Research Article

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Title: Mid-life environmental enrichment increases synaptic density in CA1 in a mouse model of Aβ-associated pathology and positively influences synaptic and cognitive health in healthy ageing

Abbreviated title: Mid-life environmental enrichment

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Abstract

Early-life cognitive enrichment may reduce the risk of experiencing cognitive deterioration and dementia in later-life. However, an intervention to prevent or delay dementia is likely to be taken up in mid to later-life. Hence, we investigated the effects of environmental enrichment in wildtype mice and in a mouse model of Aβ neuropathology ($APP_{SWE}/PS1_{dE9}$) from 6 months of age. After 6 months of housing in standard laboratory cages, $APP_{SWE}/PS1_{dE9}$ (n = 27) and healthy wildtype (n = 21) mice were randomly assigned to either enriched or standard housing. At 12 months of age, wildtype mice showed altered synaptic protein levels and relatively superior cognitive performance afforded by environmental enrichment. Environmental enrichment was not associated with alterations to Aβ plaque pathology in the neocortex or hippocampus of $APP_{SWE}/PS1_{dE9}$ mice. However, a significant increase in synaptophysin immunolabelled puncta in the hippocampal subregion, CA1, in $APP_{SWE}/PS1_{dE9}$ mice was detected, with no significant synaptic density changes observed in CA3, or the Fr2 region of the prefrontal cortex. Moreover, a significant increase in hippocampal BDNF was detected in $APP_{SWE}/PS1_{dE9}$ mice exposed to EE, however no changes were detected in neocortex or between Wt animals. These results demonstrate that mid to later-life cognitive enrichment has the potential to promote synaptic and cognitive health in ageing, and to enhance compensatory capacity for synaptic connectivity in pathological ageing associated with Aβ deposition.

Interventions to reduce the burden of dementia-causing diseases are urgently needed given global ageing and the current lack of effective therapeutic approaches. Epidemiological investigations suggest an active cognitive lifestyle may offer protection against the clinical expression of dementia (Valenzuela et al. 2011), potentially by building resilience to the underlying pathology and compressing cognitive morbidity to later stages of disease. In order to mimic the benefits of enhanced cognitive stimulation, an 'environmental enrichment' (EE) paradigm in experimental animals is often employed. An EE paradigm typically involves the manipulation of an animals' environment in order to facilitate sensory, cognitive, and motor stimulation (van Praag et al. 2000; Nithianantharajah and Hannan 2006).

There is a wealth of evidence supporting EE-induced cognitive and neural benefit in a range of animal models of neurodegenerative disease (Nithianantharajah and Hannan 2006). However, the reported effects on a hallmark pathological feature of AD, Aβ plaques, in familial Alzheimer's disease (FAD) animal models have been variable. Following an EE intervention, reduction (Lazarov et al. 2005; Costa et al. 2007; Herring et al. 2011), no change (Arendash et al. 2004; Wolf et al. 2006; Cotel, Jawhar, Christensen, Bayer, and Wirths 2012), or an exacerbation of Aβ pathological burden (Jankowsky et al. 2003, 2005) has been reported in transgenic mice expressing human FAD-related gene mutations. Notably, many of these EE studies have involved such stimulation (EE) from weaning or in early-life. Despite the variation between studies regarding Aβ pathological alterations following EE, there is a consensus of marked protection of cognitive function following this intervention.

In this regard, the underlying mechanism allowing for cognitive protection is elusive. EE in other models may influence synaptic connectivity in order to provide cognitive benefit (Nithianantharajah and Hannan 2006). Moreover, brain-derived neurotrophic factor (BDNF), a protein expressed widely throughout the central nervous system, vital for the maintenance,

survival, and growth of neurons (Mattson, Maudsley and Martin, 2004) has been implicated in both AD and EE. As BDNF mediates synaptic plasticity and cognitive function (e.g. Murer et al, 2001; Lu, 2003), it is thought that BDNF may be critically involved in the pathophysiology underlying cognitive decline in AD. Moreover, reduced levels of BDNF are found in the hippocampus and frontal and parietal cortices of the AD brain (Ferrer et al, 1999; Hock et al, 2000). BDNF promotes synapse formation (Park and Poo, 2013) and thus could be a potential target for diseases of synaptic plasticity failure, such as in AD. To date, BDNF has not been able to be delivered across the blood-brain barrier (Lu et al, 2013). However, some evidence suggests EE may be able to increase endogenous levels of BDNF in healthy animals (Ickes et al., 2000; Ramirez-Rodriguez et al, 2014; Novkovic et al, 2015).

With respect to human interventions of relevance to dementia and EE, the benefit of early-life cognitive stimulation, in the form of education, on later-life cognitive function has been well reported (e.g. Anstey and Christensen 2000; Lenehan, Summers, Saunders, Summers and Vickers 2014). However, research on the potential protective effects of later-life cognitive engagement and enrichment is limited. The Tasmanian Healthy Brain Project (THBP; Summers et al. 2013) has produced the first research on the potential benefit of formal late-life education on cognitive reserve (Lenehan et al. 2015). However, investigation of later-life EE in animal models of neuropathology has been limited. Moreover, whether cognitive intervention introduced at the inception of AD-neuropathology produces benefit is unknown. This concept is difficult to test in human cases considering dementia is typically detected many years after the AD-related neuropathology forms (Braak and Del Tredici 2011).

In the current study, we investigated the effects of an EE intervention in a FAD transgenic model ($APP_{swe}PS1_{dE9}$ line; APP/PS1) from 6 months of age to model an intervention targeted to mid-life. The APP/PS1 mouse model begins to show Aβ plaque

pathology by 6 months of age (Garcia-Alloza et al. 2006) and most closely resembles earlier stages of human AD in terms of synapse loss and neuritic pathology (Mitew, Kirkcaldie, Dickson and Vickers, 2013a, b). It was hypothesized that EE would produce positive cognitive effects associated with less vulnerability of, or increased levels of synaptic markers, rather than a reduction in Aβ neuropathology.

We examined region-specific alterations in synapses following EE. The dorsomedial region of the murine prefrontal cortex, frontal area 2 (Fr2), involved in complex cognitive functions (Uylings et al. 2003) was selected. In addition, we examined the hippocampal subregions CA1 and CA3 due to their role in spatial learning and memory (Anderson et al. 2006; Kesner 2007). These regions are not only potential targets of EE evidenced by increases in working memory and spatial learning and memory in rodent models exposed to EE, but are also highly susceptible to AD (Vickers et al. 2000; Patrylo and Williamson 2007; Morrison and Baxter 2014).

Materials and Methods

Animals and EE protocol

Male transgenic mice expressing chimeric mouse/human amyloid precursor protein (APP) and mutant human presenilin1 (PS1) on C57BL/6 background [B6.Cg-Tg (APPswe, $PSEN1_{dE9}$) 85Dbo/J; RRID_MGI:3665286] (APP/PS1; Jankowsky et al. 2004) were used for the present study. This transgenic mouse model recapitulates pathology that most closely resembles the initial stages of AD, and Aβ deposits have been observed from 4-6 months of age in this model, with abundant plaques present by 9 months (Jankowsky et al. 2004; Garcia-Alloza et al. 2006; Vickers et al. 2009). All animals lived in standard housing (SH) conditions comprising group housing of 4-5 mice per 30 x 30 x 14 cm cage, *ad Libitum*

access to food and water, an igloo, one small wooden stick and one tissue. $APP_{swe}PSEN1_{dE9}$ (APP/PS1; $n = 27$) and littermate wildtype control (Wt; $n = 21$) mice were randomly assigned to SH or EE conditions at 6 months of age, for the following 6 months. The EE housing condition consisted of a larger 60 x 30 x 14 cm cage with the contents of the SH cage and the addition of enrichment objects (wooden and plastic blocks of differing shapes and sizes, platforms, a ball, running wheel, and a mouse hut). Housing conditions were maintained until the 12-month end-point.

Cognitive and behavioural assessment general protocol

Mice were handled daily and habituated to the test room for 7 days prior to testing to minimize the effects of stress due to handling at time of testing. All cognitive testing procedures occurred at the same stage of the light cycle. Black curtains surrounded the test equipment during testing, in order to prevent the mice becoming distracted, or from using extra-maze cues. Testing equipment was cleaned with 70% ethanol between trials in order to prevent odour cues. The experimenter was blind to the genotype of animals. Noise was minimized and lighting was kept constant throughout all testing. Exclusion criteria for behavioural testing were defined prior to testing, and adhered to. All testing was recorded for later analysis using a JVC digital camera mounted to the ceiling.

Y Maze spatial short-term memory

Hippocampal-dependent spatial short-term recognition memory was assessed by the Y maze. The two-trial Y maze task is a test based on the innate tendency of rodents to explore novel environments (Dellu et al. 2000; Wang et al. 2006). All mice underwent a 10-minute acquisition trial, where a randomly assigned arm of the Y maze was blocked (novel arm). The testing phase began after a 1-hour inter-trial interval, where all three arms of the

maze were open for exploration for 5 minutes. Y maze testing was recorded for later analysis, where the percentage of time spent in the novel arm was calculated as a percentage of the total time in all three arms. Testing was performed immediately before being assigned to differential housing, in order to establish baseline performance, after 3 months of differential housing and finally after 6 months at end-point.

Barnes maze spatial learning & long-term memory

The Barnes maze test of spatial learning and long-term memory was performed following 6 months of differential housing. Mice were initially habituated to the maze by being able to freely explore the maze for two 5-minute adaptation trials. Twenty-four hours after adaptation, a seven-day training period began of two trials per day. The trial ended when the mouse had climbed into the hidden escape, or after 5 minutes had elapsed. Fourteen days after the 7-day training period had elapsed, long-term memory was assessed with four trials. The latency to reach the escape box in each trial was recorded for analysis.

Tissue collection

Following the final day of behavioural testing, mice were terminally anaesthetized first with gas anaesthesia (isoflurane) followed by sodium pentobarbitone (100 mg/kg delivered intraperitoneally). For histology (Wt n = 11; APP/PS1 n = 17), animals were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS pH 7.4). Postmortem brains were transferred to 18% then 30% sucrose solutions overnight. Brains for histology were serially sectioned on a cryostat (Leica CM 1850) in 40 µm coronal sections. Sections used for analysis were from bregma 2.0 - ¯3.0 mm according to the stereotaxic mouse brain atlas (Paxinos and Franklin 2008). For Western blotting procedures, animals (Wt n = 10; APP/PS1 n = 10) were transcardially perfused with PBS

(0.1M). Postmortem brains were removed and the neocortex and hippocampus were dissected and immediately frozen in liquid nitrogen, and samples were stored at -80°C for later analysis.

Antibody characterization

All antibodies used in the present study are commercially available and have been previously characterized (see Table 1 for references). Optimal antibody concentrations were determined for each antibody. Control experiments were performed by omitting primary antibodies, which eliminated all immunoreactivity. Aβ deposits were detected by immunohistochemistry using a mouse monoclonal antibody (MOAB-2) with an epitope at residues 1-4 (manufacturer information). The MOAB-2 antibody is raised against human Aβ, and recognises human unagreggated, oligomeric, and fibrillar forms of $A\beta_{42}$, but not APP (Youmans et al., 2012). Previous work from our lab has confirmed the specificity and labelling pattern of the MOAB-2 antibody, which co-labels with the commonly used anti-Aβ antibody, 6E10 (Collins et al., 2015). Synaptic puncta were visualized by immunolabelling with the pan-presynaptic marker, synaptophysin (RRID:AB_570874). The synaptophysin antibody is a polyclonal antibody raised in rabbit, the immunogen is a full-length (313 amino acids) synthetic peptide from human synaptophysin, and labels a single band at approximately 38 kDa on western blots (manufacturer's information).

The following antibodies were used for Western blot in the present study, and recognize the expected band on a Western blot of mouse brain tissue. The molecular sizes of the immunoreactive bands are: mouse anti-VGlut1 (RRID:AB_262185), 60 kDa; mouse anti-PSD-95 (RRID:AB_303248), 95 kDa; Rabbit anti-Gephyrin (RRID:AB_1860491), 80 kDa; mouse anti-GAD65 (RRID:AB_2263126), 65 kDa; mouse GAD67 (RRID:AB_2278725), 67 kDa; mouse anti-β-actin (internal control; RRID:AB_476743), 42 kDa. The antibody rabbit

anti-BDNF (RRID:AB_630940) was used for ELISA, the antibody detects precursor and mature BDNF, and labels bands at 32 kDa (precursor) and 14 kDa (mature) (manufacturer information). Table 1 shows full antibody information.

Quantitation of Aβ plaques

For the quantitation of Aβ deposits, antigen retrieval by formic acid treatment was performed in order to enhance immunoreactivity for the visualization of Aβ deposits (Kai et al. 2012). Ten sections evenly spaced across the rostrocaudal extent of the brain (bregma 2.0 - ¯3.0 mm) were incubated in formic acid (Sigma-Aldrich) at room temperature for 8 minutes, followed by 6 x 10 minute PBS washes. Free-floating sections were washed for 3 x 10 minutes in 0.25% Triton-X-100 and incubated with serum-free protein block (Dako) for 15 minutes at room temperature. The sections were immunolabelled with the MOAB-2 antibody (1:2000; Novus Biologicals, Table 1) which specifically labels mouse and human un-aggregated Aβ, following the protocol outlined in Collins, King, Woodhouse, Kirkcaldie and Vickers (2015). MOAB-2 labelling was visualized by incubation in Alexa-fluorophore conjugated secondary antibody (1:1000; Molecular Probes, goat anti-mouse IgG2b-546) and cover-slipped using Dako fluorescent mounting medium.

Quantitation of synaptophysin immunoreactive puncta

To determine region-specific alterations in synaptic density, synaptophysin immunoreactive puncta were quantitated histologically in the dorsomedial region of the murine prefrontal cortex, frontal area 2 (Fr2; Van De Werd, Rajkowska, Evers and Uylings 2010) and the hippocampal subregions CA1 and CA3 (Paxinos and Franklin 2008). Three sections containing Fr2 (bregma 1.98 – 0.38 mm) and three sections that contained CA1 and CA3 (bregma ¯1.28 - ¯2.12 mm) were immunostained with primary anti-synaptophysin

antibody (1:200; Millipore, Table 1; RRID:AB_570874) according to standard procedures (Collins et al. 2015) using Alexa-fluorophore secondary antibody, goat-anti-rabbit IgG 594 (Molecular Probes). To highlight architecture, sections were also incubated with the nuclear stain DAPI (5 μg/mL; Invitrogen) for 5 minutes at room temperature. Sections were mounted using fluorescent mounting medium (Dako).

Image acquisition

In order to determine Aβ plaque load in the neocortex and hippocampus, images were obtained with a Leica DM fluorescence microscope on a 10x objective and NIS Elements imaging software. Ten sections evenly spaced from the rostral to caudal extent of the neocortex from bregma position 2.0 to -3.0 mm were imaged for MOAB-2 Aβ plaque load. The left side of the neocortex was imaged from the midline to the rhinal fissure. Images of the whole hippocampus were taken between bregma position -1.22 and -2.46 mm of 3-5 sections per animal. Aβ plaque load (defined as percentage area occupied by MOAB-2 immunoreactivity) was determined by applying a custom plugin for ImageJ (RRID:SCR_003070) to the plaque images, which automatically segmented images as plaques or background pixels by random forest classification, as described by Sommer et al. (2011). The classifier was trained with a random selection of example plaque images from the data set, which were annotated in order to distinguish plaques from background pixels.

Imaging of synaptophysin labelling was performed on a Perkin-Elmer Ultraview VOX confocal imaging system with Volocity 6.3 (RRID:SCR_002668) imaging software. All images were acquired with the same laser power and exposure settings, using a 60x objective. Five images (image window size = 118.15 x 118.15 μm)/section were taken within Fr2, CA1, and CA3 subregions across three sections/animal. For APP/PS1 animals, Aβ plaque free regions were included in the analysis. These images then underwent image

segmentation and random forest classification in order to distinguish synaptic puncta from background pixels using the custom ImageJ plugin as described for plaque load analysis. An ImageJ watershed algorithm with Gaussian blurring ($\sigma = 1$) was applied to the segmented images. Particles ranging from 0.15 to 2.0 μm^2 were quantified (Mitew et al. 2013a). Synaptic density was calculated as the number of synaptophysin immunolabelled puncta per field (118.15 x 118.15 μm), corrected for cell body area.

Western blotting

The right side of the neocortex and hippocampus were homogenized in RIPA buffer (Sigma) containing a protease (Roche diagnostics) and phosphatase inhibitor cocktail (AG Scientific). The samples were centrifuged at 13000 RPM for 15 minutes, rotated for a further 30 minutes, and centrifuged again at 4°C for 15 minutes at 13000 RPM. The resulting supernatant was removed and stored at -80°C for protein analysis. The protein concentrations of samples were determined using the Bradford assay. Samples were prepared as a total volume of 10μl containing 10μg of protein per lane. The samples were separated (3 repeats/sample) on a 12% NuPage Novex Bis-Tris gel (Invitrogen) by electrophoresis at 200V for 20 minutes at room temperature. Following, proteins were transferred to an activated PVDF membrane at 20V for 60 minutes. Membranes were blocked for 2 hours in 5% commercial skim milk powder. Membranes were incubated in primary antibodies overnight at 4°C in combinations of rabbit anti-synaptophysin (1:2000, Millipore; RRID:AB_570874); mouse anti-VGlut1 (1:1000, Millipore; RRID:AB_262185); mouse anti-PSD-95 (1:1000, Abcam; RRID:AB_303248) mouse anti-GAD65 (RRID:AB_2263126); mouse anti-GAD67 (RRID:AB_2278725) (1:1000, Millipore), rabbit anti-Gephyrin (1:1000, Abcam; RRID:AB_1860491) and anti-β-actin (1:5000, Sigma-Aldrich; RRID:AB_476743) (Table 1). Membranes were washed in Tris-buffered saline with 0.1% Tween-20 (Sigma) and

incubated in species-appropriate secondary antibodies (1:7000, Dako). Bands were visualized by staining with a chemiluminescent substrate kit (Millipore).

BDNF Enzyme-linked immunosorbent assay (ELISA)

Neocortex and hippocampal samples were prepared as described for Western blotting. Briefly, each sample was prepared in duplicate and diluted in coating buffer (1:100; 60% $NaHCO_3$, 30% $Na2CO_3$ in distilled water), and 50 µl of diluted sample was added per well to a 96-well flat-bottomed plate (Costar 5395, Sigma-Aldrich) and incubated at 4°C overnight. Following overnight incubation, the plate was washed five times with washing buffer (0.05% tween-20 in 0.01M PBS). Following, 100 µl of blocking buffer (5% fetal calf serum in 0.01M PBS) was added to each well and incubated at 37°C for 30 minutes. Following five washes, 50 µl of diluted primary BDNF antibody (1:500; Santa Cruz, Table 1; RRID:AB_630940) was added to each sample well, and incubated at room temperature for 1 hour. Five washes were undertaken and the secondary HRP antibody was added (1:2000; anti-Rabbit, Dako, Table 1) and incubated at room temperature for 45 minutes. Following washing, 100 µl of freshly prepared Tetramethylbenzidine (TMB; Sigma-Aldrich) substrate was applied to each well for 10 minutes, and 0.1M Sulphuric acid was added to stop the colour reaction. Optical densities were read at 450 nm on a microplate reader (SpectraMax, Molecular Devices), and concentrations of BDNF were determined by comparison to the standard curve using a 4-parameter algorithm. Values were averaged between the duplicate samples, and expressed as a percentage relative to Wt controls.

Table 1. List of primary antibodies

Statistical analysis

Analyses were performed using IBM SPSS (Version 20; RRID:SCR_002865). Statistical analyses were performed using independent t-tests, two-way ANOVA, and repeated measures ANOVA. A statistically significant two-way ANOVA was followed up by separate independent t-tests. Variables considered were genotype (Wt or Tg) and housing condition (SH or EE). Values of $p < .05$ for differences between group means were classified as statistically significant.

Results

Pre-intervention and follow-up short-term memory

In order to assess cognitive changes following exposure to EE, we first established baseline short-term memory (STM) function using the Y maze at six- months of age. An independent t-test revealed APP/PS1 animals spent significantly less time in the novel arm of the maze compared to the Wts ($t_{(46)} = 3.15$, $p = 0.003$), an indication of a STM deficit. Following 3 months of differential housing, a two-way ANOVA demonstrated no significant genotype x housing effect on Y maze performance, $F_{(1, 42)} = 2.26$, $p = 0.14$. However, when Wt and APP/PS1 groups were analysed separately, an independent t-test demonstrated for APP/PS1 mice, those in EE spent significantly more time in the novel arm of the maze than those in SH ($t_{(18)} = 3.75$, $p = 0.002$) (Figure 1A) (Table 2).

Post-intervention short-term memory

Following 6 months of differential housing, at 12 months of age, no significant genotype x housing effect on Y maze performance was detected by two-way ANOVA ($F_{(1,}$

$_{43)}$ = 0.20, p = 0.66). Moreover, a two-way repeated measures ANOVA of the three testing time-points revealed that Y maze performance over time was not significantly affected by housing x genotype ($F_{(2, 82)}$ = 2.07, p = 0.13) (Figure 1A) (Table 2).

Table 2. Mean percentage time in novel arm of the Y maze

Post-intervention learning & long-term memory

A Greenhouse-Geisser corrected repeated measures ANOVA of learning on the Barnes maze demonstrated no significant main effect of genotype x housing on learning ($F_{(9.19, 280.18)}$ = 0.56, p = 0.87) (Figure 1B) (Table 3). In addition, a two-way ANOVA demonstrated no significant genotype x housing effect on long-term memory performance ($F_{(1, 46)}$ = 2.78, p = 0.10) (Figure 1C). A separate independent t-test of Wt animals, demonstrated that those in EE when compared to those in SH, demonstrated a significantly reduced latency to reach the escape on the LTM trial ($t_{(25)}$ = 2.07, p = 0.049). However, analysis of the Tg animals revealed EE did not have a significant effect on LTM ($t_{(10.94)}$ = 0.39, p = 0.71).

Table 3. Latency (s) to reach escape on the Barnes Maze learning trials

Aβ plaque load

In order to determine whether EE introduced after the onset of Aβ neuropathology alters β-Amyloid load, we analysed MOAB-2 immunolabelling, an antibody that recognises human and mouse unaggregated, oligomeric, and fibrillar forms of Aβ42 and unaggregated Aβ40 (Youmans et al. 2012). An independent t-test of Aβ plaque load in the neocortex measured by MOAB-2 immunolabelling, did not differ significantly according to housing condition at 12 months ($t_{(15)} = 0.65$, $p = 0.53$) (Table 4). Additionally, no significant difference in hippocampal MOAB-2 immunolabelling was detected by independent t-test ($t_{(15)} = 0.18$, $p = 0.86$) (Table 4) (Figure 2).

Table 4. MOAB-2 load (% area) of Neocortex and Hippocampus

Western blots of synaptic markers

In order to assess the effect of EE on synapses in APP/PS1 and Wt mice, we quantified levels of synaptic proteins in the neocortex and hippocampus by Western blotting, and analysed group differences by two-way ANOVA. A significant housing x genotype effect was detected for labelling with the pan-presynaptic protein, synaptophysin, in the neocortex ($F_{(1, 16)} = 8.12$, $p = 0.01$) (Figure 3B), and in the hippocampus ($F_{(1, 16)} = 9.43$, $p = 0.008$) (Figure 4B). Subsequent independent t-tests showed that Wt animals in EE, as compared to Wts in SH, had relatively increased cortical ($t_{(8)} = 3.22$, $p = 0.01$) and hippocampal ($t_{(8)} = 3.51$, $p = 0.008$) synaptophysin levels.

To further examine whether these effects could be attributed to excitatory or inhibitory synaptic changes, antibody markers VGlut-1, PSD-95, Gephyrin, and GAD65/67

were examined. No housing x genotype effect was detected in the excitatory synaptic marker VGlut-1 in the cortex ($F_{(1, 16)} = 0.15$, $p = 0.70$) (Figure 3C), or in the hippocampus ($F_{(1, 16)} = 0.007$, $p = 0.94$) (Figure 4C). No significant housing x genotype effect was detected for levels of the excitatory post-synaptic marker, PSD-95 in the cortex ($F_{(1, 16)} = 0.003$, $p = 0.95$) (Figure 3D). However, there was differential expression in the hippocampus ($F_{(1, 16)} = 8.99$, $p = 0.009$). When genotypes were analysed separately, Wt EE animals had higher hippocampal levels of PSD-95 compared to the Wts in SH ($t_{(8)} = 2.84$, $p = 0.02$) (Figure 4D). Gephyrin labelling was not significantly altered by the effect of housing x genotype in neocortex ($F_{(1, 16)} = 1.42$, $p = 0.25$) (Figure 3E) or in hippocampus ($F_{(1, 16)} = 1.13$, $p = 0.31$) (Figure 4E). However, analyzing the Wt and APP/PS1 animals separately demonstrated that enriched Wt animals had significantly lower hippocampal Gephyrin labelling as compared to those in SH ($t_{(8)} = 3.06$, $p = 0.02$). The inhibitory synaptic marker GAD65/57 was significantly affected by housing x genotype ($F_{(1, 16)} = 5.54$, $p = 0.03$). Analysing genotypes separately demonstrated increased GAD65/57 protein levels in neocortex of Wt EE animals ($t_{(7.45)} = 2.64$, $p = 0.03$) (Figure 3F). However, this effect was not detected in the hippocampus ($F_{(1, 16)} = 0.02$, $p = 0.90$) (Figure 4F).

Synaptophysin immunolabelling in Fr2, CA1, and CA3

Quantification of immunolabelled synaptophysin puncta of the dorsomedial region of the prefrontal cortex, Fr2, showed no significant housing x genotype alterations in synaptophysin ($F_{(1, 24)} = 0.07$, $p = 0.79$) (Figure 5). Housing x genotype did not significantly influence CA1 synaptophysin immunolabelling ($F_{(1, 24)} = 0.94$, $p = 0.34$). However, an independent t-test demonstrated that, for the APP/PS1 animals, EE produced a significant increase in synaptophysin density in CA1 ($t_{(16)} = 2.54$, $p = 0.02$) (Figure 6). However, no

significant effect of housing x genotype on levels of synaptophysin density was found within CA3, ($F_{(1, 24)} = 2.79$, $p = 0.12$) (Figure 7).

BDNF protein levels in neocortex and hippocampus

A two-way ANOVA demonstrated relative levels of BDNF were not altered in neocortex across genotype or housing condition ($F_{(2, 23)} = 0.88$, $p = 0.43$) (Figure 8A). A two-way ANOVA also revealed no genotype x housing effect on hippocampal BDNF ($F_{(2, 23)} = 1.04$, $p = 0.37$). However, when genotypes were considered separately, an independent t-test demonstrated a significant increase in hippocampal BDNF in APP/PS1 mice housed in EE compared to mice housed in SH ($t_{(8)} = 3.39$, $p = 0.009$) (Figure 8B).

Discussion

The global scale related to the predicted rise in the prevalence of AD (Prince et al. 2015) has led to increased research strategies aimed at delaying the onset of dementia. A substantial literature supports the proposal that a stimulating lifestyle beginning in early-life has beneficial later-life effects on cognitive ageing, potentially by buffering against pathological damage, compressing cognitive morbidity to later years. However, the effect of a cognitive intervention targeted at mid-life is not well understood. Moreover, whether cognitive stimulation can enhance the compensatory capacity of synaptic connectivity for existing pathology is unknown. Here, we have investigated the effect of mid-life EE in experimental models of normal ageing and Aβ neuropathology.

The results of the present study demonstrate that EE initiated after Aβ plaque deposition has begun does not modify the course of subsequent amyloid pathology. However, EE differentially produced changes in cognitive function and synaptic markers. The data demonstrated the relationship between EE and synaptic health and cognitive function was not

straightforward. Rather, we found EE in healthy ageing mice led to increased inhibitory synaptic markers in the cortex and elevated excitatory receptor markers in the hippocampus, along with superior long-term memory function. However, relative to Wt mice, and perhaps due to increased mutant APP/PS1 expression or AD-related pathology, the APP/PS1 mice did not show as extensive changes in synaptic protein levels or in cognitive function afforded by EE. However, EE produced an increase in synaptic density in CA1 in this model of Aβ neuropathology, and ameliorated some aspects of cognitive dysfunction associated with ageing and the APP/PS1 genotype.

With regards to the current study, there have been conflicting reports regarding the age of cognitive impairment onset in the APP/PS1 mouse model of AD. In the current report, a STM deficit was observed in APP/PS1 mice at 6 months of age. Other reports support the finding of the current study, where a STM deficit was observed at 6 months of age (Zhang et al. 2006; Aso et al. 2012; Izco et al. 2014). One explanation for an early STM deficit might be the dramatic increase in Aβ deposition at this time-point (Izco et al. 2014), resulting in neural disruption at the microcellular level.

Y maze testing after 3 months of EE demonstrated improved STM performance by the APP/PS1 animals. This finding suggested EE can abate the initial STM deficit in this mouse line. However, this beneficial effect did not persist at 12 months of age, with STM performance being similar across all animal groups. Moreover, Barnes maze testing at 12 months demonstrated superior LTM following 6 months of EE in Wt mice. This finding suggests that LTM function in ageing can benefit from EE, however the presence of Aβ pathology may diminish this benefit. Although the present study had a relatively large sample size for FAD transgenic studies, our enrichment paradigm may have induced more subtle cognitive changes that we did not have power to detect in the current study. One other interpretation for the lack of cognitive benefit to the APP/PS1 mice, is the increasing burden

of Aβ pathology by 12 months attenuated the effect of EE. The results must also be interpreted with the features of this mouse model in mind, this APP/PS1 model being most closely aligned to the long, preclinical stage of human AD (Mitew et al. 2013a, b).

Consistent with several studies, the current data showed that Aβ plaque burden was not modified by EE (Jankowsky et al. 2003, 2005; Arendash et al. 2004; Wolf et al. 2006; Cotel et al. 2012), indicating that this intervention does not modify disease process progression. However, other studies have reported EE to directly attenuate Aβ neuropathology (Lazarov et al. 2005; Cracchiolo et al. 2007). Potential discrepancies may be due to the time-point at which enrichment was initiated. The majority of studies have investigated EE introduced at weaning, or in early-life, and before the deposition of Aβ plaque pathology. Arendash et al. (2004) in an APP_{SW} model of AD, also demonstrated that later-life EE did not reduce Aβ load. Relatedly, Verret et al. (2013) reported decreased Aβ load following early-life EE, whereas EE introduced later in life did not lead to reductions in Aβ burden. These findings together suggest that EE does not have a modifying effect on plaque formation once Aβ deposition has commenced.

EE as a potential non-pharmacological intervention may have effects on the structural plasticity of synapses. In this respect, there have been a number of studies that have demonstrated synaptic changes as a result of EE (Nithianantharajah and Hannan 2006). An increase in the expression of synaptophysin has been demonstrated following EE in healthy Wt animals in the neocortex and hippocampus (Nithianantharajah et al. 2004; Lambert et al. 2005; Birch et al. 2013). However, these results were obtained in young animals, and the relationship between enrichment, synaptophysin, and cognitive function in ageing is less clear. The present study demonstrated EE in ageing promotes synaptophysin expression in cortical and hippocampal regions, and is associated with relatively superior LTM performance. It has been suggested that, as synaptophysin is a component of

neurotransmitter-containing presynaptic vesicle membranes, the increase in synaptophysin is potentially reflective of an increase in neurotransmission, that may in turn lead to improved spatial LTM (Frick and Fernandez 2003).

The Wt mice that experienced EE exhibited relatively increased levels of inhibitory synaptic proteins in the cortex, and both increased excitatory and decreased inhibitory synaptic protein levels in the hippocampus. Cortical inhibition is vital in coordinating network activity and maintaining cortical function (Isaacson and Scanziani 2011). In contrast, the APP/PS1 mice from both housing conditions exhibited relatively high levels of both inhibitory and excitatory synaptic protein, suggesting an imbalance of the interplay between excitatory and inhibitory activity. A high level of Aβ is associated with aberrant excitatory network activity and compensatory inhibitory responses (Palop and Mucke 2010). Aβ may affect excitatory and inhibitory synapses differently, thereby creating complex imbalances in neuronal circuit activity.

While both AD and EE induce widespread alterations to the brain, in AD some brain regions are more affected than others. One notable finding of the present study was the increase in the number of synaptophysin labelled puncta within the CA1 region of EE APP/PS1 mice. Consistent with these findings, CA1 activity has been found to increase when rodents are exposed to novel environments (Nitz and McNaughton 2004; Cracchiolo et al. 2007; Csicsvari et al. 2007; Karlsson and Frank 2008). Similarly, an increase in CA1 synaptic density in healthy rodents following EE has been previously reported (Moser, Trommald, & Andersen, 1994; Moser, Trommald, Egeland and Andersen, 1997; Rampon et al. 2000; Malik and Chattarji, 2012).

Although the finding of increased CA1 synaptic connectivity following EE has been previously reported, the novelty here is that we found an increase in CA1 synaptic density in a model of Alzheimer's pathology, suggesting that this region retains capacity for experience-

dependent plasticity in response to EE, despite the presence of accumulating Aβ neuropathology. An increase in the number of synapses in plaque-free brain regions in this mouse model at 12 months of age has been previously reported (West, Bach, Søderman and Jensen, 2009; Mitew et al. 2013a). While the CA1 region is not plaque free, Aβ plaques occupy only a negligible fraction of CA1 in FAD mouse models (Merino-Serrais et al. 2011). Early-stage AD is characterized by attenuated synaptic plasticity, triggering a compensation response by the formation of new synapses in an attempt to preserve synaptic connectivity (King and Arendash 2002; Selkoe 2002; Boncristiano et al. 2005; Jansen et al. 2012). Here, the heightened number of synaptic contacts in the APP/PS1 mice may be a compensatory response to synaptic dysfunction (West et al. 2009). As EE targets CA1, it follows that the combination of this compensation response paired with stimulation of CA1 by EE would lead to an increase in synaptic density within this region.

The finding of an increase in hippocampal BDNF in APP/PS1 mice exposed to EE might suggest BDNF to be a mediator of the beneficial effects of EE. Stimulation of CA1 has been observed to increase hippocampal BDNF levels (Kealy and Commins, 2010). Moreover, hippocampal BDNF plays a critical role in learning and memory processes (Cowansage, Ledoux, and Monfils, 2010), potentially allowing for the APP/PS1 mice with unaltered Aβ plaque burden to demonstrate superior STM following 3 months of EE.

The findings presented here indicate EE initiated in mid to later-life promotes an array of beneficial effects in healthy ageing in terms of promoting synaptic and cognitive health. Such findings are remarkable considering the lack of stimulation in early-life, suggesting later-life interventions may be able to overcome some of the negative effects of minimal stimulation earlier in life. However, when ageing also involves Aβ neuropathology, EE does not influence global positive effects as seen in healthy ageing. However, EE after Aβ deposition has commenced, was associated with a more specific alteration, an increase in the

number of synaptic contacts in the AD-vulnerable CA1 subregion, and an increase in hippocampal BDNF protein levels. These data demonstrate that plasticity processes are afforded by EE in specific brain regions despite the presence of accumulating Aβ neuropathology. Hence, non-pharmacological interventions such as EE introduced during the preclinical stage of disease may promote compensatory mechanisms that enhance synaptic connectivity. Moreover, the present study demonstrates that a mid to later-life intervention based on EE has the potential to promote synaptic and cognitive health in healthy ageing.

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Figure legends

Figure 1. Y maze and Barnes maze performance. **A** Mean percentage of time spent in the novel arm of the Y maze (± SEM) at baseline, and after 3 and 6 months of differential housing. Baseline testing at 6 months of age demonstrated APP/PS1 mice spent significantly less time in the novel arm of the Y maze, $t_{(46)} = 3.15$, $p. = .003$. After 3 months of housing, no genotype x housing effect on Y maze performance was detected, $F_{(1, 42)} = 2.26$, $p. = .14$. Following 6 months of differential housing, no significant genotype x housing effect on Y maze performance was detected, $F_{(1, 43)} = 0.20$, $p. = .66$. **B** Mean latency (± SEM) to reach

the escape on the Barnes maze over 7 days of learning trials at 12 months of age. No significant main effect of genotype x housing on learning was detected, $F_{(9.19, 280.18)} = 0.56$, $p. = .87$. **C** Long-term memory performance on the Barnes maze expressed as the mean latency (± SEM) to reach the escape over four trials. The EE Wt animals on average had significantly reduced latency to reach the escape, $t_{(25)} = 2.07$, $p. = .049$.

Figure 2. Representative APP/PS1 mouse brain sections stained with the MOAB-2 antibody. **A** APP/PS1 mouse brain coronal section with MOAB-2 positive plaques from SH. **B** APP/PS1 mouse brain coronal section with MOAB-2 positive plaques from EE.

Figure 3. Mean synaptic protein levels expressed as a percentage relative to Wt control (± SEM) from neocortex samples. A Representative Western blot bands per group for each synaptic marker. **B** Synaptophysin: EE Wt mice had higher relative levels of cortical synaptophysin, $t_{(8)} = 3.22$, $p.= .01$. **C** VGlut-1: No significant differences across housing x genotype were detected, $F_{(1, 16)} = 0.15$, $p. = .70$. **D** PSD-95: No significant differences across housing x genotype were detected, $F_{(1, 16)} = 0.003$, $p. = .95$. **E** Gephyrin: No significant differences across housing x genotype were detected, $F_{(1, 16)} = 1.42$, $p. = .25$. **F** GAD65/67: Wt EE mice showed significantly increased levels of GAD65/67 compared to those in SH, $t_{(7.45)} = 2.64$, $p. = .03$.

Figure 4. Mean synaptic protein levels expressed as a percentage relative to Wt control (± SEM) from hippocampus samples. **A** Representative Western blot bands per group for each synaptic marker. **B** Synaptophysin: EE Wt mice had significantly higher hippocampal synaptophysin compared to SH Wt mice, $t_{(8)} = 3.51$, $p. = .008$. **C** VGlut-1: There was no significant housing x genotype effect on VGlut-1 expression in the hippocampus, $F_{(1, 16)} =$

0.007, *p.* = .94. **D** PSD-95: Wt EE mice had significantly higher levels of PSD-95 compared to SH Wt $t_{(8)} = 2.84$, *p.* = .02. **E** Gephyrin: EE Wt mice had significantly lower levels of hippocampal gephyrin as compared to SH Wt, $t_{(8)} = 3.06$, *p.* = .02. **F** GAD65/67: No significant differences were detected across housing x genotype in the hippocampus, $F_{(1, 16)} = 0.02$, *p.* = .90.

Figure 5. Synapse density in Fr2 of neocortex. Representative 60x (118.15 x 118.15 μm) synaptophysin staining in Fr2 per group, Scale bar = 20μm.. Mean synaptophysin density (± SEM) of Fr2 showed no significant housing x genotype effect, $F_{(1, 24)} = 0.07$, *p.* = .79.

Figure 6. Synapse density in hippocampal subregion CA1. Representative 60x (118.15 x 118.15 μm) synaptophysin staining in CA1, Scale bar = 20μm. Mean synaptophysin density of CA1 (± SEM), the APP/PS1 mice from EE had significantly higher synaptophysin density in CA1, $t_{(16)} = 2.54$, *p.* = .02, relative to the other groups.

Figure 7. Synapse density in hippocampal subregion CA3. Representative 60x (118.15 x 118.15 μm) synaptophysin staining in CA3, Scale bar = 20μm. Mean synaptophysin density CA3 (± SEM) showed no significant housing x genotype effect, $F_{(1, 24)} = 2.79$, *p.* = .12.

Figure 8. BDNF protein levels relative to Wt control (% expression ± SEM). **A** BDNF expression in neocortex is not altered by genotype or housing, $F_{(2, 23)} = 0.88$, $p = 0.43$. **B** BDNF expression in hippocampus was elevated in APP/PS1 mice housed in EE relative to the SH condition, $t_{(8)} = 3.39$, $p = 0.009$.

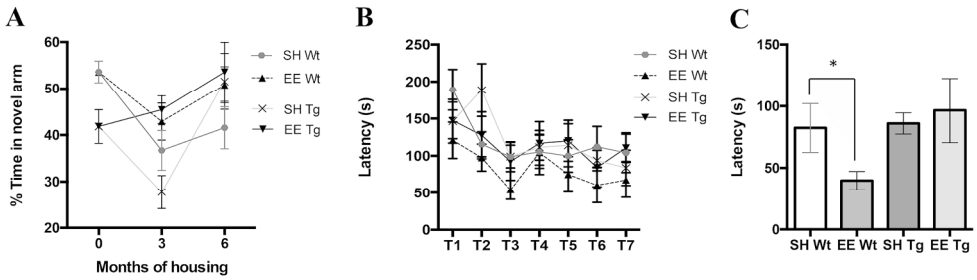

Figure 1. Y maze and Barnes maze performance. A Mean percentage of time spent in the novel arm of the Y maze (± SEM) at baseline, and after 3 and 6 months of differential housing. Baseline testing at 6 months of age showed genotype related differences, t (46) = 3.15, p = 0.003. After 3 months of housing, no genotype x housing effect on Y maze performance was detected, F (1, 42) = 2.26, p = 0.14. Following 6 months of differential housing, no significant genotype x housing effect on Y maze performance was detected, F (1, 43) = 0.20, p. = .66. B Mean latency (± SEM) to reach the escape on the Barnes maze over 7 days of learning trials at 12 months of age. No significant main effect of genotype x housing on learning was detected, F (9.19, 280.18) = 0.56, p. = .87. C Long-term memory performance on the Barnes maze expressed as the mean latency (± SEM) to reach the escape over four trials. The EE Wt animals on average had significantly reduced latency to reach the escape, t (25) = 2.07, p = 0.049.

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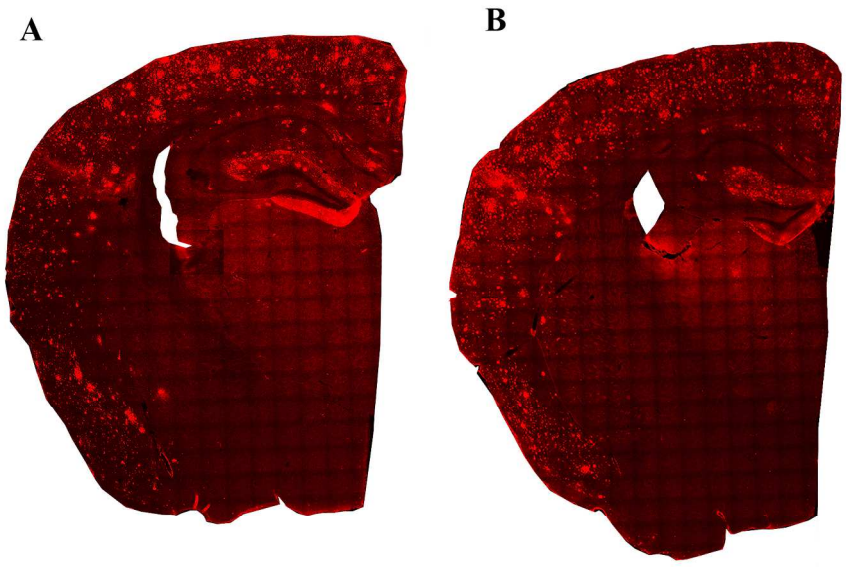
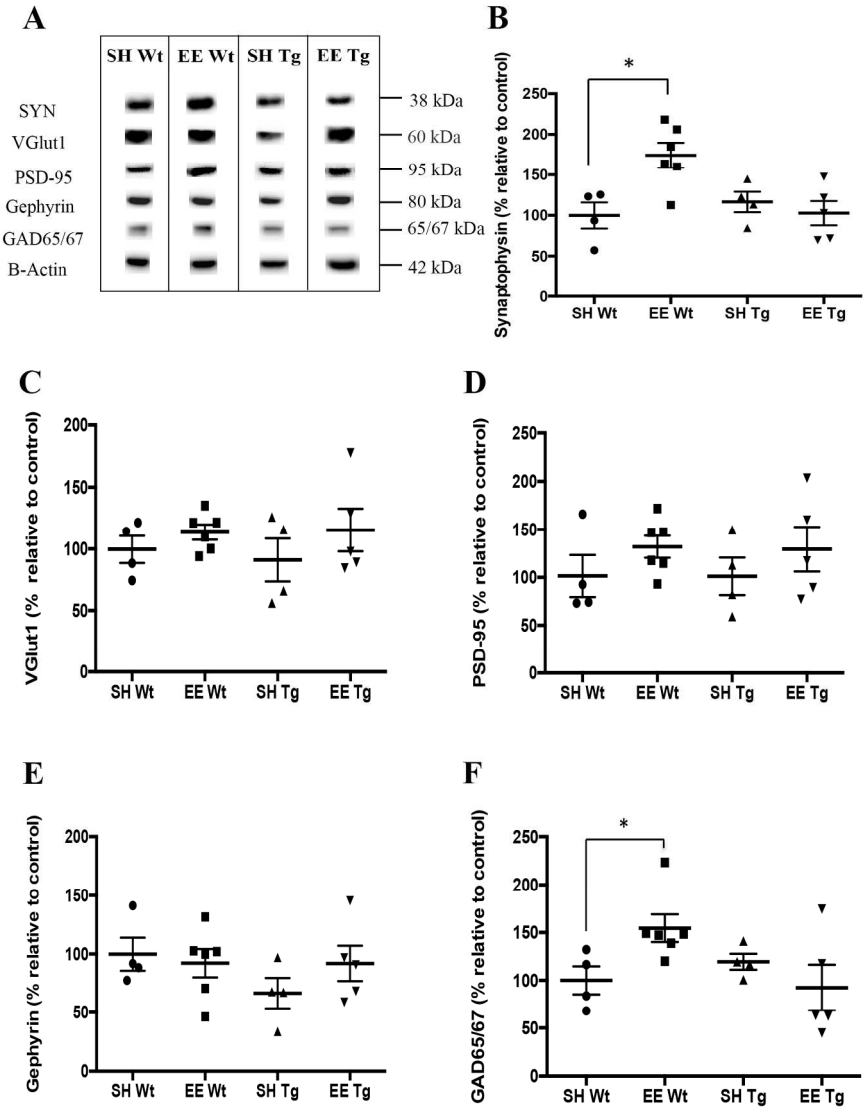


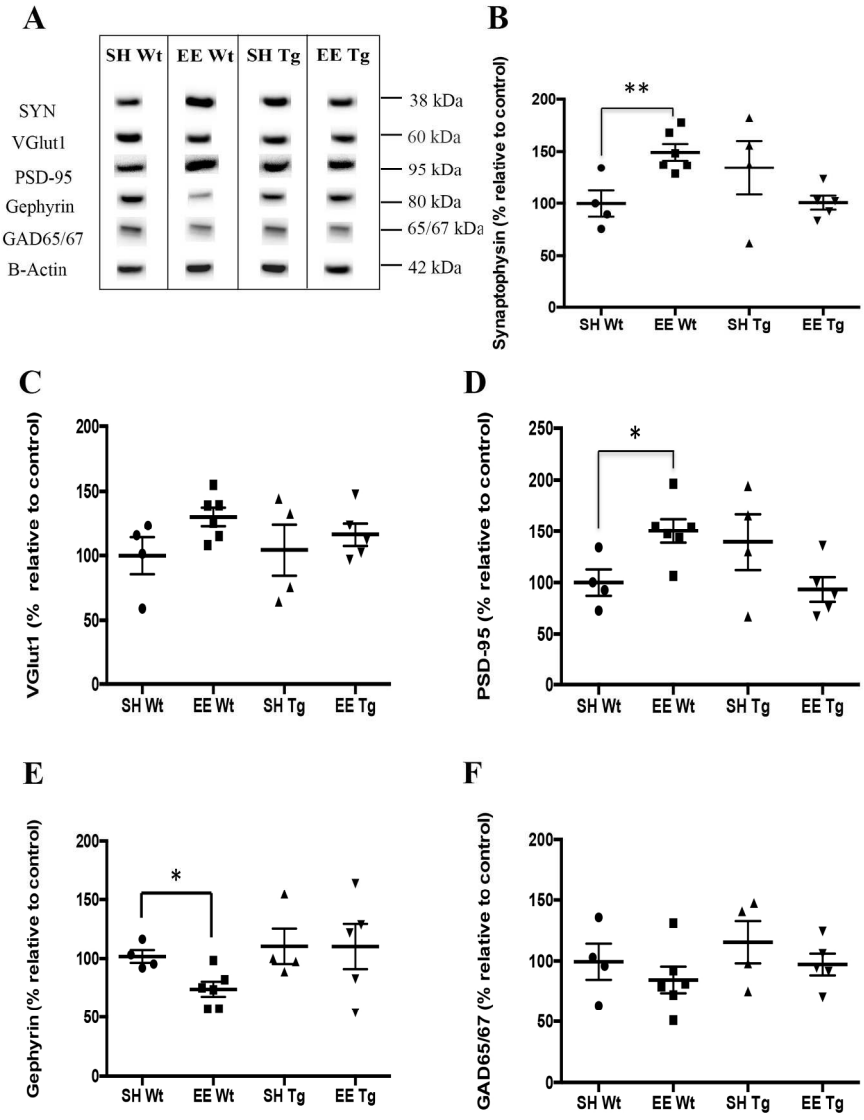
Representative APP/PS1 mouse brain sections stained with the MOAB-2 antibody. A APP/PS1 mouse brain coronal section with MOAB-2 positive plaques from SH. B APP/PS1 mouse brain coronal section with MOAB-2 positive plaques from EE.

171x230mm (300 x 300 DPI)



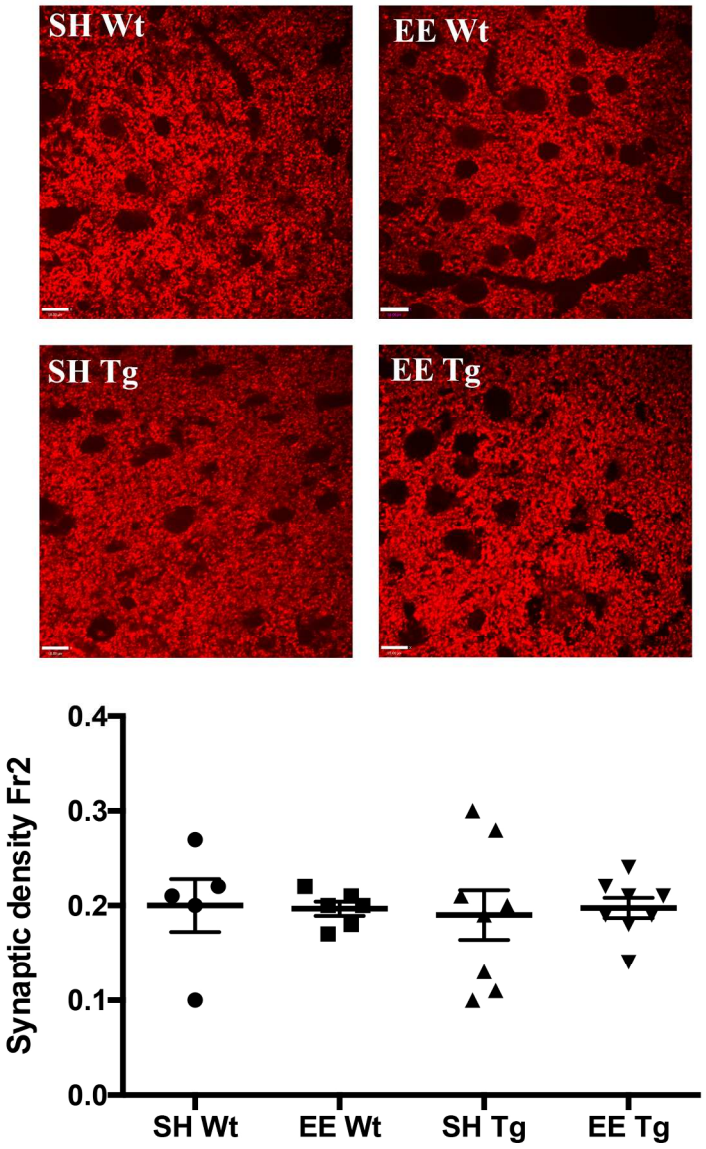
Mean synaptic protein levels expressed as a percentage relative to Wt control (± SEM) from neocortex samples. A Representative Western blot bands per group for each synaptic marker. B Synaptophysin: EE Wt mice had higher relative levels of cortical synaptophysin, t (8) = 3.22, p= 0.01. C VGlut-1: No significant differences across housing x genotype were detected, F (1, 16) = 0.15, p = 0.70. D PSD-95: No significant differences across housing x genotype were detected, F (1, 16) = 0.003, p = .95. E Gephyrin: No significant differences across housing x genotype were detected, F (1, 16) = 1.42, p = 0.25. F GAD65/67: Wt EE mice showed significantly increased levels of GAD65/67 compared to those in SH, t (7.45) = 2.64, p = 0.03.

170x230mm (300 x 300 DPI)



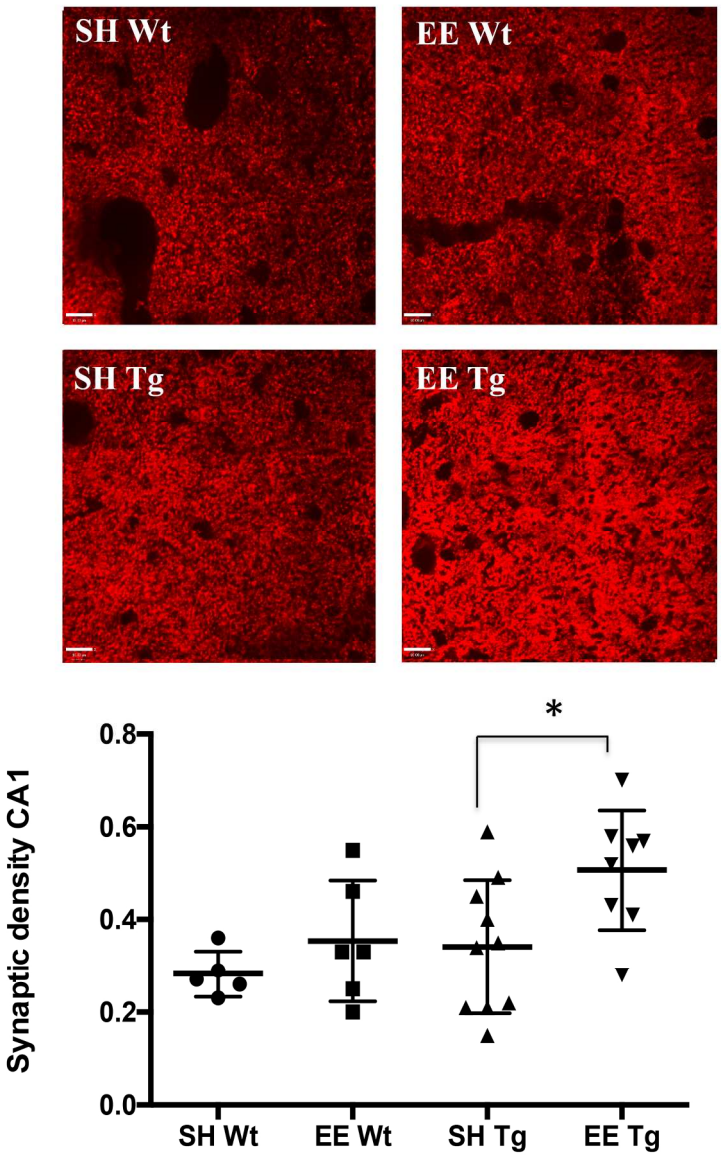
Mean synaptic protein levels expressed as a percentage relative to Wt control (± SEM) from hippocampus samples. A Representative Western blot bands per group for each synaptic marker. B Synaptophysin: EE Wt mice had significantly higher hippocampal synaptophysin compared to SH Wt mice, $t(8) = 3.51$, $p = 0.008$. C VGlut-1: There was no significant housing x genotype effect on VGlut-1 expression in the hippocampus, $F(1, 16) = 0.007$, $p = 0.94$. D PSD-95: Wt EE mice had significantly higher levels of PSD-95 compared to SH Wt $t(8) = 2.84$, $p = 0.02$. E Gephyrin: EE Wt mice had significantly lower levels of hippocampal gephyrin as compared to SH Wt, $t(8) = 3.06$, $p = 0.02$. F GAD65/67: No significant differences were detected across housing x genotype in the hippocampus, $F(1, 16) = 0.02$, $p = 0.90$.

170x230mm (300 x 300 DPI)



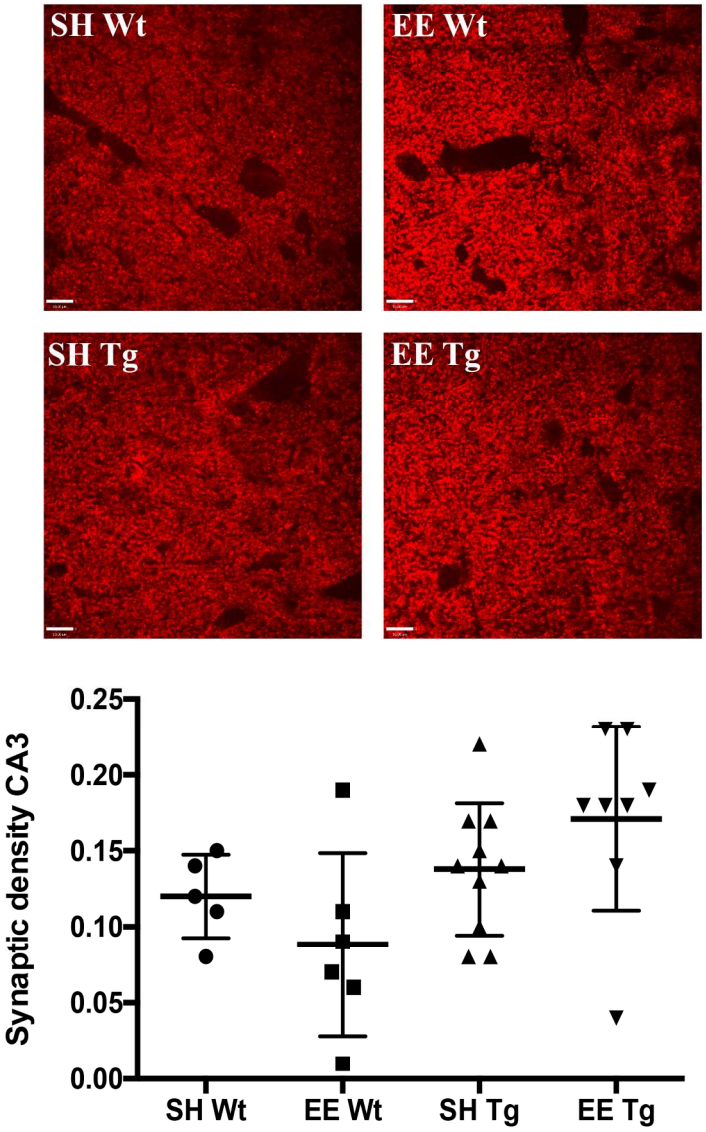
Synapse density in Fr2 of neocortex. Representative 60x (118.15 x 118.15 m) synaptophysin staining in Fr2 per group, Scale bar = 20m. Mean synaptophysin density (± SEM) of Fr2 showed no significant housing x genotype effect, F (1, 24) = 0.07, p = 0.79.

171x230mm (300 x 300 DPI)



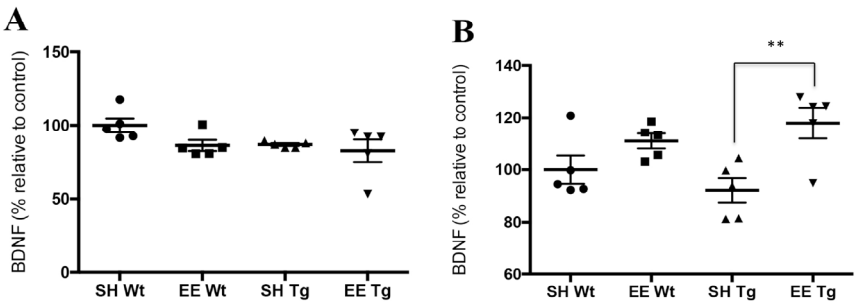
Synapse density in hippocampal subregion CA1. Representative 60x (118.15 x 118.15 m) synaptophysin staining in CA1, Scale bar = 20m. Mean synaptophysin density of CA1 (± SEM), the APP/PS1 mice from EE had significantly higher synaptophysin density in CA1, t (16) = 2.54, p = 0.02, relative to the other groups.

171x230mm (300 x 300 DPI)



Synapse density in hippocampal subregion CA3. Representative 60x (118.15 x 118.15 m) synaptophysin staining in CA3, Scale bar = 20m. Mean synaptophysin density CA3 (± SEM) showed no significant housing x genotype effect, F (1, 24) = 2.79, p = 0.12.

171x230mm (300 x 300 DPI)



BDNF protein levels relative to Wt control (% expression ± SEM). A BDNF expression in neocortex is not altered by genotype or housing, $F(2, 23) = 0.88$, $p = 0.43$. B BDNF expression in hippocampus was elevated in APP/PS1 mice housed in EE relative to the SH condition, $t(8) = 3.39$, $p = 0.009$.

171x230mm (300 x 300 DPI)

Antibody name	*Host organism (Clone)*	*Isotype*	*Immunizing agent*	*Source (catalogue number)*	*RRID*	*Dilution*
MOAB-2	Mouse monoclonal	IgG2b	C-terminal for Aβ40 and Aβ42 (Youmans et al., 2012)	Novus Biologicals (NBP2-13075)	N/A	1:2000 (IHC)
Synaptophysin	Rabbit polyclonal	IgG	Synthetic peptide of human synaptophysin (313 amino acids) (Rehm, Wiedenmann, & Betz, 1986)	Millipore (AB9272)	RRID:AB_570874	1:200 (IHC) 1:2000 (WB)
VGlut1	Mouse monoclonal	IgG1	Recombinant protein from rat VGlut1 (560 amino acids) (Fazzari et al., 2014)	Millipore (MAB5502)	RRID:AB_262185	1:1000 (WB)
PSD-95	Mouse monoclonal (clone 6G6-1C9)	IgG2a	Recombinant protein from rat PSD-95 (Li et al., 2010)	Abcam (Ab2723)	RRID:AB_303248	1:1000 (WB)
Gephyrin	Rabbit polyclonal	IgG	Synthetic peptide of human Gephyrin (amino acids 396-445) (Harvey et al., 2004)	Abcam (Ab83401)	RRID:AB_1860491	1:1000 (WB)
GAD65	Mouse monoclonal (clone GAD-6)	IgG2a	Purified GAD65 from rat (585 amino acids) (Besser et al., 2015)	Millipore (MAB351)	RRID:AB_2263126	1:1000 (WB)
GAD67	Mouse monoclonal (clone 1G10.2)	IgG2a	Recombinant GAD67 protein (594 amino acids) (Fong et al., 2005)	Millipore (MAB5406)	RRID:AB_2278725	1:1000 (WB)
β-actin	Mouse monoclonal (clone AC-74)	IgG2a	Modified β-cytoplasmic actin N-terminal peptide (Arellano et al., 2012)	Sigma-Aldrich (A5316)	RRID:AB_476743	1:5000 (WB)
BDNF	Rabbit polyclonal	IgG	Epitope mapping, internal region of Human BDNF (amino acids 128-147) (Flores-Otero & Davis, 2011)	Santa Cruz (SC-546)	RRID:AB_630940	1:500 (ELISA)

Table 2. Mean percentage time in novel arm of the Y maze

	SH Wt			EE Wt			SH Tg			EE Tg		
	M	SD	*n*	M	SD	*n*	M	SD	*N*	M	SD	*n*
Baseline	51.63	12.00	12	53.33	9.89	14	41.02	15.79	10	41.02	13.56	9
6 months												
9 months	36.72	14.80	12	42.89	14.84	14	45.41	11.24	10	45.41	9.04	9
12 months	41.56	15.59	12	50.83	14.70	14	53.59	19.14	10	53.59	19.15	9

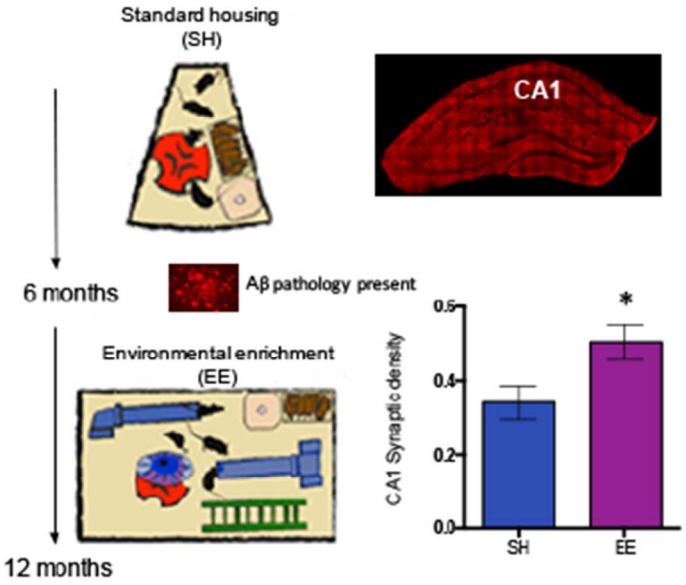
Table 3. Latency (s) to reach escape on the Barnes Maze learning trials

Trial	SH Wt	EE Wt	SH Tg	EE Tg
	M (SD)	M (SD)	M (SD)	M (SD)
1	179.42 (81.67)	121.75 (86.16)	133.85 (78.96)	147.55 (78.21)
2	125.96 (65.14)	97.54 (62.53)	171.85 (99.52)	127.60 (99.85)
3	114.67 (86.33)	53.96 (44.96)	104.40 (46.80)	92.35 (80.99)
4	109.88 (70.04)	104.38 (90.87)	121.15 (75.10)	116.65 (92.72)
5	106.00 (73.37)	74.96 (83.07)	109.10 (78.01)	120.25 (87.36)
6	118.71 (87.78)	60.46 (82.12)	87.55 (43.27)	84.40 (49.37)
7	116.38 (92.00)	67.58 (48.01)	94.80 (73.18)	110.65 (58.37)

Table 4. MOAB-2 load (% area) of Neoortex and Hippocampus

	SH	EE
	$n = 9$	$n = 8$
	M (SEM)	*M* (SEM)
Neocortex	29.44 (2.75)	25.76 (5.15)
Hippocampus	25.42 (2.35)	24.74 (2.92)

A transgenic mouse model of Alzheimer's disease exposed to an enriched environment following Aβ deposition, showed an increase in CA1 synaptic density and elevated hippocampal BDNF, despite no changes to Aβ pathological burden. Thus, stimulation from the environment in mid-life may enhance the capacity for synaptic connectivity in pathological ageing.



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