

Structure-dependent effects of amyloid- β on long-term memory in *Lymnaea stagnalis*

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Amyloid- β ($A\beta$) peptides are implicated in the causation of memory loss, neuronal impairment, and neurodegeneration in Alzheimer's disease. Our recent work revealed that $A\beta$ 1–42 and $A\beta$ 25–35 inhibit long-term memory (LTM) recall in *Lymnaea stagnalis* (pond snail) in the absence of cell death. Here, we report the characterization of the active species prepared under different conditions, describe which $A\beta$ species is present in brain tissue during the behavioral recall time point and relate the sequence and structure of the oligomeric species to the resulting neuronal properties and effect on LTM. Our results suggest that oligomers are the key toxic $A\beta$ 1–42 structures, which likely affect LTM through synaptic plasticity pathways, and that $A\beta$ 1–42 and $A\beta$ 25–35 cannot be used as interchangeable peptides.

Keywords: amyloid beta; classical conditioning; long-term memory; *Lymnaea*; oligomer

Amyloid β ($A\beta$) is cleaved from the amyloid precursor protein (APP) to produce a range of $A\beta$ isoforms of which $A\beta$ 1–40 and $A\beta$ 1–42 are the most common. This APP cleavage process is well-defined, with $A\beta$ peptides predominantly being produced *via* α - or β - and γ -secretases [1,2]. Alongside $A\beta$ peptides, other APP fragments are produced in the cleavage process and are believed to play neuroprotective and neurotrophic roles in the brain [1,2]. The function of APP and its peptides are unknown, but appear to have an important role in neuromuscular junction formation, synaptic transmission, and ion channel function [3]. In

Alzheimer's disease (AD), the shift from healthy protective function to pathogenic cell death arises from an increase in $A\beta$ 1–42 production and oligomerization [1,4,5]. Other $A\beta$ peptides of various lengths have also been found in both AD brains and cerebrospinal fluid [6,7], being produced *via* caspases and proteolytic degrading enzymes [1]. These fragments have been suggested to play an important role in pathology [1].

One of these shorter fragments, $A\beta$ 25–35, has been detected in AD plaques and is a known cleavage product of $A\beta$ 1–40 racemized at D-Ser26 [8]. $A\beta$ 25–35 may represent the toxic core of the more

Abbreviations

$A\beta$, amyloid- β ; AD, Alzheimer's disease; APP, amyloid precursor protein; CGC, cerebral giant cell; HFIP, hexafluoroisopropanol; LTM, long-term memory; SEM, standard error mean; TEM, transmission electron microscopy.

physiologically prevalent toxic species A β 1–42 [9], and it displays similar fibrillization through β sheet formation [10]. For this reason, many labs utilize A β 25–35 as a cost-effective means of studying A β . Of the different length peptides, available both synthetically and in AD-related tissues, it is generally agreed that these peptides are toxic when they exist as small, prefibrillar oligomers [11–15]. Specifically, dimers and dodecamers have been directly linked to toxicity and behavioral disruptions [12,13,16–18], although others suggest that all soluble low-n oligomers could be toxic [19,20]. These toxic oligomers produce AD pathology by first disrupting synapse function in memory-encoded neuronal circuitry, further developing into synaptic degeneration, and finally full cell death [12,13,21].

Amyloid precursor protein is highly evolutionarily conserved, with > 95% sequence homology existing across mammalian species and high homology within invertebrate species [22,23]. Many invertebrate model organisms have been used for A β and AD studies [24,25]. For example, *Drosophila* has an APP ortholog, APPL [26], an α -secretase ortholog [27], and components of γ -secretase [28–30]. This γ -secretase can process human APP [31,32] and human APP can be cleaved to produce A β in flies, suggesting an endogenous β -secretase-like protease in *Drosophila* [32]. APP and the protease processing system (presenilin 1 and 2) are well conserved across the animal kingdom and APP mRNA expression has been shown in the ganglia of *Aplysia californica* [33], which is closely related to *Lymnaea*.

A β and AD research has only rarely branched into molluscan model systems, although these offer a wealth of information on cellular and molecular mechanisms of memory function and dysfunction by providing uniquely tractable models in the field [34]. Indeed, the use of mollusks such as the sea slug *A. californica* and the pond snail *Lymnaea stagnalis* helped build much of the molecular and electrophysiological understanding of learning and memory [34–40]. The first group to utilize a mollusk in A β memory studies considered A β 25–35 in the land snail *Helix lucorum* [41]. In these experiments, the researchers reported that the animals' conditioned food aversion reflex was inhibited when A β was administered before training [41]. Our lab expanded the A β studies in mollusks, finding that A β 1–42 and A β 25–35 disrupted consolidated long-term memory (LTM) in the pond snail *L. stagnalis* [42].

Our studies brought about a very intriguing question: Are the two peptides affecting memory consolidation *via* similar pathways? Although both peptides ultimately disrupted consolidated long-term memory prior to neuronal death, there were significant

differences in: (a) peptide production, (b) morphology, (c) quantity of oligomers in the hemolymph, and (d) effects on neuron electrical properties.

- 1 Firstly, although A β 1–42 and A β 25–35 both disrupted consolidated long-term memory, the two peptides were produced under very different conditions [42]. A β 1–42 was administered at 1 μ M directly into the hemolymph, with an expected final concentration of 1 nM. Moreover, A β 1–42 was solvent prepared. In this preparation method, the lyophilized peptide is solubilized in a fluorinated alcohol for disaggregation, dried, and resolubilized in DMSO. The solubilized peptide then undergoes removal of solvent *via* a desalting column and buffer-exchange into a final normal saline solution, and is centrifuged to remove any insoluble aggregates. This solvent preparation method (see Materials and methods) has been well studied [42–44] and produces maximal soluble A β 1–42. In contrast, lyophilized A β 25–35 was solubilized directly into saline solution (as for *H. lucorum* [41]), incubated for 2 h at room temperature, and then administered at 0.1 mM with an expected final concentration of 0.1 μ M [42]. When injected at 1 μ M, memory was no longer disrupted which suggests that a significantly higher concentration of A β 25–35 was necessary for similar behavioral effects [42].
- 2 Comparison of the morphological features of A β 1–42 and A β 25–35 peptides over a 24-h *in vitro* assembly by transmission electron microscopy (TEM) revealed significant differences between the structures formed by the two peptides under these conditions. A β 1–42 followed a self-assembly pathway from oligomeric, to protofibrillar, and finally fibrillar states while A β 25–35 was predominantly crystalline in morphology and aggregated further over time [42].
- 3 Hemolymph of both A β 1–42- and A β 25–35-treated animals underwent formic acid extraction, immunogold labeling with A β oligomer antibody Nu1 [12], and were visualized with TEM. Both samples contained more A β oligomer labeling than buffer-treated controls, but with a 600-fold more labeling in A β 1–42-treated animals compared to A β 25–35 [42].
- 4 Finally, the two peptides disrupted properties of the *Lymnaea* nervous system differently. A β 25–35 caused a decrease in input resistance and an abolition of the learning-induced depolarization of the membrane potential of the cerebral giant cell (CGC), a key neuron underlying memory [45,46], while A β 1–42 had no detectable effect on CGC electrical properties [42].

These findings led us to consider whether the observed behavioral and electrophysiological differences in the A β peptides are due to the different lengths and sequences of the peptides, or to the structure the peptides adopted by the 24-h postinjection time point when the memory test was conducted. If the primary structure is the critical difference between the two peptides, the method of preparation should not alter the peptide's effect on behavior or electrophysiology. However, if the peptide's effect on behavior is related to its structure at the 24-h postinjection time point, then a difference in peptide preparation should have drastic effects on the resulting behavior and electrophysiology. To address these questions, we prepared A β 25–35 using the previously mentioned solvent preparation method [44], and report the resulting changes in LTM, electrical neuronal properties, peptide morphology, and quantity of oligomers in the hemolymph after 24 h of *in vivo* incubation. Here, we reveal significant differences in the effects of peptides formed under different conditions and with different structures; expanding knowledge of the effects of oligomeric A β on memory and cellular functions in the brain.

Materials and methods

Experimental animals

Pond snails, *L. stagnalis*, were bred and maintained in 18–22 °C copper-free water in large holding tanks, with a 12 : 12 h light–dark cycle. The animals were fed twice a week with Tetra-Phyll (TETRA Werke, Melle, Germany) and with lettuce three times a week. Three days before each experiment an appropriate number of animals were transferred into the behavioral laboratory where they were kept in smaller tanks in a food-deprived state before the experiments commenced.

Preparation and systemic application of A β peptides

A β peptides were solvent prepared, as described previously [42,44]. Briefly, 0.2 mg A β Fragment 25–35 (Sigma-Aldrich, Irvine, UK) or A β 1–42 (rPeptide; Bogard, GA, USA) were solubilized in hexafluoroisopropanol (HFIP; Sigma-Aldrich) to disaggregate the peptides, and then dried completely to remove HFIP. This protocol has been optimized [43] and has been shown to reproducibly produce soluble, oligomeric A β 1–42 [42,44,47]. Once HFIP was completely evaporated, dry DMSO (Sigma-Aldrich) was added to the A β . The A β was then added to a prepared Zeba buffer-exchange column (ThermoFisher Scientific, Paisley, UK)

with a normal saline solution (50 mM NaCl, 1.6 mM KCl, 2 mM MgCl₂ · 6H₂O, 3.5 mM CaCl₂ × 2H₂O, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], pH 7.9) [48] and centrifuged for 30 min at 16 000 *g*, 4 °C to remove insoluble structures and all remaining solvents [49]. This final step is critical for removing fibrillar species, leaving only soluble, oligomeric A β [44]. Protein concentration was calculated by measuring optical density at 280 nm using a NanoDrop spectrophotometer (Thermo Fisher, Paisley, UK) and correcting for the molar absorption coefficient of each peptide. A β peptides were then diluted to the 1 μ M working concentration in 100 μ L using normal saline solution at 20 °C, and were systemically injected into the animals directly after preparation using a 1-mL syringe with 30-gauge precision glide needles (Becton Dickinson, Oxford, UK). For vehicle control animals, 100 μ L of normal saline solution was injected.

Formic acid-extracted hemolymph preparation

After 24 h *in vivo* incubation of solvent-prepared A β 25–35, roughly 1 mL of hemolymph was extracted from each snail and submitted to formic acid extraction, as described previously [42,50]. Briefly, the hemolymph was mixed with equal volumes 0.4% diethylamine/100 mM NaCl. About 400 μ L was then centrifuged at 16 000 *g* for 1 h, 4 °C. Supernatant was aspirated and 200 μ L 1 M Tris pH 7.4 was added to the pellet. Four hundred microliters of cold formic acid was added and the sample was sonicated and then centrifuged at 16 000 *g* for 1 h, 4 °C. The supernatant was neutralized in 4 mL 1 M Tris, 0.5 M Na₂HPO₄, which was again centrifuged at 16 000 *g* for 1 h, 4 °C. The supernatant was neutralized with 1/10 volume 1 M Tris, pH 6.8. The samples were stored at –80 °C until used for imaging by TEM.

Transmission Electron Microscopy

As previously described [42], 4 μ L of the formic acid-extracted hemolymph sample was pipetted on to Formvar/carbon coated 400-mesh copper grids (Agar Scientific, Essex, UK), washed with Milli-Q water (EMD Millipore, Waton, UK), and negative stained with 2% uranyl acetate for 1 min. Grids were allowed to air dry. After initial imaging the samples were immunogold labeled with 1 μ g·mL^{–1} Nu1 (Klein Laboratory) [12], a mouse conformational antibody raised against oligomeric A β , and then labeled with goat anti-mouse 10 nm gold-conjugated secondary antibody (BBI Solutions OEM Ltd., Cardiff, UK) to label oligomeric structures. All grids were examined in a Hitachi 7100 TEM at 100 kV and digital images acquired with an axially mounted (2K × 2K pixel) Gatan Ultrascan 1000 CCD camera (Gatan UK, Oxford, UK).

Negative staining of solvent-prepared A β 25–35 was used to monitor peptide morphology over the incubation time.

Aliquots of 100 μM A β 25–35 were allowed to incubate in normal saline solution (50 mM NaCl, 1.6 mM KCl, 2 mM MgCl₂ \times 6H₂O, 3.5 mM CaCl₂ \times 2H₂O, 10 mM HEPES, pH 7.9 at 20 °C) in a closed Eppendorf tube for 0, 3, or 24 h. This *in vitro* incubation method previously produced reliable and reproducible results for buffer-prepared A β 25–35 and solvent-prepared A β 1–42 [42]. Samples were prepared and images acquired as stated above. This experiment was conducted three times, to ensure assembly was consistent.

Single-trial food-reward classical (CS+US) conditioning

Using well-established methods [45], *L. stagnalis* underwent single-trial food-reward classical conditioning in which the conditioned stimulus (amyl acetate) and the unconditioned stimulus (sucrose) were paired. An unpaired control was not used, as naive controls show no difference from unpaired controls behaviorally [51,52] or electrophysiologically [53]. Both the vehicle-injected control and A β -injected groups were trained. The naïve groups were not trained and were not injected, but underwent the same food-deprivation/feeding schedule and handling as the experimental groups.

Electrophysiology

The two-electrode current-clamp-based electrophysiology method employed to test the electrical properties of the CGCs has been described in detail elsewhere [45]. Briefly, the cerebral ganglia (location of the CGCs) were desheathed and treated with a solid protease (Sigma type XIV; Sigma-Aldrich) to soften the inner sheath for intracellular recording. Sharp electrodes (5–20 M Ω) were filled with 4 M potassium acetate. Signals from the intracellular electrodes were amplified using Axoclamp 2B (Axon Instrument, Molecular Devices, Sunnyvale, CA, USA) and NL 102 (Digitimer, Hertfordshire, UK) amplifiers and digitized at 2 kHz using a micro 1401 Mk II interface and analyzed using SPIKE 2 software (version 5.21; Cambridge Electronics Design, Cambridge, UK). The CGC membrane potential and input resistance as well as action potential characteristics (frequency, amplitude, half-width, and after-hyperpolarization amplitude) were analyzed over a 100-s period recorded 120 s after the initial electrode impalement. This is sufficient time to allow the cell to recover from impalement [45,53,54].

Statistical analysis

Data were analyzed using GRAPHPAD PRISM software (version 4.03; GraphPad Software Inc., San Diego, CA, USA). Normality was tested using D'Agostino and Pearson omnibus

normality test. Data were first analyzed with one-way ANOVA followed by Tukey's multiple comparison to establish significance (criterion, $P < 0.05$).

Results

Solvent-prepared A β 25–35 has no significant effect on *Lymnaea* LTM recall or electrical neuronal properties

Two important questions in the A β and AD field still demand elucidation: (a) Can synthetic A β 25–35 reliably be used in place of synthetic A β 1–42?; (b) Are the observed behavioral effects of various A β peptides on consolidated long-term memory due to different primary structures, or due to the final conformation of these peptides? We speculated briefly about the answers to these questions previously after we discovered that both A β 1–42 and A β 25–35 disrupted consolidated LTM in *Lymnaea* at different concentrations and potentially *via* different pathways [42]. Here, we aim to directly address each question by comparing synthetic peptide preparation methods and observing the resulting effect on behavior. For solvent-prepared A β , peptides are solubilized in HFIP and undergo column purification and centrifugation to remove aggregated species. This method produces soluble A β [42,44,47], which is then diluted into normal saline. Previously, buffer-prepared A β peptides had been solubilized in normal saline and vortexed briefly [41,42].

To tackle these questions, we used a single-injection and single-trial behavioral paradigm in a tractable animal model of long-term memory. *Lymnaea stagnalis* were trained using single-trial food-reward classical conditioning [45], injected with 1 μM solvent-prepared A β 25–35 or A β 1–42 24 h post-training, and tested 48 h post-training (Fig. 1A). A β -treated animals were compared to vehicle-treated control animals and naïve animals, shown in Fig. 1. A β 25–35 (1 μM) did not cause behavioral deficits; instead, the animals in A β 25–35 (1 μM) group exhibited similar behavioral responses to vehicle-injected control animals. Both A β 25–35 (1 μM)-treated and vehicle-injected animals exhibited a significantly greater feeding response to the conditioned stimulus compared to naïve and A β 1–42 (1 μM)-treated animals (Fig. 1B). Thus, solvent-prepared A β 25–35 (1 μM) does not disrupt memory in contrast to solvent-prepared A β 1–42 (1 μM), when applied at equal concentrations.

We continued our investigation to determine whether solvent-prepared A β 25–35 could alter spike characteristics and two of the key electrical properties of the CGCs, membrane potential and membrane

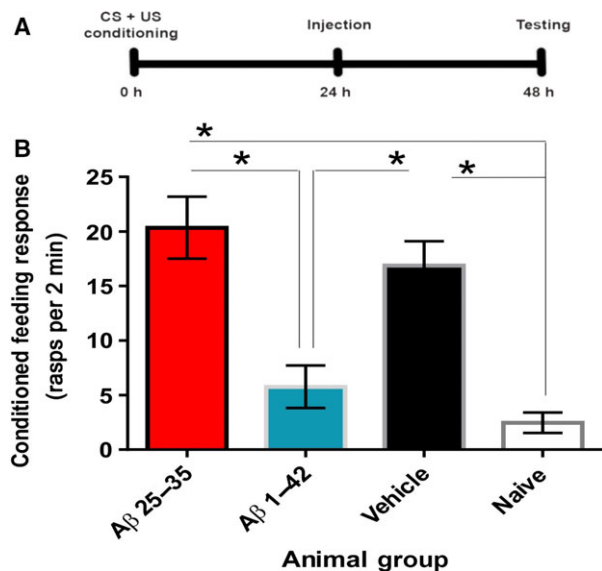


Fig. 1. Solvent-prepared A β 25–35 (1 μ M) does not cause memory impairment when allowed to incubate *in vivo* for 24 h. (A) Timeline of the experiment. CS, conditioned stimulus; US, unconditioned stimulus. (B) Four starved animal groups [solvent prepared A β 25–35 ($n = 23$) and A β 1–42 ($n = 13$), buffer-only vehicle ($n = 18$), naïve (untreated and untrained) ($n = 17$)] were tested for rasp rate to amyloid acetate, a measure of the feeding response to the conditioned stimulus. Means \pm standard error mean (SEM) values are shown. Asterisks indicate behavioral responses that are significantly different between groups. ANOVA, $P < 0.0001$. Tukey's multiple comparisons with $P < 0.05$: A β 25–35 vs. Naïve, A β 25–35 vs. A β 1–42, Vehicle vs. Naïve, Vehicle vs. A β 1–42.

resistance, both of which were shown to be affected by buffer-prepared A β 25–35 [42]. Of the measured parameters, learning-induced depolarization of the CGC soma membrane was linked to long-term memory in previous studies [46] with the other parameters remaining unaffected by single-trial classical conditioning [45]. The hypothesis we were testing here was that the solvent-prepared A β 25–35's inability to disrupt memory was predominantly due to a lack of effect on the CGC's membrane potential. The other parameters were measured because it could not be ruled out that similar to its buffer-prepared version, solvent-prepared A β 25–35 would abolish learning-induced depolarization of the CGC soma membrane but the memory impairing effects of this change would be compensated for by homeostatic changes in spike characteristics or input resistance. In accordance with previous findings [45], no change was observed in key parameters of the CGC action potentials, such as spike frequency, amplitude, half-width, or after-hyperpolarization after classical conditioning (Fig. S1). The input resistance of the CGC soma membrane was also unaffected by solvent-

prepared A β 25–35 (Fig. 2). Importantly, the CGC's membrane potential remained depolarized, similar to vehicle controls (Fig. 2).

Characterization of solvent-prepared A β 25–35

From the combined behavioral and electrophysiological experiments from Figs 1 and 2, we were very curious to understand the conformation and state of oligomerization of the apparently benign, solvent-prepared A β 25–35. We predict that by altering the method of peptide preparation from buffer prepared [41,42] to solvent prepared, the conformational state of A β 25–35 has been altered and this has affected A β 25–35's ability to disrupt nonsynaptic plasticity in *Lymnaea* central nervous system and LTM [42,45]. An intriguing point is thus raised: a simple alteration of peptide preparation, and thus conformation of the protein, is directly related to its function. We continued our investigation into this benign A β 25–35 to understand what morphological change occurred as a result of solvent preparation.

A β 1–42 self-assembles from soluble monomer, to soluble low- n oligomers and large- n oligomers, and finally to cross- β fibrils [9]. Along this pathway, morphologically distinct oligomeric, protofibrillar, and fibrillar states can be observed using negative stain TEM. To investigate the assembly of the solvent-prepared A β 25–35, the peptide was allowed to assemble *in vitro* at room temperature in a closed Eppendorf tube over a 24-h period and samples were examined by TEM after 0, 3, and 24 h of incubation. There were no observable species at either the 0- or 3-h time points, suggesting the peptide remains in an unassembled or low- n oligomeric state. Due to negative stain method constraints, a resolution limit of about 3 nm exists [54]. Therefore, it is unlikely that a monomer or low- n oligomer of A β 25–35 structure can be visualized using this method. By 24 h, amyloid-like fibrils had formed (Fig. 3). These fibrils have a pronounced curved appearance. There was no evidence of higher order oligomer formation at any observed time point (Fig. 3A,B).

The resolution limitations of negative stain may be the reason why no oligomeric species were observed. To examine whether oligomeric A β 25–35 is found *in vivo* following administration of solvent preparation samples, A β 25–35 was extracted from the animals' hemolymph 24 h after treatment using formic acid and prepared for immunogold labeling and imaging using TEM [50]. Soluble fractions were added to a TEM grid, negative stained, immunogold labeled using the anti-A β oligomer antibody Nu1 [12] and a gold-conjugated secondary antibody, and imaged using TEM.

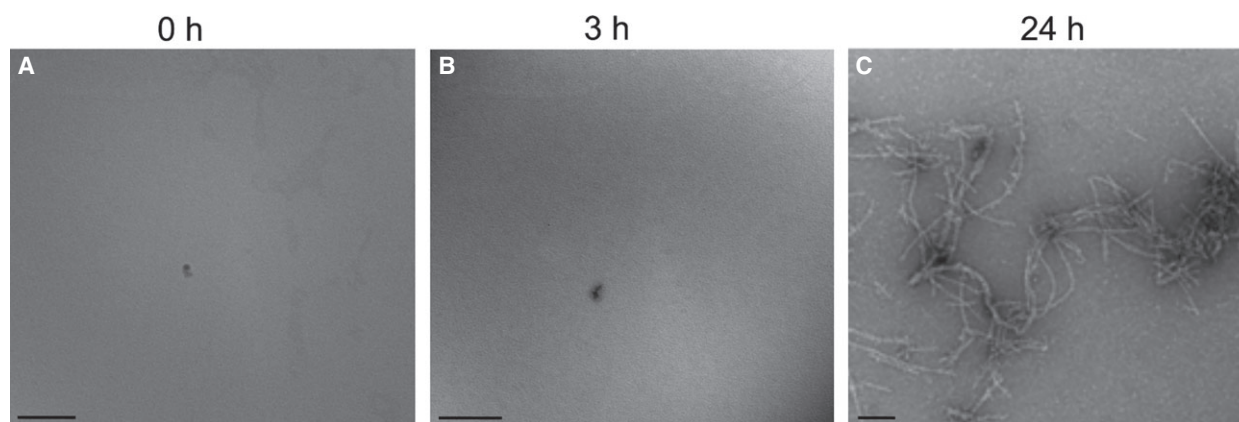
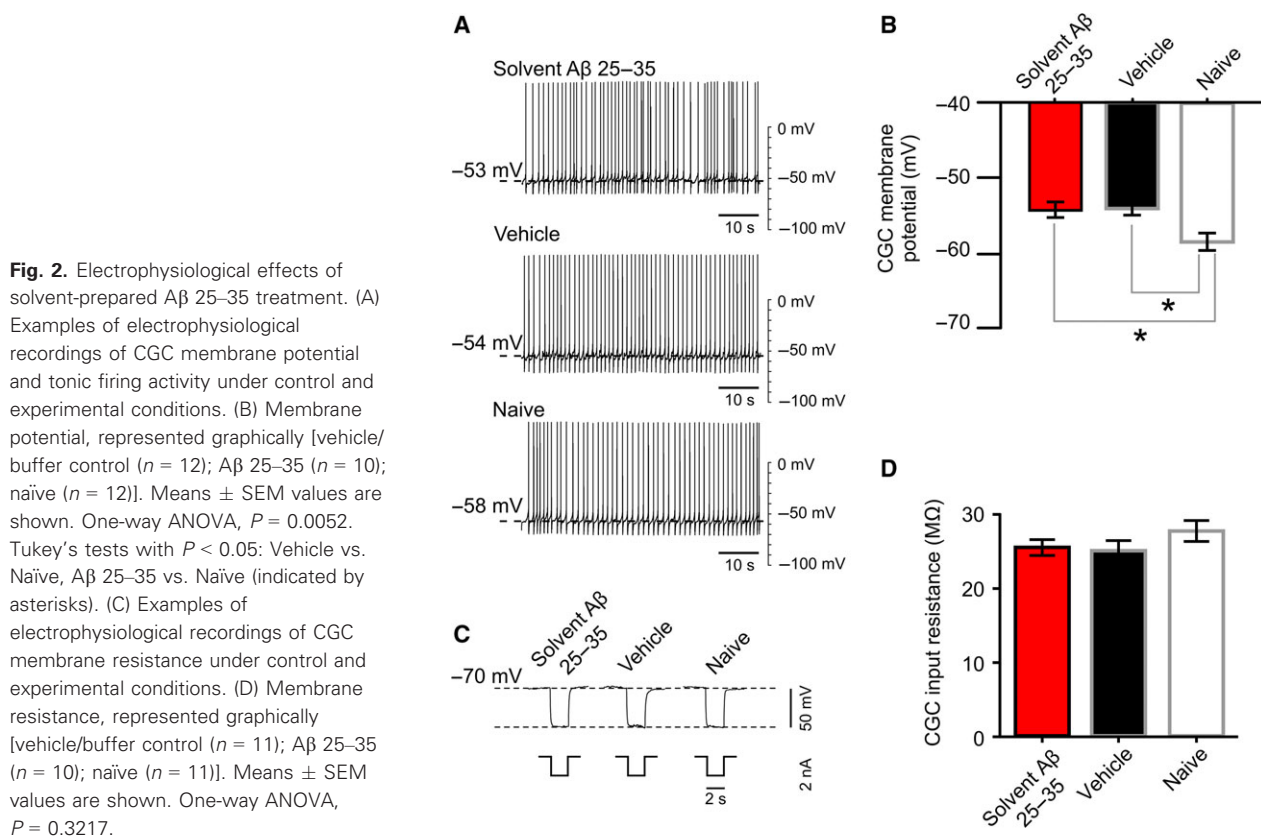


Fig. 3. Solvent-prepared A β 25–35 fibrilizes when allowed to incubate in normal saline solution for 24 h. About 100 μ M solvent-prepared A β 25–35 was prepared as described in Materials and methods and allowed to aggregate in normal saline solution over a 24-h period. Samples were taken at 0, 3, and 24 h, negative stained, and imaged using the TEM. The peptide self-assembles over the 24-h period. (A) No observable A β 25–35 species are found at the 0-h time point. (B) No observable A β 25–35 species are found at the 3-h time point. (C) A β 25–35 fibrils are found at the 24-h time point. Scale bars represent 100 nm.

Even if the oligomers are too small for visualization by TEM, the antibody gold particles will indicate areas where A β 25–35 oligomers are present. Extracts from animals treated with solvent-prepared A β 25–35 expressed negligible labeling, less than 1 gold label per

micrograph (Fig. 4A). This was similar to the vehicle-injected (buffer only) animal oligomer levels (Fig. 4B). A β 1–42-injected positive controls labeled very well with this immunogold-labeling method (Fig. 4C) and the Nu1 antibody was validated by a lack of labeling

in the secondary antibody-only method control (Fig. 4D). The only sample with significant immunogold labeling was the positive control (Fig. 4). This suggests that nonspecific antibody labeling was very low and that oligomeric A β 25–35 species were not present at detectable levels in the sample extracted from treated animals.

Discussion

Solubilized A β peptides are commonly used in amyloid studies. A β 1–42 is the predominant toxic species, as it has been linked to AD and the accompanying memory loss and neuronal death [9]. However, A β 25–35 is still commonly utilized as an affordable alternative to A β 1–42, as it has been shown to be toxic to cells and retains similar structural properties [1,6–10]. Much focus has gone into appropriate preparation of A β peptides, and standardization of peptide preparation

will likely decrease experimental variability between research groups. This research is critical, as varying the preparation of synthetic peptides is known to result in morphologically and functionally distinct A β [50,55,56]. We intended to study the effect of this variability in A β peptide preparation by comparing previously published work on solvent-prepared A β 1–42 and buffer-prepared A β 25–35, with our current work on solvent-prepared A β 25–35. The effect of A β preparation on downstream memory mechanisms remains unclear. Here, we considered how A β preparation using solvent affects the range of conformational species produced and their effects on a highly tractable model animal. We considered A β 's effect on *Lymnaea* LTM, a number of electrical properties of a key neuron in *Lymnaea*'s central nervous system, the presence of oligomers in *Lymnaea* hemolymph, and the peptide's morphological change over time *in vitro*. This work not only identifies preparation methods of

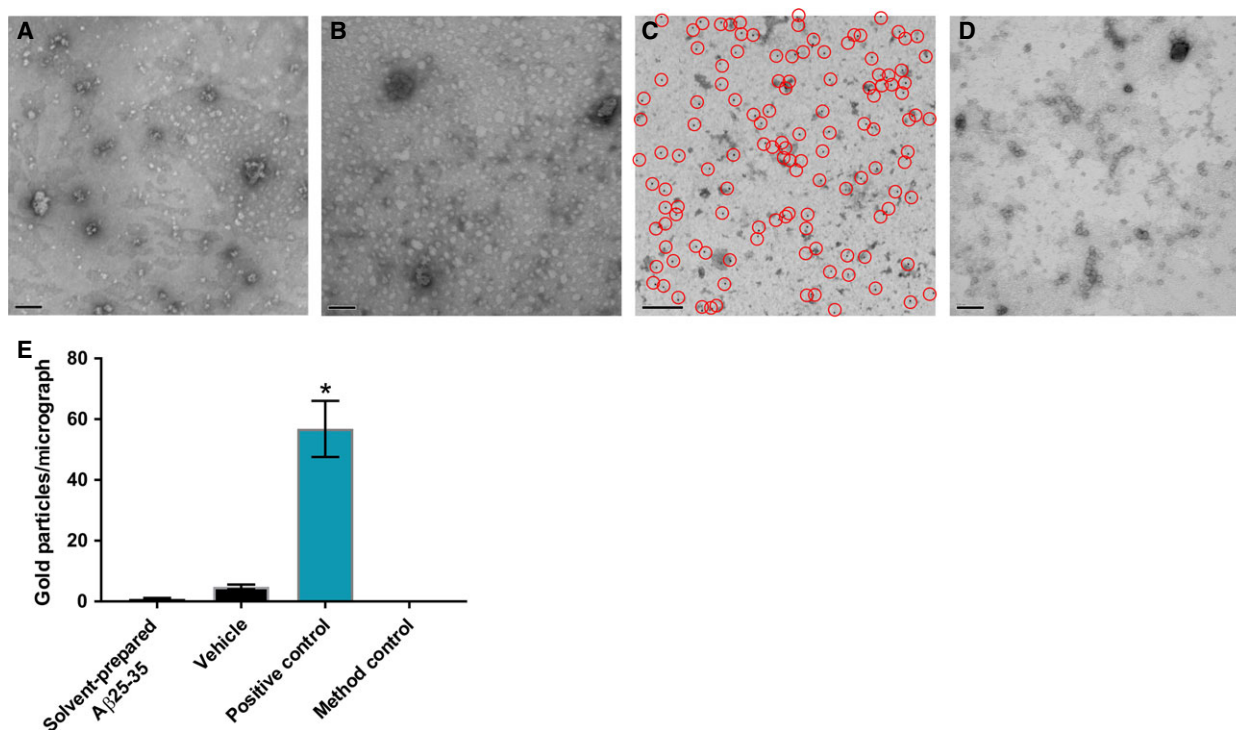


Fig. 4. Oligomeric A β is not found in the hemolymph of animals after 24 h *in vivo* incubation with solvent-prepared A β 25–35. (A) Micrograph of negative stained and Nu1 immunogold-labeled, formic acid-extracted hemolymph from animals treated with 1 μ M solvent-prepared A β 25–35 after 24 h *in vivo* incubation. (B) Micrograph of negative-stained and Nu1 immunogold-labeled, formic acid-extracted hemolymph from animals treated with vehicle after 24 h *in vivo* incubation. (C) Positive control: Micrograph of negative stained and Nu1 immunogold-labeled, formic acid-extracted hemolymph from animals treated with 1 μ M solvent-prepared A β 1–42 after 24 h *in vivo* incubation. Red circles indicate gold particles. (D) Method control: Micrograph of negative stained and secondary antibody-only labeled, formic acid-extracted hemolymph from animals treated with 1 μ M solvent-prepared A β 1–42 after 24 h *in vivo* incubation. Scale bars in A–D represent 100 nm. (E) Graphical representation of immunogold labels present in micrographs. A β 25–35 $n = 20$, Vehicle $n = 16$, positive control $n = 11$, antibody control $n = 16$. Means \pm SEM values are shown. Asterisk indicates significant differences in gold particles per image between the positive control and each of the other groups (One-way ANOVA, $P < 0.0001$; Tukey's, $P < 0.05$).

functionally relevant A β 1–42 and A β 25–35, but may indicate that care should be taken when replacing A β 1–42 with A β 25–35. Indeed, the two peptides have drastically differing effects on *Lymnaea* behavior and nonsynaptic plasticity when prepared and applied under memory-disrupting conditions and concentrations.

We observed here and in previous work that memory recall 24 h postinjection, 48 h post-training is only disrupted in A β -treated animals that retain oligomeric species in their hemolymph after 24 h *in vivo* incubation [42]. This is unsurprising, as other labs have suggested that the solvent used to dissolve synthetic A β affects the initial conformation and aggregation kinetics [57]. Importantly, the difference in primary structure of A β 1–42 and A β 25–35 drastically alters how these peptides fold in different environments, as revealed by TEM studies by comparing solvent-prepared A β 1–42 and A β 25–35 with buffer-prepared A β 25–35 [42]. This emphasis on environmental influence is critical to AD research, as A β of varying lengths have been identified in the disease [6,7]. The research presented here suggests that the formation of intermediates by A β peptides is heavily influenced by preparation method, and when A β 25–35 is solvent prepared, it does not form pathological species. Only when prepared to form

nonamyloid-like crystalline structures does A β 25–35 have the pathologically relevant effect of impairing LTM [42].

Importantly, only specific phases of memory seem to be vulnerable to A β oligomers in these studies. We found that the 24–48-h postconditioning time point is vulnerable to A β oligomers [42]. This time point is not vulnerable to A β nonoligomeric species [42]. The 0–24 h post-training time point is also not disrupted by A β treatment (Fig. 5), regardless of whether oligomeric species are present or not [42]. This supports previous studies that showed that A β oligomers are the memory-disrupting species [13,14,16,18] and research that suggests that memory phases dependent upon synapse structure remodeling are vulnerable to A β [55,58,59]. Our work supports the emerging hypothesis that oligomeric A β affects memory by altering new synaptic growth or synaptic rearrangement [60] (Fig. 5), which is necessary for the persistence of long-term memory [61]. Our exploration of structure-dependent effects of A β on behavioral function critically enhances the field in a novel way. Due to the nonamyloid-like crystallization of memory-disrupting A β 25–35, we believe this could be an artifact of improperly folded peptide, which can then disrupt the electrical properties of neurons in a non-native way. However, further

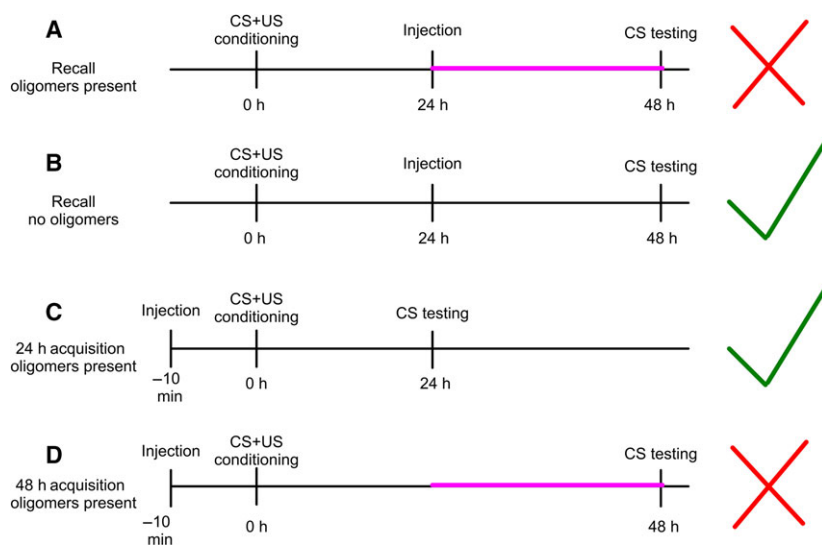


Fig. 5. Behavioral memory time lines. (A) 24-h *in vivo* incubation, memory recall time point with A β treatments that result in the presence of *in vivo* oligomers after 24 h. Memory is inhibited. (B) 24-h *in vivo* incubation, memory recall time point with A β treatments that exhibit no *in vivo* oligomers after 24 h. Memory functions correctly. (C) 24-h *in vivo* incubation, memory acquisition time point with A β treatments that result in the presence of *in vivo* oligomers after 24 h. Memory functions correctly. (D) 48-h *in vivo* incubation, memory acquisition time point with A β treatments that result in the presence of *in vivo* oligomers after 24 h. Memory is inhibited. The red 'x' indicates experiments where memory is inhibited. The green 'check' indicates experiments where memory functions correctly. The pink line indicates the 24–48-h postconditioning time point that may be vulnerable to A β oligomers.

experiments are needed to be certain. This in-depth focus of A β structure, and its influence on neuronal circuitry and memory time points, narrows the scope for future studies investigating molecular memory and drug targeting.

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Author contributions

LF, GK, and LCS conceived the project. LF and LCS designed the imaging experiments, which were performed by LF and DV, and analyzed by LF. LF designed, performed, and analyzed the behavioral experiments. LF and MC designed the electrophysiology experiments, which were performed and analyzed by MC. LF, GK, and LCS wrote the paper and all authors edited the manuscript.

Data sharing statement

The datasets supporting the conclusions of this article are included within the article and its supporting information.

References

- Kaminsky YG, Marlatt MW, Smith MA and Kosenko EA (2010) Subcellular and metabolic examination of amyloid-beta peptides in Alzheimer disease pathogenesis: evidence for A beta (25–35). *Exp Neurol* **221**, 26–37.
- Walsh DM and Selkoe DJ (2007) A beta oligomers – a decade of discovery. *J Neurochem* **101**, 1172–1184.
- Hoe HS, Lee HK and Pak DTS (2012) The upside of APP at synapses. *CNS Neurosci Ther* **18**, 47–56.
- Gong YS, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Krafft GA and Klein WL (2003) Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc Natl Acad Sci USA* **100**, 10417–10422.
- Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA *et al.* (2008) Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med* **14**, 837–842.
- Naylor R, Hill AF and Barnham KJ (2008) Neurotoxicity in Alzheimer's disease: is covalently crosslinked A beta responsible? *Eur Biophys J* **37**, 265–268.
- Maddalena AS, Papassotiropoulos A, Gonzalez-Agosti C, Signorell A, Hegi T, Pasch T, Nitsch RM and Hock C (2004) Cerebrospinal fluid profile of Amyloid beta peptides in patients with Alzheimer's disease determined by protein biochip technology. *Neurodegener Dis* **1**, 231–235.
- Kubo T, Nishimura S, Kumagai Y and Kaneko I (2002) *In vivo* conversion of racemized beta-amyloid (D-Ser(26) A beta 1–40) to truncated and toxic fragments (D-Ser(26) A beta 25–35/40) and fragment presence in the brains of Alzheimer's patients. *J Neurosci Res* **70**, 474–483.
- Cárdenas-Aguayo MC, Silva-Lucero MC, Cortes-Ortiz M, Jimenez-Ramos B, Gomez-Virgilio L, Ramirez-Rodriguez G, Vera-Arroyo E, Fiorentino-Perez R, Garcia U, Luna-Munoz J *et al.* (2014) Physiological role of amyloid beta in neural cells: the cellular trophic activity, Chapter 9. In *Neurochemistry* (Heinbockel T, ed.). InTech, Mexico City.
- Naldi M, Fiori J, Pistolozzi M, Drake AF, Bertucci C, Wu R, Mlynarczyk K, Filipek S, De Simone A and Andrisano V (2012) Amyloid β -peptide 25–35 self-assembly and its inhibition: a model undecapeptide system to gain atomistic and secondary structure details of the Alzheimer's disease process and treatment. *ACS Chem Neurosci* **3**, 952–962.
- Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA and Finch CE (1994) Purification and characterization of brain clusterin. *Biochem Biophys Res Commun* **204**, 1131–1136.
- Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL *et al.* (1998) Diffusible, nonfibrillar ligands derived from A beta(1–42) are potent central nervous system neurotoxins. *Proc Natl Acad Sci USA* **95**, 6448–6453.
- Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ and Selkoe DJ (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416**, 535–539.
- Lesne S, Koh MT, Kotilinek L, Kaye R, Glabe CG, Yang A, Gallagher M and Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature* **440**, 352–357.

- 15 Millucci L, Raggiacchi R, Franceschini D, Terstappen G and Santucci A (2009) Rapid aggregation and assembly in aqueous solution of A beta (25–35) peptide. *J Biosci* **34**, 293–303.
- 16 Bernstein SL, Dupuis NF, Lazo ND, Wyttenbach T, Condron MM, Bitan G, Teplow DB, Shea JE, Ruotolo BT, Robinson CV *et al.* (2009) Amyloid- β protein oligomerization and the importance of tetramers and dodecamers in the aetiology of Alzheimer's disease. *Nat Chem* **4**, 326–331.
- 17 O'Nuallain B, Freir DB, Nicoll AJ, Risse E, Ferguson N, Herron CE, Collinge J and Walsh DM (2010) Amyloid beta-protein dimers rapidly form stable synaptotoxic protofibrils. *J Neurosci* **30**, 14411–14419.
- 18 Reed MN, Hofmeister JJ, Jungbauer L, Welzel AT, Yu C, Sherman MA, Lesne S, LaDu MJ, Walsh DM, Ashe KH *et al.* (2011) Cognitive effects of cell-derived and synthetically derived A beta oligomers. *Neurobiol Aging* **32**, 1784–1794.
- 19 McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Vbeyreuther K, Bush AI and Masters CL (1999) Soluble pool of A beta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann Neurol* **46**, 860–866.
- 20 Hepler RW, Grimm KM, Nahas DD, Breese R, Dobson EC, Acton P, Keller PM, Yeager M, Wang H, Shughrue P *et al.* (2006) Solution state characterization of amyloid beta-derived diffusible ligands. *Biochemistry* **45**, 15157–15167.
- 21 Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. *Science* **298**, 789–791.
- 22 Finch CE and Sapolsky RM (1999) The evolution of Alzheimer disease, the reproductive schedule, and apoE isoforms. *Neurobiol Aging* **20**, 407–428.
- 23 Tharp WG and Sarkar IN (2013) Origins of amyloid-beta. *BMC Genom* **14**, 290.
- 24 Prussing K, Voigt A and Schulz J (2013) *Drosophila melanogaster* as a model organism for Alzheimer's disease. *Mol Neurodegener* **8**, 25.
- 25 Lublin AL and Link CD (2013) Alzheimer's disease drug discovery: in vivo screening using *Caenorhabditis elegans* as a model for beta-amyloid peptide-induced toxicity. *Drug Discov Today Technol* **10**, 115–119.
- 26 Rosen DR, Martinmorris L, Luo LQ and White K (1989) A *Drosophila* gene encoding a protein resembling the human beta-amyloid protein-precursor. *Proc Natl Acad Sci USA* **86**, 2478–2482.
- 27 Allinson TMJ, Parkin ET, Turner AJ and Hooper NM (2003) ADAMs family members as amyloid precursor protein alpha-secretases. *J Neurosci Res* **74**, 342–352.
- 28 Boulianne GL, Livne-Bar I, Humphreys JM, Liang Y, Lin C, Rogaev E and St George-Hyslop P (1997) Cloning and characterization of the *Drosophila* presenilin homologue. *NeuroReport* **8**, 1025–1029.
- 29 Francis R, McGrath G, Zhang J, Ruddy DA, Sym M, Apfeld J, Nicoll M, Maxwell M, Hai B, Ellis MC *et al.* (2002) aph-1 and pen-2 are required for notch pathway signaling, gamma-secretase cleavage of beta APP, and presenilin protein accumulation. *Dev Cell* **3**, 85–97.
- 30 Hong CS and Koo EH (1997) Isolation and characterization of *Drosophila* presenilin homologue. *NeuroReport* **8**, 665–668.
- 31 Fossgreen A, Bruckner B, Czech C, Masters C, Beyreuther K and Paro R (1998) Transgenic *Drosophila* expressing human amyloid precursor protein show gamma-secretase activity and a blistered-wing phenotype. *Proc Natl Acad Sci USA* **95**, 13703–13708.
- 32 Greeve I, Kretschmar D, Tschape JA, Beyin A, Brellinger C, Schweizer M, Nitsch RM and Reifeberger R (2004) Age-dependent neurodegeneration and Alzheimer-amyloid plaque formation in transgenic *Drosophila*. *J Neurosci* **24**, 3899–3906.
- 33 Moroz LL and Kohn AB (2010) Do different neurons age differently? Direct genome-wide analysis of aging in single identified cholinergic neurons. *Front Aging Neurosci* **2**, 2.
- 34 Bailey CH, Kandel ER and Harris KM (2015) Structural components of synaptic plasticity and memory consolidation. *Cold Spring Harb Perspect Biol* **7**, a021758.
- 35 Schacher S, Castellucci VF and Kandel ER (1988) cAMP evokes long-term facilitation in *Aplysia* sensory neurons that requires new protein synthesis. *Science* **240**, 1667–1669.
- 36 Dale N, Schacher S and Kandel ER (1988) Long-term facilitation in *Aplysia* involves increase in transmitter release. *Science* **239**, 282–285.
- 37 Montarolo PG, Goelet P, Castellucci VF, Morgan J, Kandel ER and Schacher S (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. *Science* **234**, 1249–1254.
- 38 Brunelli M, Castellucci V and Kandel ER (1976) Synaptic facilitation and behavioral sensitization in *Aplysia*: possible role of serotonin and cyclic AMP. *Science* **194**, 1178–1181.
- 39 Carew TJ and Kandel ER (1973) Acquisition and retention of long-term habituation in *Aplysia*: correlation of behavioral and cellular processes. *Science* **182**, 1158–1160.
- 40 Byrne J, Hochner B and Kemenes G (2017) Cellular and molecular mechanisms of memory in molluscs. In *Learning and Memory: A Comprehensive Reference* (Byrne J, ed.) *I. Mechanisms of Memory* (Sara S, ed.), pp. 133–148. Elsevier, Cambridge, UK.
- 41 Samarova EI, Bravarenko NI, Korshunova TA, Gulyaeva NV, Palotas A and Balaban PM (2005) Effect of beta-amyloid peptide on behavior and synaptic plasticity in terrestrial snail. *Brain Res Bull* **67**, 40–45.

- 42 Ford L, Crossley M, Williams T, Thorpe JR, Serpell LC and Kemeenes G (2015) Effects of A β exposure on long-term associative memory and its neuronal mechanisms in a defined neuronal network. *Sci Rep* **5**, 10614.
- 43 Broersen K, Jonckheere W, Rozneski J, Vandersteen A, Pauwels K, Pastore A, Rousseau F and Schymkowitz J (2011) A standardized and biocompatible preparation of aggregate-free amyloid beta peptide for biophysical and biological studies of Alzheimer's disease. *Protein Eng Des Sel* **24**, 743–750.
- 44 Soura V, Stewart-Parker M, Williams TL, Ratnayaka A, Atherton J, Gorringer K, Tuffin J, Darwent E, Rambaran R, Klein W *et al.* (2012) Visualization of co-localization in A beta 42-administered neuroblastoma cells reveals lysosome damage and autophagosome accumulation related to cell death. *Biochem J* **441**, 579–590.
- 45 Kemeenes I, Straub VA, Nikitin ES, Staras K, O'Shea M, Kemeenes G and Benjamin PR (2006) Role of delayed nonsynaptic neuronal plasticity in long-term associative memory. *Curr Biol* **16**, 1269–1279.
- 46 Watson SN, Risling TE, Hermann PM and Wildering WC (2012) Failure of delayed nonsynaptic neuronal plasticity underlies age-associated long-term associative memory impairment. *BMC Neurosci* **103**, 103.
- 47 Marshall KE, Vadukul DM, Dahal L, Theisen A, Fowler MW, Al-Hilaly Y, Ford L, Kemeenes G, Day IJ, Staras K *et al.* (2016) A critical role for the self-assembly of amyloid-beta 1-42 in neurodegeneration. *Sci Rep* **6**, 30182.
- 48 Benjamin PR and Winlow W (1981) The distribution of three wide-acting synaptic inputs to identified neurons in the isolated brain of *Lymnaea*. *Comp Biochem Physiol* **70**, 293–307.
- 49 Williams TL, Day IJ and Serpell LC (2010) The effect of Alzheimer's A β aggregation state on the permeation of biomimetic lipid vesicles. *Langmuir* **26**, 17260–17268.
- 50 McDonald JM, Cairns NJ, Taylor-Reinwald L, Holtzman D and Walsh DM (2012) The levels of water-soluble and triton-soluble A beta are increased in Alzheimer's disease brain. *Brain Res* **1450**, 138–147.
- 51 Kemeenes I, O'Shea M and Benjamin PR (2011) Different circuit and monoamine mechanisms consolidate long-term memory in aversive and reward classical conditioning. *Eur J Neurosci* **33**, 143–152.
- 52 Naskar S, Wan H and Kemeenes G (2014) pT305-CaMKII stabilizes a learning-induced increase in AMPA receptors for ongoing memory consolidation after classical conditioning. *Nat Commun* **5**, 3967.
- 53 Nikitin ES, Vavoulis DV, Kemeenes I, Marra V, Pirger Z, Michel M, Feng J, O'Shea M, Benjamin PR and Kemeenes G (2008) Persistent sodium current is a nonsynaptic substrate for long-term associative memory. *Curr Biol* **18**, 1221–1226.
- 54 Yeoman MS, Pieneman AW, Ferguson GP, Termaat A and Benjamin PR (1994) Modulatory role for the serotonergic cerebral giant-cells in the feeding system of the snail, *Lymnaea*. 1. Fine wire recording in the intact animal and pharmacology. *J Neurophysiol* **72**, 1357–1371.
- 55 Takadera T, Sakura N, Mohri T and Hashimoto T (1993) Toxic effect of a beta-amyloid peptide (beta-22-35) on the hippocampal neuron and its prevention. *Neurosci Lett* **161**, 41–44.
- 56 Pike CJ, Burdick D, Walencewicz AJ, Glabe CG and Cotman CW (1993) Neurodegeneration induced by beta-amyloid peptides in vitro- the role of peptide assembly state. *J Neurosci* **13**, 1676–1687.
- 57 Williams TL and Serpell LC (2011) Membrane and surface interactions of Alzheimer's A beta peptide – insights into the mechanism of cytotoxicity. *FEBS J* **278**, 3905–3917.
- 58 Borlikova GG, Trejo M, Mably AJ, Mc Donald JM, Sala Frigerio C, Regan CM, Murphy KJ, Masliah E and Walsh DM (2013) Alzheimer brain-derived amyloid beta-protein impairs synaptic remodeling and memory consolidation. *Neurobiol Aging* **34**, 1315–1327.
- 59 Klyubin I, Ondrejcek T, Hayes J, Cullen WK, Mably AJ, Walsh DM and Rowan MJ (2014) Neurotransmitter receptor and time dependence of the synaptic plasticity disrupting actions of Alzheimer's disease A beta in vivo. *Philos Trans R Soc Lond B Biol Sci* **369**, 20130147.
- 60 Freir DB, Fedriani R, Scully D, Smith IM, Selkoe DJ, Walsh DM and Regan CM (2011) A beta oligomers inhibit synapse remodelling necessary for memory consolidation. *Neurobiol Aging* **32**, 2211–2218.
- 61 Casadio A, Martin KC, Giustetto M, Zhu H, Chen M, Bartsch D, Bailey CH and Kandel ER (1999) A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. *Cell* **99**, 221–237.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Solvent-prepared A β 25–35 does not affect certain intrinsic neuronal properties.