# Serial Reversal in 3xTg-AD Mice

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## 1 Methods

### 1.1 Subjects

Male and female 3xTg-AD mice (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 are pooled together in this analysis (Table 1). There are 33 mice included

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 |

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 was able to divert the air flow either 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 Sigma-Aldritch 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.

### 1.5.1 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.

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 |

#### 1.5.2 Odour discrimination training

Mice were then introduced to the unrewarded (S-) odour. During this stage of training the mice were presented with a stimulus odour when they inserted their head into the odour sampling port, either a rewarded stimulus (S+), or an unrewarded stimulus (S-). When the mice were presented with the S+, they received a water reward (XX $\mu$l) 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. If they did respond to at least 85% of the S+ presentations, 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 Discrimination and reversal learning

Mice were then moved to the testing 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 Retest

One, two, or three months after finishing the reversal of odour pair 18, mice were retested on that reversal.

### 1.6 Statistical analysis

R version 4.2.2 (R Core Team, 2022) was used for all analysis, 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. 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 $\chi^2$ 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

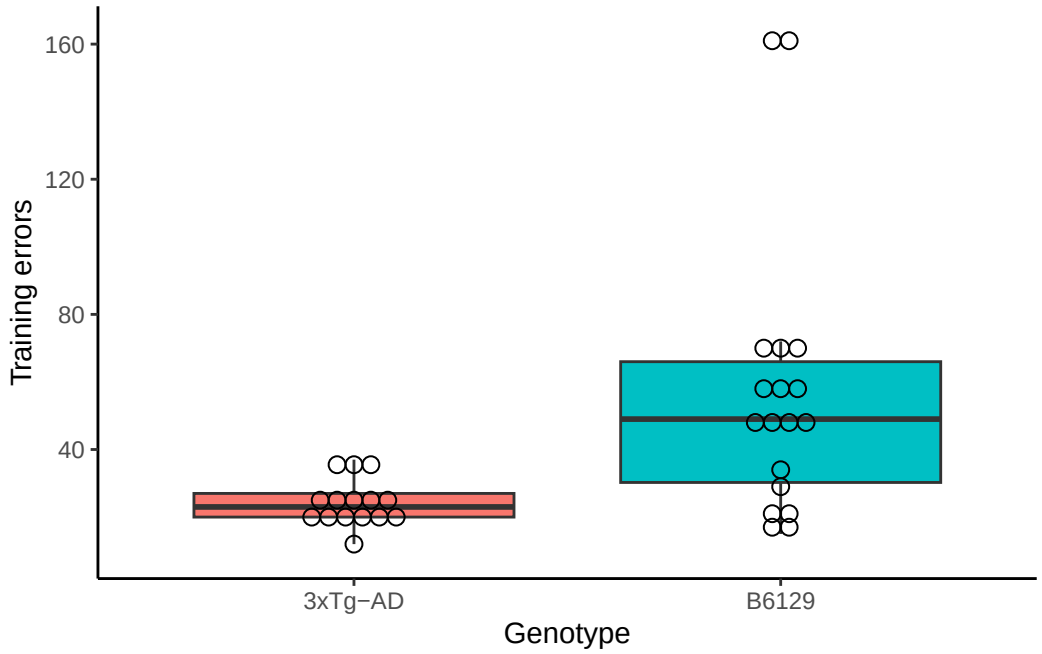


Figure 1: Errors made during training.

## 2.1 Training

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

## 2.2 Difference scores

The sequence of odour pairs was divided into thirds, and ANCOVAs, with age as the covariate, examining the differences between the errors on the discrimination trials and the reversal trials were run on each third. 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 ($F_{(1,22)} = 15$, $p < 0.001$, $\eta^2_G = 0.094$), with the B6129 mice (44.52885 ± 63.51423) having greater differences scores than the 3xTg-AD mice (19.54762 ± 40.96852). There was no significant effect of odour pair ($F_{(2.3,50)} = 1.4$, $p = 0.25$, $\eta^2_G = 0.051$; Figure 2), nor a significant interaction ($F_{(2.3,50)} = 0.68$, $p = 0.53$, $\eta^2_G = 0.025$).

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

On the last third of odour pairs (13 - 18) there was a significant interaction between age and odour pair ($F_{(2.2,55)} = 5.7$, $p = 0.004$, $\eta^2_G = 0.16$), but no significant effects of genotype ($F_{(1,25)} = 3.1$, $p = 0.09$, $\eta^2_G = 0.02$), odour pair ($F_{(2.2,55)} = 2.5$, $p = 0.09$, $\eta^2_G = 0.076$), nor an interaction between genotype and odour pair ($F_{(2.2,55)} = 0.34$, $p = 0.73$, $\eta^2_G = 0.011$).

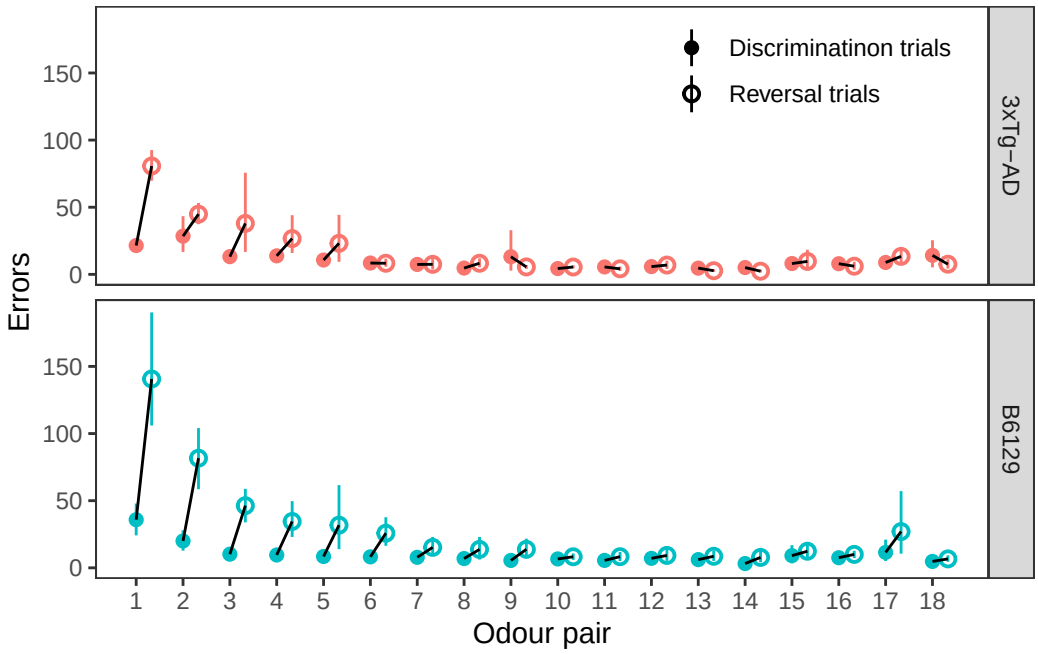


Figure 2: Errors (±95% CI) made by the mice at on each odour pair.

## 2.3 Discrimination

The sequence of odour pairs was divided into thirds, and ANCOVAs, with age as the covariate, examining the errors on the discrimination trials were run on each third (Figure 3). Greenhouse-Geisser corrections were applied when Mauchly's test for sphericity detected that within-subjects factors violated sphericity.

There were no significant effects in the first (1 - 6), second (7 - 12) or last thirds (13 - 18) of trials ($p$'s >= 0.058).

### 2.3.1 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 passing 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 $\chi^2$ tests with Yate's continuity corrections were run on the number of mice showing errorless learning. The $\chi^2$ on the effect of odour pair was significant ($\chi^2_{17} = 43$, $p < 0.001$), with the majority of errorless discriminations occuring 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 $\chi^2$ on the effect of genotype was not significant ($\chi^2_1 = 1.5$, $p = 0.214$).

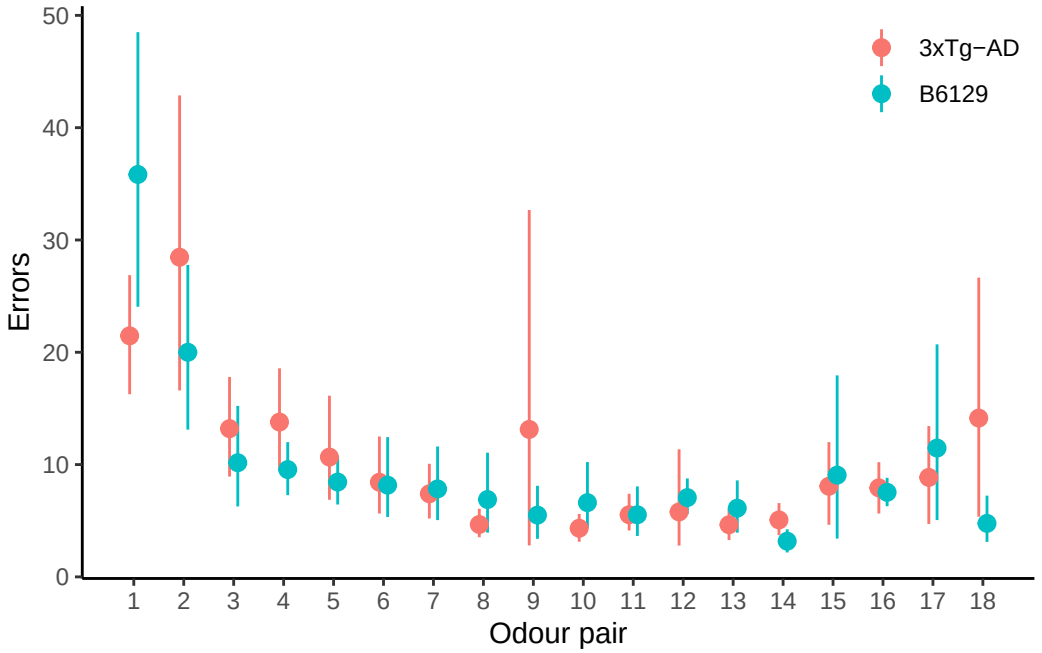


Figure 3: Errors (±95% CI) made by the mice at on each odour pair during the discrimination stages.

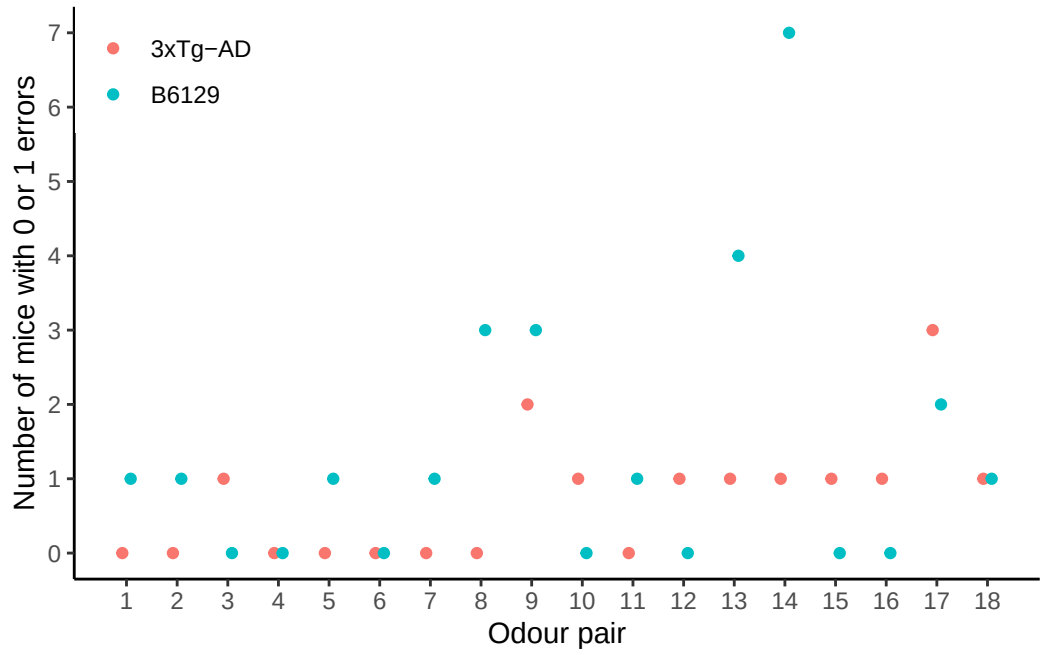


Figure 4: Number of mice showing errorless learning on each odour pair during discrimination.

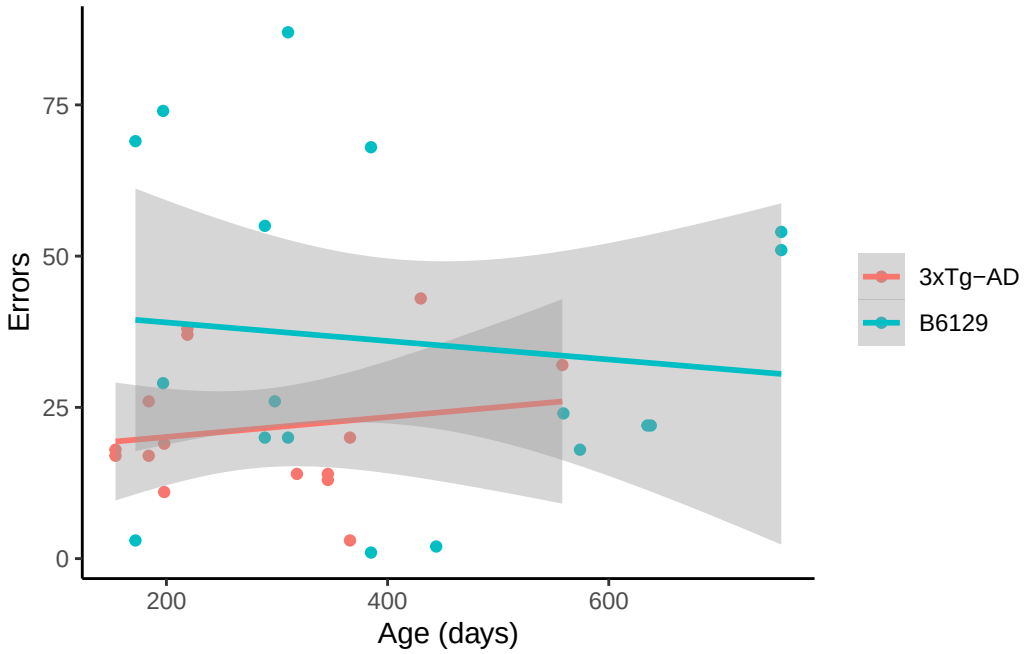


Figure 5: Correlation between age and errors made on odour pair one discrimination.

### 2.3.2 Age effects

Pearson's correlations were used to compare the number of errors made on odour pair one to the age of the mice for each genotype (Figure 5). The correlations were not significant for either the B6129 ($r$ = -0.11, $p$ = 0.655), nor the 3xTg-AD ($r$ = 0.17, $p$ = 0.543) mice, thus there was no significant change in errors with age.

## 2.4 Reversal

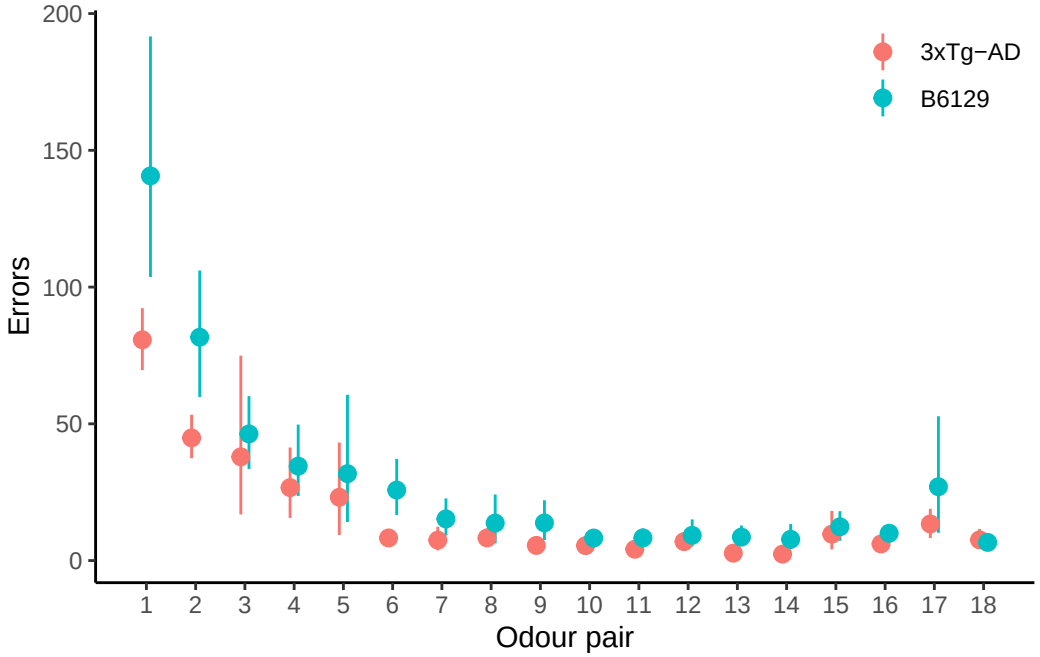


Figure 6: Errors (±95% CI) made by the mice at on each odour pair during the reversal stages.

The sequence of odour pairs was divided into thirds, and ANCOVAs, with age as the covariate, examining the errors on the reversal trials were run on each third (Figure 6). 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 were significant effects of genotype ($F_{(1,23)} = 8.2$, $p = 0.009$, $\eta^2_G = 0.057$), with the B6129 mice ($60 \pm 67$) making more errors than the 3xTg-AD mice ($36 \pm 41$). 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 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 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 ($F_{(1,25)} = 0.8$, $p = 0.38$, $\eta^2_G = 0.006$), odour pair ($F_{(1.4,36)} = 2.8$, $p = 0.09$, $\eta^2_G = 0.082$), nor an interaction between genotype and odour pair ($F_{(1.4,36)} = 0.13$, $p = 0.81$, $\eta^2_G = 0.004$).

### 2.4.1 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 passing at least one reversal with just one or zero errors. One mouse, a 3xTg-AD, had one or fewer errors on the reversal of 5 odour pairs.

Pearson's $\chi^2$ tests with Yate's continuity corrections were run on the number of mice showing errorsless learning. The

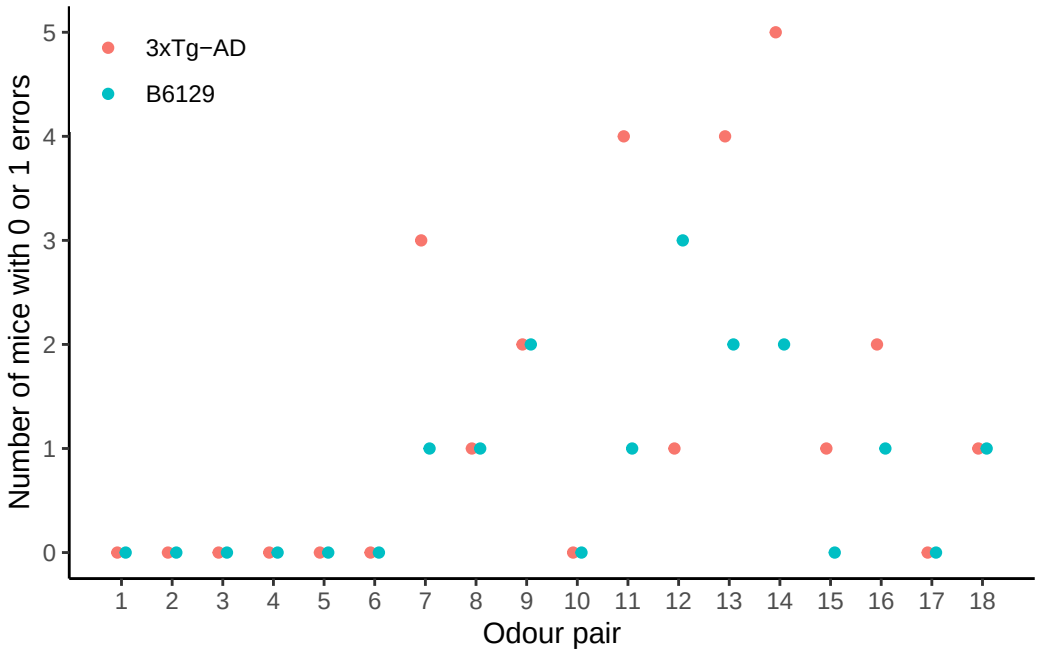


Figure 7: Number of mice showing errorless learning on each odour pair during reversal

$\chi^2$ on the effect of odour pair was significant ($\chi^2_{17} = 49$, $p < 0.0001$), with the majority of errorless discriminations occuring on odour pairs 8 - 18 as the number of errorless odour pairs increased from 0 to 19 to 19 in each third of the odour pairs. The $\chi^2$ on the effect of genotype was also significant ($\chi^2_1 = 4.6$, $p = 0.0323$), with the 3xTg-AD mice having more errorless reversals than the B6129 mice.

### 2.4.2 Age effects

Pearson's correlations were used to compare the number of errors made on odour pair one to the age of the mice for each genotype. The correlations were not significant for either the B6129 ($r = 0.23$, $p = 0.372$), nor the 3xTg-AD ($r = 0.12$, $p = 0.689$) mice (Figure 8).

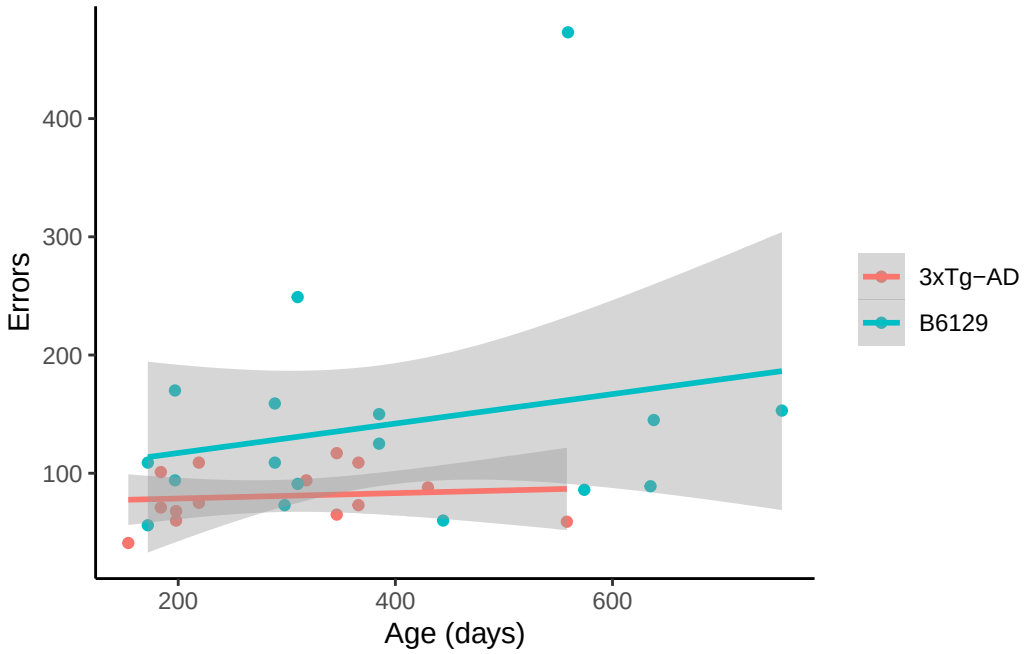


Figure 8: Correlation between age and errors made on odour pair one reversal.

## 2.5 Total errors

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).

### 2.5.1 Age effects

Pearson correlations were used to compare the total number of errors made per mouse to the age of the mice 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.

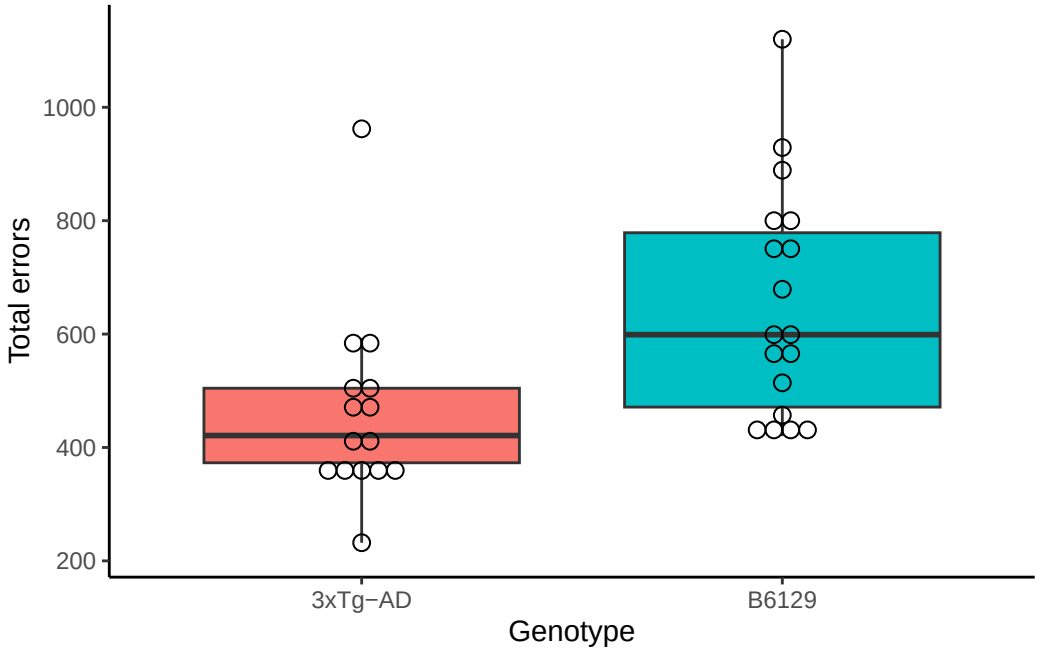


Figure 9: Total errors made, excluding retest.

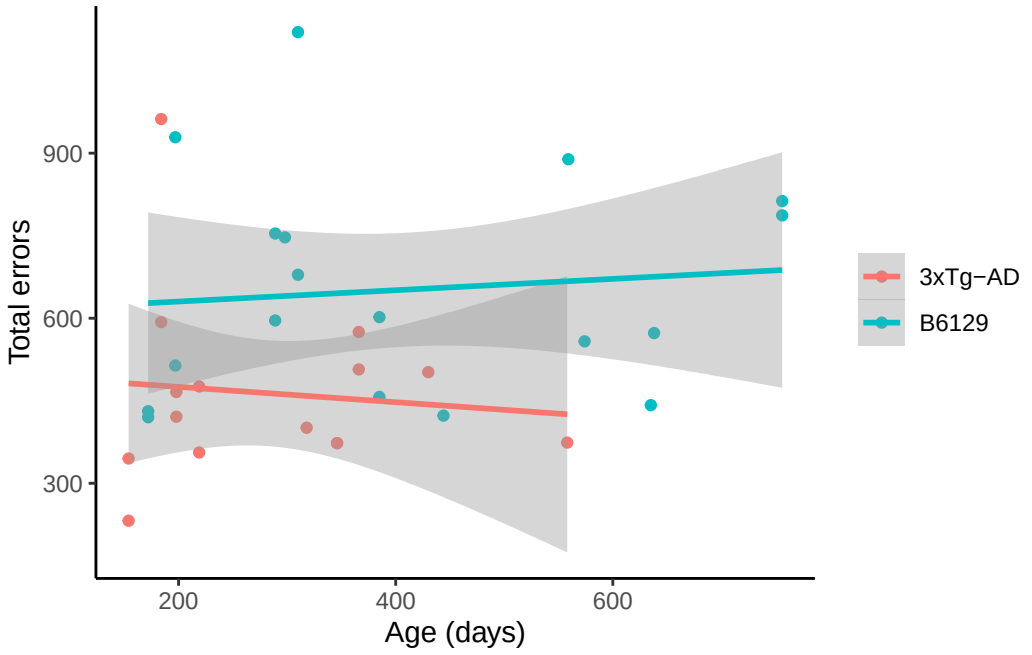


Figure 10: Correlation between age and total errors made.

## 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 between the days until the retest for the B6129 (55 ± 24) and 3xTg-AD (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 (40 ± 24) was significantly higher than the errors made on the final odour discrimination (6.8 ± 8.4; $t_{23} = 8.3$, $p < 0.0001$).

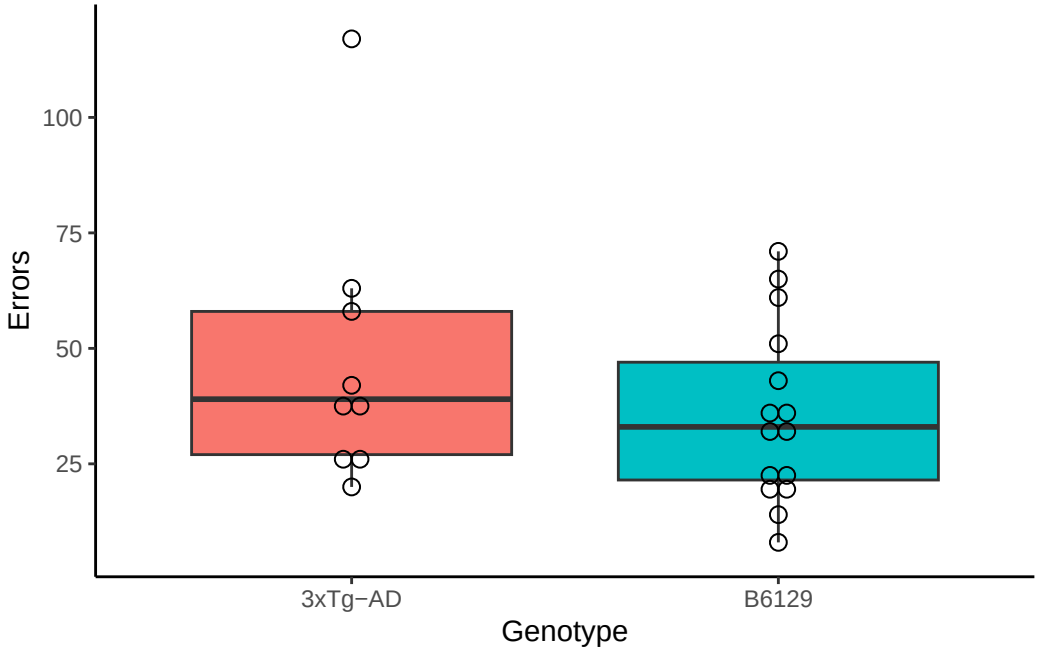


Figure 11: Total errors made during retest.

### 2.6.1 Age effects

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

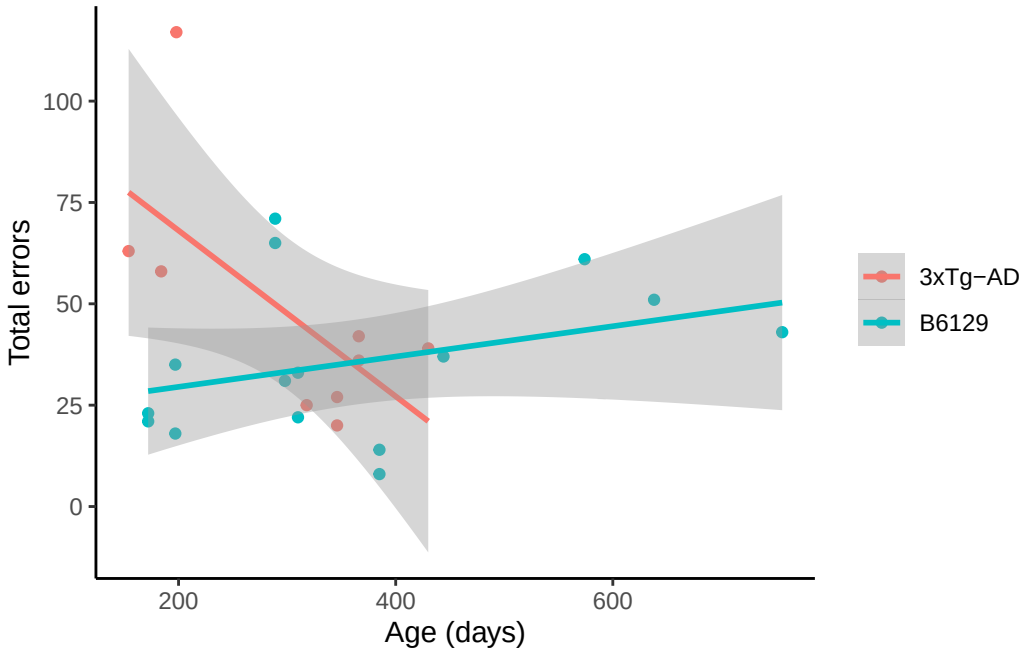


Figure 12: Correlation between age and errors made on the retest.

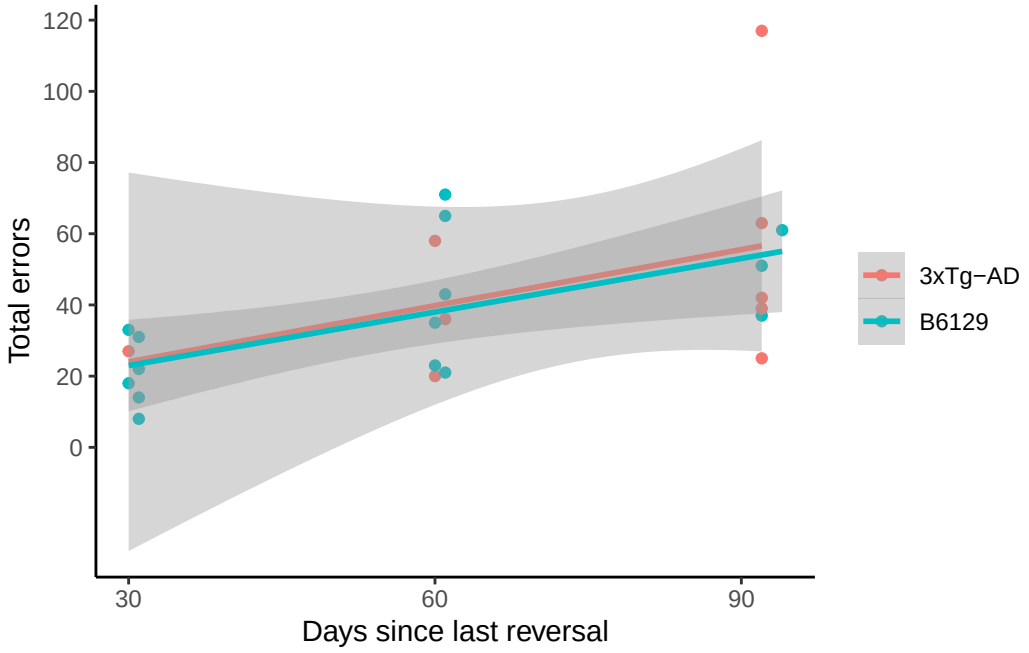


Figure 13: Correlation between days after last reversal and errors made on the retest.

### 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 effects ($p$'s >= 0.14).

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