Selective reduction of APP-BACE1 activity improves memory via NMDA-NR2B receptor-mediated mechanisms in aged PDAPP mice

Charles E. Evans a,b, Rhian S. Thomas b,c, Thomas J. Freeman a,b, Martha Hvoslef-Eide d, Mark A. Good a,c*, Emma J. Kidd b

a School of Psychology Cardiff University, Cardiff, UK  
b School of Pharmacy & Pharmaceutical Sciences, Cardiff University, Cardiff, UK  
c Department of Applied Sciences, University of the West of England, Bristol, UK  
d Department of Biosciences, University of Oslo, Oslo, Norway

1. Introduction

The excess accumulation of β-amyloid (Aβ) in the brain with age is considered an important factor in the cascade of cellular, neural network, and cognitive changes that characterize the early stages of Alzheimer’s disease (AD) (Hardy and Selkoe, 2002; Selkoe, 2001). Aβ is produced by the proteolytic cleavage of amyloid precursor protein (APP) by the β-secretase cleavage enzyme (BACE1). BACE1 is located in the presynaptic terminals of neurons and is thought to be critical for the production of Aβ and subsequent disruption of synaptic connectivity that characterizes early-stage AD (Sadleir et al., 2016).

One approach to altering APP metabolism is through the use of BACE1 inhibitors (Yan and Vassar, 2014). However, this approach has been problematic because BACE1 has multiple substrates other than APP (Hemming et al., 2009). Although BACE1 modulation in people with clinically diagnosed dementia appears to lack therapeutic efficacy, the targeting of this pathway in the elderly with cognitive decline or those at high risk of dementia remains an area of current interest. Therefore, understanding the effects of selectively modifying BACE1-APP processing on synaptic function and cognition is vital to understanding the mechanism and the conditions under which such an intervention may have therapeutic value.

In the present study, we used a monoclonal antibody, 2B3, directed against the BACE1 cleavage site of APP to reduce BACE1 cleavage without influencing other BACE1 substrates (Thomas et al., 2013, 2011b). We hypothesized that in vivo administration of 2B3 would (1) reduce Aβ production, (2) improve aberrant synaptic processes in the hippocampus, and (3) improve visuospatial associative recognition memory. In experiment 1, we first established the age of onset of a selective associative (object-in-place [OIP]) recognition memory deficit in transgenic platelet-derived growth factor promoter hAPP717V (PDAPP) mice; a task that relies on a brain network that includes the hippocampus (Barker and Warburton, 2011). In experiment 2a, we

* Corresponding author at: School of Psychology, Cardiff University Park Place, Cardiff CF10 3AT, UK. Tel.: (+44) 02920 875867; fax: (+44) 2920 874858.
E-mail addresses: Good@cardiff.ac.uk, Good@cf.ac.uk (M.A. Good).
used the mice from experiment 1 to assess the effects of intracerebroventricular (ICV) administration of the antibody, 2B3, on associative memory dysfunction in aged PDAPP mice. In experiment 2b, we addressed the question of whether disruption of endogenous APP processing by 2B3 in normal mice influenced associative recognition memory. In experiment 3, we investigated the effects of chronic intraperitoneal (IP) administration of 2B3, initiated before the onset of behavioral impairment, on the subsequent development of cognitive dysfunction in aged PDAPP mice. Finally, experiment 4 used a within-subject Latin-square design to test the hypothesis that disruption of glutamate signaling through N-methyl-D-aspartate (NMDA) NR2B receptors, evident in PDAPP mice from experiments 2 and 3, was required for normal associative recognition memory in WT mice. Our results show for the first time that selective reduction in APP metabolism by BACE1 using steric hindrance both improved and protected mice from memory dysfunction and altered synaptic NMDA-NR2B expression in PDAPP mice.

2. Materials and methods

2.1. Animals

Male PDAPP mice (Games et al., 1995), and their WT littermates, were bred and maintained on a C57Bl/6 genetic background as previously described (Evans et al., 2018). Mice were housed either individually (if they showed signs of aggressive behavior) or in groups. One WT and one transgenic mouse were housed separately. All animals were housed using standard environmental and cage conditions, including nesting cardboard tubes and clean bedding. Behavioral testing was carried out during the light hours of the cycle. Animals were maintained according to the UK Home Office under the Animal Scientific Procedures Act (1986) and EU regulations.

Before assignment to groups, the mice were genotyped according to protocols described previously (Evans et al., 2018). Briefly, an ear biopsy sample was collected from each mouse at 6–8 weeks of age as part of animal husbandry identification procedures, which was then digested and DNA extracted using DNeasy Blood and Tissue kits (Qiagen). A polymerase chain reaction was used to amplify the human APP V717F transgene DNA. PDAPP-specific primers forward: 5′-ATCTGGCCCGGCTGGAAAAAAG-3′ and reverse: 5′-GATGTCCCCCTCTCTGGTTC-3′ (Eurofins, Wolverhampton, UK) amplified the hAPP V717F mutation. Control primers for MusA-Actin forward: 5′-CACCACACCTTCTCACATGACTG-3′ and reverse: 5′-TCTACAGGTAGTCAGGTGGTCG-3′ (Eurofins) targeted MusA-Actin.

Experiment 1 established the age of onset of cognitive impairment, and experiment 2 assessed the effects of ICV infusions of 2B3 on cognition in the same animals. ICV administration was considered the most appropriate means to ensure the delivery of the antibody in the brain and thus provide a test of its putative actions in vivo as the most appropriate means to ensure the delivery of the antibody in the brain and thus provide a test of its putative actions in vivo.

In experiments 1 and 2a, mice received 2 different object-based memory tasks; a novel object recognition task and a visuospatial OIP task (Barker and Warburton, 2011; Good and Hale, 2007; Hale and Good, 2005) (Fig. 2A and B). Briefly, before testing object memory, animals were habituated to the arena (60 cm × 60 cm square × 40 cm high). Mice were allowed to explore the arena freely for 10 minutes on day 1. Mice were then habituated for 2 consecutive days to the arena containing 2 identical objects for 10 minutes each day. Each mouse received 2 rounds of testing on each task, 1 day with a 5-minute and 1 day with a 24-hour delay period (in a counterbalanced order). The objects were a collection of everyday items and ornaments as described previously (Hale & Good, 2005).

2.2. Behavior

In experiments 1 and 2, mice received 2 different object-based memory tasks; a novel object recognition task and a visuospatial OIP task (Barker and Warburton, 2011; Good and Hale, 2007; Hale and Good, 2005) (Fig. 2A and B). Briefly, before testing object memory, animals were habituated to the arena (60 cm × 60 cm square × 40 cm high). Mice were allowed to explore the arena freely for 10 minutes on day 1. Mice were then habituated for 2 consecutive days to the arena containing 2 identical objects for 10 minutes each day. Each mouse received 2 rounds of testing on each task, 1 day with a 5-minute and 1 day with a 24-hour delay period (in a counterbalanced order). The objects were a collection of everyday items and ornaments as described previously (Hale & Good, 2005).

2.2.1. Novel object recognition

to assess object novelty versus familiarity discriminations, 2 identical objects were placed in the center of the arena and mice were allowed to explore the objects for 10 minutes during the sample phase. Mice were given a total of 3 sample phases, each...
Figure 1. Schematic illustration detailing experimental designs used in this study. (A) Experiment 1 assessed cognitive performance (object-novelty and OiP performance) of PDAPP and WT littermate controls at 3 age points across the study. Experiment 2a used the same mice to assess whether 2B3 (icv) infusion by osmotic minipumps could improve OiP memory and pathology in PDAPP mice. (B) A separate cohort of PDAPP mice was used to exclusively determine the age-related changes of β-amyloid pathology in the hippocampus of PDAPP mice using time points comparable to the behavioral time points used in separate experiments. (C) A separate cohort of WT mice was administered 2B3 to assess its effect on normal endogenous mouse APP processing and memory. These mice first received behavioral testing (object-novelty and OiP memory) prior to implantation of osmotic minipumps. Subsequently, they were administered 2B3 or IgG control antibody for 14 days. Memory function was then reassessed before culling and assessment of endogenous APP metabolism. (D) The effects of 2B3 administered longitudinally via a peripheral (i.p.) route on cognition was assessed in a separate cohort of PDAPP mice. These mice first received pretreatment behavioural testing (object novelty, OiP, and foraging behavior tasks) at 11 months of age. After which, the mice were administered 2B3 or vehicle i.p. for 15 weeks. During treatment weeks 13–15, memory function was reassessed, following which, the mice were culled and brain pathology was analysed. (E) Experiment 4 assessed the role of the NR2B receptor in OiP memory in normal WT mice. This was carried out in a Latin-square design whereby the cohort was split into 2 groups (A and B; each group n = 5). Group A were initially assessed on the task after Ro25-6981 administration, while group B after vehicle treatment. After this initial assessment, Ro25-6981 was then administered to group B mice, while group A mice now received vehicle. Abbreviations: OiP, object-in-place; WT, wild type.
Ennaceur and Delacour (1988). In brief, object contact was de-
scribed as the time spent exploring the novel object (or objects in novel
locations) as when an animal was within a 2 cm radius of the object and
not climbing or sitting on, the objects. A discrimination ratio (DR) was used to index the animals’
discriminative performance in the test phase that was independent of individual differences in object contact times; this was calculated as the time spent exploring the novel object (or objects in novel locations)/the time spent exploring all objects. All objects were
cleaned before each phase of testing to reduce the use of odor cues introduced from handling the objects.

2.2.4. Foraging behavior

To establish the generality of the cognitive changes promoted by
2B3 administration, mice that received longitudinal IP 2B3 in-
jections were also assessed on a foraging-based spatial working
memory task sensitive to age-dependent changes in PDAPP mice
(Evans et al., 2018). Briefly, throughout the training and test phase, mice were water-deprived to approximately 90% of their pre-
training weight. Water was given for 4 hours immediately after
training or testing each day. Mice were trained to forage from white
ceramic pots (6.5 cm diameter, 3.5 cm depth; Lakeland, UK), which
were mounted on a wooden cube base. Pots were secured to the
floor of the cage/arena with blue-tack. In the initial training stages
(day 1-3), pots containing 10 mL of water were placed in the home
cage of mice for 1 hour to encourage mice to consume liquid re-
wards from within the pot. The following 3 days (day 4-6), 2 pots
were baited with a 30 µL liquid reward (1:3 sweetened condensed milk [Nestle] solution, prepared in water: H₂O) in an open arena
with 1-cm-deep sawdust covering the arena floor. Mice were placed in the center of the arena and given 3 minutes to forage from
each pot. Mice were removed once the liquid reward was consumed from each pot or the 3-minute limit was reached. Mice were given 1
training session/day. On each day, the location of the pots was
moved to a new location to prevent the development of any sys-
tematic search bias in the test phase.

Mice were then tested over the next 4 consecutive days with 1
session per day. During these sessions, the arena was set up with 6
pots arranged in a circular pattern, each 20 cm apart. The mouse
was placed in the center of the arena and allowed to forage pots
until they had consumed all 6 rewards or until a 10-minute period
had elapsed from when the first pot was foraged. Following the trial, mice were returned to their home cage. The pots were then
wiped clean with 70% ethanol wipes, and the milk solution
replenished before the next mouse was tested. All test sessions
were recorded onto a DVD player using an overhead camera.

Foraging behavior was defined as a mouse jumping onto the rim
of a pot and directing its nose in toward the bottom to consume a
reward. An error was defined as a mouse returning to forage in a pot
that had previously been foraged. A full description of errors can be
found in Evans (2018).

2.3. Antibody production and administration

Full details of the immunization protocol, hybridoma develop-
ment, antibody characterization, production, and purification are
provided elsewhere (Thomas et al., 2006, 2011b, 2013).

In Experiments 2a and 2b, purified and sterilized 2B3 (1.2 mg/
ml), control monoclonal IgG1x antibody (experiment 2b) and
vehicle (experiment 2a) were administered intra-
cerebroventricularly via a 28G cannula surgically implanted into
the left lateral ventricle (Alzet; 0004760). The minipumps were
attached to the infusion cannula via a catheter and implanted
subcutaneously between the scapulae. Minipumps were filled with
200 µL of antibody or vehicle (Model number 1002; Alzet, Califor-
nia, USA; flow rate 0.25 µL/hour for a period of 14 days). In exper-
iment 2a, the vehicle was sterile PBS and was administered to both
PDAPP mice (n = 11) and WT littermate control groups (n = 10). In experiment 2b, the WT control group received ICV infusion of a
monoclonal IgG1x.
In experiment 3, mice received a weekly IP injection of 2B3 for 15 weeks. 2B3 was produced by ascites (ProMab, California, USA), before being purified and sterilized as described previously. PDAPP mice were administered 2B3 (IP) at a dose of 20 mg/kg at an average concentration of 3 mg/mL. Sterile PBS was administered at the same volume to vehicle PDAPP and WT mice control groups. After the completion of behavioral testing and 3 days after the final IP injection of 2B3, brain tissue was collected from all mice for biochemical analyses.

2.4. Surgical procedure

During stereotaxic surgery, the mouse was anesthetized using an isoflurane/O₂ mix, and a small hole was drilled through the skull of the animal 0.5 mm posterior and 1.2 mm lateral to Bregma. A 28G cannula was then inserted 3.0 mm ventral to the skull surface and fixed in place by dental acrylic. The minipump was carefully inserted into a subcutaneous pocket between the scapulae, the skin incision sutured, and the mouse allowed to recover in an incubator until independently mobile. Following standard postoperative care procedures, the mice were then housed individually for the duration of the study.

Postoperative behavioral testing on the OiP associative recognition task occurred 9 days after surgery and used the procedure described in experiments 1 and 2. On day 14, following behavioral testing, the animals were culled and brain tissue collected for analyses.

2.5. Ro25-6981 administration

Ro25-6981 (Tocris, Abingdon, UK) was dissolved in sterile saline at a final concentration of 5 mg/mL. Mice were administered a single 10 mg/kg dose of Ro25-6981 or vehicle IP, 30 minutes before the start of the associative recognition OiP task. This dose was selected based on previously published behavioral work (e.g., Mikics et al., 2017).

2.6. Pathology: protein extraction and immunobLOTS

The hippocampus and cortex were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C. Soluble proteins were extracted from brain samples as previously described (Thomas et al., 2011a). For the ICRV study, all blots quantifying changes in soluble protein levels for the ICV study assessed 10 WT vehicle, 11 PDAPP vehicle mice, and 10 PDAPP mice administered 2B3 intra-cerebroventricularly. For the IP study analysis, 9 WT vehicle, 10 PDAPP vehicle, and 9 PDAPP 2B3-administered mice were assessed. Nonsynaptosomes were extracted by using Syn-PER synaptic protein extraction reagent (Thermo Fisher, UK). Western blotting was performed using standard methods as described previously (Thomas et al., 2011a) using samples from all animals undergoing treatment and behavioral testing. Briefly, after protein quantification, 20 µg of protein/sample was resolved on either a 10% or 7.5% polyacrylamide gel and detected with the relevant antibody (Table 1). The right hippocampus of all 10 WT vehicle, 11 PDAPP vehicle, and 10 PDAPP mice administered 2B3 intra-cerebroventricularly were used to assess synaptic protein changes.

2.7. Sandwich ELISA for detection of APP metabolites

ELISAs for the quantification of Aβ were carried out as previously described (Thomas et al., 2011a,b), or as recommended by the manufacturer: APP (R&D Systems, Abingdon, UK), β-amyloid derived C-terminal fragment (β2CTF; IBL, Hamburg, Germany), and Aβ40 and Aβ42 (human and mouse; Invitrogen, California, USA).

| Primary antibody | Species | Dilution | Source |
|------------------|---------|----------|--------|
| APP (22C11)      | Mouse   | 1:1000   | Milipore |
| BACE1            | Rabbit  | 1:1000   | Cell Signalling Technology |
| NMDA-NR1         | Mouse   | 1:1000   | BD Biosciences |
| NMDA-NR2B        | Rabbit  | 1:500    | Milipore |
| pY1472           | Rabbit  | 1:750    | Milipore |
| PSD95            | Rabbit  | 1:1000   | Abcam |
| PS1              | Rabbit  | 1:500    | Santa Cruz |
| Fyn              | Mouse   | 1:750    | BD Biosciences |
| STEP             | Mouse   | 1:750    | Novus |
| ERK              | Rabbit  | 1:2000   | Cell Signalling Technology |
| Phospho-ERK      | Rabbit  | 1:1000   | Cell Signalling Technology |
| GAPDH            | Pre-conjugated | 1:50,000 | Sigma |
| β-Actin          | Pre-conjugated | 1:20,000 | Sigma |

Key: ERK, extracellular signal–regulated kinase; NMDA, N-methyl-D-aspartate; STEP, striatal-enriched phosphatase.

Data are presented as ng or pg/mg total protein concentration. With exception of experiment 2b, which assessed endogenous mouse Aβ40 and Aβ42, all further ELISAs were sensitive to human protein only. Therefore, the PDAPP mice that expressed the human APPV717F were used. The ICV study used 11 PDAPP vehicle mice and 10 PDAPP 2B3 mice (however, 1 mouse was removed as an outlier as explained below). The IP study used 10 PDAPP vehicle mice and 9 PDAPP 2B3 mice to quantify protein levels by ELISA.

2.8. Statistical analyses

All statistical analyses were performed using IBM SPSS statistics. The behavioral data conformed to the assumptions of analysis of variance (ANOVA) and were analyzed using a mixed measures design. Significant interactions were assessed using tests for simple main effects. Western blot data were analyzed using one-way ANOVA followed by Tukey post hoc analysis. WT data were collapsed across treatment (untreated and vehicle) for the analysis of the cognitive and behavioral effects of 2B3 administration as no group differences were observed between WT groups (p > 0.5 (data not shown)). ELISA data were analyzed using either Student’s t-test or a Kruskal-Wallis test with Dunn’s test along with a Bonferroni correction for multiple comparisons. All data were subject to Levene’s and Shapiro-Wilk tests for data normality before analysis. Appropriate transformations were carried out when necessary.

Data generated from ELISA assays were quantified by comparing data to standard curves from each plate using GraphPad Prism 4 and normalized to total protein concentration. One PDAPP 2B3-treated mouse was found to be an extreme outlier across the ELISA analyses of the 2B3 ex vivo tissue, as determined by SPSS Tukey box plots and more conservative methods for labeling outliers, as described previously (Carling, 1998; Hoaglin and Iglewicz, 1987). No transformation was able to normalize this outlier for either parametric or nonparametric analysis. Therefore, the data from this 1 mouse were removed from all ELISA analyses but no other analysis. The result of this exclusion was that all remaining ELISA data sets were normally distributed, and its exclusion did not change the pattern of results. Western blots were quantified using Image J software (www.imagej.nih.gov/).

3. Results

Experiment 1 used object novelty and OiP tasks to determine the nature and age of onset of memory deficits in PDAPP mice. In the second stage of the study, experiment 2a, the same animals were administered the APP antibody 2B3, at 17–18 months of age, via osmotic minipumps for a period of 14 days. Having established that
2B3 reversed pre-existing associative recognition memory impairment, experiment 3 determined whether longitudinal peripheral administration of 2B3 prevented the onset of cognitive decline in PDAPP mice.

3.1. Experiment 1

During development, WT mice showed a greater overall object contact time during sample phases across both tasks and at all ages, than PDAPP mice, \( p < 0.01 \) (Supplementary Table 1). However, analysis showed that both WT and PDAPP mice habituated normally across the 3 sample phases, as indicated by a decline in object contact times (Supplementary Table 1). In the test phase, WT mice showed a greater contact time with objects than PDAPP mice, \( p < 0.001 \) across all ages (Supplementary Table 2). However, both WT and PDAPP mice showed a preference to explore objects in novel locations more than familiar locations both at 6-8 and 10-12 months of age, \( p < 0.01 \). However, at 14-16 months of age, only WT but not PDAPP mice showed this preference, \( p < 0.001 \) and \( p > 0.05 \), respectively; data collapsed across both delays. There were no significant interactions involving genotype and delay for either task (see Supplementary Analysis). In contrast, both WT and PDAPP mice showed a preference to explore novel objects across all ages and delays, \( p < 0.01 \), and showed no genotype difference, even at 14-16 months of age (\( p > 0.05 \); Supplementary Table 2).

An analysis of DRs similarly showed an identical pattern with WT mice only at 14-16 months of age, \( p < 0.01 \) (Supplementary Table 1). However, all ages as indicated by no main effect of genotype, \( F(1, 27) = 2.5, p > 0.1 \), and no genotype \( \times \) age interaction, \( F(1, 27) = 2.8, p > 0.1 \). In contrast, PDAPP mice were significantly impaired relative to control mice only at 14-16 months of age for the OiP task (see Fig. 3A and 3B). Analysis of the OiP task revealed a significant age \( \times \) genotype interaction, \( F(2, 54) = 9.2, p < 0.05 \). Subsequent tests of simple main effects revealed that PDAPP mice showed a significant main effect of age with a reduction in OiP memory performance across age ranges, \( F(2, 26) = 9.8, p < 0.001 \). Further analysis revealed that PDAPP mice showed a significant memory deficit compared with WT mice only at 14-16 months of age, \( F(1, 27) = 49.9, p < 0.001 \). There was no significant interaction involving delay, \( F < 1 \). The analysis of DR values confirmed that PDAPP mice showed an age-dependent deficit in associative recognition memory, without affecting object novelty/familiarity discriminations.

An analysis of age-related changes in hippocampal A\( \beta \) levels in a separate group of PDAPP mice confirmed a numerical 3.6-fold increase in soluble A\( \beta \)40 levels (Fig. 3C; nonsignificant following analysis by the Kruskal-Wallis test, \( X^2(3) = 2.7, p > 0.1 \)) and a significant, \( X^2(3) = 10.5, p < 0.05 \), 32-fold increase in soluble A\( \beta \)42 levels by 15 months of age (Fig. 3D). Dunn’s test for multiple comparisons showed a significant increase in the levels of soluble A\( \beta \)42 when comparing mice at 3 months and 15 months of age, \( p < 0.05 \). Thus, PDAPP mice showed an age-related increase in amyloid pathology with a marked increase in A\( \beta \) production at the same age as behavioral deficits emerged in a separate cohort of mice.

3.2. Experiment 2

3.2.1. Experiment 2a

After 2B3 or vehicle administration, PDAPP mice continued to explore all 4 objects less than WT mice, \( p < 0.01 \). However, all

![Figure 3](image-url)

Figure 3. PDAPP mice show an age-dependent decline in OiP memory performance and an increase in amyloid levels. (A) PDAPP mice (\( n = 14 \)) showed no deficits in object novelty memory across any age or delay compared to WT littermates (\( n = 15 \)). (B) However, a significant age \( \times \) genotype interaction revealed that PDAPP mice showed an age-dependent impairment in OiP memory at 14-16 months of age across both delays compared to WT littermates. There was also a significant decline in the OiP performance of PDAPP mice at 14-16 months of age compared to their performance at 6-8 and 10-12 months of age. DR scores were analyzed using 3-way ANOVA with Bonferroni corrected post hoc analysis for significant interactions. (C-D) Levels of soluble A\( \beta \)40 and A\( \beta \)42 were then assessed in a separate colony of mice at 3 months (\( n = 5 \)), 7 (\( n = 7 \)), 11 (\( n = 7 \)), and 15 months (\( n = 7 \)) of age. (C) Levels of soluble A\( \beta \)40 and (D) A\( \beta \)42 increased with age in the hippocampus of PDAPP mice. A\( \beta \) levels were quantified by ELISA assays. Data were analyzed using the Kruskal-Wallis test with Dunn’s post hoc analysis with Bonferroni corrections. \( * p < 0.05 \); \( *** p < 0.001 \). Error bars represent the standard error of the mean (SEM). Abbreviations: ANOVA, analysis of variance; DR, discrimination ratio; OiP, object-in-place; WT, wild type.
mice continued to show reduced contact times across sample trials, $p^s < 0.001$ (Supplementary Table 3A). During the test phase, overall mice showed a preference to explore objects in novel locations over familiar, $p < 0.001$ (Supplementary Table 4A). However, WT mice had higher contact times with objects both in familiar and novel locations than vehicle PDAPP mice, $p$ values $< 0.01$, and PDAPP 2B3-treated mice, $p$ values $< 0.05$. No significant differences in contact times were observed between PDAPP treatment groups, $p > 0.1$.

An analysis of the DRs, however, showed a significant treatment x time point interaction, $F(2, 39) = 3.27, p < 0.05$. Tests of simple main effects revealed that PDAPP mice administered ICV 2B3 showed a significant improvement in performance compared to both their pre-administration, $p < 0.01$, and to vehicle PDAPP mice performance, $p < 0.001$. In contrast, vehicle PDAPP mice remained significantly impaired relative to WT controls, $p < 0.001$, and 2B3 PDAPP mice were not significantly different to WT mice, $p > 0.5$ (see Fig. 4A). Thus, ICV infusion of 2B3 in PDAPP mice reversed an age-dependent deficit in associative recognition memory.

### 3.2.2. Experiment 2b
To investigate further how inhibition of APP metabolism at the BACE1 cleavage site affected healthy WT control animals, a separate group of mice were directly administered ICV 2B3. Similar to PDAPP mice, 2B3 administration in WT mice showed no overall changes in object contact times across sample phases for object novelty and OiP tasks relative to WT IgG1 controls, $p^s > 0.1$ nor during the test phases, $p^s > 0.1$ (Supplementary Table 3C and 4C, respectively). Interestingly, while both 2B3 and control WT IgG1 mice explored novel objects in preference to familiar objects, a numerical reduction in contact times with objects in novel locations was observed in 2B3 mice (Supplementary Table 4C).

**Figure 4.** 2B3 administration prevents the onset of and reverses age-dependent cognitive deficits in PDAPP mice. (A) PDAPP mice treated with 2B3 (n = 10) by ICV administration showed a significant improvement in OiP memory compared with their pretreatment scores (***$p < 0.01$) and performed significantly better than vehicle-treated PDAPP mice (n = 11; ***$p < 0.001$). No difference in DR scores were reported when comparing WT mice (untreated n = 11, vehicle n = 10) to PDAPP 2B3 mice. However, vehicle PDAPP mice remained impaired compared with WT mice (###$p < 0.001$). (B) Peripheral administration of 2B3 to PDAPP mice (n = 9) before the onset of impaired OiP performance showed that no decline in OiP performance occurred after 15-week 2B3 administration, $p > 0.05$. 2B3 PDAPP mice further showed a greater OiP performance than vehicle PDAPP mice (n = 10), (###$p < 0.001$), which did show an age-dependent decline in OiP performance (**$p < 0.01$). PDAPP vehicle mice were further impaired compared with WT mice (untreated n = 10, vehicle n = 10; ###$p < 0.001$), whereas 2B3-administered PDAPP mice were not. (C) No significant differences were reported between groups in peripheral 2B3 administration when comparing novel object recognition memory, indicating all mice were able to encode object information. (D) Peripheral PDAPP vehicle mice showed an age-related increase in foraging errors compared with WT mice (###$p < 0.001$). PDAPP mouse administered 2B3, however, made less foraging errors than PDAPP vehicle controls (**$p < 0.01$) and less errors than their pre-2B3 administration scores (###$p < 0.001$). (E) Peripheral vehicle PDAPP mice showed an age-dependent increase in repeat errors made in the foraging task (**$p < 0.01$), which was not observed following 2B3 administration (p > 0.05). Vehicle PDAPP mice made significantly more repeat errors than 2B3 PDAPP mice (**p < 0.05) and WT littermate controls (###$p < 0.001$). DR scores and foraging errors were analyzed using a 3-way ANOVA with Bonferroni corrected post hoc analysis for significant interactions. Error bars express the SEM. Abbreviations: ANOVA, analysis of variance; DR, discrimination ratio; ICV, intracerebroventricular; OiP, object-in-place; SEM, standard error of the mean; WT, wild type.
however, this failed to reach statistical significance. When the data from both tasks were converted to DRs, there was no significant difference between 2B3 and control IgG1 mice on the novelty test ($t < 1$) but a significant deficit in 2B3 mice on the OiP task ($t(13) = 2.66, p < 0.05$; Supplementary Figure 1B). Furthermore, both groups performed the novel object recognition memory task above chance (0.5), $p's < 0.01$ (Supplementary Figure 1A). In contrast, 2B3 mice did not perform above chance level (0.5) on the OiP task, $p > 0.5$, unlike WT IgG1 control mice, $p < 0.01$ (Supplementary Figure 1B). These results indicate that 2B3 (but not a control IgG1) administered to normal WT mice leads to a selective disruption of associative recognition memory.

### 3.3. Experiment 3

Analysis of contact times with objects across sample trials for both recognition memory tasks showed that all groups explored the objects at similar levels, $p > 0.05$ (Supplementary Table 3B). All mice also showed habituation of exploratory activity to objects, as indicated by a decrease in contact times when comparing sample trial 1 to sample trial 3, $p's < 0.001$. Following contact time analysis within the test phase of the object novelty and OiP tasks, mice showed no significant differences in overall contact times with all objects $p's > 0.05$ (Supplementary Table 4B). In the OiP task, despite treatment groups showing numerical differences in contact times
between objects in novel and familiar locations, all mice continued to show a general preference to explore objects in novel locations over familiar, $p < 0.001$. The same pattern was also observed in the object novelty task.

However, analysis of the OiP test DRs revealed a significant time × treatment group interaction, $F(2, 34) = 3.41, p < 0.05$. Testing for simple main effects further revealed that, although vehicle PDAPP mice showed a decline in OiP performance across pre- and post-treatment time points, $p < 0.01$, IP administration of 2B3 prevented this decline, $p > 0.05$. Moreover, both WT and 2B3 PDAPP mice showed comparable performance ($p > 0.05$) and both groups performed better than vehicle PDAPP mice, $p < 0.001$ (Fig. 4B). Analysis of the object novelty task DRs showed no effect of treatment group, $F(2, 34) = 0.16$, $p > 0.1$, or treatment group × time interaction, $F(2, 34) = 0.27$, $p > 0.1$, (Fig. 4C). To summarize, the results showed that peripheral administration of 2B3 before the onset of cognitive decline prevented the age-dependent deficit in associative recognition, without influencing object novelty detection, in PDAPP mice.

To establish the generality of the improvement in associative OiP memory with 2B3, PDAPP mice were also tested on a spatial working memory foraging task (Evans et al., 2018). Time to complete the foraging task after vehicle or 2B3 administration was similar between all groups, $p > 0.05$ (Supplementary Table 5), which indicated that neither transgene expression nor 2B3 administration influenced the motivation to complete the task. However, foraging accuracy was improved in 2B3 PDAPP relative to vehicle PDAPP mice (Fig. 4D and E). More specifically, analysis of the mean total number of errors (a measure of overall foraging accuracy) revealed a significant treatment time × group interaction, $F(2, 34) = 3.68$, $p < 0.05$. Tests of simple main effects showed that 2B3 PDAPP mice made less errors than their pre-drug assessment at 11 months of age, $p < 0.05$ and less errors than vehicle PDAPP mice, $p < 0.01$ (Fig. 4D). In contrast, vehicle PDAPP mice made more errors than WT vehicle controls, $p < 0.001$ (Fig. 4D). WT mice and 2B3 PDAPP mice performed at a comparable level, $p > 0.05$. A similar pattern of results was observed when analyzing repeat errors, a measure of working memory (Fig. 4E). An ANOVA revealed a significant treatment time × group interaction, $F(2, 34) = 3.85$, $p < 0.05$. Simple main effects analysis revealed that 2B3 PDAPP mice made fewer repeat errors than vehicle PDAPP mice, $p < 0.05$, and that there was no significant change in the number of repeat errors compared to pre-drug assessment, $p > 0.05$. In contrast, vehicle PDAPP mice showed an age-dependent increase in the total number of repeat errors compared to pre-drug assessment, $p < 0.05$. Vehicle PDAPP mice also made more repeat errors than WT mice, $p < 0.01$. This impairment was not present in 2B3 PDAPP mice, $p > 0.05$.

3.4. Biochemical analyses: experiments 2a, 2b, and 3

In experiment 2a, Western blot analysis showed that ICV 2B3 administration caused no change in total levels of BACE1, $F(2, 28) =$
NR1, evident in experiment 3 after IP 2B3 administration (maximum NR2B, receptor subunits, NR1, or NR2B in hippocampal synaptosomes of ICV total expression levels of the synaptic marker PSD95 or NMDA receptor phosphorylation. Previous work in normal animals has explored object contact times of WT mice in either the sample or test phases when compared with vehicle contact times, p's > 0.05 (Supplementary Tables 6 and 7). Moreover, despite numerical differences, both vehicle (p < 0.001) and Ro-25-6981 (p < 0.01) mice explored objects in novel locations more than those in familiar locations (Supplementary Table 7). However, an analysis of the test DR scores showed that Ro25-6981 impaired performance relative to vehicle administration, t(9) = 2.47, p < 0.05 (Fig. 6F). These data confirm that, under normal physiological conditions, the NR2B receptor plays a key role in associative recognition memory processes in normal aged WT mice.

4. Discussion

Male PDAPP mice showed a selective age-dependent impairment in associative recognition memory, while sparing object novelty detection. Similar to previous studies, the age-dependent decline in associative recognition memory coincided with a rise in hippocampal Aβ levels (Barker and Warburton, 2013; Good and Hale, 2007; Hale and Good, 2005; Selkoe, 2001). ICV administration of the anti-APP antibody, 2B3, directed against the BACE1 cleavage site, reduced APP metabolism and lowered Aβ and βCTF levels in the hippocampus. The reduction in Aβ was accompanied by restoration of associative recognition memory in aged PDAPP mice. Furthermore, longitudinal peripheral administration of 2B3 prevented the onset of associative recognition memory impairment as well as a decline in spatial working memory in PDAPP mice at 15 months of age. A critically important aspect of our results is that both ICV and IP administration of 2B3 normalized a deficit in NMDA receptor phosphorylation. Previous work in normal animals has shown that hippocampal NMDA receptors are required for associative recognition memory but not object novelty/familiarity discriminations (Barker and Warburton, 2013). Our results are,
Vehicle performance (**intraperitoneal; OiP, object-in-place; SEM, standard error of the mean; WT, wild type. Error bars represent the SEM. Abbreviations: ANOVA, analysis of variance; DR, discrimination ratio; ERK, extracellular signal regulating kinase; ICV, intracerebroventricular; IP, intraperitoneal; OiP, object-in-place; SEM, standard error of the mean; WT, wild type.

Scores of the OiP task after Ro25-6981 administration, a selective NR2B antagonist, showed impaired OiP performance in 17- to 18-month-old WT mice (n = 10) compared with WT (***p < 0.001) and 2B3 PDAPP mice (****p < 0.001). (C) No significant changes were reported in total levels of the Src kinase Fyn, or STEP61 (p values > 0.05); however, PDAPP vehicle mice showed a significant increase in total levels of STEP46 compared with WT mice (**p < 0.01). (D) Total levels of ERK were increased in vehicle PDAPP mice compared with WT (**p < 0.01) and 2B3 PDAPP mice (****p < 0.001). Despite an apparent numerical reduction in phosphorylated (active) ERK in vehicle PDAPP mice, no significant differences were reported in total levels of ERK; vehicle PDAPP mice had a significant reduction in phosphorylated ERK compared with WT (**p < 0.05) and 2B3 PDAPP mice (****p < 0.001). (E) A similar result was observed in PDAPP mice administered chronically with 2B3 by IP injection. A representative blot demonstrates a reduction in NR2B pY1472. No significant change was observed in total levels of NR2B (p values > 0.1). Vehicle PDAPP mice showed a significant reduction in pY1472 compared with WT controls (**p < 0.05). When pY1472 was expressed as a ratio of total NR2B, vehicle PDAPP mice had a significant reduction in phosphorylated NR2B (**p < 0.01) and 2B3 PDAPP mice (*p < 0.05). There was no significant difference when comparing WT and 2B3 mice. (F) DR scores of the OiP task after Ro25-6981 administration, a selective NR2B antagonist, showed impaired OiP performance in 17- to 18-month-old WT mice (n = 10) compared with vehicle performance (**p < 0.01). All Western blot data were analyzed using 1-way ANOVA with Tukey post hoc analysis. DR scores were analyzed using paired-samples t-tests. Error bars represent the SEM. Abbreviations: ANOVA, analysis of variance; DR, discrimination ratio; ERK, extracellular signal—regulated kinase; ICV, intracerebroventricular; IP, intraperitoneal; OiP, object-in-place; SEM, standard error of the mean; WT, wild type.

Figure 6. Impaired NMDA-NR2B signaling causes OiP impairment, which is reversed by 2B3 in PDAPP mice. (A) Representative Western blots of hippocampal synaptosomes from PDAPP mice administered 2B3 by ICV (n = 10) or vehicle (n = 11) and WT vehicle control (n = 10). Blots demonstrate changes in NR2B phosphorylation and subsequent downstream signaling cascades. No changes in total levels of PSD95, NR1 (total NMDA receptors) (relative to WT control) were reported p > 0.05. (B) No significant changes were reported in total levels of NR2B or pY1472, p’s > 0.05. However, when pY1472 was expressed as a ratio of total NR2B, PDAPP vehicle mice showed a significant reduction in NR2B phosphorylation compared with WT (**p < 0.001) and 2B3 PDAPP mice (****p < 0.001). (C) No significant changes were reported in total levels of the Src kinase Fyn, or STEP61 (p values > 0.05); however, PDAPP vehicle mice showed a significant increase in total levels of STEP46 compared with WT mice (**p < 0.01). (D) Total levels of ERK were increased in vehicle PDAPP mice compared with WT (**p < 0.01) and 2B3 PDAPP mice (****p < 0.001). Despite an apparent numerical reduction in phosphorylated (active) ERK in vehicle PDAPP mice, no significant differences were reported in total levels of ERK; vehicle PDAPP mice had a significant reduction in phosphorylated ERK compared with WT (**p < 0.05) and 2B3 PDAPP mice (****p < 0.001). (E) A similar result was observed in PDAPP mice administered chronically with 2B3 by IP injection. A representative blot demonstrates a reduction in NR2B pY1472. No significant change was observed in total levels of NR2B (p values > 0.1). Vehicle PDAPP mice showed a significant reduction in pY1472 compared with WT controls (**p < 0.05). When pY1472 was expressed as a ratio of total NR2B, vehicle PDAPP mice (n = 10) showed a significant reduction in comparison to WT control (n = 9); **p < 0.05) and 2B3 PDAPP mice (n = 9; p < 0.05). There was no significant difference when comparing WT and 2B3 mice. (F) DR scores of the OiP task after Ro25-6981 administration, a selective NR2B antagonist, showed impaired OiP performance in 17- to 18-month-old WT mice (n = 10) compared with vehicle performance (**p < 0.01). All Western blot data were analyzed using 1-way ANOVA with Tukey post hoc analysis. DR scores were analyzed using paired-samples t-tests. Error bars represent the SEM. Abbreviations: ANOVA, analysis of variance; DR, discrimination ratio; ERK, extracellular signal—regulated kinase; ICV, intracerebroventricular; IP, intraperitoneal; OiP, object-in-place; SEM, standard error of the mean; WT, wild type.

Therefore, consistent with the view that the age-related accumulation of Aβ changes hippocampal synaptic events that underpin memory (Guntupalli et al., 2016).

Previous work with PDAPP mice has reported an age-related deficit in object novelty detection (Dodart et al., 1999). However, this result has not been replicated across laboratories (Chen et al., 2000). This discrepancy may be related to the test procedure. In contrast to our own study and that conducted by Chen et al., Dodart et al. (1999) exposed mice to a single object in the sample phase and presented a familiar and novel object in the test phase. This test procedure confounded object novelty with object-location novelty. Thus, the deficit in PDAPP mice reported by Dodart et al. may have reflected impaired processing of location information. Importantly, with the exception of the present experiments, no study has
compared the impact of aging on object novelty and associative OiP memory in the same PDAPP mice longitudinally, and our evidence indicates an age-related sensitivity to associative object-location memory or mismatch detection in PDAPP mice.

Before discussing the effects of 2B3 on APP processing and cognition, it is worth highlighting 2 drawbacks of the study. One drawback is that only male mice were tested. This strategy was undertaken to minimize extraneous sources of variability in both the behavioral and biological measures and thus maximize the detection of cognitive and synaptic changes induced by 2B3. Nevertheless, given the positive results of this study in male mice and the fact that over 60% of patients with AD are female (“2015 Alzheimer’s disease facts and figures,” 2015), it would be important clearly to test the assumption that aged female PDAPP mice would also demonstrate cognitive and pathology benefits from reducing APP cleavage by BACE1.

A second drawback of the study is that the half-life of 2B3 has yet to be established in vivo. Although a single weekly injection of 2B3 was sufficient to alter NR2B expression and cognition in PDAPP mice, there was no evidence of changes in brain amyloid levels, unlike the ICV administration study. However, this lack of sensitivity to any changes probably reflects the much lower levels of 2B3 reaching the brain after peripheral IP administration, (because of the blood-brain barrier) compared with direct ICV administration. Further research is required to assess the temporal dynamics of the interactions of 2B3 with APP processing in vivo. Nevertheless, our study is the first to show that anti-APP-BACE1 and immunization approaches have been investigated. Thus, Arbel et al. (2005) reported the activity of the antibody BBS1 also directed against the BACE1 cleavage site of APP. In vivo administration of BBS1 by osmotic minipumps to 3xTg mice for 28 days resulted in a significant improvement in object-novetly memory at 17–18 month of age (Rabinovich-Nikitin et al., 2012). These improvements in behavior were complemented by a significant reduction in plaque size, Aβ load, and a 24% reduction in soluble Aβ42 (Rabinovich-Nikitin et al., 2012). Peripheral administration of BBS1 also resulted in improved object-novetly memory and reduced inflammatory markers in Tg2576 mice (Rakover et al., 2007) as well as reduced Aβ plaques and intracellular Aβ load in mice with the hAPP V717L London mutation (Arbel-Ornath et al., 2009). Despite the changes in Aβ production, BBS1 did not improve spatial reference memory, as assessed in the water maze. Our current findings confirm that steric hindrance of APP processing by BACE1 can have beneficial effects on amyloid levels and associative spatial recognition memory processes that rely upon the hippocampus. Unlike the present study, however, the effect of BBS1 on synaptic processes sensitive to amyloid accumulation was not examined. A related study by Chang et al. (2007) immunized mice against memapsin-2 (BACE1) with the hypothesis that anti-memapsin-2 antibodies would bind to memapsin-2 located on the surface of neurons and, when endocytosed, would interfere with the cleavage of APP and thus lower Aβ production. Immunization of Tg2576 with anti-M2 saw a reduction in amyloid load and improvement in performance in a water maze reference memory task. Unlike the present study, however, the extent of the cognitive change was not indexed against WT control mice but only vehicle-treated Tg2576 mice. However, taken together, our results, and those of Chang (ibid), confirm that selectively targeting BACE cleavage of APP can have positive effects on amyloid production and memory function. Importantly, however, our study is the first to show that an anti-APP antibody targeting the BACE1 cleavage site improved associative recognition memory and spatial working memory and reduced phosphorylation of NMDA-NR2B receptors. In addition, it is worth noting that 2B3 caused a significant reduction in levels of βCTF, an effect that has not previously been reported with BBS1 or anti-M2. Increased levels of βCTF have been observed in patients with AD, and recent research has revealed an Aβ-independent mechanism causing dysregulation of endocytosis (Kim et al., 2015; Pimplikar et al., 2010). Consistent with this evidence, genetic reduction in BACE1 activity in a mouse model of Down syndrome improved endosomal volume and improved cholinergic markers without lowering Aβ levels (Jiang et al., 2016). It is possible, therefore, that the reduction in βCTF levels by 2B3 may have had a beneficial impact on endocytic pathways, and this possibility requires further investigation.

In terms of the clinical relevance of our findings, evidence is beginning to emerge that some antibody-based therapies have a positive impact on amyloid pathology and may be disease-modifying in amyloid-positive patients (e.g., BAM2401, https://www.elsevier.com; aducanumab, Sevigny et al., 2016). Although these findings are preliminary, the idea that such immunotherapies may be useful in the context of treating individuals who are amyloid positive in old age is gaining traction. However, our evidence that 2B3 disrupted associative (but not object novelty) recognition memory in normal young mice confirms that metabolism of APP by BACE1 is an important physiological process that contributes to memory in healthy controls (see also Blume et al., 2018; Ou-Yang et al., 2018; and review by Zhu et al., 2018 for further discussion).
Therefore, targeting this pathway in healthy presymptomatic aged participants should be combined with close monitoring for deleterious changes in memory function.

As highlighted by Piton et al. (2018), modulation of BACE activity remains a viable strategy in trials on prodromal and early AD. Our data add to a growing body of preclinical evidence that selectively modifying APP processing at an early stage of amyloid accumulation in the aging amyloid-positive brain has a beneficial effect on cognition. It also confirms that APP processing by BACE1 has a role in normal memory function and that disruption to this equilibrium is detrimental. An antibody therapy that targets APP processing by BACE1 may therefore only have clinical relevance in the context of amyloid-related cognitive changes in the elderly (see Farrell et al., 2017; Hedden et al., 2012).

Conclusion

In conclusion, the present study has shown that selectively influencing APP metabolism by reducing BACE1 activity by steric hindrance with an antibody, reversed and protected against an age-dependent associative recognition memory deficit in PDAPP mice. ICV administration of 2B3 reduced levels of soluble AβI and jCTF without affecting total levels of APP, and both ICV and chronic IP administration normalized the phosphorylation of NMDA-NR2B receptors. These novel findings provide important evidence that selective inhibition of APP processing at the BACE1 cleavage site can improve both memory and markers of synaptic pathology in a mouse model of age-related amyloid accumulation. Finally, the results contribute to other findings suggesting that modification of APP processing, and associated downstream glutamatergic signaling cascades, may be beneficial in populations with altered APP metabolism, including aged individuals (Rodrigue et al., 2012) and those at high risk of AD.

Disclosure statement

All authors declare that they have no competing financial interests.

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Authors’ contributions: MAG, EJK, and RST conceived the study and subsequent funding. MH-E obtained pilot data used for obtaining funding. CEE, TJF, and MAG designed animal experiments and performed surgical procedures. CEE, MH-E, TJF, and RST prepared 2B3. CEE and MH-E bred all mice used in this study. CEE and TJF performed animal experiments and carried out antibody administration, and CEE performed the biochemical analysis. CE analyzed data. CEE, MAG, and EJK wrote the manuscript. All authors reviewed and approved the manuscript.

Declarations

Ethical approval and consent to participate: All procedures for animal use were approved according to UK Home Office under the Animal Scientific Procedures Act (1986) and EU regulations.

Consent for publication: All authors have approved of the contents of this manuscript and provided consent for publication.

Availability of supporting data: The data and materials are available from corresponding authors on reasonable request.

Appendix A. Supplementary data

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