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Timing impairments in early Alzheimer's Disease: Evidence from a mouse model

--Manuscript Draft--

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Dear Dr Burwell,

I am pleased to present the revised manuscript *Timing impairments in early Alzheimer's Disease: evidence from a mouse model* (BNE-2019-0981) for the consideration of the editors. In the revision we have done our best to address the final remaining comment which was raised by Reviewer 3, which is clearly highlighted (in red text) in the submission.

We hope the revision succeeds in satisfying the reviewers, and that the article is now acceptable for publication in Behavioural Neuroscience.

Yours sincerely,

Paul Armstrong (Dr)

Reviewer's comments

Reviewer #3:

The authors have adequately addressed by concerns, except for one that was perhaps not adequately explained in my first review.

More than one reviewer was confused by the use of the term "replication" in this manuscript.
I think the authors statement in response to comment 31 explains the problem: "we intended to replicate the finding rather than the experiment". The standard definition of a replication study is repeating the same experimental method on different subjects by the same or different experimenters. Therefore, experiment 2 is not a replication of experiment 1. The subjects are different, but so are the test parameters.
The language should be corrected throughout the MS to reflect that the results of experiments 1 and 2 show consistency. Transgenic mice showed an increase in timing variability (peak spread) in delay conditioning when the CS was 15s (exp1), and 20s (exp 2), but not 10s (exp 2). Perhaps the rationale for using CS lengths in exp2 that are shorter and longer than the CS used in exp 1 was to determine sensitivity of the genotypic differences to different CS delay lengths? i.e. how impaired the mice are. This was achieved, but hidden by the incorrect use of the term "replicate".

On rereading the manuscript we agree with the reviewer that the use of the term "replication" could be misleading to the audience. We have corrected this by no longer using the term "replication" and making it clear that the aim of Experiment 2 was to see if delay conditioning in general produced timing impairments and that the results of Experiments 1 and 2 are consistent with each other but are not replications (p.19).

Timing impairments in early Alzheimer's Disease: Evidence from a mouse model

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Author contributions

Paul Armstrong: co-designed and conducted the experiments, data analysis, interpretation of results, manuscript preparation. Marie-Christine Pardon: interpretation of results, critical revision of the article. Charlotte Bonardi: co-designed the experiment, data analysis, interpretation of results, manuscript preparation.

Disclosure

The authors disclose that they have no actual or potential conflicts of interest, financial or otherwise, related to the present work. All animal procedures were carried out in accordance with the UK Animals Scientific Procedures Act and approved by the Home Office under Project Licence 40/3444.

Author Notes

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ABSTRACT

A key characteristic of Alzheimer's disease (AD) is loss of episodic memory - memory for what happened, where and when; this final aspect - *timing* - is the focus of this paper. Although timing deficits have been reported in AD patients, few parallel studies have been performed in animals, compromising the translational potential of these findings. We looked for timing impairments in the APPswe/PS1dE9 mouse model of AD at 4-5 months of age, before significant plaques have developed. In Experiments 1 and 2a mice were trained with auditory stimuli that were followed by food, either immediately (delay stimulus; Experiments 1 & 2a) or after a short interval (trace stimulus; Experiment 1). In Experiment 1 APPswe/PS1dEdE9 mice conditioned normally, but showed more variable timing of the delay-conditioned cue. Experiment 2 examined timing of two delay-conditioned CSs, with Experiment 2a using mice 4-5 months old, and Experiment 2b mice at 6-8 months. With the longer CS the transgenic mice showed both more variable timing, and earlier timed peak responding than wild-type mice, these effects were not influenced by age. Our results bear similarity to those seen in AD patients, raising the possibility that they have diagnostic potential. They also resemble deficits in animals with dorsal hippocampal lesions, suggesting that they could be mediated by this area. Activated microglia, a component of the immune response thought to be driven by the elevated levels of β-amyloid, were elevated in both dentate gyrus and striatum of young transgenic mice, providing some support for this proposal.

Keywords: Alzheimer's disease, APPswe/PS1dE9, interval timing, appetitive, Pavlovian conditioning, peak procedure

Abbreviations: Alzheimer's disease (AD), dentate gyrus (DG), mild cognitive impairment (MCI), conditioned stimulus (CS), unconditioned stimulus (US), APPswe/PS1dE9 (APP/PS1), wild type (WT).

Introduction

Alzheimer's disease (AD) is a neurodegenerative condition characterized by the abnormal accumulation of amyloid plaques, neurofibrillary tangles and neuronal loss, especially of cholinergic neurons in the basal forebrain (Auld et al., 2002, Schliebs and Arendt, 2011, Selkoe, 2001, Thal et al., 2002, Braak and Braak, 1995). As these pathological symptoms start years before the emergence of clinical symptoms, the best time to treat AD would be in the preclinical stages - yet current cognitive test batteries that could help screen for AD are insufficiently sensitive to detect it (Aizenstein et al., 2008, Baudic et al., 2006, Fiandaca et al., 2015, Rentz et al., 2013, Spaan and Dolan, 2010). Developing translational tests sensitive to the cognitive changes in preclinical stages of AD is thus critical, both for development of screening tests that could facilitate early diagnosis, and testing potential treatments.

Impairment in *episodic memory* - the recall of previous experiences in a spatio-temporal context - is a cognitive hallmark of AD, and also detectable in patients with Mild Cognitive Impairment (MCI), which in a proportion of cases converts to AD. This suggests episodic memory failure could be a good diagnostic indicator of AD. However, the existence of episodic memory in non-human species has been questioned (Tulving and Markowitsch, 1998, althought see Clayton et al., 2001, Crystal, 2010) and translational demonstrations of episodic memory deficits in transgenic mouse models of AD are rare (But see Davis et al., 2013a, Davis et al., 2013b).

An alternative is to focus on the spatio-temporal components of episodic memory. For example, *spatial* learning is impaired in both AD and MCI (Allison et al., 2016, Verghese et al., 2017, Laczo et al., 2009, Vlcek and Laczo, 2014), an effect mirrored in virtually all transgenic mouse models of AD (Stewart et al., 2011, Webster et al., 2014). *Temporal* processing, on the other hand, has received much less attention, especially in animal work. The limited studies examining temporal processing in AD patients show increased variability in their ability to judge time intervals (Carrasco et al., 2000, Caselli et al., 2009, El Haj et al., 2013, Hellstrom & Almkvist, 1997, Nichelli et al., 1993, Rueda & Schmitter-Edgecombe, 2009, but see Heinik et al., 2012), although no consistent effect on timing accuracy was found. Despite the number of studies showing timing impairments in patients, only two studies have

examined timing in transgenic mouse models of AD (Gur et al., 2019a; 2019b); the first found no effect in 10-month-old 3xTg AD mice, while the second found significant underestimation of the target interval in 9-month-old 5xFAD mice, but did not reproduce the effect on variability found in patients. Given the inconsistency of these findings, and the fact that in both the mice were quite old and their neuropathology potentially advanced, the potential implications of these findings for early diagnosis are arguably limited. Accordingly the aim of the present experiments was to further examine temporal processing, in a different, younger transgenic model of AD.

We began by examining timing in an appetitive Pavlovian conditioning task, in which a conditioned stimulus (CS) is paired with a motivationally significant unconditioned stimulus (US). The CS then comes to elicit a conditioned response indicating anticipation of the US that follows – *delay conditioning* (Pavlov, 1927). We also examined *trace conditioning*, a variant in which CS and US are temporally separated by a trace interval. This temporal manipulation retards learning (the *trace conditioning deficit*), and recruits additional neural substrates including hippocampus -- indeed, disruption of trace conditioning is regarded as indicative of hippocampal impairment (Bangasser et al., 2006, Chowdhury et al., 2005, McEchron et al., 1998, Solomon et al., 1986, but see Rawlins & Tanner, 1998; Rogers et al., 2006, Tam & Bonardi, 2012a). Hippocampus is affected early in the development of AD ((Thal et al., 2002, Braak and Braak, 1995, van der Flier and Scheltens, 2009, Halliday, 2017, Setti et al., 2017, Henneman et al., 2009, Allison et al., 2016), and has been implicated in temporal processing. For example, pioneering work by Meck and colleagues demonstrated systematic differences in timing accuracy after hippocampal damage (Meck et al., 1984, Meck et al., 2013, Meck, 1988), which later studies reproduced with lesions confined to dorsal hippocampus (Tam et al., 2015, Tam et al., 2013, Tam and Bonardi, 2012a, Tam and Bonardi, 2012b, Balci et al., 2009, Yin and Meck, 2014, Merchant et al., 2013, Meck et al., 2013). Thus it seemed possible that timing impairments would be more pronounced in this hippocampal-dependent trace conditioning task.

We measured timing using the *peak procedure*. This technique involves training animals that presentations of a fixed-duration *conditioned stimulus* (CS; e.g. a 15-s click) are followed by food

delivery; then the animals' ability to estimate the point at which food is presented is assessed in intermittently presented *peak* trials, during which the CS is presented for an extended duration (e.g. 45s) without reinforcement. The animals' pattern of responding makes it possible to determine both the accuracy and variability with which they anticipate the food (Balci et al., 2008, Buhusi et al., 2009, Buhusi and Meck, 2005, Matell et al., 2006, Tam and Bonardi, 2012a, Tam and Bonardi, 2012b). In Experiment 1 this procedure was adapted for trace conditioning by extending the trace interval rather than the CS on peak trials (e.g. Balci et al., 2009).

The experiments were conducted using a well characterized double transgenic mouse model of AD – the APPswe/PS1dE9 (APP/PS1; Borchelt et al., 1997). APP/PS1 mice co-express the mutated Swedish APP gene and exon-9 deleted variant of the presenilin 1 gene, and show the age-related cognitive decline characteristic of AD. These cognitive deficits were initially attributed to the presence of amyloid plaques; however more recent work questioned this interpretation, suggesting that in fact the elevated levels of β-amyloid that precede plaque formation are responsible. For example, in this strain cognitive deficits begin to manifest at 3.5 months (Bonardi et al., 2011, Pistell et al., 2008, Vegh et al., 2014, Zhang et al., 2012, Ramirez-Lugo et al., 2009) the exact point at which levels of soluble β-amyloid start to be elevated in cortex and hippocampus (Hu et al., 2010). Amyloid plaques, in contrast, although first detected at 4 months, are only widespread at 12 months of age, along with significant neuronal loss (Kuhla et al., 2017, Huang et al., 2016, Savonenko et al., 2005, Ruan et al., 2009). Moreover, manipulations that increase plaque levels can *improve* performance on spatial tasks (Jankowsky et al., 2005), while the spatial learning deficits seen in older mice are correlated with levels of soluble amyloid rather than plaque load (Zhang et al., 2011). Thus our animals were tested at 4 months of age, which we judged to be equivalent to a preclinical stage of AD, at which point both cognitive impairments and elevation of β-amyloid begin to emerge.

Experiment 1

In Experiment 1 four-month-old APP/PS1 and WT mice were trained on an appetitive conditioning task with two 15-s auditory stimuli. The *delay* CS was followed immediately by a sucrose pellet, whereas sucrose occurred five seconds after offset of the *trace* CS; levels of conditioned responding are typically lower in the latter type of trial, an effect termed the trace conditioning deficit. This paradigm allowed us to examine timing by examining responding on non-reinforced *peak trials*. On delay peak trials the CS was extended to 45s, and responding recorded in each 1-s bin of the CS. Timing would be evident as a progressive increase in response rate to 15s after CS onset, at which point sucrose was previously delivered, followed by a gradual decline to baseline levels. Timing on trace peak trials was examined in a similar manner, but by recording responding during the 15-s CS presentation and also during an extended 15-s trace interval that followed it.

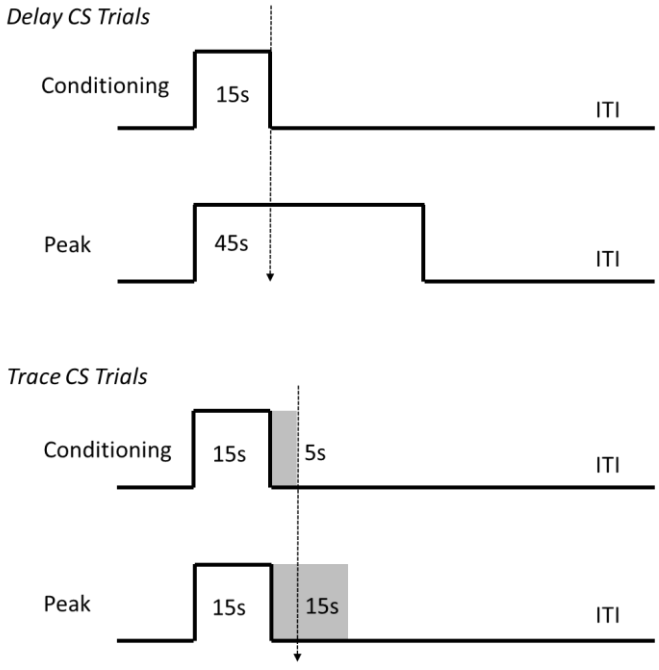


Figure 1 Pavlovian conditioning and peak trials. Conditioned stimuli and trace intervals are drawn to scale and the vertical dashed line represents the time point of US delivery on the conditioning trials.

Materials and Methods

Ethical statement

Procedures were authorized under UK law, carried put in accordance with the Animals (Scientific Procedures) Act (1986), and also the EU Directive 2010/63/EU for animal experiments. This work was funded by the School of Psychology, University of Nottingham, who otherwise had no involvement in this research.

Subjects

The experiment employed 24 naïve male APP/PS1 and littermate wild-type (WT) control mice; 12 APP/PS1 and 12 WT conducted in two replications (mean *ad libitum* weight ranged between 20 – 30 grams). All animals were bred in the University of Nottingham's transgenic animal facility from stock purchased from the Jackson laboratory. The APP/PS1 mouse expresses the chimeric mouse/human APP (Mo/HuAPP695swe) and Presenilin (PS1-dE9) mutations crossed onto a C57BL/6 background. All animals were group-housed and maintained on a 12/12 hour light/dark cycle; holding room temperature, humidity and air exchange were automatically controlled. Animals had ad-libitum access to water, and were provided with a play tube and nesting materials. All experimental procedures occurred during the light cycle. Seven days before the start of testing each mouse was gradually reduced to 85% of its ad-libitum weight by restricting its food ration; the mice were maintained at this level throughout testing.

Behavioral apparatus

The behavioral experiments were conducted in six identical sound-attenuating conditioning chambers equipped with ventilation fans (Med Associates). Each had a floor made from 24 stainless steel bars separated by a 7.9-mm gap, above a stainless steel waste pan. Two chamber sides were made from stainless steel panels (15.9cm x 14.0cm x 12.7cm); a transparent polycarbonate back wall, ceiling and front-loading door made up the remaining sides. A 1.9-cm deep foodcup, into which 45-mg sucrose pellets (Formula P, Test Diet) could be delivered, was housed in a 2.5cm x 2.5cm opening in the center of the right wall, 1cm above the floor. An infra-red photo-beam crossed the food cup opening, and each

beam break was recorded as a response. A 28-V, 12-W houselight, mounted in the center of the left chamber wall, was illuminated throughout the experiment. An audio generator delivered a 74-dB white noise, a 2-Hz 74-dB click and a 75-dB, 2-Khz tone through loudspeakers mounted in the right-hand wall. The equipment was controlled by Med-PC for Windows (Tatham and Zurn, 1989).

Procedure

Behavioral testing: Habituation. Before training all mice were given sucrose pellets in their home cage. The day before conditioning all animals spent 30 minutes in the conditioning chambers with access to five sucrose pellets.

Behavioral testing: delay and trace conditioning. On days 1-6 all mice received conditioning sessions (1 session per day), each consisting of 20 delay and 20 trace trials presented in a semi-random order. Delay trials comprised a 15-s pre-CS period, followed immediately by a 15-s CS presentation terminating with delivery of a sucrose pellet. Trace trials were identical except for the insertion of a 5-s trace interval between CS offset and pellet delivery. An intertrial interval (ITI), consisting of a fixed 120s plus a variable period (drawn from an exponential distribution) with a mean of 60 sec, which separated US delivery from the start of the next pre-CS period (Fig 1). In each replication half of each genotype had the white noise as the delay CS and the clicker as the trace CS, and the remainder the reverse.

Behavioral testing: interval timing. On days 7-8, two test sessions were administered (1 session per day). These sessions were identical to the conditioning sessions, except that 10 delay and 10 trace trials were replaced by 10 delay peak and 10 trace peak trials respectively. In delay peak trials the delay CS was presented for 45s, and in trace peak trials the 15-s trace CS was followed by a 15-s trace interval; neither was reinforced (Figure 1).

Histology

β-amyloid pathology. To confirm that plaque load conformed with previous work on this strain (e.g. Huang et al, 2016; Kuhla et al., 2017; Ruan et al., 2009; Savonenko et al., 2005), two of the APP/PS1 mice were culled after testing, and their brains removed and processed exactly as in Pardon et al. (2016). Results are presented in the Supplementary material (Figure S1)

Data Analysis

Delay and trace conditioning. Mean response rates (in responses per minute; rpm) during the pre-CS, CS and trace intervals for each trial type were computed for each session. Average response rates during pre-CS periods were used to index background responding, while conditioning was measured as a *difference score* - the rate of responding during the target period (delay or trace CS, or trace interval) after subtraction of the rate of responding during the corresponding pre-CS period. This gave a measure of the degree to which responding during the target period was elevated over background levels. The results are presented in the supplementary materials (Table S1).

Interval timing. One APP/PS1 mouse died after the first test session, so only data from the first test session were used for this animal. Timing ability was evaluated from peak trial responding, using the method employed by Tam and colleagues (Tam et al., 2015, Tam et al., 2013, Tam and Bonardi, 2012a, Tam and Bonardi, 2012b), following that adopted in previous work (Balci et al., 2008, Buhusi et al., 2009, Buhusi and Meck, 2005, Matell et al., 2006, Tam and Bonardi, 2012a, Tam and Bonardi, 2012b). Response rates during each 1-s bin of each type of peak trial, pooled over all test sessions, were computed for each mouse; in the trace peak trials of Experiment 1 responding during the 15-s trace interval was appended to responding during the preceding 15-s CS to produce a single 30-s distribution. Using this data, the mean response rate was then calculated over the full peak trial response distribution (45s for the delay CS, 30s for the trace CS), and compared between genotypes. The resulting 1s bin distributions were then smoothed, by calculating response rates over a 5-bin moving average (i.e., trials 1-5, 2-6, 3-7 etc), to avoid bin-by-bin variability obscuring meaningful differences (Harris et al., 2011).

The smoothed data were then normalised by dividing each response rate in each 1s time bin by the maximum response rate for each mouse e.g. (Carvalho et al., 2001, Fox and Kyonka, 2015, Kim et al., 2017, Narayanan et al., 2012). A Gaussian function with three parameters (a = peak rate, b = spread, and c = peak time) was then fitted onto each animal's smoothed and normalised response function (Figure 2) where i indicates 1-s time bins. Peak time indicates timing accuracy, while spread indicates precision: the greater the spread, the less precise the timing. The coefficient of determination, R^2, was computed as a measure of the degree to which the Gaussian function fitted the data. Animals with fits less than 0.2 were omitted only from the Gaussian analyses; no animals were omitted from this experiment. The peak rate parameter was not analyzed due to the Gaussian function being fitted onto the normalized response distributions, which normalized each animal's maximum response rate to 1.

Timing on peak trials was assessed by computing the area under the curve (AUC) on each animal's normalized distribution, using GraphPad Prism version 8 (GraphPad Software, La Jolla California USA), which employs the trapezoidal method for calculating the area under the curve. In brief, between each 1s bin the connecting segment makes the shape of a trapezoid, using the trapezoid rule the area of each trapezoid is the same as the equivalent rectangle; the AUC is then the sum of all the areas of each rectangle. Separate AUC values were calculated from CS onset to expected US delivery (*Start* AUC: delay 0s – 15s; trace 0s – 20s;) and also from expected US delivery to the end of the peak trial (*Stop* AUC: delay 15s – 41s; trace 20s – 25s).

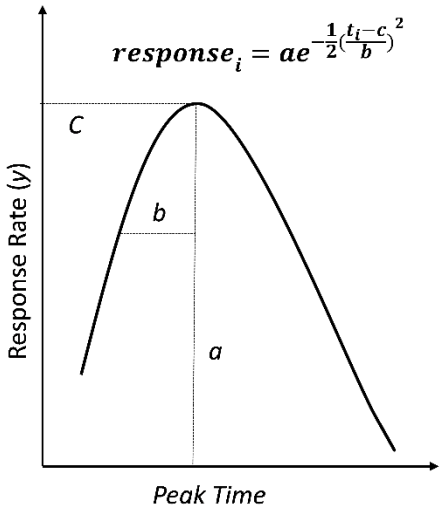


Figure 2. The Gaussian model with three parameters (a = peak time, b = peak spread, c = peak rate) which was fitted to the peak trial response distributions.

Statistical analysis

Data were analysed using repeated measures ANOVA. The Greenhouse-Geisser correction was applied when the assumption of sphericity was violated; η_p^2 is given as a measure of effect size for significant effects and interactions, and the significance level was set at $p < .05$. Significant two-way interactions were explored with simple main effects analysis using the pooled error term, and significant three-way interactions by theoretically meaningful 2-way ANOVAs. Post-hoc tests on main effects with more than two levels were performed with t-tests, and one-sample t-tests compared mean peak times with the target time; in both cases the Bonferroni correction was applied.

Results

Delay and trace conditioning. Figure 3 shows genotype mean difference scores for delay and trace CSs over the conditioning sessions (Panels A and B respectively). In order to visualise the trace conditioning deficit, Panel D shows these data collapsed across genotype, and suggests that the difference scores were lower in the trace than the delay CS – the expected trace conditioning deficit. Panels A and B show that difference scores were slightly higher in the APP/PS1 mice. ANOVA with genotype (APP/PS1 vs WT), CS type (delay/trace) and sessions (1 to 6) compared responding in delay

and trace CSs between genotypes. The effects of CS type and session were significant, $F(1, 22) = 21.1$, $MSe = 23.57$, $p = .002$, $\eta_p^2 = .355$, and $F(3, 66) = 34.29$, $MSe = 63.1$, $p < .001$, $\eta_p^2 = .61$ respectively, as was their interaction, $F(5, 110) = 5.87$, $MSe = 5.842$, $p < .001$, $\eta_p^2 = .211$: difference scores were greater for the delay CS than the trace CS on sessions 3 – 6 inclusive (smallest $F(1, 132) = 9.10$, $MSe = 8.79$, $p = .003$, $\eta_p^2 = .064$ for session 6). No effect or interaction involving genotype was significant, largest $F(1, 22) = 2.14$, $MSe = 141.74$, $p = .158$; nothing else was significant, $Fs < 1$. Thus a trace conditioning deficit developed as training progressed, and the apparent genotype differences suggested by Panels A and B were not statistically significant.

Mean difference scores during the trace interval are shown in Panel C, and preCS response rates in Table S1 in the supplementary material; neither differed between genotypes; ANOVAs with genotype and session as factors revealed only main effects of session, $F(2.04, 44.9) = 44.60$, $MSe = 109.40$, $p < .001$, $\eta_p^2 = .649$ and $F(3.05, 67.16) = 24.59$, $MSe = 8.41$, $p < .001$, $\eta_p^2 = .53$ for trace and preCS scores respectively; nothing else was significant, $Fs < 1$.

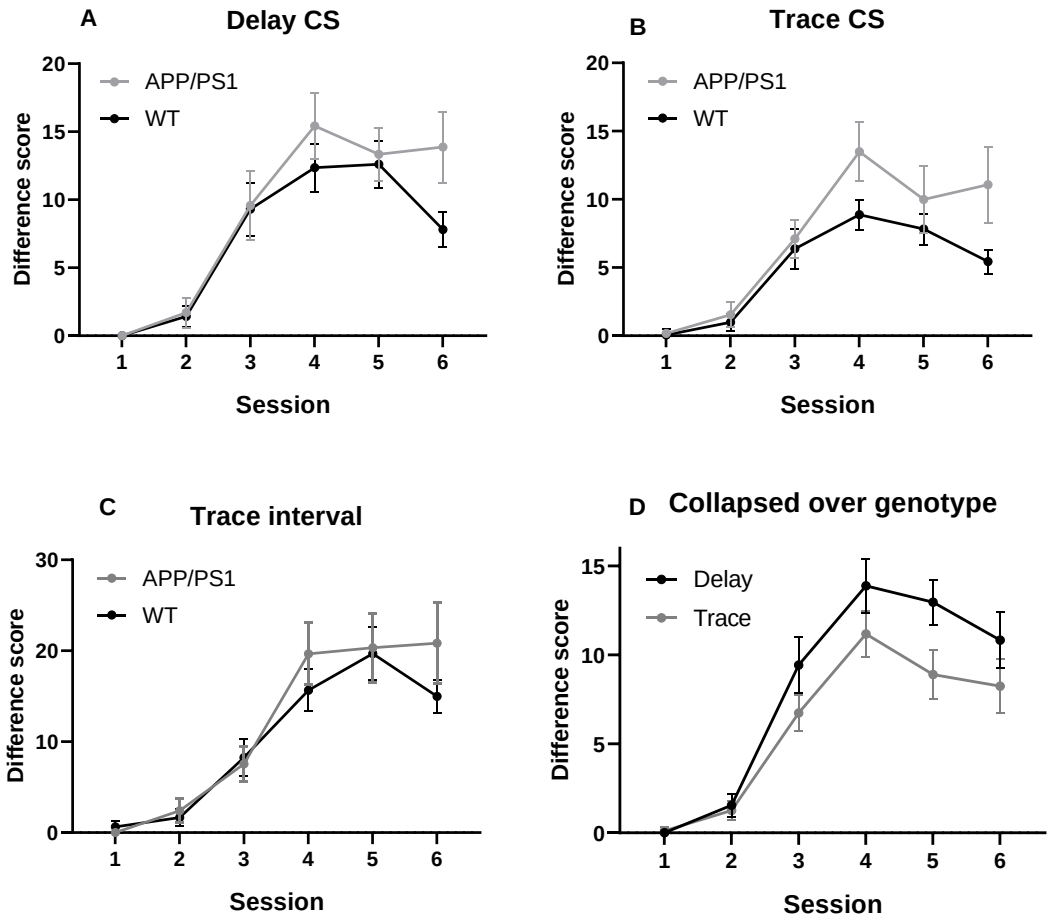


Figure 3: Genotype mean difference scores ± SEM for the delay CS (A) trace CS (B) and trace interval (C), and mean difference scores for delay and trace CSs (D) during the six acquisition sessions of Experiment 1. A trace conditioning deficit (greater difference scores for the delay than the trace CS) was present on sessions 3-6 inclusive; no genotype effects were observed. APP/PS1: N = 12, WT: N = 12.

Interval Timing: response rate. Genotype mean (smoothed) rates of responding during each 1-s bin of the delay and trace peak trials are presented in Figure 4 (Panels 1a & 2a respectively); it can be seen that the APP/PS1 mice responded numerically more than WT animals; however this proved not to be significant. For the purposes of comparison we computed the average response rate across all 1-s time bins for each CS; the resulting values for the delay and trace peak trials respectively were 8.7 (*SEM = 1.4)*) and 8.2 (*SEM = 1.5*) for the WT mice, and 12.02 (*SEM = 2.3*) and 12.67 (SEM = 3.0) for the

APP/PS1 animals; ANOVA with genotype and CS type as factors revealed no significant differences ($Fs < 1$).

Interval Timing – area under the curve (AUC). Both the smoothed (Figure 4 Panels 1a & 2a for delay and trace respectively) and normalized (Figure 4 Panels 1b & 2b for delay and trace respectively) response distributions appeared broader and flatter in the APP/PS1 mice than in the WT mice, and this was especially evident on the ascending part of the function. This was quantified by AUC analysis (Elcoro, 2016): *Start* AUC values for delay and trace trials respectively are shown in Panels 1c & 2c for each group, and the corresponding *Stop* AUC values in Panels 1d & 2d. Both *Start* and *Stop* AUC values were higher on trace than delay trials; also *Start* AUC values appeared to be higher for the APP/PS1 mice, although the *Stop* AUC values did not differ systematically between genotypes. ANOVA on the *Start* AUC data confirmed this description, revealing a main effect of CS type $F(1,22) = 55.694$, $MSE = 8.73$, $p = .019$, $\eta_p^2 = .225$ and of genotype $F(1,22) = 15.410$, $MSE = 6.113$, $p = .001$, $\eta_p^2 = .412$, but no interaction, $F(1,44) = .046$, $MSE = 8.738$, $p > .05$. Analysis of the *Stop* AUC values only revealed a main effect of CS type $F(1,22) = 425.70$, $MSE = 4.003$, $p < .001$, $\eta_p^2 = .951$. Thus the APP/PS1 mice showed weaker temporal control of responding *before* US delivery on both delay and trace trials.

Interval Timing – Gaussian functions. Genotype mean values of the Gaussian parameters are shown in Figure 5. The values of spread (Panel A), which indicate timing precision, were numerically higher for the APP/PS1 than WT mice, and also higher on delay than trace trials. ANOVA with genotype and CS type as factors confirmed that the APP/PS1 mice had higher values of spread on delay trials: there were main effects of genotype, $F(1, 22) = 6.29$, $MSe = 32.9$, $p = .02$, $\eta_p^2 = .22$, trial type, $F(1, 22) = 22.7$, $MSe = 19.40$, $p < .001$, $\eta_p^2 = .51$, and a significant interaction, $F(1, 22) = 5.15$, $MSe = 19.40$, $p = .033$, $\eta_p^2 = .19$; APP/PS1 mice had higher values of spread on delay trials, $F(1, 22) = 7.03$, $MSe = 42.31$, $p = .015$, $\eta_p^2 = .242$ but not on trace trials, $F < 1$. Figure 5 also shows that the peak times (Panel B) were higher on delay than on trace trials, but did not differ systematically between genotypes.

ANOVA confirmed this with a significant effect of CS type, $F(1, 22) = 4.54$, $MSe = 35.12$, $p = .045$, $\eta_p^2 = .171$, but nothing else significant, largest $F(1, 22) = 1.50$, $MSe = 35.12$, $p = .235$. One sample *t*-tests showed peak times (for APP/PS1 and WT respectively 22.45s and 21.12s for delay trials, and 16.71s and 19.57s for trace trials) did not differ from the target value of 20s on trace trials *ps* > .05, but did differ from the target value of 15s on delay trials ($p = .01$ and $p < .001$ for APP/PS1 and WT respectively). It is paradoxical that peak times were higher for *delay* trials - on which sucrose was delivered after 15s - than for trace trials, where it was delivered after 20s. This may reflect the mice using the 5-s interval following *offset* of the trace CS to time the occurrence of sucrose delivery, rather than the 20-s interval from CS onset.

Mean values of R^2 (Panel C) were higher on trace trials, and for the WT mice. ANOVA revealed a main effect of genotype, $F(1, 22) = 4.70$, $MSe = .041$, $p = .041$, $\eta_p^2 = .18$, reflecting greater R^2 values for WT mice, and a main effect of CS type, $F(1, 22) = 6.51$, $MSe = 018$, $p = .018$, $\eta_p^2 = .23$, reflecting greater values for the delay CS; the interaction was not significant, $F(1, 22) = 1.98$, $MSe = 018$, $p = .17$, $\eta_p^2 = .082$.

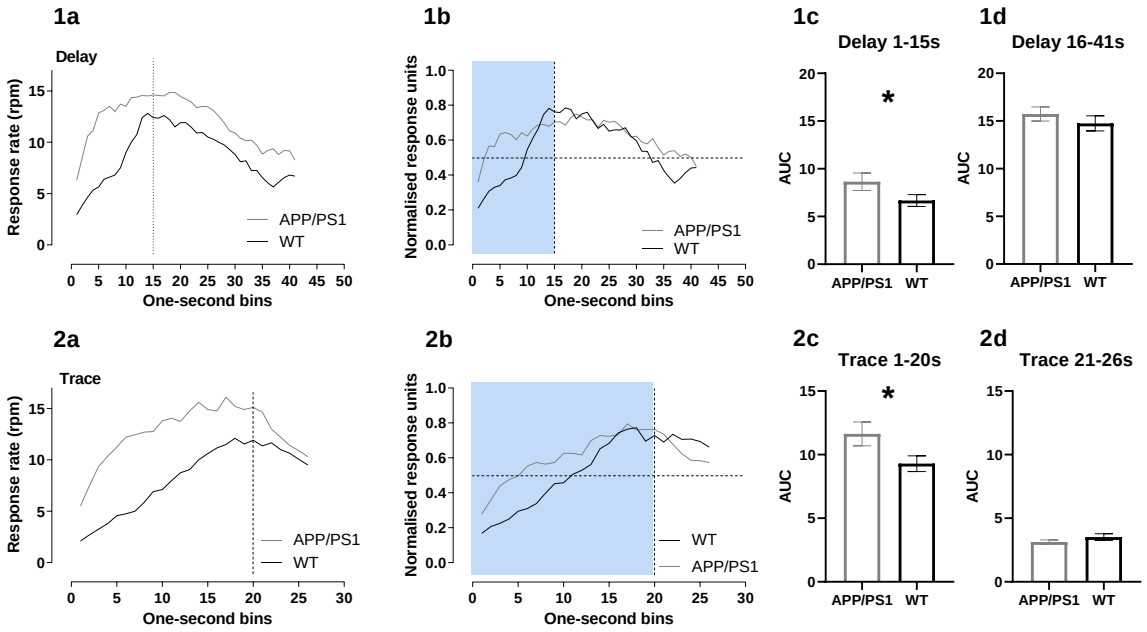


*Figure 4. Data from test sessions of Experiment 1. Genotype mean (smoothed) rates of responding during each 1-s bin of the delay (Panel 1a) and trace peak trials (Panel 2a), and corresponding normalized functions for delay (Panel 1b) and trace CSs (Panel 2b). Start AUC scores (±SEM), presented for delay and trace trials in Panels 1c and 2c respectively, reflect the genotype difference in AUC for the light blue area in Panels 1b and 2b. Stop AUC scores, presented for delay and trace trials in Panels 1d and 2 respectively, derive from the remainder of these functions. * denotes a significant genotype difference. APP/PS1: N = 12; WT: N = 12.*

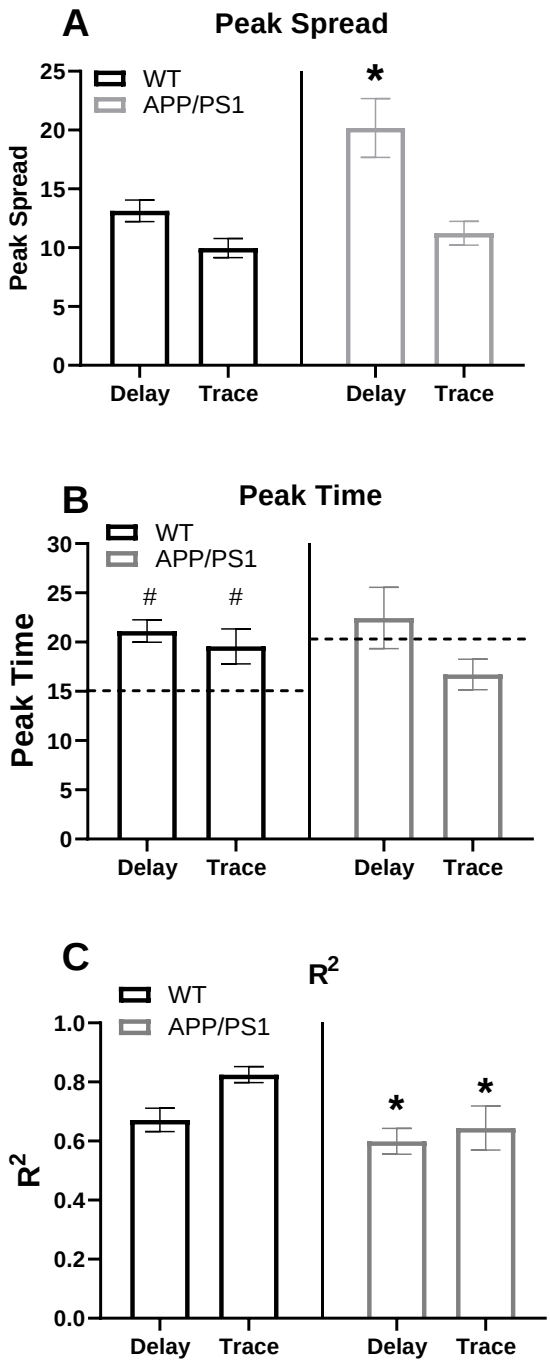


*Figure 5 Genotype mean values of peak spread (A), peak time (B) and R^2 (C) ±SEM for the delay and trace trials. APP/PS1 mice had significantly greater values of spread on delay trials - indicating less precise timing - than WT mice. * denotes statistically significant genotype effect, # denotes a significant difference between observed peak time and time of expected US delivery.* (*WT; N = 12; APP/PS1; N = 12).*

Discussion

While there was no effect of genotype on learning or the size of the trace conditioning deficit, APP/PS1 mice showed signs of impaired interval timing. APP/PS1 had higher *Start* AUC scores than the WTs (Figure 4c), suggesting broader and hence less precise timing functions, before the point of US delivery. This was supported by the Gaussian analysis, which also revealed greater peak spreads for the delay CS in the APP/PS1 than in WT mice; accuracy was unaffected.

Experiment 2a & 2b

Experiment 1 revealed an impairment in timing the delay CS in the 4-month-old APP/PS1 mice, and the first aim of Experiment 2 was to see if this novel finding could be detected again with altered delay parameters (Experiment 2a); as no effect was found in timing the trace CS, this condition was omitted from the present experiment. A second aim of Experiment 2 was to examine whether there was any change in these timing deficits as pathology worsened with age, by examining performance in mice that were 6-8 months old (Experiment 2b). Our aim was to explore the suggestion that the deficits observed in Experiment 1 are attributable to elevated levels of β-amyloid rather than plaques. Elevated β-amyloid begins to emerge at 4 months in this strain, whereas plaque deposition is only sporadic until the mice are at least 6 months of age (Hu et al., 2010); thus if plaques were responsible for the findings of Experiment 1, we would expect the deficits to be much more profound at 6-8 months, by which time plaque load is substantial; conversely, if β-amyloid were responsible, then one would not necessarily make such a prediction.

Both Experiments 2a and 2b comprised identical training with three auditory CSs; two of these, one 10s in duration and the other 20s, were immediately reinforced with a sucrose pellet; the third stimulus, which was 10s for half its presentations, and 20s for the remainder, was never reinforced. Timing of the reinforced CSs was then examined in peak trials, as in Experiment 1.

Materials and methods

All aspects of the method that are not specified were identical to those of the previous experiment.

Subjects

Experiment 2a employed 36 mice (18 APP/PS1 & 18 WT) all 4-5 months of age. Experiment 2b employed 35 mice, 16 APP/PS1 and 19 WT aged between 6.5 - 8 months. Both experiments were conducted in three replications.

Behavioural apparatus

Identical to Experiment 1 except for the addition of a 75-dB, 2-Khz tone.

Behavioral testing: delay conditioning. This occurred on days 1-6; each session comprised 42 trials: 14 *10s* trials, 14 *20s* trials, and 14 nonreinforced *CS-* trials. CS presentations on the *10s* and *20s* trials were 10s and 20s respectively; in both, CS offset was accompanied by sucrose pellet delivery. On nonreinforced CS- trials the cue was either 10-s or 20-s long (7 trials of each per session). The *10s* and *20s* CSs were either click or noise (counterbalanced across genotype in each replication); CS- was the tone for all animals.

Behavioral testing – Interval timing. On days 7-9, three test sessions were administered (1 session per day); these were identical to the conditioning sessions except that three of each of the *10s* and *20s* trials were replaced with 10s and 20s peak trials respectively. 10s peak trials comprised a 30-s presentation of the *10s* CS, and 20s peak trials a 60-s presentation of the *20s* CS; neither was reinforced.

Histology

β-amyloid pathology. Once Experiment 2a was completed, brains were taken from two of the APP/PS1 mice, one of 4 months and one of 5 months; these were processed exactly as in Experiment

1, again to confirm the mice showed levels of plaques consistent with previous reports in this strain (e.g. Huang et al, 2016; Kuhla et al., 2017; Ruan et al., 2009; Savonenko et al., 2005). No brains were taken from mice in Experiment 2b, as it is clear from previous work that plaques are evident at this age in this strain (e.g. Garcia-Alloza et al., 2006). Results are presented in the supplementary material (Figure S1)

Data treatment

The peak trial data were pooled over all three test sessions, but in all other respects data treatment was identical to that of Experiment 1. For the Gaussian function analysis the criterion of excluding animals with a response distribution for which R^2 was less than 0.2 resulted in elimination of 2 mice from each genotype in Experiment 2a (leaving N = 16 for each genotype), and 4 of each genotype in Experiment 2b (leaving N = 12 and 15 for APP/PS1 and WT respectively). The remaining analyses included all the animals.

Results

Delay conditioning. The genotype mean difference scores for the conditioning sessions are shown in Figure 6: Panels A and C show these data for the WT mice for Experiments 2a and 2b respectively, and Panels B and D show the corresponding APP/PS1 data. It is evident that all mice learned to respond more to the reinforced than nonreinforced CSs as training progressed, and difference scores for the *10s* CS tended to be slightly higher than those for the *20s* CS, especially in the APP/PS1 animals (although this proved not to be reliable). Finally, there was no obvious difference in pattern between Experiments 2a and 2b.

For Experiment 2a ANOVAs on the difference scores, with genotype, CS type (*10s*, *20s*, *CS-)* and session as factors, revealed main effects of session, $F(5, 170) = 46.144$, $MSe = 76.0$, $p < .001$, $\eta_p^2 = .567$ and CS type $F(2, 68) = 70.27$, $MSe = 68.66$, $p < .001$, $\eta_p^2 = .674$, and an interaction between these two factors, $F(10, 340) = 20.42$, $MSe = 22.08$, $p < .001$, $\eta_p^2 = .38$, due to the higher scores for the reinforced *10s* and *20s* CSs than the non-reinforced CS, which was significant from session 2 onwards

(smallest $p < .001$ for session 2). Differences scores for the *10s* and *20s* CSs did not differ during session six (smallest $p = .12$). There was also a significant interaction between session and genotype, $F(5, 170) = 5.884$, $MSe = 76.06$, $p = .001$, $\eta_p^2 = .148$; scores were higher for the WT than the APP/PS1 mice on session 2 $F(1, 34) = 5.543$, $MSe = 34.7$, $p = .023$, $\eta_p^2 = .140$, but this pattern had reversed by session 6, $F(1, 34) = 5.36$, $MSe = 25.12$, $p = .027$, $\eta_p^2 = .136$. No significant genotype differences were seen during the remaining sessions (smallest $F(1, 34) = 2.77$, $MSe = 43.9$, $p = .105$) No interactions involving genotype and CS type were significant, $Fs < 1$, suggesting that the groups did not differ in learning the task; nothing else was significant, $Fs < 1$.

ANOVA on the mean rates of preCS responding (*Table S1, in the supplementary material*), with genotype and session as factors, revealed a main effect of session, $F(5, 170) = 53.77$, $MSe = 2.09$, $p < .001$, $\eta_p^2 = .613$ which interacted with genotype $F(5, 17) = 4.33$, $MSe = 2.09$, $p = .001$, $\eta_p^2 = 113$: APP/PS1 mice responded more than WT mice on sessions 2 and 4, smallest $F(1, 34) = 5.94$, $MSe = 4.6$, $p = .02$, $\eta_p^2 = .149$ (session 2); these genotype differences had dissipated by session 6, $F < 1$.

A parallel ANOVA on the data from Experiment 2b revealed significant effects of session $F(5, 165) = 25.22$, $MSe = 122.82$, $p < .001$, $\eta_p^2 = .433$, CS type, $F(2, 66) = 46.25$, $MSe = 54.97$, $p < .001$, $\eta_p^2 = .584$ and an interaction between the two $F(10, 330) = 15.1$, $MSe = 29.70$, $p < .001$, $\eta_p^2 = .314$; both the *10s* and *20s* CSs had significantly greater difference scores than the non-reinforced CS from session 2 onwards (smallest $ps < .001$ for session 3-6). Nothing else was significant (smallest $F(10, 330) = 2.31$, $MSe = 29.70$, $p = .059$, $\eta_p^2 = .066$, for the three-way interaction).

Analysis of preCS response rates, shown in Table S1 in the supplementary material, revealed significantly higher rates in APP/PS1 mice, $F(1, 33) = 6.59$, $MSe = 3.26$, $p = .015$, $\eta_p^2 = .17$, and session, $F(2.60, 85.75) = 17.90$, $MSe = 3.19$, $p < .001$, $\eta_p^2 = .35$; the interaction was not significant, $F(2.60, 85.75) = 1.33$, $MSe = 3.19$, $p = .27$.

In summary, despite a tendency of APP/PS1 mice to have higher response rates and greater difference scores, there was no evidence of an effect of genotype on learning the discrimination in either age-group.

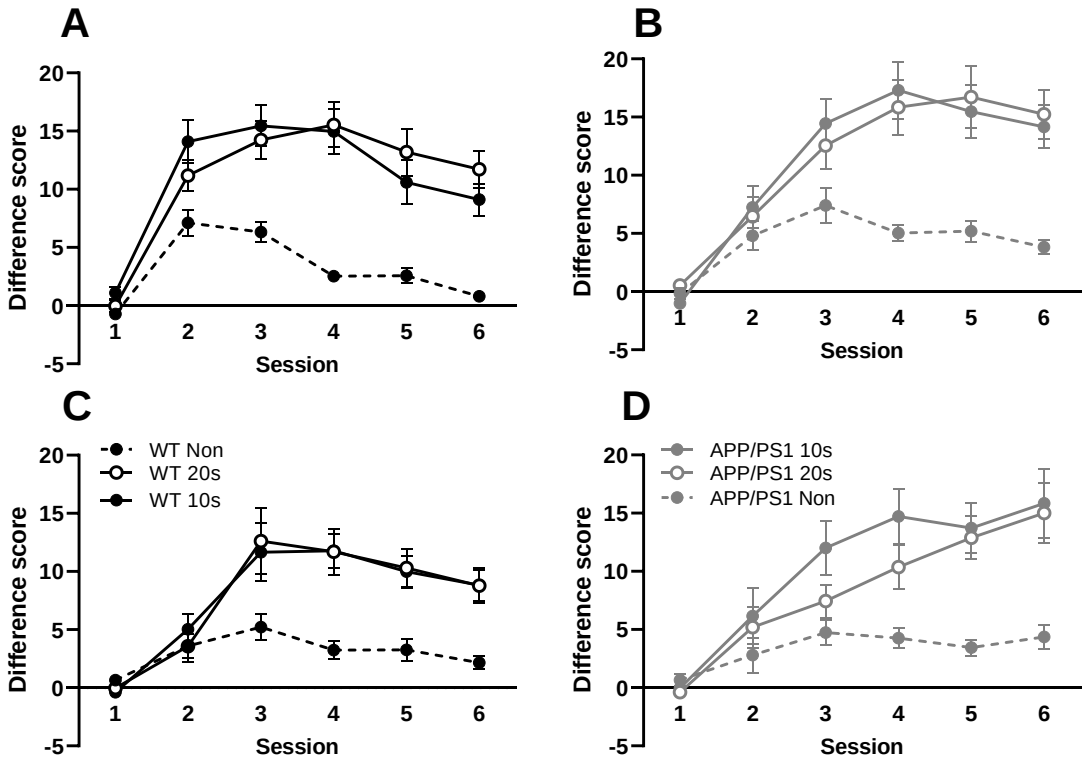


Figure 6: Genotype mean difference scores ±SEM for the 10-s CS, 20-s CS and CS- in the six acquisition sessions of Experiment 2a (Panel A & B for WT and APP/PS1 respectively), Experiment 2b (Panels C & D for WT and APP/PS1 respectively). There was no effect of genotype on acquisition of the discrimination between reinforced and nonreinforced CSs. Experiment 2a APP/PS1: N = 18, WT: N = 18, Experiment 2b APP/PS1: N = 16, WT: N = 19.

Interval timing

Interval Timing: response rate. Genotype mean response rates per 1-sec bin of the peak trials are shown in Figure 7 (Panels 1a, 2a, 3a and 4a); it appears that response rates were again higher in the APP/PS1 mice. Response rates averaged across all the time bins for both CS types are shown in Table 2; ANOVA conducted on these data with genotype, experiment and CS type as factors revealed a main effect of CS type $F(1,67) = 107$, $MSe = 14.16$, $p < .001$, $\eta_p^2 = .615$, genotype $F(1,67) = 13.3$, $MSe = 83.87$, $p = .001$, $\eta_p^2 = .166$ and an interaction between the two $F(1,67) = 4.08$, $MSe = 14.16$, $p = .047$, $\eta_p^2 = .057$: APP/PS1 mice showed greater average response rates for the *10s* CS, $F(1, 67) = 11.42$,

MSe = 73.76, $p = .001$, $\eta_p^2 = .146$, and the *20s* CS $F(1, 67) = 13.75$, *MSe* = 24.27, $p = .001$, $\eta_p^2 = .170$. Mean response rates were also higher for the 10s CS than to the 20s CS in both WT and APP/PS1 mice (*ps* < .001). No other main effect or interaction was significant, *F*s < 1

Table 2

Genotype mean response rate across all time bins for the smoothed 10-s and 20-s CS response distributions in Experiments 2a and 2.

Experiment	Genotype	CS Type	
2a	APP/PS1	*10s* CS	19.55 (2.45)
		20s CS	12.63 (1.4)
	WT	*10s* CS	13.05 (.98)
		20s CS	7.89 (.75)
2b	APP/PS1	*10s* CS	19.19 (2.97)
		20s CS	10.44 (1.29)
	WT	*10s* CS	11.71 (1.4)
		20s CS	6.3 (1.1)

Standard errors of the mean are shown in parentheses. Experiment 2a APP/PS1: N = 18, WT: N = 18; Experiment 2b APP/PS1: N = 16, WT: N = 19.

Interval Timing: area under the curve (AUC). The normalized response distributions (Figure 7 Panels 1b, 2b, 3b and 4b) showed a similar pattern to Experiment 1, with APP/PS1 mice showing a broader distribution of responding; in addition there was an accompanying tendency for APP/PS1 animals to peak *earlier* than the WT mice. The *Start* AUC scores, shown in Panels 1c, 2c 3c and 4c, appeared higher in the APP/PS1 mice, especially for the *20s* CS; ANOVA with genotype, experiment and CS type revealed a main effect of CS type $F(1, 67) = 178.92$, *MSe* = 4.21, $p < .001$ $\eta_p^2 = .727$,

genotype $F(1, 67) = 6.16$, $MSe = 13.99$, $p = .016$, $\eta_p^2 = .084$ and an interaction between the two, $F(1, 67) = 4.68$, $MSe = 4.12$, $p = .034$, $\eta_p^2 = .065$; nothing else was significant (smallest $F(1, 67) = 2.09$, $MSe = 13.99$, $p = .152$, for a main effect of experiment). *Start* AUC scores were greater for the APP/PS1 mice for the longer *20s* CS, $F(1, 67) = 6.51$, $MSe = 14.47$, $p = .013$, $\eta_p^2 = .089$, although not for the shorter CS ($p = .081$). In addition *Start* AUC scores were greater for the longer CS in both genotypes ($ps < .001$), reflecting the broader distributions for longer CSs anticipated by Weber's law. *Stop* AUC scores are shown in Panels 1d, 2d 3d and 4d, which do not suggest any clear genotype differences; ANOVA revealed only a main effect of CS type, $F(1, 67) = 133.18$, $MSe = 7.37$, $p < .001$, $\eta_p^2 = .665$, reflecting higher *Stop* AUC scores for the longer *20s* CS, nothing else was significant $F(1, 67) = 1.23$, $MSe = 9.8$, $p = .271$, for the genotype x experiment interaction)

Interval Timing – Gaussian function

The Gaussian parameters of peak spread and peak time, along with the values of R^2, are shown in Figure 8 (Panels A, B and C respectively).

Peak spread. Spreads (Figure 8 Panel A) appeared significantly higher for the *20s* CS, as Weber's law predicts, but in addition APP/PS1 mice in both age-groups seemed to show higher values of spread than the WT mice for this longer CS. ANOVA with genotype, experiment and CS type as factors confirmed this impression, revealing main effects of genotype, $F(1, 55) = 5.92$, $MSe = 39.57$, $p = .018$, $\eta_p^2 = .097$, and CS type, $F(1, 55) = 61.28$, $MSe = 31.58$, $p < .001$, $\eta_p^2 = .52$, and an interaction between these factors $F(1, 55) = 4.16$, $MSe = 31.58$, $p = .046$, $\eta_p^2 = .07$; spread for the *20s* CS was significantly greater in the APP/PS1 mice, $p = .019$. No other main effect or interaction was significant, largest $F(1, 55) = 1.75$, $MSe = 31.58$, $p = .191$ (CS x experiment).

Peak time. Peak times (Figure 8 Panel B) appeared higher for the *20s* than the *10s* CS as one would expect, but in addition seemed lower in the APP/PS1 mice for the longer CS. ANOVA with genotype, experiment and CS type as factors confirmed this, revealing an effect of CS type, $F(1, 55) = 38.63$, $MSe = 36.00$, $p < .001$, $\eta_p^2 = .41$ which interacted significantly with genotype, $F(1, 55) = 5.32$,

MSe = 36.00, $p = .025$, $\eta_p^2 = .088$: peak times were significantly lower in the APP/PS1 mice for the *20s* CS, $p = .040$, but not the *10s* CS, $F < 1$. Again no effect or interaction involving age was significant, *Fs* < 1, and nothing else was significant, largest $F(1, 55) = 3.06$, *MSe* = 93.76, $p = .08$. Group mean values of peak time for the *10s* CS were, averaged over both age-groups, 14.80s and 15.54s for APP/PS1 and control mice respectively; both differed from the target time of 10s, *ps* < .01. Corresponding values for the *20s* CS were 19.17s and 25.20s; the WT mice significantly overestimated the target time, $p = .002$, while the APP/PS1 mice did not, $p = .71$.

Goodness of fit. The values of R^2 values are shown in Figure 8 Panel C; ANOVA revealed only a main effect of CS type, $F(1, 55) = 16.61$, *MSe* = .022, $p < .001$, $\eta_p^2 = .23$; nothing else was significant, largest $F(1, 55) = 1.42$, *MSe* = .036, $p = .23$. Thus neither genotype nor age affected goodness of fit in this experiment.

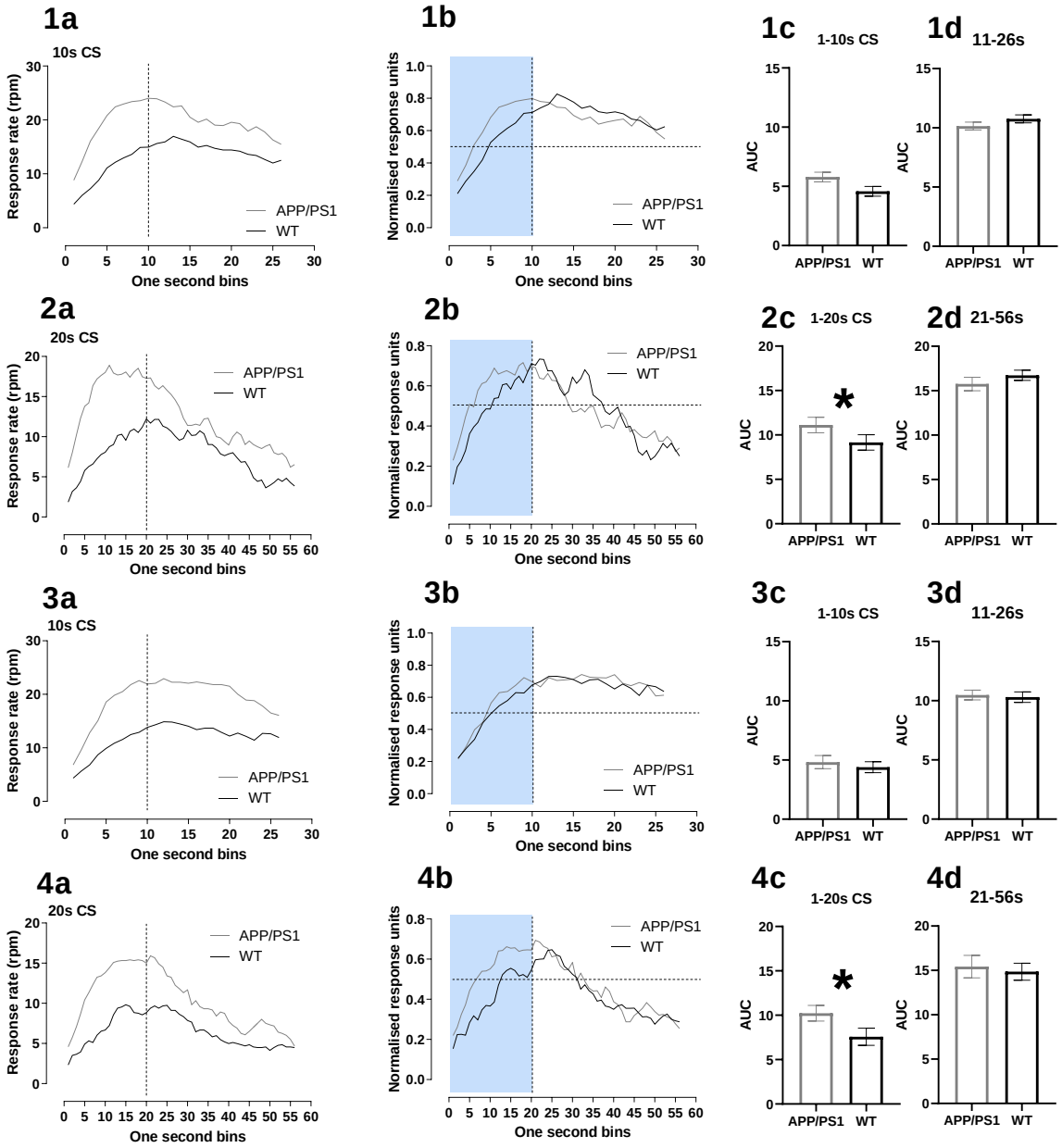
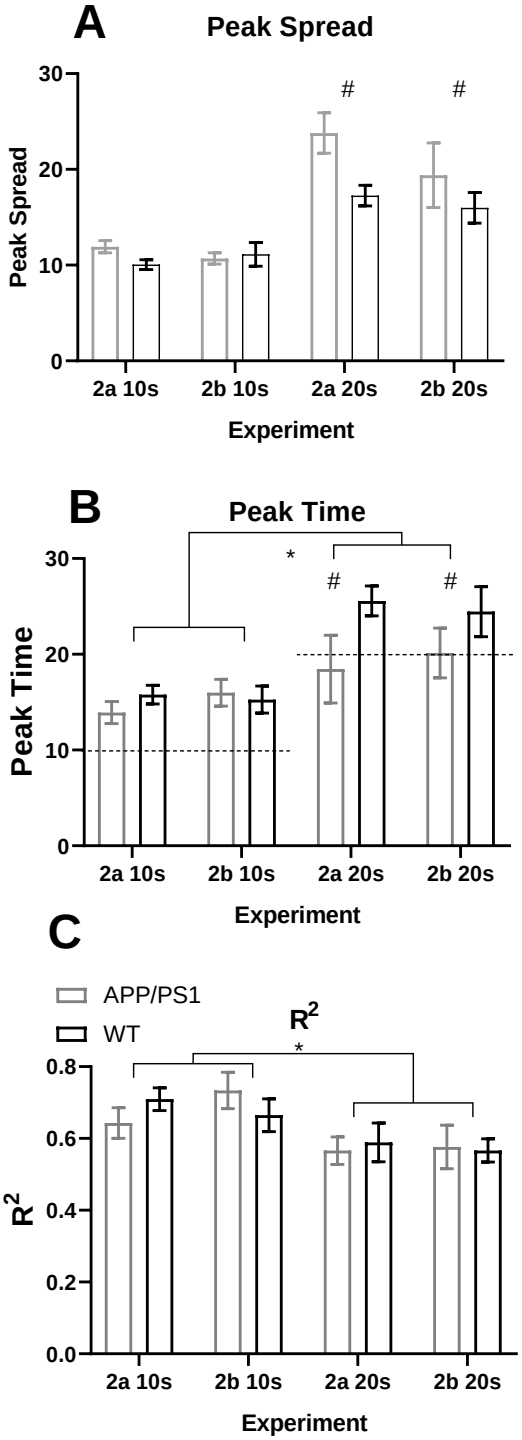


Figure 7: *Panels 1 & 2 show data from Experiment 2a, Panels 3 and 4 data from Experiment 2b. Genotype mean (smoothed) response rates during each 1-s bin of the 10-s (Panels 1a, 3a) and 20-s (Panels 2a, 4a) CSs; corresponding normalized rates shown in Panels 1b & 3b for the 10-s CS, and Panels 2b & 4b for the 20-s CS. Start AUC scores (±SEM), presented for 10-s and 20-s CSs in Panels 1c & 3c, 2c & 4c respectively, reflect the genotype difference in AUC for the light blue area in Panels 1b & 3b, and 2b & 4b. Stop AUC scores, presented for 10-s and 20-s CSs in Panels 1d & 3d, and 2d & 4d respectively, derive from the remainder of these functions. * reflects a significant genotype*

difference. Experiment 2a APP/PS1: N = 18, WT: N = 18; Experiment 2b APP/PS1: N = 16, WT: N = 19.



F*igure 8. Genotype mean values of peak spread (A), peak time (B) and* R^2 *(C) ± SEM for the 10-s and 20-s CSs in Experiments 2a and 2b. The APP/PS1 mice had significantly higher spreads and lower peak times than the WT mice for the 20-s CS; neither was affected by age. # = significant genotype*

*difference, * denotes statistical significance between CS types, Experiment 2a APP/PS1: N = 16, WT: N = 16; Experiment 2b APP/PS1: N = 12, WT: N = 15.*

Discussion

As in Experiment 1 the APP/PS1 mice showed some evidence of impaired interval timing. They had greater *Start* AUC scores and values of peak spread than the wild types for the longer, *20s* CS, indicating broader response functions and hence poorer timing precision. However, as in Experiment 1 the *Stop* AUC scores did not differ the genotypes, suggesting that the APP/PS1 response functions were broader only on the ascending section that preceded food delivery. A novel finding in this second experiment was a difference in timing accuracy – APP/PS1 peak times for the *20s* CS were significantly earlier than those of the WT mice; however, this was not obviously a genotype impairment in accuracy, as although the WT mice significantly overestimated the time of food delivery, the APP/PS1 peak times did not differ from the corresponding target times. Finally, none of these effects was influenced by age. The fact that the timing abnormality was evident in both the younger and older mice is consistent with the suggestion that it was caused by elevated levels of β-amyloid, which would have been present in both young and old mice, rather than plaque deposition, which would have been substantial in the older mice only. However, the fact the younger and older mice did not seem to differ in performance deserves some comment – one might imagine that this elevation would be accentuated as the mice aged, magnifying the behavioural effects. But this suggestion may be over-simplistic. First, there is evidence that levels of soluble β-amyloid do not in fact increase between 6 and 12 months of age in this strain (Xiong et al., 2011). But even if they did, there is no particular to suppose that the relationship between these indirect physiological indices of neural damage and behavioural performance is linear, or that the relative sensitivity of their measures is sufficient to detect changes over this time period. In short, if a relationship between the two were present this would be compelling – but the absence of one cannot be truly informative, as it could arise for any number of reasons.

There was no sign that genotype had any effect on the animals' ability to learn the discrimination between reinforced and non-reinforced CSs in either age-group. There was, however, a tendency

throughout for APP/PS1 mice to respond more, and have higher difference scores, than WTs in both experiments, which might reflect increased motivation; moreover, although the analysis of mean non-normalised response rates during peak trials revealed that this difference was not significant in Experiment 1, it was in Experiment 2. However, in Experiment 2 this same analysis revealed higher response rates in the APP/PS1 mice in *both* the *10s* CS and the *20s* CS - and inspection of the data shown in Table 2 reveals that this genotype difference in response rate was numerically greater for the *10s* CS. Thus if response rate alone had been driving the greater spread seen in the APP/PS1 animals, then one would also predict they would have shown greater spread for the *10s* CS, whereas in fact there was no sign of such a difference. The entire pattern of behavioural findings is therefore difficult to explain in terms of response rate alone.

As in Experiment 1, the R^2 measure of fit to the Gaussian model was low relative to the R^2 values found in human studies, although consistent with those found in the animal literature (Tam and Bonardi, 2012b, Drew et al., 2007, but see Yin and Meck, 2014). Critically, although genotype differences in R^2 were observed in Experiment 1, no such differences were observed in Experiment 2, meaning that the transgenic effect on timing cannot be attributed to this factor.

Experiment 3

Experiment 3 explored the neural basis of our behavioral findings. It has been argued that the mechanism by which elevated levels of β-amyloid produce cognitive impairment is via neuroinflammatory responses, which are now widely thought to drive the symptoms of AD (Parachikova, 2007). While it has long been established that neuroinflammation can be triggered by plaque deposition, more recent work suggests that soluble β-amyloid can have the same effect (Bruno et al., 2009; Lue et al., 1999; Okello et al., 2009) and this has also been demonstrated in our APP/PS1 mice. For example, there have been reports of elevated levels of inflammatory markers in this strain from 2 months of age, well before the start of plaque deposition (e.g. Ruan et al., 2009; Zhang et al.,

2012). Thus in the present study we attempted to evaluate neuroinflammation markers in our animals, to evaluate the possibility that neuroinflammation could underlie the behavioural effects that we observed.

The inflammatory response in the brain involves increased production of reactive astrocytes and activated microglia, whose role is to detect and repair damage, and stimulate production of a variety of substances involved in the immune response. Accordingly we examined density of activated microglia in APP/PS1 and WT mice from Experiment 2a, by staining with the iba1 antibody, a protein that is expressed in microglia and up-regulated during microglial activation. The primary site of interest was the hippocampus (Ruan et al., 2009, Xiong et al., 2011, Zhang et al., 2012), but we also examined the striatum, an area affected early in familial AD (Pievani et al., 2013, Selden et al., 1994) and heavily implicated in timing (Meck, 2005). In the APP/PS1 mice, clusters were also analyzed: microglia cells group around aggregated amyloid plaques to form a single cluster (Shemer and Jung, 2015, Puli et al., 2012, Stalder et al., 1999, Ruan et al., 2009, Marlatt et al., 2014), which can be used as an indirect marker for plaque deposition.

Materials and methods

Subjects

The experiment employed 12 APP/PS1 and 12 wild type mice, which had previously taken part in Experiment 2a. Half of each genotype were 4 months of age, and the remainder 5 months.

Tissue preparation. The brains used to estimate microglial pathology were post-fixed in 4% paraformaldehyde and stored at 4-8^{o}C for at least 48 hours. They were then placed into increasing concentrations of ethanol (70% ethanol for 1 hour, twice; 90% ethanol for 1 hour; 90% ethanol for 1.5 hours; 100% ethanol for 1.5 hours, twice; and finally 100% ethanol for 2 hours). The samples were then placed into 100% chloroform for 2 hours, before being put into liquid paraffin wax, twice for 1 hour, and finally embedded in paraffin wax with a tissue embedding station (Leica TP1020). All sections were cut on the coronal plane and mounted on 3-Aminopropyltriethoxysilane (APES) slides.

Hippocampal sections were taken from between bregma 1.18mm and -2.92mm, and striatal sections from between bregma 1.32mm and -0.82mm.

Immunohistochemistry. Immunohistochemistry for iba1 staining was carried out according to a standardised procedure (Pardon et al., 2016). Two slides per brain region, averaging 6 slices per slide, were taken. After staining all slides were then mounted with Clearvue mountant (Thermo scientific, cat. Nr. 4212), and cleaned the following day with acetone. Sample images were taken using a Hamamatsu NanoZoomer-XR 2.0-RS C10703 digital scanning system using a TDI NanoZoomer camera (Hamamatsu Photonics K.K. Systems, Japan) with a 20 X magnification, and viewed with NDP.view2 software (NanoZoomer Digital Photography).

Data treatment

Microgliosis in APP/PS1 and WT mice was compared by counting the number of stained microglia and, in the APP/PS1 mice, microglial clusters. Eight striatal and six hippocampal slices (dorsal to ventral) per animal were used; regions of interest were defined in both hemispheres for dorsal striatum, CA1, CA2, CA3 and dentate gyrus (DG). These regions were .2mm^2 for all but CA2, for which they were 0.1mm^2. The number of iba1-stained microglia in these regions was then counted blind to genotype. Clusters (in the APP/PS1 mice; there were no clusters in WT animals) were counted separately in hippocampal (bregma 1.18mm and -2.92mm) and dorsal striatal sections (bregma 1.32mm / -0.82mm), and also in the surrounding cortical tissue. In both cases counts were averaged across hemisphere and slice, and transformed into counts per mm^2.

Results

Microgliosis. Figure 9 (Panel A) shows the genotype mean number of counts per mm^2 in CA1, CA2, CA3 and also DG regions of interest; representative examples of DG microglia are shown in Figure 10. Counts were numerically higher in the APP/PS1 mice, especially in the DG. ANOVA on counts in hippocampus proper, with area (CA1, CA2, CA3), genotype (APP/PS1, WT) and age (4 and 5 months) as factors revealed a main effect of area, largest $F(1.39, 33.68) = 13.02$, $MSe = 49.26$, $p <$

.001, η_p^2 = .39; t-tests revealed higher counts in CA1 than in CA3, t (23) = 6.37; no other comparisons were significant. There was also a significant effect of age, $F(1, 20) = 23.81$, $MSe = 148.54$, $p < .001$, η_p^2 = .54, counts being higher at 5 months in every condition; nothing else was significant, largest $F(1, 20) = 3.04$, $MSe = 148.54$, $p = .10$. Although there was no genotype effect on counts in hippocampus proper, a difference in DG was evident: ANOVA revealed a main effect of genotype, $F(1, 20) = 10.42$, $MSe = 111.06$, $p = .004$, η_p^2 = .34, and age, $F(1, 20) = 8.73$, $MSe = 111.06$, $p = .008$, η_p^2 = .30, but no interaction, $F(1, 20) = 1.35$, $MSe = 111.06$, $p = .26$. Thus in the DG microglia counts were significantly higher in the APP/PS1 mice, as well as in the older mice. Panels C and D of Figure 9 show a similar pattern in dorsal striatum, with counts being higher in the APP/PS1 animals and in the older mice: ANOVA with genotype and age as factors revealed main effects of both genotype, $F(1, 20) = 4.93$, $MSe = 53.66$, $p = .038$, η_p^2 = .20 and age, $F(1, 20) = 16.54$, $MSe = 53.66$, $p = .001$, η_p^2 = .45, but no interaction, $F < 1$. Thus APP/PS1 mice had greater numbers of microglia than WTs in dentate gyrus and dorsal striatum, but not in CA1, CA2 and CA3.

The total number of microglial clusters in the entire cortex and hippocampal areas taken from the hippocampal sections are shown in Figure 9 (Panel E). Cluster levels appeared to be higher at five months than at four, and more so in cortex than hippocampus. ANOVA confirmed main effects of both age, $F(1, 10) = 20.19$, $MSe = 13.43$, $p = .001$, η_p^2 = .67, and area, $F(1, 10) = 51.12$, $MSe = 5.62$, $p < .001$, η_p^2 = .84, and a significant interaction, $F(1, 10) = 23.00$, $MSe = 5.62$, $p = .001$, η_p^2 = .70: more clusters were found in cortex at five months than at four, $F(1, 20) = 40.66$, $MSe = 9.52$, $p < .001$, but levels in the hippocampus did not differ with age, $F(1, 20) = 1.37$, $MSe = 9.52$, $p = .26$. We observed no clusters in the striatum itself, but in the corresponding cortical slices the mean numbers of clusters were 3.54 and 9.19 at 4 and 5 months respectively, and these values differed, $F(1, 10) = 17.10$, $MSe = 5.59$, $p = .002$, η_p^2 = .63.

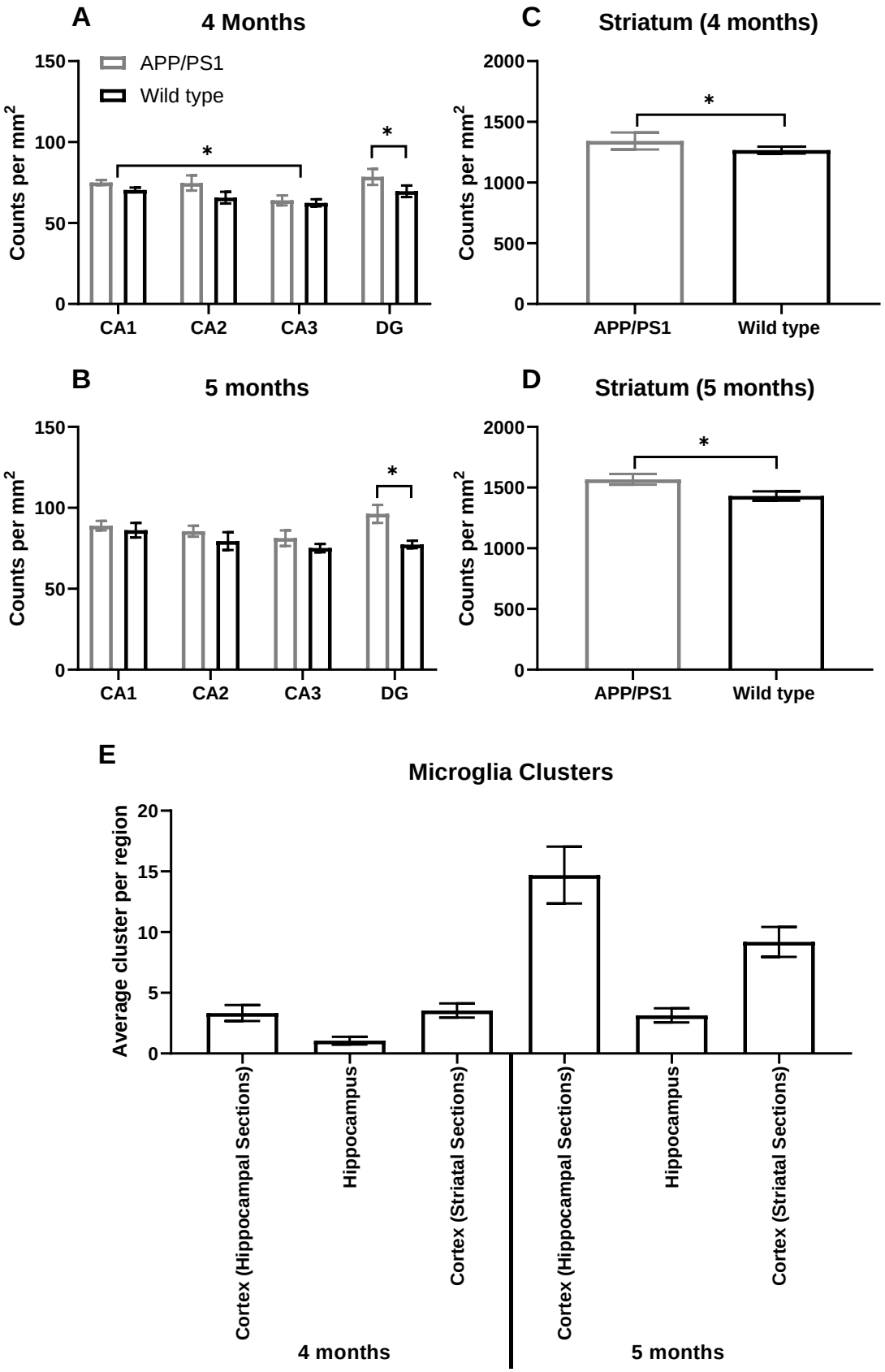

A
4 Months
APP/PS1
Wild type
Counts per mm2
150
100
50
0
CA1
CA2
CA3
DG
*
*
C
Striatum (4 months)
2000
1500
1000
500
0
APP/PS1
Wild type
*
B
5 months
D
Striatum (5 months)
E
Microglia Clusters
Average cluster per region
20
15
10
5
0
Cortex (Hippocampal Sections)
Hippocampus
Cortex (Striatal Sections)
4 months
5 months

*Figure 9: Iba1 staining: genotype mean counts per mm^2 ± SEM in CA1 CA2 CA3 and DG at 4 month (Panel A) and 5 months (Panel B), and in striatum at 4 months (Panel C) and 5 months (Panel D). Panel E shows the average number of clusters in the cortex, hippocampus and striatum of 4 and 5 month old APP/PS1 mice. APP/PS1 mice had significantly greater numbers of microglia than WTs in dentate gyrus and dorsal striatum, but not in CA1, CA2 and CA3. In the APP/PS1 mice the number of microglial clusters increased between fout and 5 months in cortex, but not hippocampus. * denotes statistical significance*

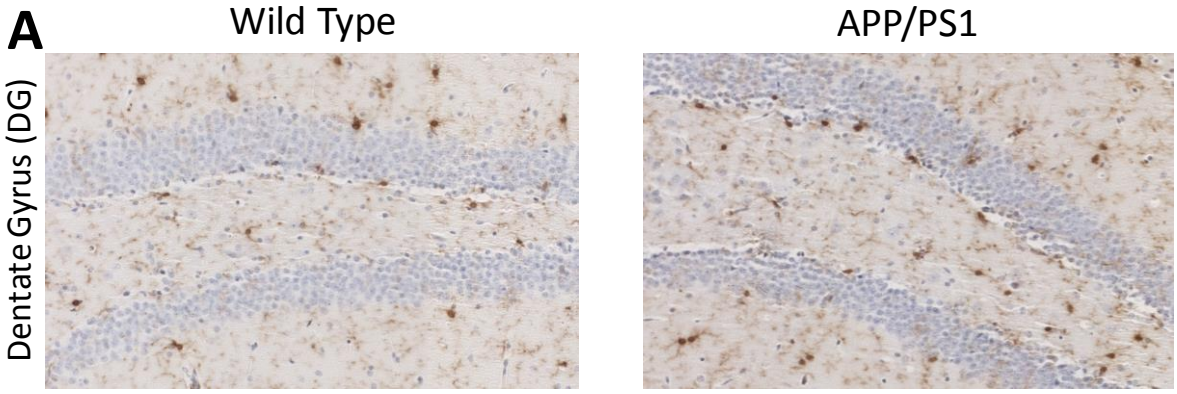


Figure 10: Representative samples of iba1-stained microglia (20x magnification) in the dentate gyrus in wild type and APP/PS1 mice at 5 months of age.

Discussion

APP/PS1 mice had greater numbers of microglia than WTs in dentate gyrus and dorsal striatum, but not in CA1, CA2 and CA3; numbers increased in all areas with age. In addition the number of microglial clusters in the APP/PS1 mice increased between 4 and 5 months in cortex, but not hippocampus; no clusters were seen in striatum, or in the WT mice.

General Discussion

The results of the present experiments suggest that the APP/PS1 mice showed abnormalities in their timing behavior. First, they had greater values of spread than the WT mice for the delay CS in

Experiment 1 and for the *20s* CS in Experiment 2, an observation which is consistent with more variable timing - an exact parallel of what is found in human participants (Caselli et al., 2009, Nichelli et al., 1993, Carrasco et al., 2000, Rueda and Schmitter-Edgecombe, 2009, El Haj et al., 2016, Hellstrom and Almkvist, 1997) It is unclear why the effect on spread was not evident for the trace CS in Experiment 1 or the shorter *10s* CS in Experiment 2, although both might be due to ceiling effects making differences in spread more difficult to detect. In Experiment 1 the pattern of results suggested that the mice were using trace CS *offset* to time food delivery 5s later, making food delivery easier to time than for the 15-s delay CS. Similarly, in Experiment 2 the *10s* would have been easier to time than the longer, *20s* CS, again making ceiling effects in timing more likely.

The APP/PS1 mice also had greater *Start* AUC scores for both CSs in Experiment 1, and for the *20s* CS in Experiment 2, although the *Stop* AUC scores did not differ. This is consistent with the effect on spread in suggesting reduced precision of timing, at least before the criterion time. This asymmetric effect of genotype on the ascending and descending section of the response functions also bears on an alternative interpretation of our results. Although it has been supposed that peak procedure performance is independent of motivational factors such as US magnitude (e.g. Roberts, 1981), there is evidence that the resulting response distributions may be influenced not only by timing ability, but also *impulsivity*. Single trial analysis reveals that animals' response rates abruptly switch from a state of low to high responding near the criterion time, and then abruptly drop again. A tendency to respond impulsively could bring forward the transition to the high state in this low/high/low pattern, raising the ascending arm of the average response function – and in principle explain the pattern of results we observed. However, this effect should be symmetrical and also delay the second transition to the low response state –elevating the descending section of the averaged functions as well. Matell & Portugal (2007) have provided evidence in support of this. In an elegant study they attempted to remove the contribution of impulsive responding on FI schedule performance by superimposing an additional behaviour-dependent variable-interval reinforcement schedule on a different manipulandum, in which the animal only had the possibility of obtaining food if it was making a second response. They observed that this decreased the spread of the FI response functions, effectively increasing the precision of timing

– and critically this effect was symmetrical: single trial analysis revealed that both low/high and high/low transition points moved closer to the criterion time (although the differences in the averaged functions did appear numerically larger before the criterion time). On this basis we think it unlikely the asymmetrical effect we observed can be accounted for in terms of impulsivity.

Another aspect of our findings that is difficult to explain in terms of impulsivity is the effect we observed of genotype on timing *accuracy*: in Experiment 2 the APP/PS1 mice showed significantly earlier peak times than the wild types for the *20s* CS (cf. Gür et al., 2019b). This may help explain the asymmetric effect on *Start* and *Stop* AUC values – shifting the function to the left in this way would selectively increase *Start* AUC values even without any concomitant effect on spread (cf. Figure 6 Panel 2b). Of course, a leftward shift in the timing function should also reduce the *Stop* AUC values, which we did not observe, but this may have been offset by an increase in spread. Although effects of AD on timing accuracy have not yet been reported in human participants, they are consistent with the effect on hippocampal function we anticipated. The overall profile in the APP/PS1 mice of greater values of spread accompanied by earlier peak times bears a close resemblance to the effects of dorsal hippocampal damage on timing (Tam and Bonardi, 2012a, Tam and Bonardi, 2012b, Bonardi, 2001, Meck, 1988, Meck et al., 2013). Meck's original studies (Meck et al., 1984, Meck, 1988) demonstrated reliably shortened peak times after fimbria-fornix lesions, and various authors have reproduced these findings in animals given chemical lesions of dorsal hippocampus (Tam et al., 2013, Tam and Bonardi, 2012a, Balci et al., 2009, Yin and Meck, 2014) - an effect which forms the basis of a recently proposed computational model of hippocampal function (Oprisan et al., 2018). In addition Tam et al., (2015) conducted a set of generalised linear mixed-effects models on their data which revealed, in addition to the effect on timing accuracy, an impairment in timing precision that was systematically related to the degree of dorsal hippocampal damage sustained. This previously unidentified effect resembles the effects found in the present experiments, and is further supported by the significantly higher levels of microgliosis in the DG area of hippocampus in the brains of our young APP/PS1 animals.

The suggestion of hippocampal involvement in the early cognitive deficits of AD is supported by the results of previous studies, showing a variety of hippocampal impairments emerging around 3 months of age in this transgenic model, such as a reduction in *c-fos* and *arc* (which are known to

upregulate in learning and memory) and the reduction in cyclic-AMP response element binding protein (CREB) (Christensen et al., 2013, Pedros et al., 2014, Ettcheto et al., 2018). In addition impaired LTP (Vegh et al., 2014, Volianskis et al., 2010) in hippocampal slices, reduction in dentate resting membrane potentials, action potential threshold (Minkeviciene et al., 2009), and a decrease in theta and increase in gamma activity, via EEG recording (Papazoglou et al., 2016) have all been reported. Moreover, this strain also shows impairments in hippocampal-dependent spatial memory before amyloid plaque deposition (Bonardi et al., 2011, Vegh et al., 2014, Edwards et al., 2014).

Nonetheless, it is also possible the results reflect the cholinergic disruption that accompanies AD (Francis et al., 1999, Ferreira-Vieira et al., 2016). For example, Balci and colleagues (2008), in a peak procedure with mice, found that the cholinergic antagonist scopolamine reduced timing precision, while physostigmine (a cholinesterase inhibitor) enhanced it. Moreover, APP/PS1 mice show hippocampal-specific alterations in cholinergic function. APP/PS1 mice at 3.5 months show a reduction in acetylcholine and choline acetyltransferase, which are both positively correlated with impaired performance on a spatial memory task, whilst soluble β-amyloid was inversely correlated with acetylcholine and choline acetyltransferase levels (Zhang et al., 2012). By 5-6 months APP/PS1 mice show a reduction in the release of preformed and newly synthesised acetylcholine in the hippocampus (Machova et al., 2010). It has been suggested that this cholinergic dysfunction is caused by soluble β-amyloid, which in turn causes the cognitive deficits (but see Goto et al., 2008, Perez et al., 2007). Thus early soluble β -amyloid-induced disruption of hippocampal cholinergic function could in principle be a driving force behind the effects on timing precision and accuracy observed in these experiments.

Another possibility is that the effects on timing stem from damage to the medial prefrontal cortex (mPFC). Buhusi et al (2018) recently reported local GABAergic inactivation of mPFC with muscimol infusions resulted in more variable timing on a dual-peak interval task, just as was seen in our experiments. However, they found timing accuracy remained intact, while here effects on accuracy, albeit limited to the longer CS, were found in Experiment 2. The prefrontal cortex is an area of early plaque deposition (Bonardi et al., 2011, Kim et al., 2012) and undergoes impairments in synaptic plasticity (Christensen et al., 2013) and cortical hyper-excitability (Gurevicius et al., 2013) in the

APP/PS1 mouse. Alternatively, the effects on timing observed here and by Buhusi et al. (2018) might be due to a functional connection between mPFC and hippocampus. Glutamatergic projections are found between ventral hippocampus and mPFC, while mPFC also connects to the CA1 and CA3 regions of dorsal hippocampus (Sigurdsson and Duvarci, 2015), and there is also strong evidence for synchronous communication between these two regions during learning and recall (Sigurdsson and Duvarci, 2015, Wierzynski et al., 2009, Colgin, 2011).

We also reported elevated levels of microglia in the dorsal striatum of the APP/PS1 mice, which increased between 4 and 5 months; we believe this is the first study to demonstrate such an effect in striatum which, along with the dopaminergic system, have been implicated in timing behavior (Macdonald et al., 2012, Matell et al., 2003); thus this could also in principle underlie the behavioural timing effects we observed. For example, MacDonald et al (2012) showed that intracerebral infusions of anisomycin (a protein synthesis inhibitor) into the dorsal striatum affected start and stop response thresholds in a peak-interval timing task, while Parkinson's patients, who suffer degeneration of the basal ganglia, overestimated stimulus durations, but performed veridically when their dopamine levels were restored by medication (Malapani et al., 1998). However, under other conditions they both under- and over-estimated durations, making the nature of the effect on accuracy less clear. Moreover, the variability in Parkinson's patients' timing does not seem to be affected by the disease (Claassen et al., 2013). Animal studies complement these findings. Maricq and Church (1983) found that rats also overestimated elapsed time when treated with methamphetamine (neurotoxic to dopaminergic neurons), but did the opposite with haloperidol (a dopamine antagonist). Such evidence suggests that striatal dopamine plays a key role in timing, and striatum features heavily in current timing models (e.g. striatal beat frequency model; (Meck, 2005). Nonetheless, the documented effects of striatal and dopaminergic manipulations are less clearly paralleled in our results than the effects of dorsal hippocampal damage: Our most consistent finding was the increase in timing variability in the APP/PS1 mice, an effect which has not been reported in studies of dopaminergic manipulation.

In summary, these experiments demonstrated increased timing variability, and in Experiment 2 shorter peak times, in the APP/PS1 mouse. These effects were evident at 4-5 months of age, and seemed unaffected by age, at least up to 8 months. Although similar findings on timing variability have been

reported in patients, to our knowledge there are only previous two studies that have examined timing in transgenic models of AD (Gür et al., 2019a; 2019b), and neither found an effect on timing variability. This may be related to procedural differences between the two sets of studies, a marked one of which is that Gür et al. conducted extensive testing comprising 29-39 training sessions with intermixed probe trials, whereas our study - in part to avoid compromise testing the animals in the critical time window - employed far fewer. It may be that extensive testing overcomes any tendency to increased variability by allowing better learning of the target intervals. In this sense perhaps our tasks had more in common with human studies, which predominantly report increased variability in patients after brief testing (Carrasco et al., 2000, Caselli et al., 2009, El Haj et al., 2013, Hellstrom & Almkvist, 1997; Nichelli et al., 1993, Rueda & Schmitter-Edgecombe, 2009; but see Heinik et al., 2012). But further experimental work will be required to address such speculations.

Gür et al. (2019b) did, however, report significant underestimation of the target interval indicating an effect on timing accuracy, mirroring the one we reported in Experiment 2. Their study was conducted in 9-month-old female 5xFAD mice which have no tau mutation but in which β-amyloid appears in cortex and hippocampus from 1.5 months of age. These mice would thus be very likely to have developed clear hippocampal plaque pathology by the time of testing which could explain this finding because - as noted above - earlier peak time is a signature of dorsal hippocampal damage. This strengthens our confidence in the effect on accuracy we observed in Experiment 2, and also suggests that - given the absence of significant hippocampal plaque pathology in the 4-5-month old mice of Experiment 2a - the effect may be due rather to the neuroinflammation produced by elevated β-amyloid rather than plaques *per se*. Although it remains unclear why we observed no such an effect in our Experiment 1, it may be that the inclusion of trace trials interfered, for some reason, with detection of the effect.

In conclusion, this work strengthens the procedural parallel between mouse and animal research that should underpin future translational work, and may form the basis for future diagnostic screening tests, for which there is an urgent need. We also showed elevation in levels of microglia at 4-5 months, which reflects the neuroinflammation preceding plaque formation, in the dentate gyrus area of the

hippocampal formation and the dorsal striatum. Within the APP/PS1 group there was also an age-dependent increase in numbers of microglia clusters, which precede plaque formation, in cortex, and in addition evidence of clusters in hippocampus. Although either hippocampal or striatal damage could theoretically underlie the behavioural effects we observed, the pattern of findings more closely resemble that seen after damage to the dorsal hippocampus. It is to be hoped that these findings lay the basis for human translational work that will both broaden the range of cognitive diagnostic tests, and provide a firm translational basis for preclinical testing of potential treatments.

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