Serial Reversal in 3xTg-AD Mice

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1 Methods

1.1 Subjects

Male and female 3xTg-AD (B6;129-Tg(APPSwe,tauP301L)1Lfa Psen1^{tm1Mpm}/Mmjax; JAX stock #004807) and B6129SF2/J mice (JAX stock #101045) were bred at Dalhousie University from parents purchased from the Jackson Laboratory in Bar Harbour, Maine. The 3×Tg-AD mice have three mutations, the Swedish (K670N/ M671L) mutation to amyloid precursor protein (APP), a mutation to presenilin-1 (PS1) (M146V), and a tau mutation (P301L) (Oddo et al., 2003). Pups were weaned at 21 days of age and housed in same sex groups of 2-4 in transparent polyethylene cages (35 × 12 × 12 cm) with ad libitum food (Purina Rodent Chow #5001) and tap water. Housing cages contained pine chip bedding and a polyvinyl chloride tube (5 cm diameter, 8 cm long) for enrichment. The housing room was on a 12:12 h reversed light/dark cycle with lights off at 0930h. Mice were genotyped using polymerase chain reaction by Dr. Chris Sinal (Department of Pharmacology, Dalhousie University) from ear punches taken at the time of weaning for individual identification.. All test procedures were approved by the Dalhousie Committee on Animal Care (Protocol # 13-044).

Due to the small number of male mice, sexes were pooled together for analyses (Table 1). There were 33 mice included in this study. The 3xTg-AD mice (283 ± 118 days) were significantly younger than the B6129 mice (409 ± 197 days; t_{28} = -2.3, p = 0.0304).

1.2 Apparatus

Two liquid dilution olfactometers (Knosys Olfactometers Inc.) previously described (Roddick et al., 2014; Slotnick & Restrepo, 2005) were used. Air was sent through a charcoal filter after which it was split into two pathways, one with clean air, and the other through a manifold which controlled the air flow through saturation bottles and into a T-junction, where clean and odorized air flows were mixed. A final valve diverted the air flow to the exhaust, or to

the odour sampling port which was open to the animal chamber. The odour sampling port contained an infrared beam to detect nose-pokes, a reinforcement tube delivering the water reward, and a sensor that detected when the mice were licking the reinforcement tube.

1.3 Odours

All odorants (Table 2) were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI) and diluted in heavy mineral oil. For each mouse, the rewarded (S+) and unrewarded (S-) odours were randomly assigned during each discrimination.

1.4 Water restriction

Ten days prior to the start of testing, mice were individually housed and placed on water restriction. Mice were weighed daily and given measured amounts of mash (powdered food pellets mixed with water) to maintain their weight at approximately 85% of free feeding weight. They had *ad lib* access to food during water restriction.

1.5 Behavioural testing

All behavioural testing was done during the dark phase of the light/dark cycle. Behavioural testing was done in four phases: response training, odour discrimination training, odour discrimination and reversal, and a retest.

1.5.1 .

In response training, the mice were initially trained for 20 trials to lick the reinforcement tube and received a water reward for simply licking the tube. The inter-trial interval increased from 0.1 s to 12 s over the 20 trials. During the next stage of training, a rewarded stimulus (S+) odour was introduced and the mice were required to keep their head in the odour sampling port while the final valve diverted the odour into the port. The length of time the mice were required to keep their head in the odour sampling port increased from 0.1 s to 1.1 s over 120 trials. This stage of training was completed when the mice performed 20 trials with the final valve on for 1.1 s.

1.5.2 .

Odour discrimination training involved introducing the unrewarded (S-) odour. During this stage of training the mice were presented with a stimulus odour, either a rewarded odour (S+), or an unrewarded odour (S-), when they inserted their head into the odour sampling port. When the mice were presented with the S+, they received a water reward (XX μl) for licking the reinforcement tube. Trials were initiated by the mice poking their nose into the odour sampling port, with a minimum inter-trial interval of 4 s. They were first presented with 20 trials of the S+ odour. If they did not respond to at least 85% of these S+ presentations they were placed back on the initial training. They were then

presented with blocks of 20 trials consisting of 10 S+ and 10 S- trials. This continued until the mice achieved 85% correct responses on a block.

1.5.3 .

Mice were then moved to the discrimination and reversal learning stage, which consisted of a two odour discrimination problem using odour pair 1. They were given blocks of 20 trials, 10 S+ and 10 S-, until they achieved 85% correct. They were then presented with a reversal problem using odour pair 1 in which the S+ odour became the S- odour and vice-versa. They were given blocks of 20 trials (10 S+ and 10 S-) until they achieved 85% correct. This pattern of presenting a discrimination task followed by a reversal task was repeated with each of the 18 odour pairs.

1.5.4 .

One, two, or three months after finishing the reversal of odour pair 18, mice were retested on that reversal. The mice were given blocks of 20 trials (10 S+ and 10 S-) until they achieved 85% correct.

1.6 Statistical analysis

Data were analized using R version 4.2.2 (R Core Team, 2022), using the "tidyverse" (Wickham et al., 2019) and "rstatix" (Kassambara, 2022) packages. The number of errors made prior to reaching criterion was used as the measure of learning. Sue to the small number of male mice included, and the lack of a significant sex effect and small effect size in the number of errors made on training ($F_{(1,28)} = 0.12$, $p = 0.73$, $\eta^2_G = 0.004$), sexes were pooled together for analyses. Due to the significant difference in the ages of the B6129 and 3xTg-AD mice, ANCOVAs with age as a covariate were used to assess genotype differences. Greenhouse-Geisser corrections were applied when Mauchly's test for sphericity detected that within-subjects factors violated sphericity. Pearson's χ^2 tests with Yate's continuity corrections were run on the number of mice showing errorless learning, defined as making only zero or one errors on a discrimination task or reversal. To assess the effects of age, Pearson's correlations were run.

2 Results

2.1 Odour discrimination training

An ANCOVA with age as a covariate was used to compare the number of errors made during the initial odour discrimination training. The B6129 mice (58 ± 42) made more errors than the 3xTg-AD mice (24 ± 6.8; $F_{(1,30)} = 9.8$, $p = 0.004$, $\eta^2_G = 0.25$, Figure 1).

2.2 Discrimination trials

The sequence of odour pairs (Figure 2) was divided into thirds, and ANCOVAs, with age as the covariate, were used to analyze the number of errors on the odour discrimination trials in each third. Greenhouse-Geisser corrections were applied when Mauchly's test for sphericity detected that within-subjects factors violated sphericity.

There were no significant effects of genotype, odour pair, nor interactions between genotype and odour pair, in the first (1 - 6), second (7 - 12) or last thirds (13 - 18) of trials (p's >= 0.058; Figure 3).

2.2.1 Near errorless learning

There were 35 times where mice made a single error on the initial discrimination of the odour pair, and 3 times where a mouse made zero errors (Figure 4), with 21 different mice learning at least one discrimination with just one or zero errors. One mouse, a B6129, had one or fewer errors on the initial discrimination of 6 odour pairs.

Pearson's χ^2 tests were run on the number of mice showing errorless learning. The χ^2 on the effect of odour pair was significant ($\chi^2_{17} = 43$, $p < 0.001$), with the majority of errorless discriminations occurring on odour pairs 8 - 18 as the number of errorless odour pairs increased from 4 to 12 to 22 in each third of the odour pairs. The χ^2 on the effect of genotype on errorless learning was not significant ($\chi^2_1 = 3.4$, $p = 0.064$).

2.2.2 Age effects

Pearson's correlations were used to examine the relationship between the age of the mice and the number of errors made on discrimination learning across all odour pairs for each genotype (Figure 5). The correlations were not significant for either the B6129 ($r = 0.042$, $p = 0.87$), nor the 3xTg-AD ($r = -0.037$, $p = 0.895$) mice, thus there was no significant change in errors with age.

2.3 Reversal

The sequence of reversal learning odour pairs (Figure 6) was divided into thirds, and ANCOVAs, with age as the covariate, were used to analyze the number of errors on the reversal trials. Greenhouse-Geisser corrections were applied when Mauchly's test for sphericity detected that within-subjects factors violated sphericity.

In the first third (1 - 6) of odour pairs there was a significant effect of genotype ($F_{(1,23)} = 8.2$, $p = 0.009$, $\eta^2_G = 0.057$), with the B6129 mice (60 ± 67) making more errors than the 3xTg-AD mice (36 ± 41). but no significant effect of odour pair ($F_{(2.6,59)} = 1.3$, $p = 0.27$, $\eta^2_G = 0.046$), nor an interaction ($F_{(2.6,59)} = 1.1$, $p = 0.34$, $\eta^2_G = 0.039$).

There were no significant effects of genotype or odour pair in the second third (7 - 12) of odour pairs (p's >= 0.007).

In the last third (13 - 18) of odour pairs there was a significant effect of age ($F_{(1,25)} = 8.4$, $p = 0.008$, $\eta^2_G = 0.061$) and an interaction between age and odour pair ($F_{(1.4,36)} = 6.7$, $p = 0.007$, $\eta^2_G = 0.18$), but no main effects of genotype ($F_{(1,25)}$

= 0.8, p = 0.38, η^2_G = 0.006), odour pair ($F_{(1.4,36)}$ = 2.8, p = 0.09, η^2_G = 0.082), nor an interaction between genotype and odour pair ($F_{(1.4,36)}$ = 0.13, p = 0.81, η^2_G = 0.004). The significant effects involving age indicate that age does not significantly adjust the effects of genotype and odour pair.

2.3.1 Near errorless learning

There were 30 instances of mice making a single error on the reversal of the odour pair, and 8 instances of a mouse making zero errors (Figure 7), with 18 mice learning at least one reversal with just one or zero errors. One mouse, a 3xTg-AD, had one or fewer errors on the reversal learninf of 5 odour pairs.

Pearson's χ^2 tests with Yate's continuity corrections were run on the number of mice showing errorsless learning. The χ^2 on the effect of odour pair was significant (χ^2_{17} = 49, p < 0.0001), with the majority of errorless discriminations occuring on odour pairs 8 - 18 as the number of errorless odour pairs increased from 0 to 19 and 19 in each third of the odour pairs. The χ^2 on the effect of genotype was significant (χ^2_1 = 3.9, p = 0.0479), with more 3xTg-AD mice having errorless learning than the B6129 mice.

2.3.2 Age effects

Pearson's correlations were used to examine the relationship between the age of the mice and the number of errors made across all reversal learning for each genotype. The correlations were not significant for either the B6129 (r = 0.11, p = 0.66), nor the 3xTg-AD (r = -0.11, p = 0.685) mice (Figure 8).

2.4 Difference scores

The sequence of 18 odour pairs was divided into thirds, and ANCOVAs, with age as the covariate, were conducted to examine the differences between the errors on the discrimination and reversal trials on odour pairs in each third (Figure 2). Greenhouse-Geisser corrections were applied when Mauchly's test for sphericity detected that within-subjects factors violated sphericity.

In the first third of odour pairs (1 - 6), there were significant effects of genotype ($F_{(1,22)}$ = 15, p < 0.001, η^2_G = 0.094), with the B6129 mice (45 ± 64) having greater differences scores than the 3xTg-AD mice (20 ± 41). There was no significant effect of odour pair ($F_{(2.3,50)}$ = 1.4, p = 0.25, η^2_G = 0.051), nor a significant interaction ($F_{(2.3,50)}$ = 0.68, p = 0.53, η^2_G = 0.025).

On the second third of odour pairs (7 - 12) there were no significant genotype or odour pair effects (p's >= 0.12).

On the last third of odour pairs (13 - 18) there was a significant effect of age (($F_{(1,25)}$ = 11, p = 0.003, η^2_G = 0.067) and a significant interaction between age and odour pair ($F_{(2.2,55)}$ = 5.7, p = 0.004, η^2_G = 0.16), but no significant effects of genotype ($F_{(1,25)}$ = 3.1, p = 0.09, η^2_G = 0.02), odour pair ($F_{(2.2,55)}$ = 2.5, p = 0.09, η^2_G = 0.076), nor an interaction

between genotype and odour pair ($F_{(2.2,55)} = 0.34$, $p = 0.73$, $\eta^2_G = 0.011$). The significant effects involving age indicate that age does not significantly adjust the effects of genotype and odour pair.

2.5 Total errors made on all discriminations and reversals

An ANCOVA, with age as the covariate, was used to assess the total errors made per mouse during the discrimination and reversal trials. There was a significant effect of genotype ($F_{(1,30)} = 6.5$, $p = 0.016$, $\eta^2_G = 0.18$; Figure 9), as the B6129 (652 ± 202) mice made more errors than the 3xTg-AD mice (464 ± 167).

2.5.1 Age effects

Pearson correlations were used to examine the relationship between the age of the mice and the total number of errors made per mouse for each genotype (Figure 10). The correlations were not significant for either the B6129 ($r = 0.1$, $p = 0.692$), nor the 3xTg-AD ($r = -0.098$, $p = 0.727$) mice.

2.6 Retest

Because some mice died, only 15 B6129 and 9 3xTg-AD mice were given the retest. Because 3xTg-AD mice have a shorter lifespan than B6129 mice (Rae & Brown, 2015), fewer 3xTg-AD mice (9 / 15) survived to be given the retest than the B6129 mice (15 / 18).

The retest occurred between 30 and 94 days after the final reversal. There was no difference in the number of days between the last reversal and the retest for the B6129 (55 ± 24) and 3xTg-AD mice (75 ± 23; $t_{18} = 2$, $p = 0.062$).

An ANCOVA, with age as the covariate, was used to assess the number of errors made during the retest. There was no significant effect of genotype ($F_{(1,21)} = 1.3$, $p = 0.26$, $\eta^2_G = 0.06$; Figure 11)

A paired t-test was used to compare the number of errors made during the retest to the numbers of errors made on the final odour discrimination. The mean number of errors made during the retest (3xTg-AD: 47 ± 30; B6129: 36 ± 19) was significantly higher than the errors made on the final odour discrimination (3xTg-AD: 10 ± 12; B6129: 4.6 ± NA) for both genotypes (3xTg-AD: $t_8 = 4.8$, $p = 0.00143$; B6129: $t_{14} = 6.9$, $p < 0.0001$).

2.6.1 Age effects

Pearson correlations were used to examine the relationship between the age of the mice and the number of errors made on the retest for each genotype (Figure 12). The correlation was not significant for the B6129 mice ($r = 0.34$, $p = 0.214$), but was for the 3xTg-AD ($r = -0.67$, $p = 0.05$) mice.

2.6.2 Time from last test effect

Pearson correlations were used to compare the number of errors made on the retest to the days since the final reversal. There was a significant, positive correlation for the B6129 mice ($r = 0.62$, $p = 0.013$), but not for the 3xTg-AD mice ($r = 0.4$, $p = 0.286$; Figure 13).

An ANCOVA, with age as a covariate, was run examining the effects of genotype and time since last reversal on the errors made on the retest. The time since last reversal was binned into 30, 60, and 90 day groups. There were no significant genotype or time until retest effects (p's >= 0.14).

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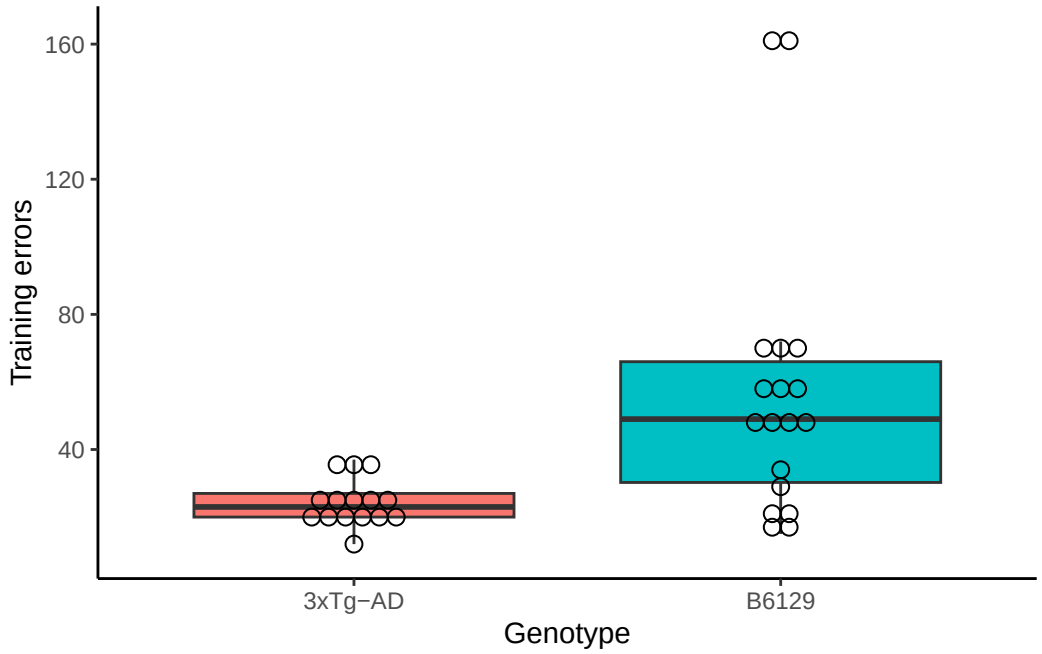

Figure 1: Errors made during the odour discrimination training. The boxes represent the inter-quartile range (IQR) with the bars in the middle showing the medians, the boxes showing the 25th and 75th percentiles and the whiskers extending to the furthest points within 1.5 interquartile ranges

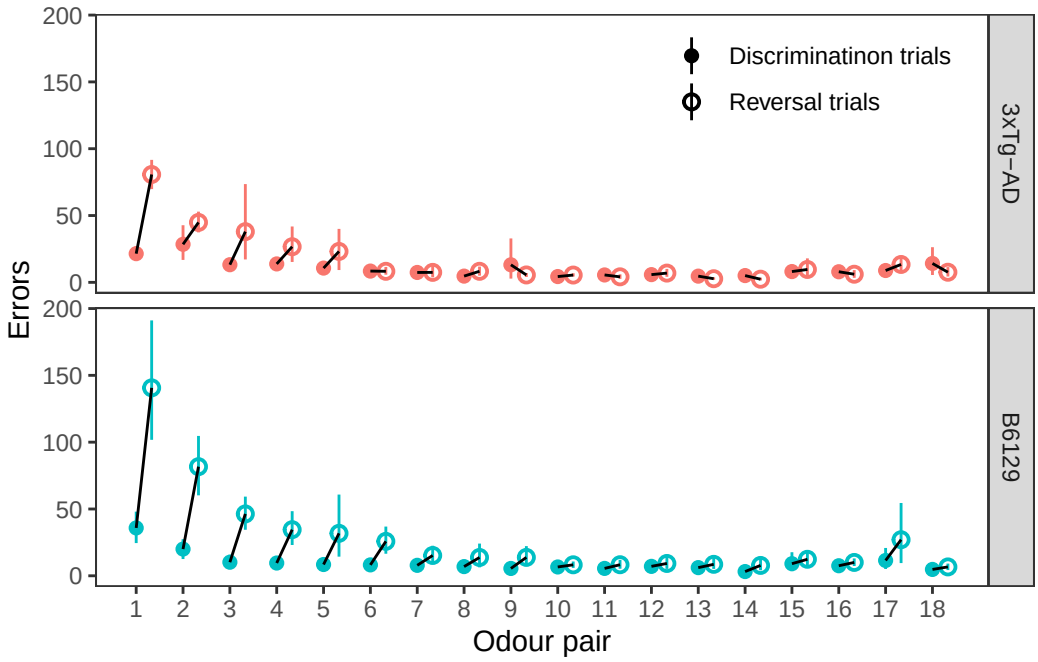

Figure 2: Errors (±95% CI) made by the mice on the discrimination and reversals of each odour pair. The difference scores (reversal errors - discriminatino errors) are indicated by the lines on each pair.

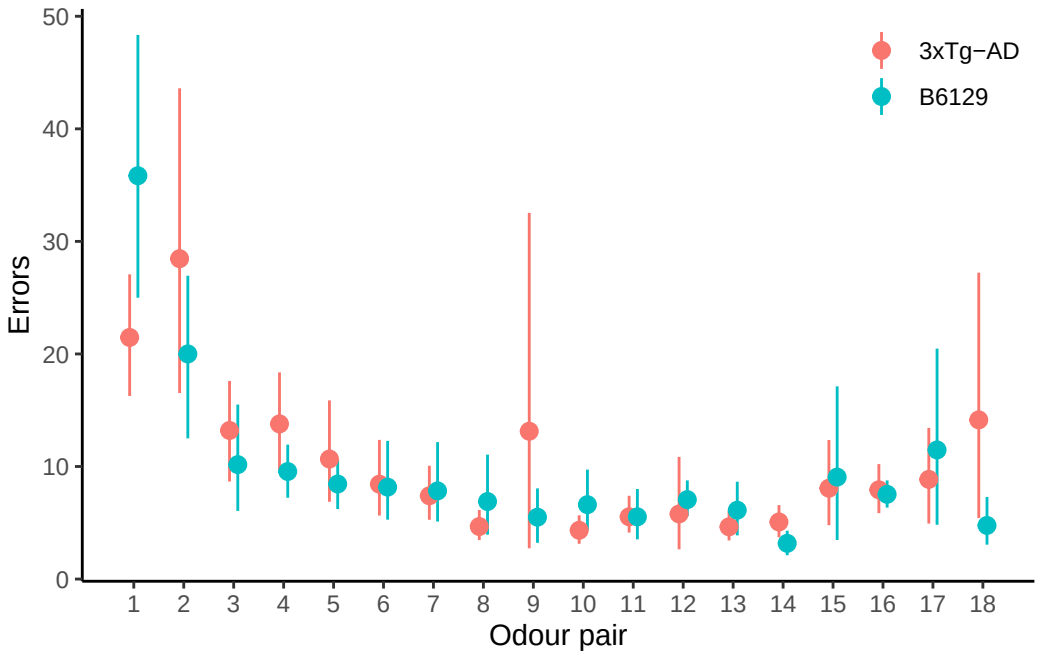

Figure 3: Errors (±95% CI) made by the mice at on each odour pair during the initial discrimination learning of each odour pair.

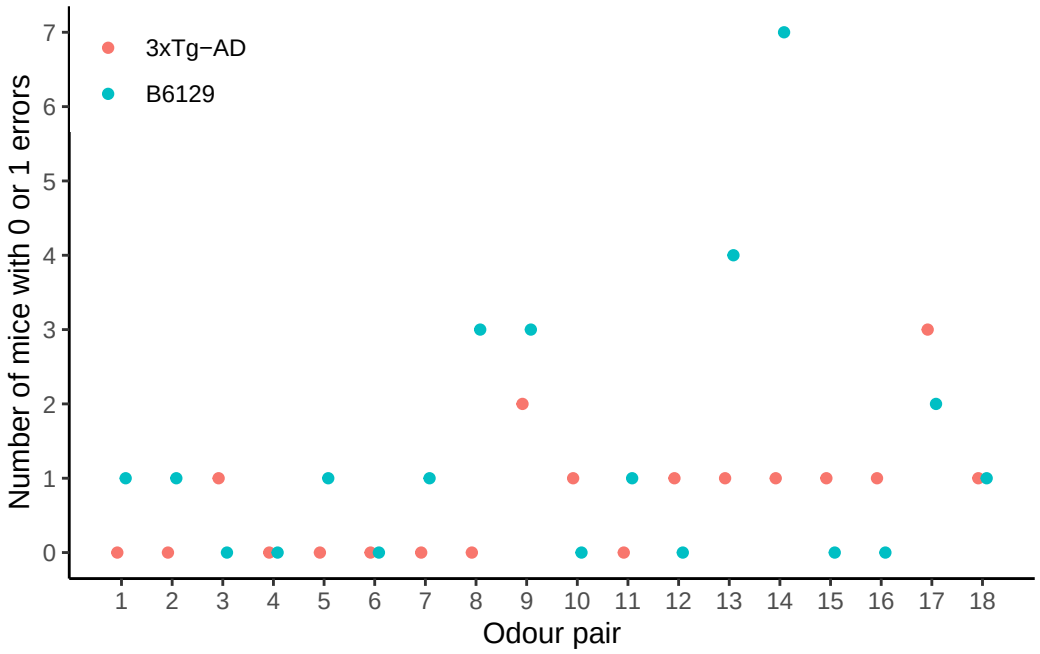

Figure 4: Number of 3xTg-AD and B6129 mice showing errorless learning on each odour pair during discrimination learning.

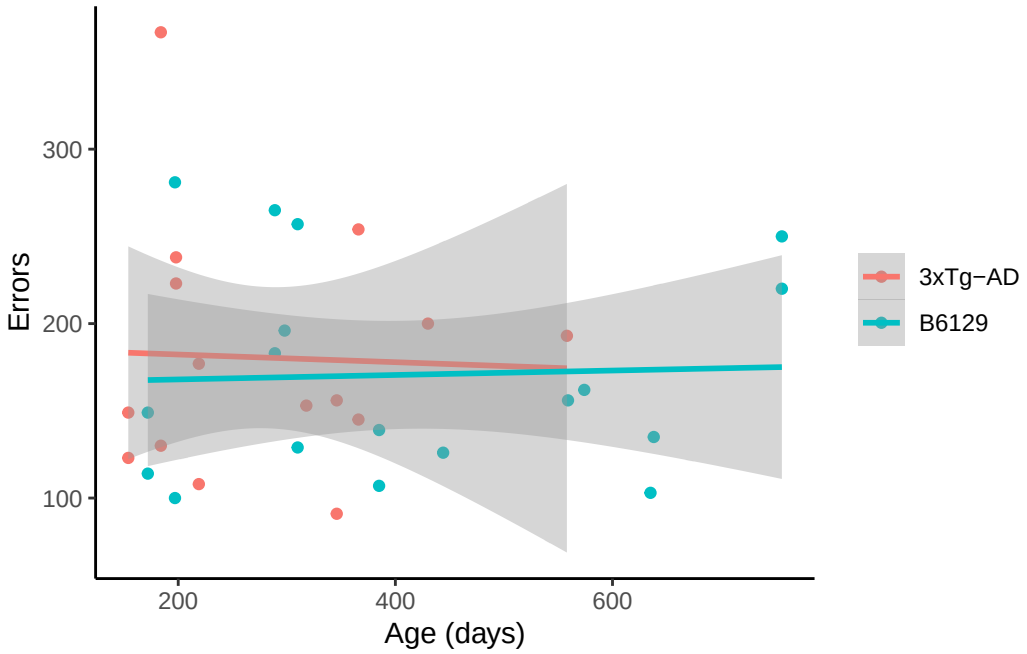

Figure 5: Correlation between mouse age and number of errors made across all 18 discrimination learning tests.

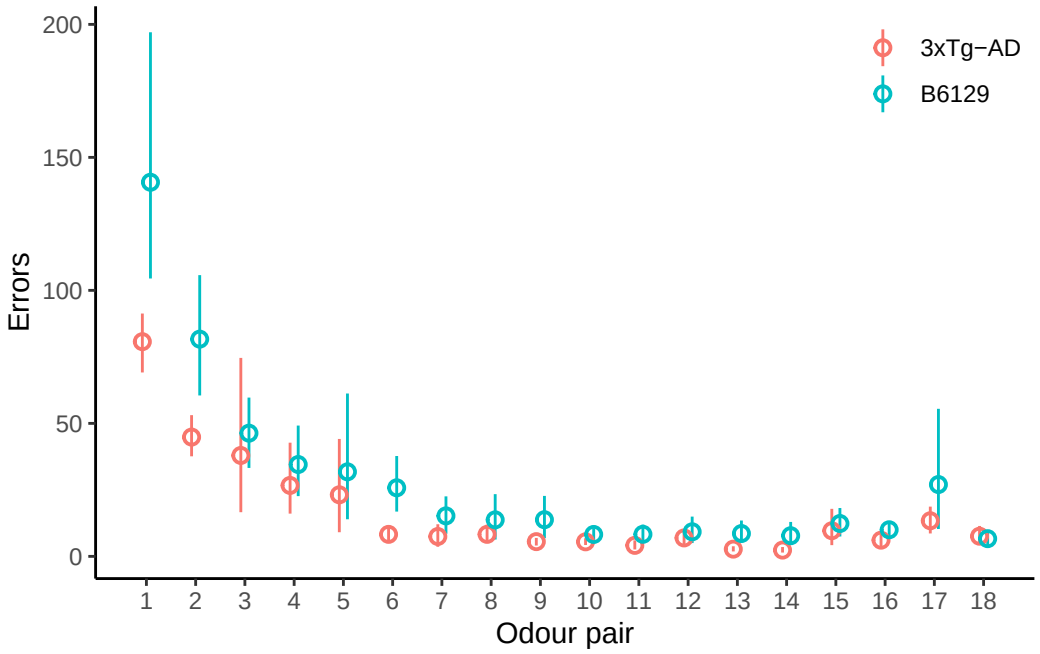

Figure 6: Errors (±95% CI) made by the mice at on each odour pair during reversal learning of each odour pair.

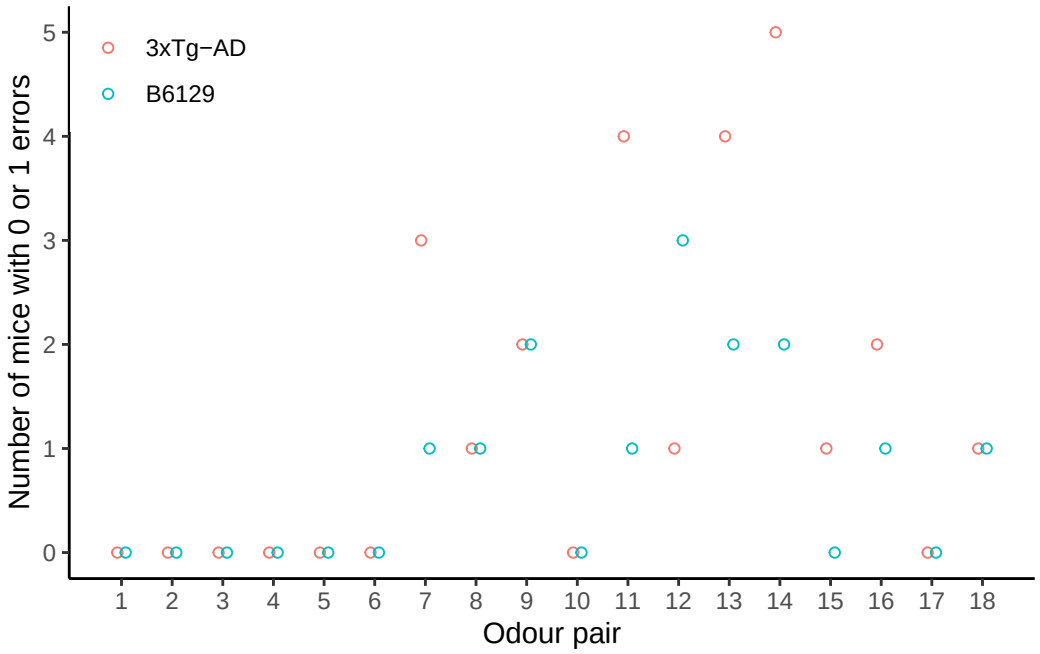

Figure 7: Number of 3xTg-AD and B6129 mice showing errorless learning on each odour pair during reversal learning

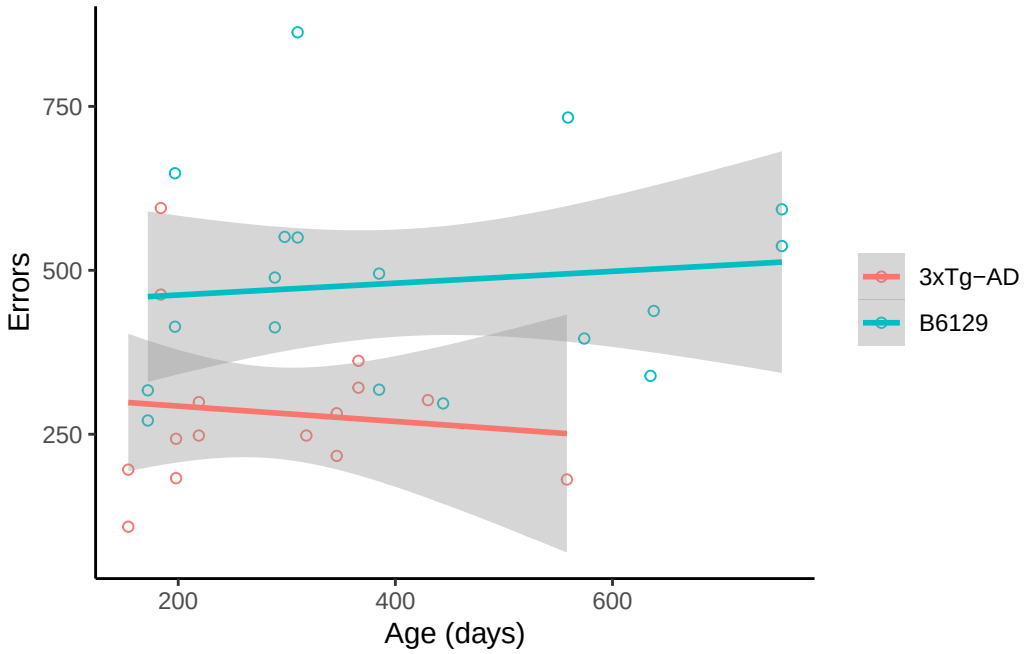

Figure 8: Correlation between mouse age and number of errors made across all 18 reversal learning tests.

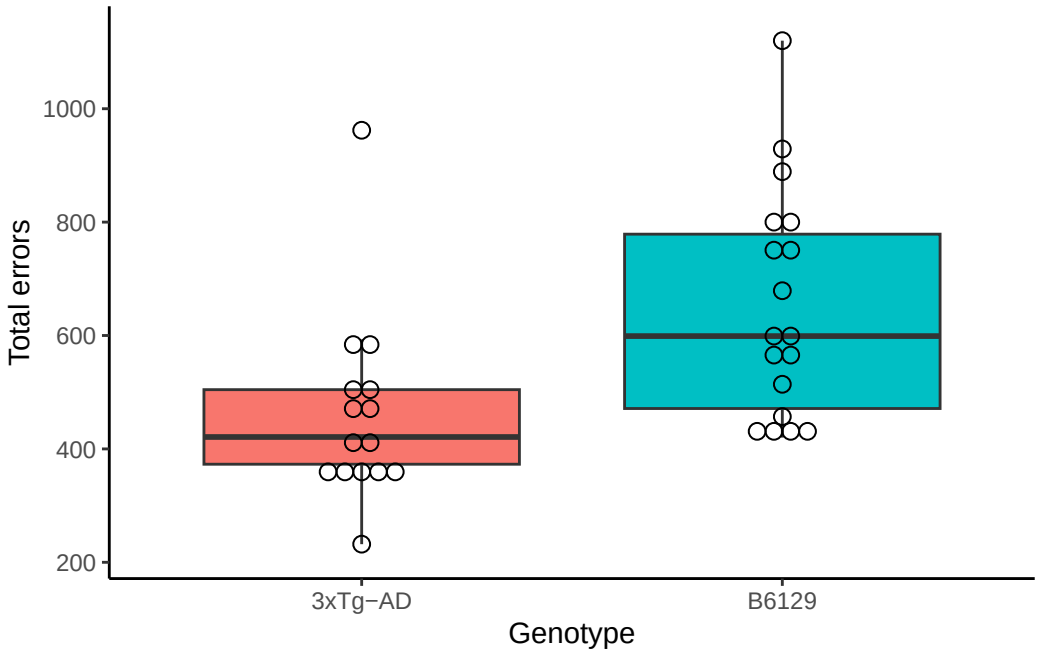

Figure 9: Total errors made by each mouse during the 18 odour discrimination and reversal learning tests.

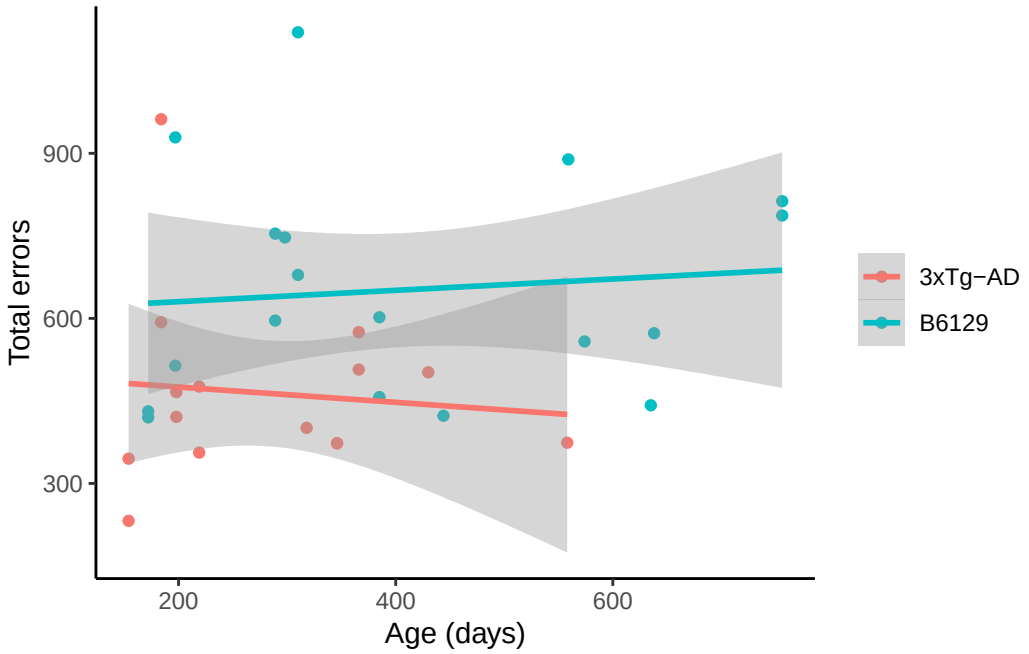

Figure 10: Correlation between mouse age and total number of errors made across all 18 discrimination and reversal learning tests..

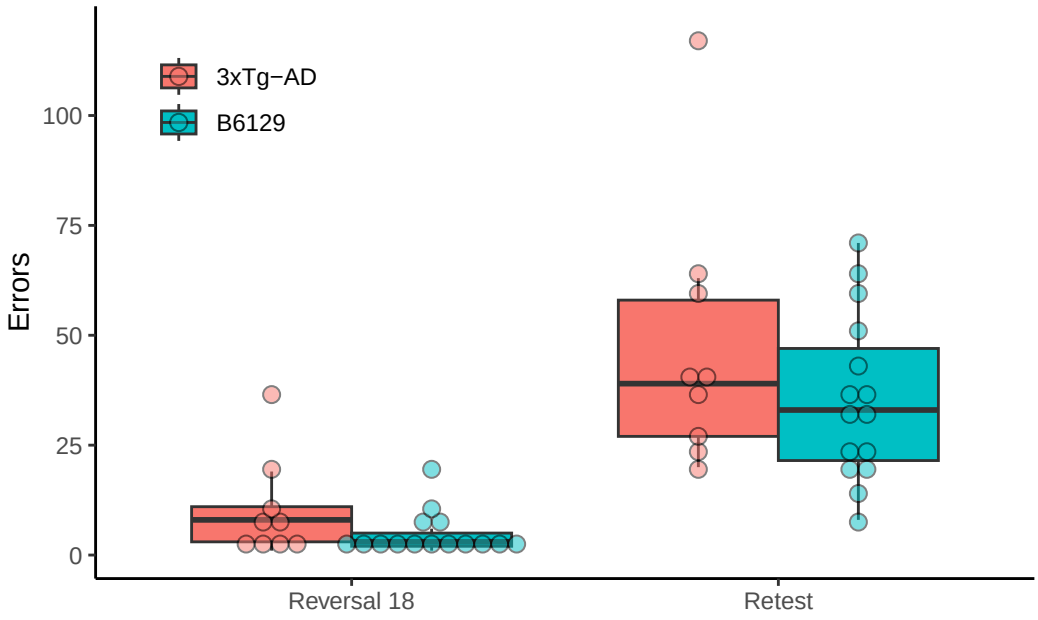

Figure 11: Total errors made during the reversal of odour pair 18 and the retest by the 3xTg-AD and B6129 mice.

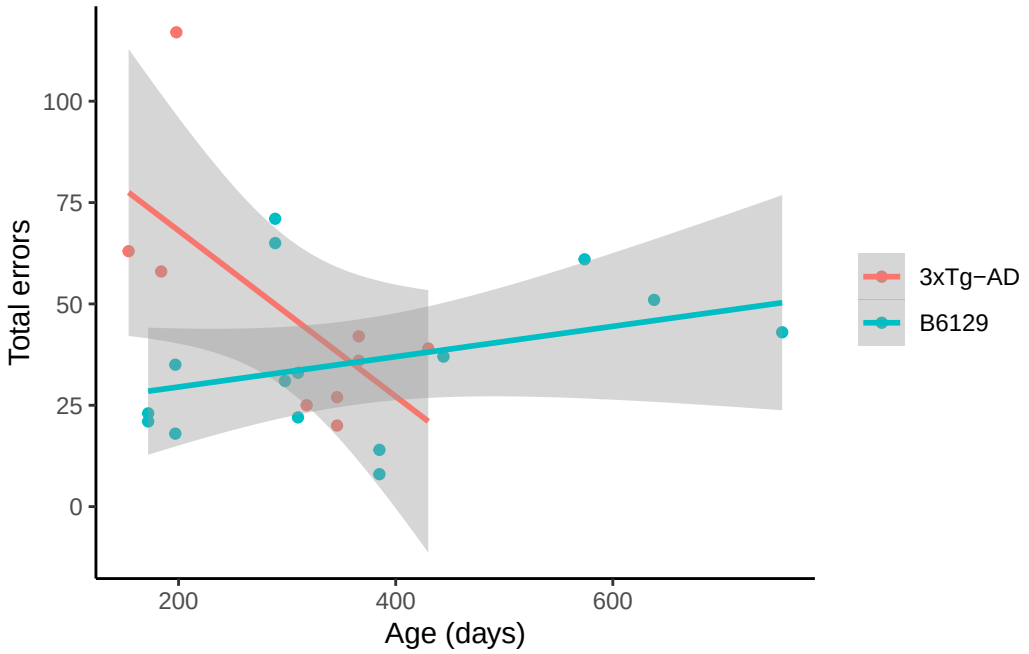

Figure 12: Correlation between age of the 3xTg-AD and B6129 mice and the number of errors made on the retest.

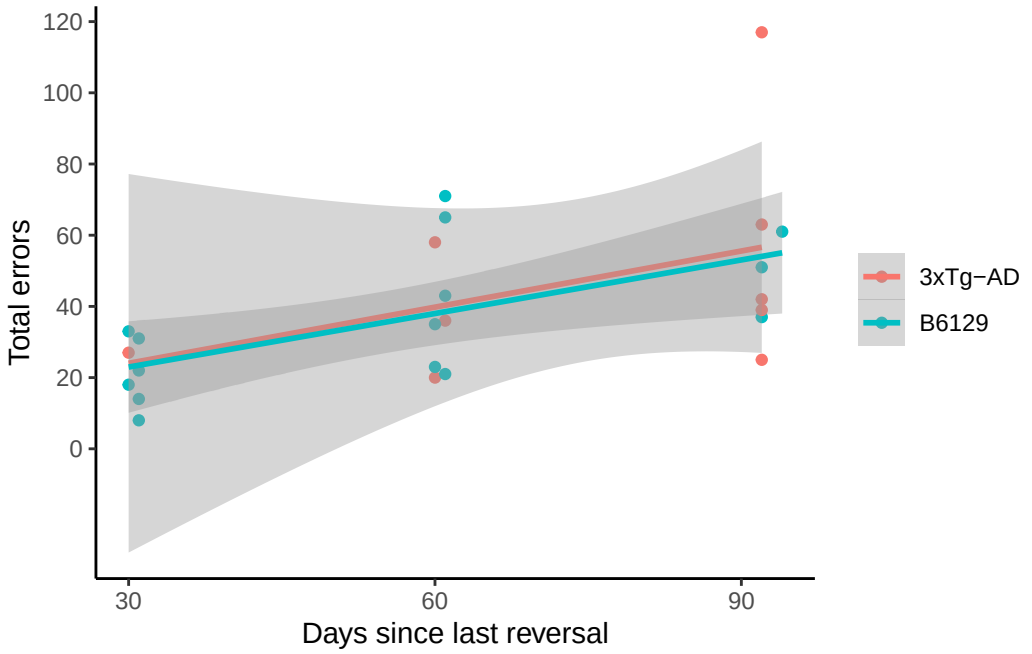

Figure 13: Correlation between days after the last reversal and ethe number of rrors made on the retest for the 3xTg-AD and B6129 mice.

Table 1: Demographics of mice tested.

Genotype	Sex	Mean age (days)	Age range	N
3xTg-AD	Female	276.71	154 - 558	14
3xTg-AD	Male	366.00	366 - 366	1
B6129	Female	403.00	172 - 756	13
B6129	Male	425.40	298 - 635	5

Table 2: Odorants used at each stage of testing.

Odour pair	Odour 1	Odour 2
Training	Orange	Lime
1	Lavender	Sage
2	Dillweed	Eucalyptus
3	Coriander	Fennel
4	Cardamom	Patchouli
5	Basil	Parsley
6	Bay	Nutmeg
7	Tarragon	Thyme
8	Clove	Ginger
9	Celery	Spearmint
10	Anise	Pimenta
11	Cassia	Cinnamon
12	Camphor	Rose
13	Litsea Cabeba	Origanum
14	Citronella	Mandarin
15	Acetophenone	Ethyl Acetate
16	Amyl Acetate	Butyl Acetate
17	Benzyl Acetate	Ethyl Acetoacetate
18	Isoamyl Propionate	Linalool