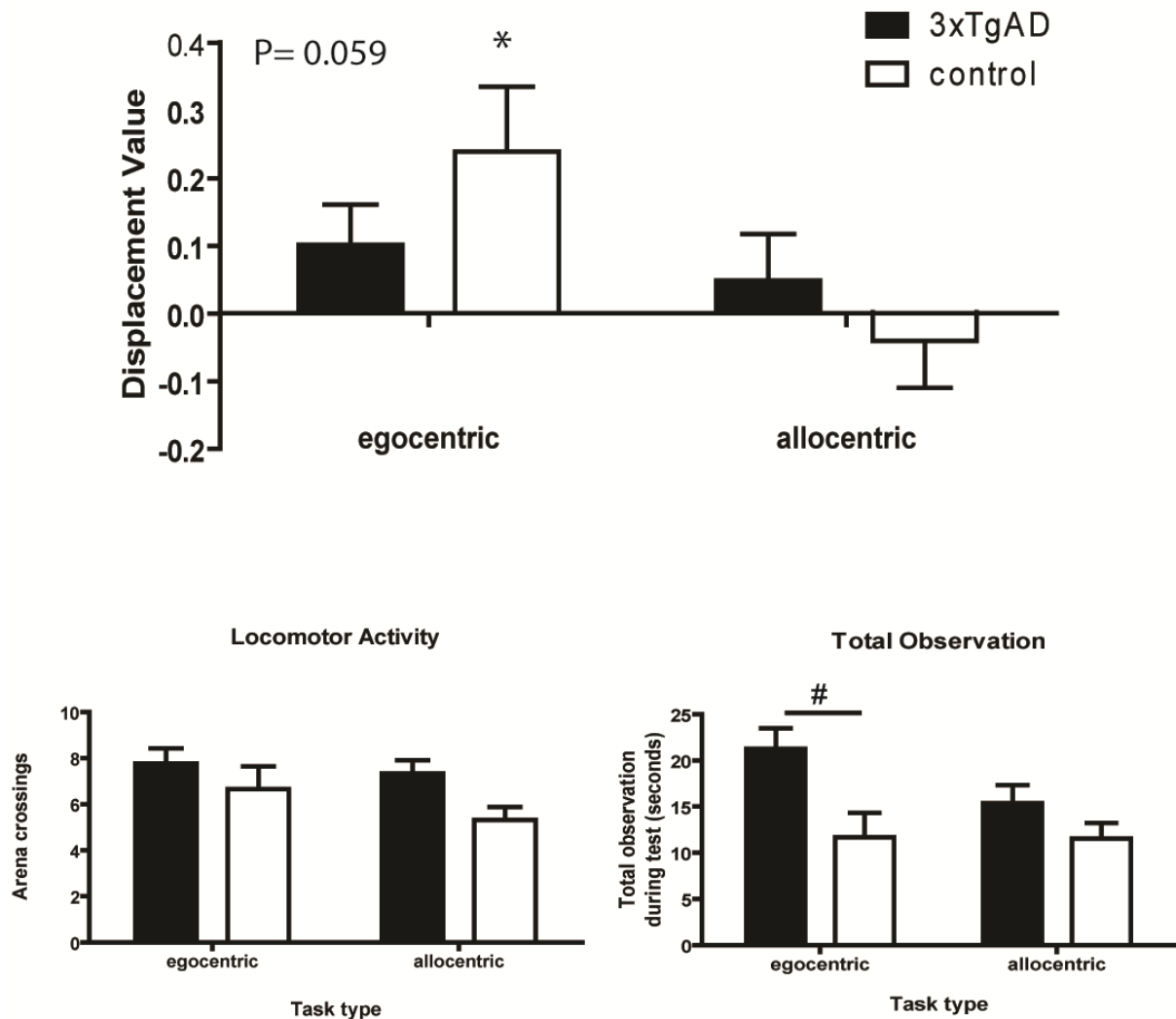
Figure 5: Egocentric and allocentric What-Where memory at a delay of 5 minutes. Top panel: What-Where D2 values for 3xTgAD and control mice at ITI 5 minutes suggest impaired object-location memory in 3xTgAD mice. D2 value for 3xTgAD egocentric performance was P=0.059 whereas neither group could perform above chance in the allocentric trials. Bottom left: LMA. Bottom Right: Total observation displayed a pair-wise genotype difference (P<0.05 #). Asterisks denote the significantly better versus chance at P<0.05 *.

What-Which Task
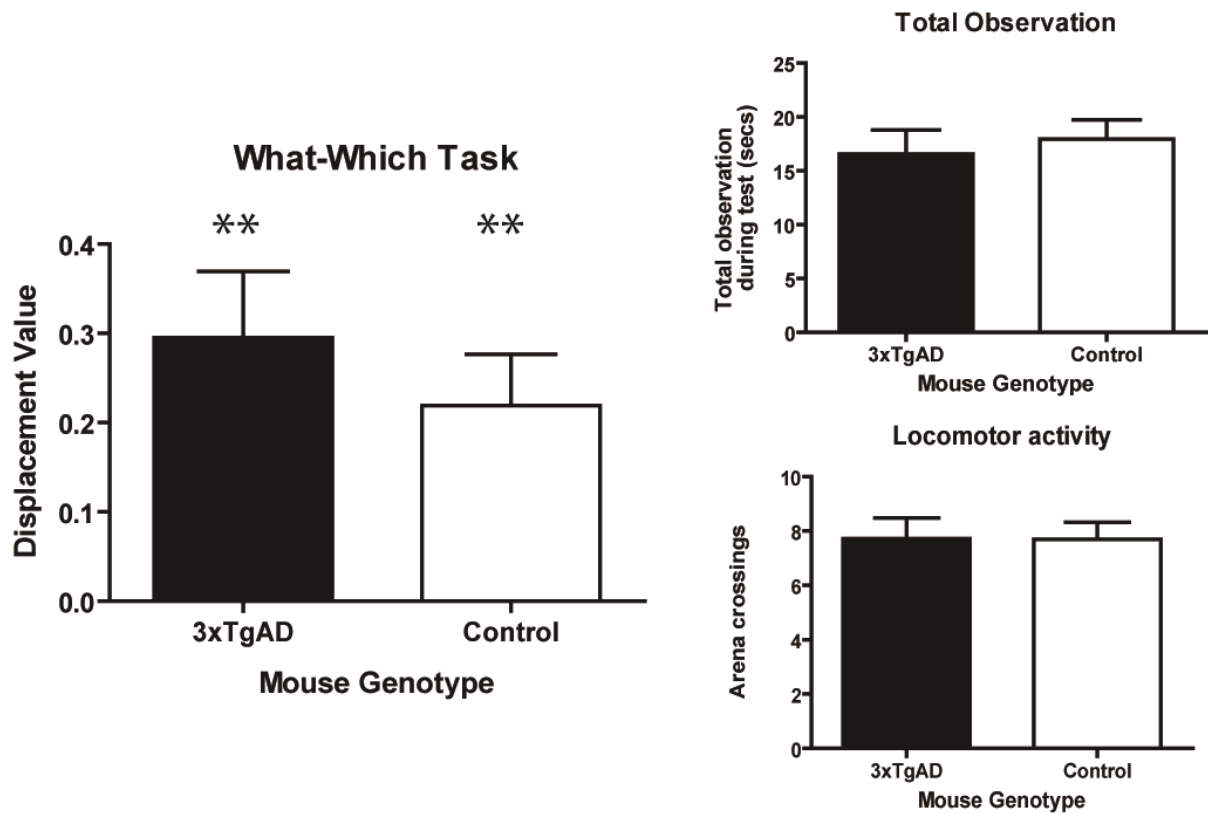
Total Observation

Locomotor activity

Figure 6: What-Which memory at a delay of 2 minutes. Left Panel: What-Which D2 values show intact object-context performance. Right panels: Exploratory measures. Asterisks denote the significantly better performance of mice versus chance at P<0.01 **.
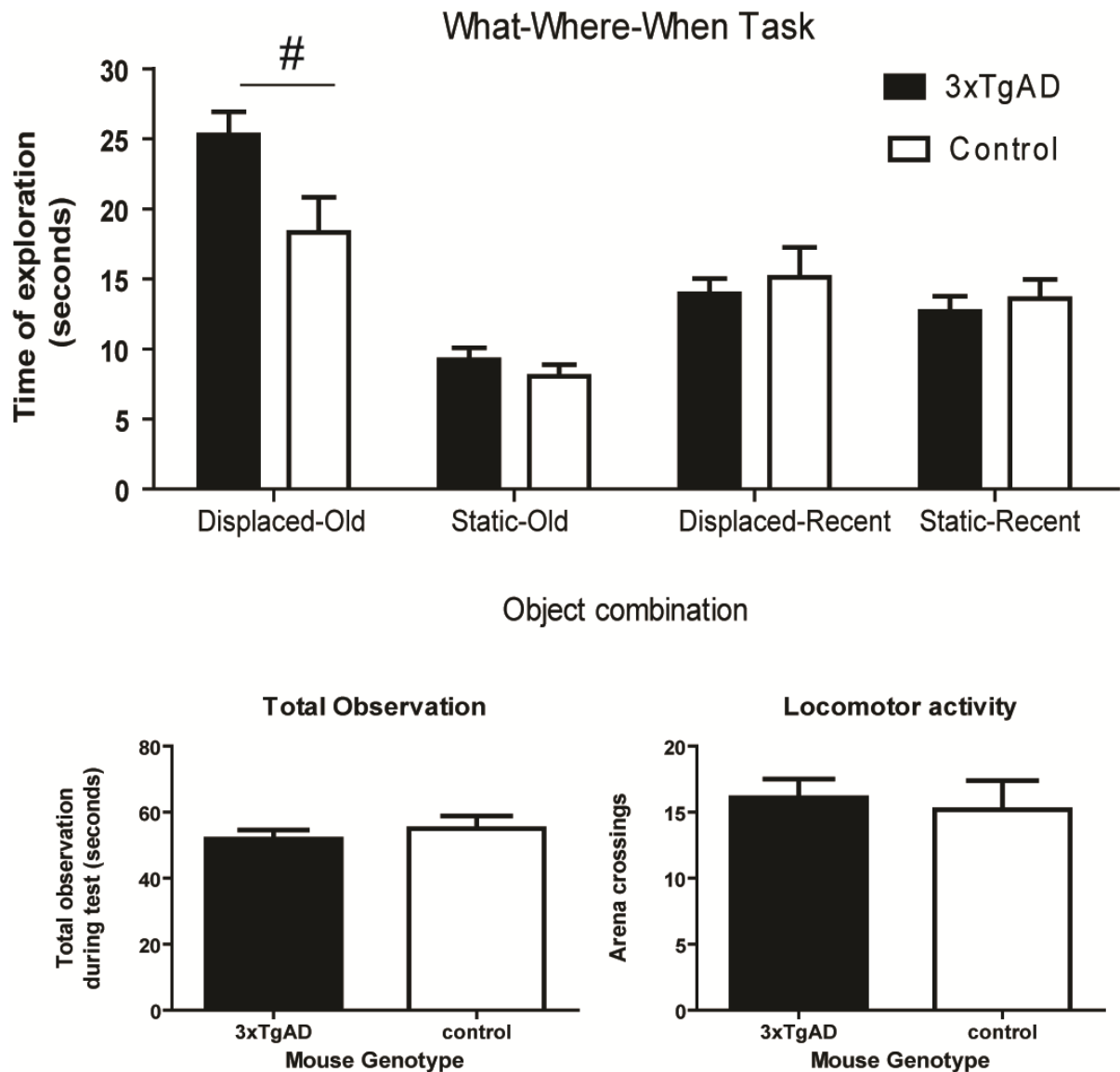
What-Where-When Task

Figure 7: What-Where-When episodic-like memory at a delay of 2 minutes. Top Panel: 3xTgAD and control mice display a significant preference for the Displaced-Old (episodic-like) object combination over other object combinations suggesting intact What-Where-When memory processing. Hash denotes genotype difference at $P<0.05$ #. See text for discussion of other post-hoc comparisons. Bottom right and left panels: Exploratory measures show no significant genotype differences.

Figure 8: APP/Aβ pathology in 3xTgAD female mouse. A: Staining in 5-month old female mice reveals intracellular Aβ within hippocampus and cortical structures (scale = 50 microns). B: Dorsal hippocampus in 3xTgAD mice of 5, 8, 11 and 15 months of age, corresponding to pre- and post-behavioural testing phases. Extracellular Aβ pathology is clear in subiculum of 15-month-old 3xTgAD mice. Control mice show no straining at any age.