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A β ₄₂-oligomer Interacting Peptide (AIP) neutralizes toxic amyloid- β ₄₂ species and protects synaptic structure and function

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The amyloid- β ₄₂ (A β ₄₂) peptide is believed to be the main culprit in the pathogenesis of Alzheimer disease (AD), impairing synaptic function and initiating neuronal degeneration. Soluble A β ₄₂ oligomers are highly toxic and contribute to progressive neuronal dysfunction, loss of synaptic spine density, and affect long-term potentiation (LTP). We have characterized a short, L-amino acid A β -oligomer Interacting Peptide (AIP) that targets a relatively well-defined population of low-n A β ₄₂ oligomers, rather than simply inhibiting the aggregation of A β monomers into oligomers. Our data show that AIP diminishes the loss of A β ₄₂-induced synaptic spine density and rescues LTP in organotypic hippocampal slice cultures. Notably, the AIP enantiomer (comprised of D-amino acids) attenuated the rough-eye phenotype in a transgenic A β ₄₂ fly model and significantly improved the function of photoreceptors of these flies in electroretinography tests. Overall, our results indicate that specifically “trapping” low-n oligomers provides a novel strategy for toxic A β ₄₂-oligomer recognition and removal.

The amyloid- β ₄₂ (A β ₄₂) peptide is considered as the main culprit in the pathogenesis of Alzheimer disease (AD)¹, postulated to impair synaptic function and initiate neuronal degeneration^{2,3}. Though the evidence for a central role of A β in the pathogenesis is very strong⁴, other models support a modulatory function for low A β concentrations on neurotransmission and memory^{5,6}.

Similar to other amyloid diseases, metastable oligomers and non-fibrillar amyloid intermediates can cause proteotoxicity in AD⁷. Intracellular tau, intracellular and extracellular A β can lead to cell death *in vivo* and *in vitro*^{8–12}. Since soluble species of A β ₄₂ are more strongly correlated with disease symptoms

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compared to amyloid fibrils¹³, these toxic oligomers are believed to underlie losses in hippocampus synapses (occurs early in the disease process) and correlate with the degree of cognitive impairment in AD patients¹⁴. Naturally secreted A β 42 oligomers can disrupt cellular models of learning and memory, hippocampal long-term potentiation (LTP) in acute slices and *in vivo*, and impair the memory of a complex learned behavior in rats¹⁵. Usually, such studies have been conducted with higher concentrations while physiological concentrations are rather in the picomolar range and may have even beneficial effects⁶. Nevertheless, *in vivo*, A β is always a mixture of various aggregates, and a defined biological concentration of a specific oligomeric conformation has not been determined yet⁴.

Soluble low-n oligomers (i.e. tetramers and hexamers) of A β 42 are neurotoxic^{15,16} and ascribed to promote the progressive loss of dendritic spines and glutamatergic synapses^{17,18}. It is plausible that the generation of toxic A β oligomers may be the result of newly formed aggregates, and/or the depolymerization of amyloid plaques. In either case, the cellular mode-of-action is not fully understood.

In the present study, we performed a peptide-based anti-amyloid approach, which differed from previously reported anti-oligomer strategies. A diverse array of biomolecules have already been identified which possess the ability to prevent amyloid fibril formation both *in vitro* and *in vivo*—large proteins such as molecular chaperones¹⁹, to small molecules such as flavonoids²⁰, polyphenols²¹, hydroxyquinoline derivatives²² and peptide-based inhibitors^{23–27}. Preferably, the compound should neither be a dissociation catalyst nor a monomer competitor but rather interact with potentially toxic A β oligomers to inhibit the conversion of low-n A β 42 oligomers into mature amyloid fibrils. Here, we demonstrate that an 8-residue long peptide (RGTFEGKF), initially designed on the framework GxFxGxF to disrupt sheet-to-sheet packings of A β 40 fibrils²⁷ can specifically target low-n A β 42 oligomers (mainly tetramers and hexamers), rather than simply inhibit A β aggregation. Compared to earlier anti-amyloid oligomer strategies, AIP is unique in that this A β -oligomer Interacting Peptide (AIP) can “trap” toxic amyloid oligomers—i.e. inhibit the conversion of low-n A β 42 oligomers into mature amyloid fibrils, neutralize their toxicity and prevent the growth of A β oligomers into larger assemblies.

Results

Characterization of AIP on A β 42 self-aggregation. To investigate the dynamics of A β 42 aggregation, the effect of AIP on fibril formation was first examined by transmission electron microscopy (TEM). After 24 hours incubation at room temperature, A β 42 wild-type (wt) peptides revealed well-structured, mature fibrils that were 18 nm wide and 300 nm long (Fig. 1A,B). In contrast, co-incubating A β 42 wt peptides with AIP yielded protofibrillar-only structures and inhibited mature fibril formation (Fig. 1C). To further assess the targeting of A β 42 aggregates by AIP, we incubated A β 42 wt peptides for 4 or 12 hours prior to adding AIP, and then analyzed the aggregates for both time points after a total incubation period of 24 hours each. For the 4 hours/20 hours treatment, TEM analysis revealed oligomeric and protofibrillar structures without any A β 42 wt mature fibrils. Interestingly, the formation of mature fibrils was not abolished in the 12 hours/12 hours treatment (Fig. 1D) suggesting that AIP prevents A β 42 wt from undergoing structural transitions to a more compact form (i.e. characteristic of pre-fibrillar structures).

As an additional TEM control, we used the A β 42 G33A substitution peptide that is known to form oligomeric beta-pleated sheet complexes more readily than A β 42 wt peptide¹⁶ and stops aggregation at premature fibril stages due to its more hydrophobic surface. The A β 42 G33A peptides did not yield mature fibrils after 24 hours, (Fig. 1A), as expected, but we did observe protofibrillar structures that were smaller compared to A β 42 wt (average length of 100 nm versus 300 nm, respectively). In contrast, we did not observe any significant differences in protofibril width between A β 42 G33A and A β 42 wt (Fig. 1B).

Since TEM is limited in its deconvolution and magnification²⁸, we also performed high-resolution atomic force microscopy (AFM) to further examine the influence of AIP on pre-fibrillar assemblies (i.e. protofibrils and oligomers). In the presence of AIP, the AFM analyses revealed globular particles and higher oligomeric structures with an average length of 100 nm for both A β 42 and A β 42 G33A peptides (Fig. S1). Validating our TEM data, this result implies that AIP might also affect oligomer formation at early stages of A β aggregation.

In order to acquire more detailed information at time points before fibrils are first observed, we performed size-exclusion chromatography (SEC) using experimental conditions to match our corresponding microscopic analyses. When freshly dissolved A β 42 wt peptides were incubated for 0, 4, or 8 hours, slow oligomerization dynamics consistently yielded superimposable tetra-/hexamer oligomer peaks (Fig. 1E). The A β 42 G33A substitution peptide exhibited faster kinetics (Fig. 1F), immediately forming high-n oligomers, which steadily increased over time, while the amount of tetra-/hexamers decreased. Thus, A β 42 G33A exhibited a faster aggregation process over 8 hours compared to the wt peptide (Fig. 1F and E, respectively). When the identical A β 42 wt experiments were repeated in the presence of AIP, consistent kinetics yielded superimposable tetra-/hexamer oligomer peaks with only a modest increase in high-n oligomers (compare Fig. 1E with 1G). Co-incubating A β 42 G33A with AIP significantly reduced the formation of high-n oligomers over time while reducing the loss in low-n oligomers (compare Fig. 1F with 1H). Importantly, our findings establish that (i) the oligomer-stabilizing effect of AIP is stronger for the more aggregation-prone A β 42 G33A peptide¹⁶ compared to A β 42 wt, and (ii) AIP has a quantitative impact on oligomers in that it can attenuate the conversion of low-n tetra-/hexamers into high-n oligomers. Moreover, AIP appears to interact with tetra-/hexameric A β complexes with a 1:1 stoichiometry

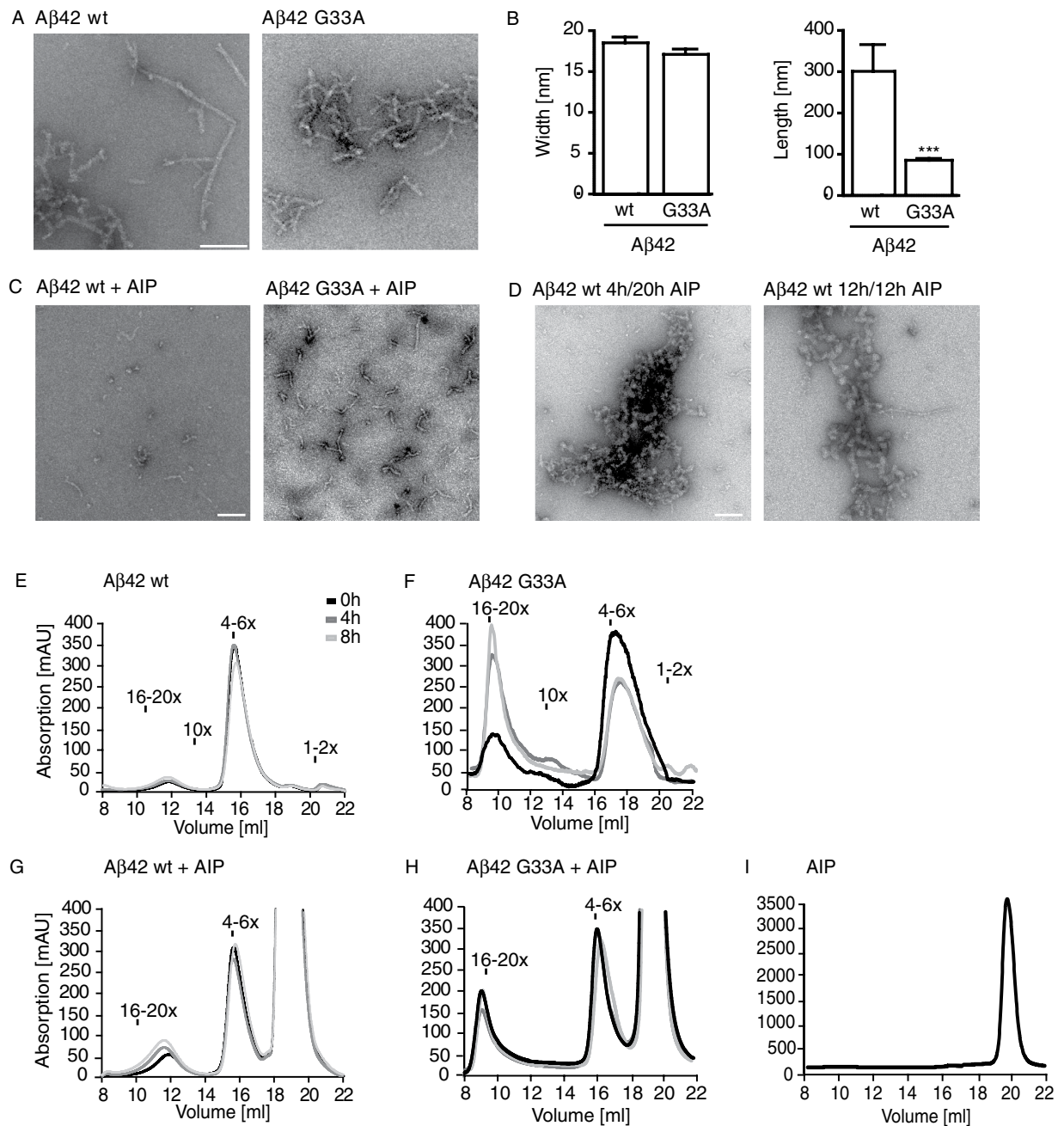


Figure 1. Aggregation studies of Aβ42 wt and Aβ42 G33A peptides. (A) TEM micrographs were acquired after 24 hours incubation. Aβ42 wt aggregated to mature fibrils whereas Aβ42 G33A solely formed globular oligomers and protofibrillar structures. Scale bar = 200 nm. The graphs in (B) depict the means ± SEM of fibril width (wt, n = 20; G33A, n = 17) and length (wt, n = 20; G33A, n = 17). ***p < 0.001, ns = not significant, Student's t-test. (C) TEM micrographs showed that neither Aβ42 wt nor Aβ42 G33A formed mature fibrils when co-incubated with the AIP for 24 hours. (D) TEM analyses of Aβ42 wt peptides aggregated for 4 and 12 hours before addition of AIP and subsequent mixture was incubated for 24 hours. AIP was capable to block fibril formation of Aβ42 wt only when low-n oligomers had formed after 4 hours pre-incubation. AIP did not abolish fibril formation when higher aggregates or protofibrils were present (12 hours pre-incubation). (C,D) Scale bar = 100 nm. SEC analyses of aggregated Aβ42 peptides within 8 hours after freshly dissolving. (E) Aβ42 wt peptides mainly existed as tetra-/hexamers whose amount slightly decreased over time whereas the quantity of high-n oligomers (16–20mers) slightly increased. (F) Aβ42 G33A is presented by a higher amount of high-n oligomers, tremendously increasing over time. (G) Freshly dissolved Aβ42 wt peptide (0h), after 4 and 8 hours of co-incubation with the AIP showed a constant amount of tetra-/hexamers and only a slight increase of high-n oligomers. (H) Co-incubation of Aβ42 G33A with the AIP abolished the shift from low aggregates to high-n oligomers over time. (I) AIP itself eluted from the column at 20 ml (for comparison see Fig. 1E,F).

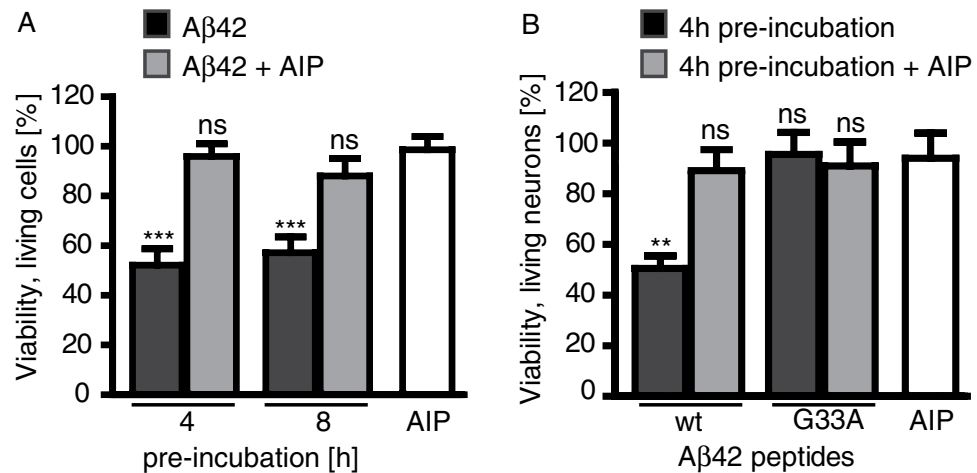


Figure 2. Cell viability of SH-SH5Y cells and primary hippocampal neurons after treatment with Aβ42 peptides. (A) SH-SY5Y cells were treated with 4- or 8-hour pre-incubated Aβ42 wt peptides (2.5 μM), in the presence and absence of AIP. Shown are graphs depicting the mean ± SEM viability as a % normalized to vehicle-treatment. *** $p < 0.001$, One-way ANOVA and Dunnett's post-hoc test (AIP treatment was used as control). Number of independent experiments in triplicate: Aβ42 4h, $n = 4$; Aβ42 + AIP 4h, $n = 7$; Aβ42 8h, $n = 6$; Aβ42 + AIP 8h, $n = 8$. (B) Hippocampal primary neurons were treated with 4-hour preincubated Aβ42 wt or Aβ42 G33A peptides, in the presence and absence of AIP. The viability (%) is shown as the mean ± SEM, normalized to vehicle-treated cells. ** $p < 0.01$, One-way ANOVA and Dunnett's post-hoc test (AIP treatment as control). Number of independent experiments in triplicate: Aβ42 wt $n = 11$, Aβ42 wt + AIP $n = 5$, Aβ42 G33A $n = 9$, Aβ42 G33A + AIP $n = 6$, AIP $n = 6$.

since we did not observe any other novel peaks eluting at different times. As an additional control, we confirmed that AIP elutes from the column in the salt fraction (Fig. 1I).

AIP inhibition of Aβ42-induced neurotoxicity. Since AIP was able to interact with low-n oligomers and attenuate mature fibril formation in the experiments above, the effect of AIP on neurotoxicity was then examined using MTT assays in SH-SY5Y cells. In the absence of AIP, freshly dissolved Aβ42 wt peptides (pre-incubated for 4 or 8 hours) were able to reduce the number of living cells by approximately 40% (Fig. 2A); under similar assay conditions, the presence of AIP was able to neutralize the Aβ42 wt-induced toxic effects. Likewise in MultiTox assays (Fig. 2B) where primary hippocampal neurons were pre-incubated with Aβ42 wt in the absence and presence of AIP for 4 hours, AIP almost completely attenuated the Aβ42-mediated loss of living cells. As a control, we also tested the non-toxic Aβ42 G33A peptide¹⁶, which behaved similarly to Aβ42 wt with AIP.

Given that soluble Aβ species can lead to dendritic spine loss²⁹ and reduce spine plasticity at dendrites¹⁷, we examined the effect of AIP on the excitatory synapse stability of dendritic spines in the presence of Aβ42. Treating organotypic hippocampal slice cultures with Aβ42 for 24 hours led to a significant ($p < 0.05$) reduction in the number of CA1 pyramidal cell dendritic spines compared to control slices (Fig. 3A,B; mean ± SEM of spine/μm are as follows: control, 1.68 ± 0.17 spine/μm; Aβ42, 1.05 ± 0.07 spine/μm). Co-incubation with AIP, however, was able to prevent the Aβ42-mediated CA1 spine density losses (AIP, 1.65 ± 0.12 spine/μm vs. Aβ42 + AIP, 1.71 ± 0.07 spine/μm). Next, we investigated whether the significant decrease in spine density was subtype-dependent (i.e. thin, stubby, mushroom) in order to assess the number of glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA)-type receptors and synapse strength³⁰, i.e. larger spine heads favor the presence of more AMPA receptors and stronger synapses³¹. Importantly, mushroom and thin spine subtypes were affected by Aβ42 treatment after 24 hours.

As LTP can be accompanied by structural modifications of dendritic spines, we next examined the effect of AIP on theta-burst stimulation (TBS)-induced LTP in the presence of Aβ42 in the CA1 region of mouse hippocampal slices. In keeping with previous findings, Aβ42 significantly inhibited LTP in the CA1 region of mouse hippocampal slices (Fig. 3C; $p < 0.05$), as compared to control (50 minutes post-TBS (mean ± SEM): control, $151 \pm 21\%$; Aβ42, $88 \pm 13\%$). Under similar conditions, LTP was not decreased when Aβ42 was co-incubated with AIP (50 minutes post TBS: AIP, $159 \pm 30\%$; Aβ42 + AIP, $148 \pm 14\%$). Figure 3D depicts averaged representative traces of field excitatory postsynaptic potentials (fEPSPs).

AIP affects the *D. melanogaster* rough-eye phenotype and electrophysiology. To assess the effect of AIP *in vivo*, we utilized our previously developed *Drosophila melanogaster* model where fly

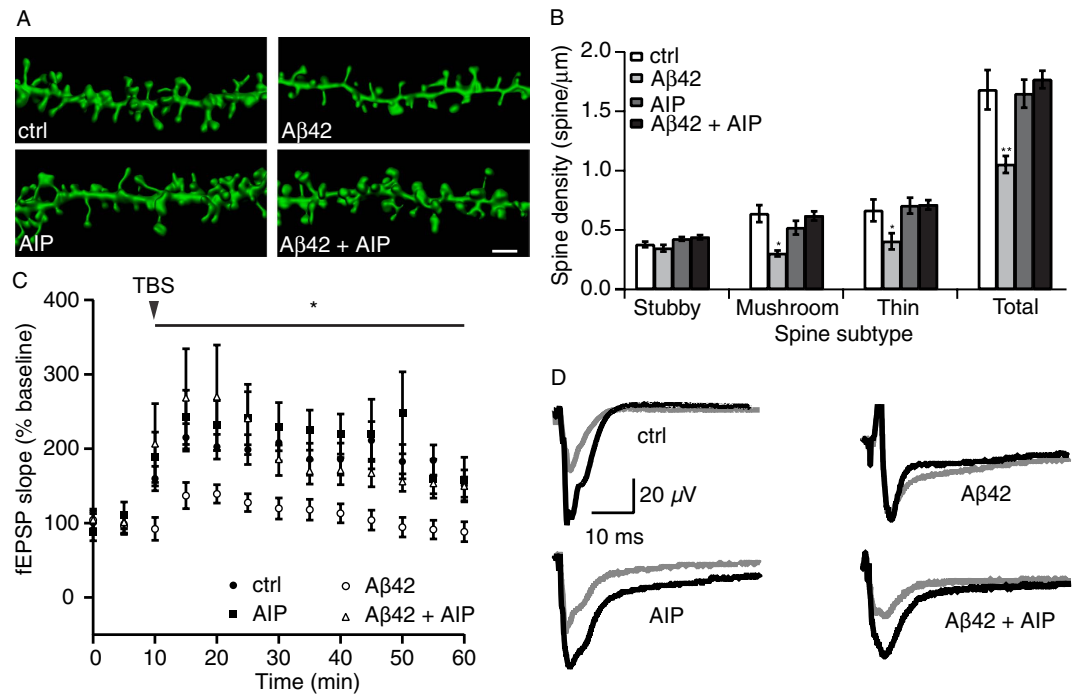


Figure 3. Quantification of dendritic spines and LTP recordings of Schaffer collateral CA1 region following A β 42 and A β 42 with AIP treatments. (A) Representative 3D reconstructions of dendritic segments from organotypic hippocampal sister slice cultures that were treated either with A β 42 (1 μM), AIP or A β 42 and AIP-containing media. Scale bar = 2 μm . (B) There is a significant decrease in the densities for spine subtypes following A β 42 treatment compared to control. Treatment with A β 42 in conjunction with AIP did not affect spine density. Total dendritic segment lengths (mean \pm SEM): Control, n = of 672 μm from 12 cells in 4 cultures; A β 42, n = 795 μm from 14 cells in 4 cultures; AIP, n = 884 μm from 16 cells in 4 cultures; A β 42 + AIP, n = 970 μm of dendrite from 18 cells in 4 cultures. * $p < 0.05$, ** $p < 0.01$ one way ANOVA with Tukey's multiple comparison test was performed for each spine subtype (* refers to A β 42 vs. A β 42 + AIP). (C) AIP treatment in the presence of oligomeric A β 42 species prevented the decrease in LTP level following TBS. Shown are quantifications of fEPSPs from control, A β 42-, AIP-, and AIP + A β 42-treated slice cultures. Following 24 hours of A β 42 treatment (1 μM), there was a marked decrease in LTP response. This deficit was prevented by AIP (AIP + A β 42). AIP alone did not have an effect on LTP. Control, n = 9 slices; A β 42, n = 6 slices; AIP, n = 6 slices; AIP + A β 42, n = 6 slices; One-way ANOVA with Tukey post-hoc comparison was used on the last time-point (* $p < 0.05$). fEPSP stimulus was delivered and recorded every 10 s; data points were averaged into five minute intervals for display and statistical analysis purposes. Averaged sample traces of fEPSPs obtained from control, A β 42-, AIP-, and AIP + A β 42-treated slice cultures are shown in (D). There was a marked decrease in the level of potentiation achieved following TBS stimulation. Black, baseline; grey, 50 minutes after TBS.

strains expressing and secreting A β 42 in the eye invokes an abnormal “rough-eye” phenotype^{16,32}. The extent of cell death can be determined by visual inspection of the eye morphology³³ and the severity of the A β 42-induced toxicity can be estimated by the ratio of photoreceptors (rhabdomeres to ommatidia)^{16,32}. TEM analysis of eye cross-sections from 5-day old non-transgenic flies revealed intact ommatidia with seven characteristic photoreceptor cells present (Fig. 4A). In contrast, TEM images from the A β 42-transgenic flies exhibited severe distortions in eye morphology (Fig. 4B); specifically, the “rough-eye” phenotype was characterized by significantly altered ommatidia (smaller and less expressed), where the photoreceptors are less expressed and rhabdomeres appear shrunken.

To test the effect of AIP, we carried out food supplementation studies, where A β 42-transgenic fly larvae were raised on food containing 5 mM AIP, which is a concentration that is in accordance to most studies examining the effects of drugs on flies³⁴. Also, since living organisms typically catabolize L-amino acid-containing proteins, for these *in vivo* studies, we tested AIPs comprised of either L- (L-AIP) or D-amino acids (D-AIP). We found that AIP-treatment led to an attenuation of A β 42-induced toxicity but not a complete rescue. For the flies that consumed AIP composed of L-amino acids (L-AIP; Fig. 4C), there still was diminished expression of photoreceptors in all ommatidia, vacuoles could be found, and shrunken rhabdomeres. For D-AIP-raised transgenic flies (Fig. 4D), eye morphology dramatically improved, including ommatidia with up to seven characteristic rhabdomeres, however, vacuoles could still be detected. As a control, the treatment of non-transgenic flies with D-AIP alone had no adverse

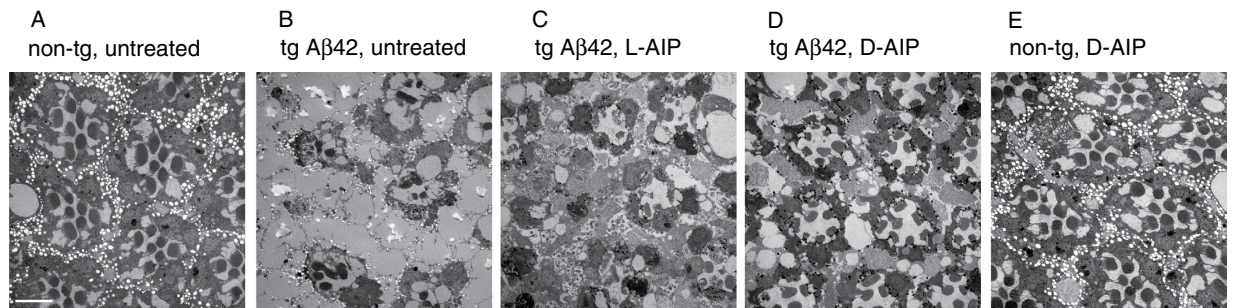


Figure 4. Ultrastructural analyses of retinas from A β 42 wt-expressing *D. melanogaster* treated with AIP. Shown are representative electromicrographs of the ultrastructure of fly retinas in cross-section. Eyes of non-tg control flies showed a highly ordered structure of rhabdomeres and ommatidia (A). In contrast, flies expressing A β 42 wt (B) have a rough-eye phenotype, with pronounced malformed ommatidia and rhabdomeres. A β 42 wt tg flies raised on 5 mM L-AIP showed an improvement of rough-eye phenotype (C), but those raised on D-AIP (D) showed a far greater retention of ommatidia and rhabdomere structures, as compared to (B). (E) Treatment of non-tg control flies with D-AIP (5 mM) did not affect eye morphology. Scale bar = 5 μ m.

effects on eye morphology (Fig. 4E). Since the D-AIP was significantly more effective at ameliorating the toxic phenotype, it can be regarded as a peptidomimetic—i.e. possessing similar selectivity and potency as the native L-AIP “parent”, as cross-validated in our *in vitro* systems (Fig. S2). The peptidomimetic was likely more effective in our A β 42-transgenic fly model since it has been reported that the D-amino acids are more protease-resistant (i.e. increased stability of AIP) as compared to their L-amino acid counterparts^{35,36}.

To determine if the protective effect of D-AIP at the structural level (i.e. retinal morphology) translated to improved eye function in the treated A β 42-transgenic flies, we then recorded electroretinograms (ERGs). All photoreceptor cells respond to a simple light pulse with a short delay, followed by a sustained depolarization (so-called “receptor potential”) that lasts as long as the stimulus³⁷. The majority of the response is from photoreceptors R1-6 and their post-synaptic targets. Rhodopsin1 (Rh1) in R1-6 responds broadly to light with a peak in the blue range, R8 rhodopsin overlaps well with the Rh1 spectral sensitivity, and UV sensitive photoreceptors (R7) fire least robust to this stimulus³⁸. Recorded with an electrode in the distal retina and a reference electrode elsewhere in the body, the ERGs measured as a change in the voltage drop across the basement membrane. Our non-transgenic flies (Fig. 5A) generated typical ERG profiles: on-transient signal (mean \pm SD: 5.45 ± 1.18 mV), followed by a receptor potential (6.75 ± 2.52 mV), and finally the negative off-transient signal (6.57 ± 1.69 mV). A β 42-expressing flies (i.e. transgenic; Fig. 5B) exhibited a significantly reduced response to light, as indicated by the missing on- and off-transient signals and receptor potential: 1.11 ± 1.06 mV, 0.94 ± 1.15 mV, and 0.94 ± 1.02 mV, respectively. A β 42 transgenic flies treated with L-AIP (Fig. 5C) generated ERG profiles similar to the untreated A β 42 flies (on-transient, 0.75 ± 0.68 mV; receptor potential, 1.16 ± 1.40 mV; off-transient, 0.83 ± 0.97 mV). Remarkably, treatment with D-AIP (Fig. 5D) significantly increased their response to light, as indicated by the enhanced on- and off-transient signals, and receptor potential (2.97 ± 0.81 mV, 3.72 ± 1.33 mV, and 4.56 ± 2.03 mV, respectively). Non-transgenic flies treated with D-AIP did not exhibit altered ERGs (Fig. 5E; on-transient, 5.88 ± 1.49 mV; receptor potential, 6.37 ± 1.18 mV; off-transient, 7.58 ± 1.04 mV), as compared to our untreated, non-transgenic flies (Fig. 5A). Figure 5F displays the quantifications of the on- and off-transient signals, as well as the receptor potential for each sample group.

Analysis of AIP interaction with A β . Based upon our biophysical AIP data (TEM, AFM, SEC) and the A β 42 structure provided by Luhrs and colleagues³⁹, we generated molecular docking models for both L-AIP (Fig. 6, Fig. S3, S4) and D-AIP (Fig. S5), and its interaction with A β 42 tetramers.

To predict how L-AIP might interact with A β 42 tetramers, 6 best-fit poses were generated (see Methods). A strong interaction was found between AIP-Arg1 and Asp23 of the terminal sheet. Due to a lateral shift within the A β 42 tetramer, Asp23 of the terminal sheet is unpaired, while within the oligomer it forms a salt bridge with Lys28 of the neighboring sheet promoting fibril growth (Fig. 6A, S3A). In our model, L-AIP-Arg1 replaces Lys28 in that interaction, implying an interference with the Asp23-Lys28 salt bridge formation. The predominant configuration of L-AIP binding is with its C-terminal portion to the groove formed at Gly33 (Fig. 6A, see Fig. S4A–E). L-AIP-Arg1 extends down to Asp23, forming a strong hydrogen bond. Additional interactions are formed between Phe4 or 8 and Gly33 plus Met35 (Fig. 6A; S4A–E). In a second binding mode, which is less frequently found, L-AIP binds to the Gly37 groove, still forming the Arg1-Asp23 hydrogen bond (Fig. S3A; S4F).

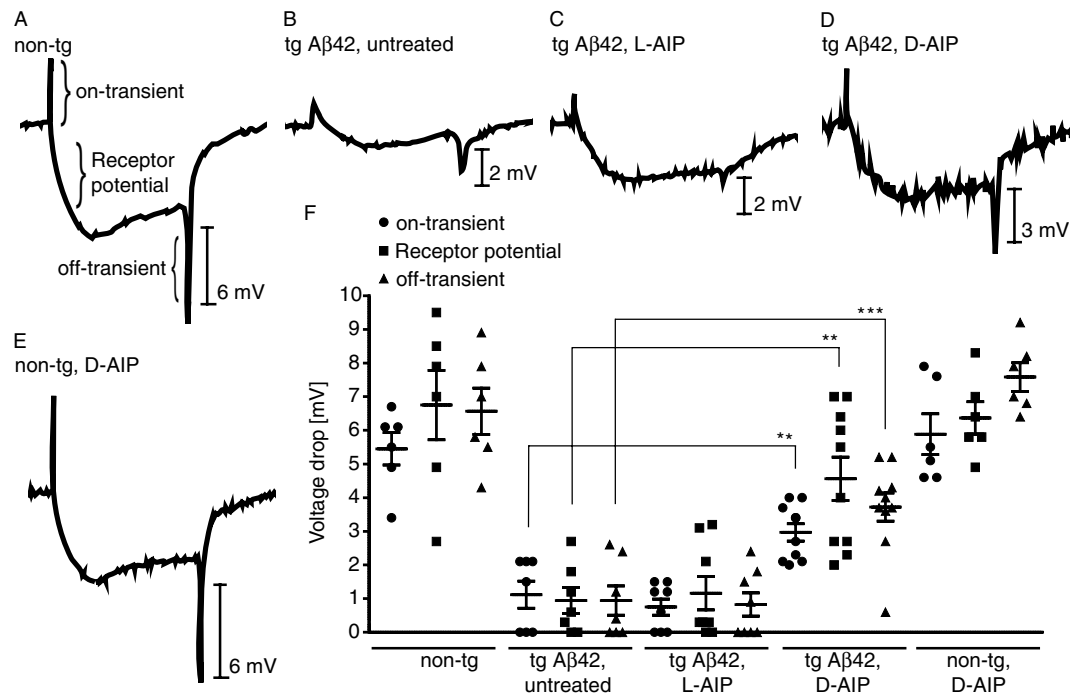


Figure 5. ERG traces in *D. melanogaster* expressing A β 42 wt in the presence and absence of AIP. Shown are representative ERG traces from non-tg (A) or tg A β 42-expressing flies in the absence (A,B) and presence of L-AIP (C), or D-AIP (D,E). (A) Untreated non-tg flies showed characteristic on- and off-transients, and receptor potentials. (B) Untreated tg A β 42 flies revealed reduced on- and off-transients, and diminished receptor potentials. (C) Tg A β 42 flies treated with 5 mM L-AIP exhibited almost no on- and off-transients, and no receptor potential. (D) Tg A β 42 flies treated with 5 mM D-AIP demonstrated a significant response to light compared to untreated tg A β 42 flies. (E) Non-tg flies treated with D-AIP showed no effect compared to untreated non-tg flies (A). Quantification of on- and off-transients, and receptor potential from each group are shown in (F). Data are represented as the mean \pm SD of $n = 6-10$. ** $p < 0.01$, *** $p < 0.001$, One way ANOVA with Tukey's multiple comparison test.

Finally, docking of D-AIP to A β 42 (Fig. S5A–G) yielded similar binding modes and specific interactions including the Arg1-Asp23 bond, as compared with the L-AIP (Fig. 6A) (Fig. S5A–C, and G). Due to the isomeric structure, however, we observe a slightly improved packing of D-AIP into each of the Gly grooves, forming similar interactions with A β 42 (Fig. S5D–F).

In our second analysis, we performed flexible docking of L-AIP to the substitution peptide A β 42 G33A to find a rationale for its faster aggregation and unique behavior compared to L-AIP (see Fig. 1E–H). The introduction of a methyl group to residue 33 changes the L-AIP binding to the Gly37 groove. Different interactions, including poses with reversed N- to C-terminal AIP topology (poses 1 and 2) are observed (Fig. S3B, S4G, H). As indicated in the third best-fit pose, configurations forming the Arg1-Asp23 hydrogen bond are still possible, although they less likely occur as with A β 42 (Fig. 6B, S4I).

The tetramer is the minimal unit required to meet the steric/spatial constraints imposed by the length of the peptidic molecule, which binds to the groove provided by Gly33 and simultaneously interacts with Asp23.

To provide further evidence of the physical interaction between AIP and A β 42 (as predicted by our SEC and molecular docking results), we performed limited proteolysis experiments. In brief, A β 42 was incubated with trypsin and aliquots taken at different time points were analyzed by MALDI mass spectrometry (MS) to test for the presence of specific cleavage products. In the absence of AIP, the cleavage of A β 42 generated A β 1–16 and A β 17–28 fragments and their corresponding C-terminal products (Fig. 7A), thus implying that this region is highly flexible and easily accessed by trypsin. To visualize the proteolytic time course, area under the curve of peaks from the acquired MALDI spectra were used to calculate the ratio of fragment A β 17–28 to uncleaved A β 42. Co-incubating A β 42 with either L- or D-AIP led to a significantly decreased ratio of A β 17–28 to A β 1–42, most likely by protecting the A β 42 peptide from trypsin by shielding the Lys28 cleavage site (Fig. 7C). Since AIP itself contains two basic residues (Arg in position 1 and Lys as the second last amino acid residue at the C-terminus), we also performed L- and D-AIP-only trypsin digestions (i.e. without A β 42) to ensure that AIP was not degraded during the three hour time-course (Fig. 7B). Overall, our proteolysis data (i.e. masking of Lys28 in A β 42 by AIP) is consistent with our previous A β 42 G33A studies where the substitution of glycine 33 to alanine

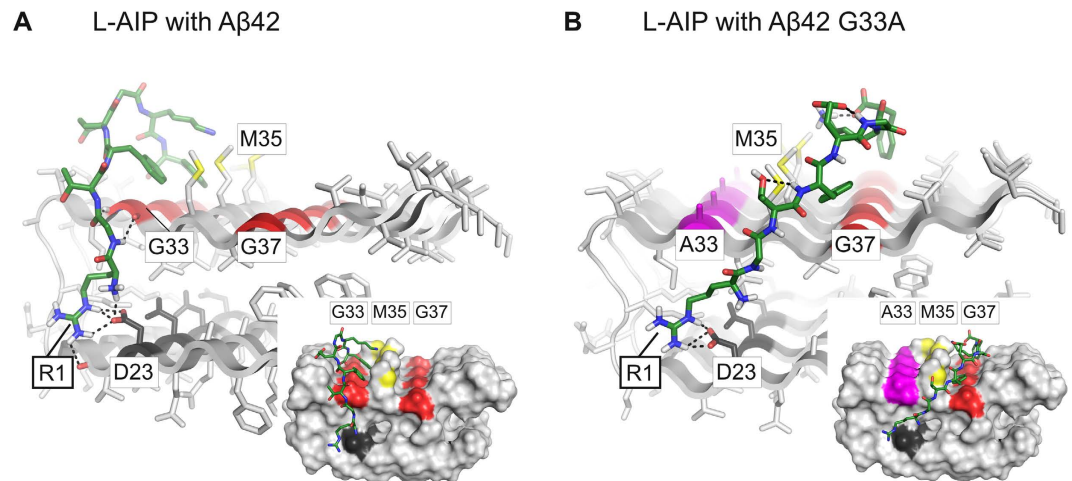


Figure 6. L-AIP docked to A β 42 and to A β 42 G33A. (A) Representative L-AIP A β 42 complex obtained from flexible docking in stick (full view) or surface representation (inset). (B) Representative L-AIP A β 42 G33A complex obtained from flexible docking in stick (full view) or surface representation (inset). In all figures, L-AIP is shown in green sticks with nitrogen colored in blue, oxygen in red, and hydrogens of polar side chains in white. Residue labels are marked by thick boundary lines. Hydrogen bonds are depicted as black dashed lines. A β 42 and A β 42 G33A are shown as gray cartoons and sticks, with G33 and G37 colored in red, Ala33 in magenta, Asp23 in black, and sulfur atoms of Met35 in yellow. Residue labels are marked by thin boundary lines.

induced a more tightly packed A β 42 conformation that masks Lys28¹⁶. Accordingly, co-incubating A β 42 G33A with L- or D-AIP did not significantly change the ratio of A β 17–28 to A β 1–42 G33A (Fig. 7C). It is also important to note that AIP did not affect general trypsin activity, as evidenced by the ratio of fragment A β 1–16 to uncleaved A β 42 which was unaltered in the absence or presence of AIP (Fig. 7D).

Since the Asp23-Lys28 salt bridge appears to be important for AIP to inhibit A β 42-mediated toxicity, we tested the A β 42 D23N Iowa mutation peptide which is associated with progressive AD-like dementia, and has been found to be more neurotoxic and to aggregate more rapidly than the wt peptide⁴⁰. In the Asp-to-Asn mutant, the wild-type Asp23-Lys28 salt bridge cannot form, thus allowing the sequence to transform either in parallel or antiparallel β -sheets which alters its folding nucleation⁴¹. Indeed, elimination of the salt bridge in the Asp23 to Asn substitution peptide abolished the ability of AIP to inhibit mature fibril formation as revealed by EM (Fig. 8). Notably, this is fully consistent with our best-fit molecular docking poses implying that AIP-Arg1 and its protonated N-terminus make H-bonds to Asp23, interfering with the Asp23-Lys28 salt bridge.

Discussion

The current A β oligomer hypothesis implies that oligomeric A β species are toxic while amyloid plaques/fibrils are not inherently toxic but a consequence of aberrant A β aggregation⁴². Accordingly, to investigate molecular mechanisms of actions that can detoxify these neurotoxic oligomers is important and highly desirable. In this study, we have demonstrated *in vitro* and *in vivo* that a non-amidated 8-residue peptide (RGTFEGKF) can function as a selective and potent A β -oligomer Interacting Peptide (AIP). AIP not only suppresses aggregation of A β oligomers into proto-fibrillar structures, but also neutralizes toxic A β oligomers. AIP directly influences the self-aggregation and *in vivo* toxicity of A β without leading to an obvious disaggregation of amyloid oligomers from fibrils. Oligomers were stable in the presence of excess AIP. In this regard, AIP might act as a chaperone, not causing an apparent dissociation of A β complexes into monomers, but competing with the addition of further monomers or oligomers to effectively prevent the growth of A β oligomers into larger assemblies. Compared to previously reported anti-amyloid oligomer strategies, AIP is unique in that it is neither a dissociation catalyst nor a monomer competitor (e.g. antibodies such as crenezumab or solanezumab⁴³) nor an aggregation enhancer (e.g. D-amino peptides such as D3⁴⁴).

Our biophysical data (EM, AFM, SEC) together with limited proteolysis/mass spectrometry results demonstrate that AIP directly interacts with A β . While the amidated predecessor of AIP was initially designed on the framework GxFxGxF to disrupt sheet-to-sheet packings of A β 40 fibrils^{26,27}, our non-amidated AIP can inhibit mature fibril formation, suppress large aggregate formation, and appears to specifically interact with A β tetramers. In contrast to other small aggregation inhibitors^{45–47}, AIP shows that peptide-based compounds can arrest further growth of the aggregates – an important and beneficial mode of action. To not only bind pre-existing low-n oligomeric complexes (with varying degrees of toxicity and solubility), AIP also may target a multitude of toxic A β oligomeric species built from potentially

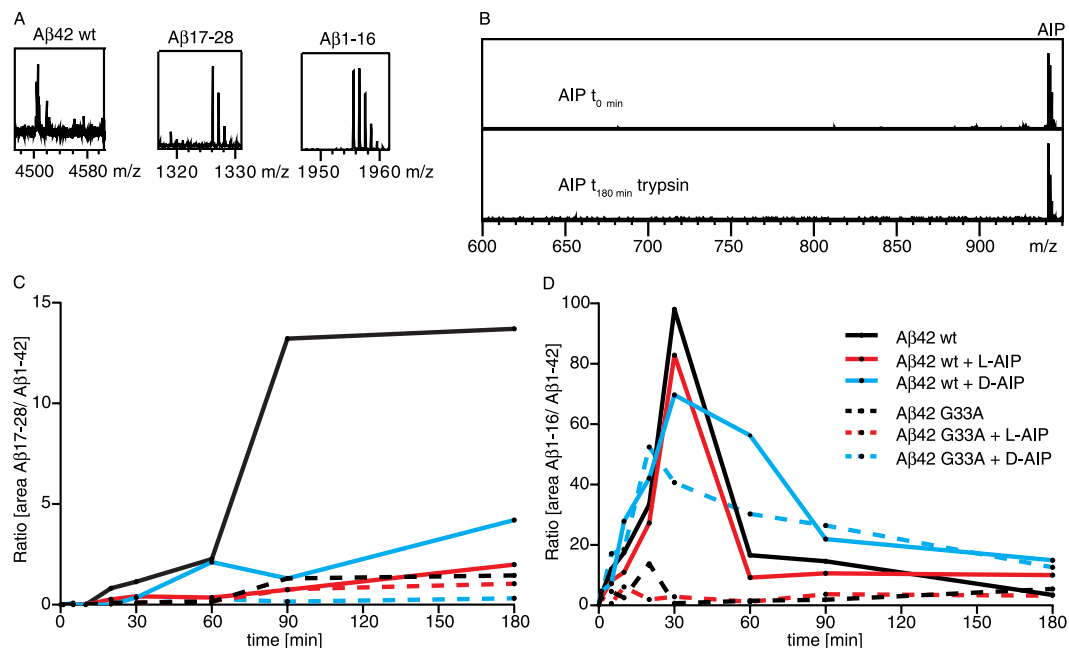
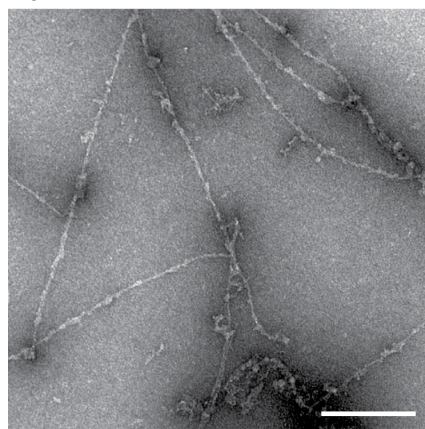


Figure 7. Co-incubation of Aβ42 with AIP affects accessibility of Lys28. (A) MALDI-MS spectra of tryptic digests yielded the following mass peaks (in Dalton (Da)): Aβ42 wt, 4512.3; Aβ17–28, 1325.7; Aβ1–16, 1954.9. (B) MALDI-MS spectra of a 3 hour tryptic digest of L- or D-AIP (941.5). Generation of Aβ17–28 (C) and Aβ1–16 (D) fragments by limited tryptic digestion of Aβ42 wt, Aβ42 G33A with or without L- or D-AIP, respectively. The values of the respective fragments were calculated as the ratio of ion peak areas of MALDI-MS spectra recorded at different time points. (C) For Aβ42 wt a rapid generation of high amounts of Aβ17–28 was observed, but hardly any cleavage of trypsin was seen at position Lys28 after co-incubation with L- or D-AIP. No difference was observed for Aβ42 G33A incubated with or without L- or D-AIP. (D) The ratios of the fragment Aβ1–16 generated from Aβ42 wt or Aβ42 G33A yielded no differences with or without L- or D-AIP, respectively.

Aβ42 D23N



Aβ42 D23N + AIP

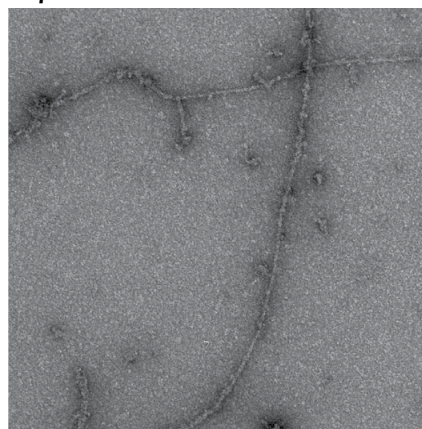


Figure 8. Aggregation studies of Aβ42 D23N peptides. TEM micrographs were acquired after 24 hours incubation. Aβ42 D23N aggregated to mature fibrils. AIP did not inhibit fibril formation of Aβ42 D23N. Scale bar = 200 nm.

toxic subunits of Aβ tetramers^{15,16,48}. As demonstrated by our EM and AFM data, co-incubating AIP with Aβ inhibits the formation of mature fibrils (ultimately the same outcome as the substitution variant Aβ42 G33A alone, as described previously¹⁶).

The Aβ42 G33A tetramer is naturally non-toxic^{16,49,50} and has a strong tendency to assemble into high-n oligomers due to its extended hydrophobic surface patch. The substitution at position 33 may

cause an altered seed structure of A β 42⁵¹ and substitution variants with either an alanine or isoleucine at position G33 of the A β sequence showed a lack of A β 42-induced neurotoxicity, but an increased aggregation of freshly dissolved peptides due to structural differences^{16,49}. Positions Ile31, Gly33 and Met35 have an overall higher solvent-excluded surface value and especially shielding of Gly33 leads to an enlarged solvent-excluded surface and promotes aggregation¹⁶. In the present study, we show that Gly33 not only impacts the aggregation of freshly dissolved A β 42 peptides, it provokes the formation of high-n oligomers over time as observed by our SEC studies. However, our EM and AFM data show that A β 42 G33A does not form mature fibrils and, in this regard, its behavior is similar to AIP/A β 42 co-incubation.

It has been shown that A β 42 oligomers can disrupt LTP in slices and *in vivo*, and also have the potential to impair the memory of a complex learned behavior in rats^{3,16,17}. Furthermore, learning models such as LTP have been shown to induce dendritic spine structural modifications^{52,53}, and LTP induction has been demonstrated to promote spine enlargement and the growth of new spines^{31,54–57}, spine branching⁵⁸, and actin-dependent remodeling^{59,60}. Notably, an increase in spine volume accompanied by insertion of AMPA receptors has been observed as well³¹. Since A β 42 low-n oligomers promote the progressive loss of dendritic spines and glutamatergic synapses^{17,18,61}, our present study (spine density, LTP, and toxicity data combined) clearly demonstrates that AIP can neutralize A β 42-induced neurotoxic effects. Mechanistically, it is likely that AIP masked the receptor recognition site of A β 42 oligomers, e.g., the cellular form of the prion protein, which has been suggested as a receptor for A β 42 oligomers⁶². Consequently, A β 42 “trapped” oligomers would not interact with the putative receptor, which may result in non-altered spine morphology and no decreased LTP, as demonstrated in our study.

The *Drosophila* “rough eye” model has become a well-established system to study toxic events as related to human cancers⁶³ and neurodegeneration⁶⁴. To assess whether the AIP has a protective effect against cell-derived A β 42, we used our previous *Drosophila* model where the transgenic expression and secretion of A β 42 in the eye generates A β aggregates¹⁶. Transgenic A β 42 flies raised on food containing D-amino acid AIP exhibited a less severe rough-eye phenotype compared to (i) transgenic A β 42 flies treated with L-amino acid AIP, or (ii) non-treated transgenic A β 42 flies. In addition to its small molecular mass (<1 kDa), the added protease resistant nature of D-AIP most likely contributed to its superior bioavailability and efficacy^{35,36}. Since D-AIP could attenuate A β 42-induced toxicity at both structural (i.e. significantly improved eye morphology by EM) and functional levels (i.e. significantly ameliorated function of photoreceptors by ERG), our findings underscore the potential of the AIP as a new therapeutic strategy.

Our *in vitro* and *in vivo* results can be explained by the interaction of AIP with the two A β 42 sites (Gly33 and Asp23) that are critical for fibril growth and modulating toxicity. The molecular docking poses indicate that both L- and D-AIP make multiple hydrogen bonds to free backbone amid nitrogens or oxygens at the oligomer edges with either polar side chains or the backbone, which further hinders A β 42 β -sheet attachment. Similarly, others have identified tau residues 306–311 (VQIVYK) as a target template for the D-amino acid hexapeptide TLKIVW that can prevent self-interaction of the VQIVYK sequence via a steric hindrance⁶⁵.

The best-fit docking poses for AIP revealed that AIP-Arg1 and its protonated N-terminus make H-bonds to A β -Asp23, which interferes with the Asp23-Lys28 salt bridge which is important for A β 42 fibril growth. A peptide carrying the familial AD (FAD) mutation A β 42 D23N (Iowa), unable to form the salt bridge by the substitution of Asp23 to Asn23⁴¹, failed to show the inhibitory effect of AIP on aggregation. This finding underscores the importance of the intramolecular contact between positions Asp23 and Lys28 in the A β sequence which alters aggregation and modulates toxicity since this FAD mutation has a 2- to 3-fold higher neurotoxicity compared to A β 42 wt⁴⁰. Thus, our data indicate that AIP contacts A β 42 peptide through the Gly33- or the Gly37-groove and that the altered three-dimensional shape between L- and D-forms retained the biological activity of AIP, i.e., by interfering with the salt bridge and inhibiting A β -induced neurotoxicity.

Numerous molecules have been identified which possess the ability to prevent amyloid fibril formation, both *in vivo* and *in vitro*. These molecules range from large proteins such as molecular chaperones¹⁹ to small molecules such as flavonoids²⁰ to polyphenols²¹. So far, the most successful structure-based approach to prevent fibril formation has been to stabilize the native tetrameric structure of transthyretin (TTR)⁶⁶ in TTR amyloidogenesis. Tafamidis meglumine, a small molecule which binds selectively to two normally unoccupied thyroxine-binding sites of the tetramer and kinetically stabilizes TTR through hydrophobic and electrostatic interactions⁶⁷, similar to AIP-A β interactions. Although A β lacks a fully ordered native structure⁶⁸, this effect proves the concept that stabilization of a native stable conformation can have beneficial outcomes in protein misfolding diseases, i.e., preparing AIP-A β complexes for removal and efficient clearance. While our present study establishes a clear biological effect of AIP on A β 42-mediated toxicity, further investigation is needed to determine the exact interaction model between AIP-like inhibitors and amyloidogenic peptides for rational therapeutic design.

Methods

Peptides. Synthetic A β peptides and AIP were purchased from Peptide Specialty Laboratories, Germany, and ChemImpex INT'L INC, USA, respectively. A β was monomerized and solubilized as described⁴⁹. Briefly, monomerized peptides were dissolved to 1 mg/ml in deionized water supplemented

with ammonia to a final concentration of 0.13% (measured pH 9.8). The AIP was applied at an A β 42-to-AIP molar ratio of 1:20.

Transmission Electron Microscopy (TEM). A β peptides were dissolved to 40 μ M and incubated for 24 h at room temperature. Aliquots (10 μ l) of matured peptide solutions were applied to glow-discharged carbon-coated copper grids, or to formvar-coated nickel grids, respectively, and negatively stained with 2% aqueous uranyl acetate as described⁶⁹. Micrographs were taken using a Philips CM100 electron microscope at 100 kV and a Fastscan CCD camera (Tietz Video and Image Processing Systems GmbH, Gauting, Germany).

Atomic Force Microscopy (AFM). AFM is an appropriate method for the characterization of pre-fibrillar protein assemblies. Here, 5 μ l of a 40 μ M A β peptide solution were applied on a 0.5 mm² freshly cleaved sheet of mica for 10–30 seconds and then removed by fast spinning off. AFM images were recorded using a MultiMode scanning probe microscope (either NanoScope IIIa, Digital Instruments Inc., or NanoScope IV, Veeco Instruments Inc., Santa Barbara, California, USA), equipped with a 10 μ m scanner (E-scanner). Height and phase images were recorded in tapping mode with scan rates of 2–4 lines per second and a resolution of 512 \times 512 pixels. Olympus etched silicon cantilevers were used with a typical resonance frequency in the range of 60–80 kHz and a spring constant of 2 N/m. All samples were investigated on dry substrates of mica (PLANO W. Plannet GmbH, Wetzlar, Germany) at room temperature open to air.

Size exclusion chromatography (SEC). SEC was performed as described¹⁶. Briefly, synthetic A β peptides were dissolved and mixed with or without AIP prior to incubation for 0, 4, or 8 hours at room temperature. Oligomers were separated by a Superose 12 10/300 GL column (GE Healthcare, Germany) with PBS as running buffer at a flow rate of 0.5 ml/min.

Limited proteolysis. Proteolysis was performed as described¹⁶ with minor modifications. Briefly, A β 42 peptides were dissolved as described above and diluted in 25 mM ammonium bicarbonate, pH 7.5 to a concentration of 20 μ g/ml. A β peptides were pre-incubated for 4 hours at room temperature without and in conjunction with the AIP, respectively. The tryptic digest was performed at 37 °C using an enzyme–substrate ratio of 1:500 trypsin (Roche) for up to 3 hours. Fragments were identified by mass spectrometry (UltrafleXtreme MALDI-TOF/TOF Bruker Daltonics, Germany) in reflector-positive mode using CCA matrix.

Molecular Modeling. A tetrameric A β 42 fibril core structure was obtained from the pentameric NMR structure 2BEG³⁹ by removing one lateral beta sheet in Pymol. A β 42 G33A tetramer was generated by point mutating the tetramer A β 42 structure at all Gly33 to Ala using Schrödingers maestro 2014–2 suite (Schrödinger Release 2014–2: Maestro, version 9.8, Schrödinger, LLC, New York, NY, 2014). For docking, both structures were prepared with the Protein Preparation Wizard⁷⁰.

A docking grid suitable for peptide docking was generated with its centre (10 Å) between the middle of two Met35, defining the center of all Gly/Ala33, Met35 and Gly37 residues by means of the receptor grid generator. This defines a binding site large enough for binding of the AIP ligand (with length <36 Å). The AIP was built as random coil in Pymol with charged Arginine, Glutamic acid and Lysine side chains and termini. Maestros build utility was used to generate its D-enantiomer (D-AIP). Both ligands were prepared with the LigPrep Wizard (LigPrep, version 3.0, Schrödinger, LLC, New York, NY, 2014) for the forcefield OPLS_2005⁷¹ including Epik protonation states⁷² and including the original state for neutral pH 7 \pm 0.5 (covering physiological pH 7.4). Glide SP-peptide docking for flexible ligand sampling⁷³ was performed using standard settings and specifically sampling nitrogen inversions, ring conformations (including input conformation), no bias sampling of torsions, and enhanced planarity of conjugated pi groups. The docking score was modified by Epik state penalties. The SP-peptide docking protocol yields around 119–200 poses per run with no more than 5–19 reasonable <-5 kcal/mol and 1–4 best docking scores <-7 kcal/mol.

Toxicity on primary hippocampal neurons and neuroblastoma cells. Primary neurons were prepared as previously described¹⁶. Briefly, hippocampi of postnatal day 0 (P0)–P1 Wistar rat pups were dissociated and cultured in neurobasal A medium. After 10 days *in vitro*, A β 42 (2.5 μ M) peptides were pre-incubated (4 or 24 hours) in the presence and absence of the AIP, respectively. Neuronal cultures were treated with the pre-incubated peptides for 48 hours. Neuronal viability was detected using the MultiTox-Fluor Multiplex Cytotoxicity assay (Promega, Germany), performed according to manufacturer's instructions.

To determine A β toxicity in SH-SH5 cells, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was performed as described⁴⁹. A β peptides were applied at a concentration of 2.5 μ M for 12 hours.

Organotypic hippocampal slice cultures, image acquisition and quantification of dendritic spines. Hippocampal slices (400 μm) from P6–8 transgenic mice expressing membrane-targeted MARCKS-eGFP under the Thy-1 promoter in a subpopulation of CA1 cells⁷⁴ were prepared as previously described using the roller-tube method⁷⁵. Slice cultures were maintained in the incubator with a roller-drum for three weeks before experimentation to allow for spine maturation⁷⁶. Slices were incubated with or without 1 μM A β 42 peptide and 20 μM AIP-containing media for 24 hours. Image acquisition and quantification was performed as described⁷⁶. Briefly, following treatment, slice cultures were transferred to a temperature-controlled chamber (30 °C) mounted on an upright confocal microscope and continuously perfused with Tyrode solution. Secondary and tertiary dendritic branches from either apical or basal dendrites of CA1 pyramidal neurons were imaged.

All animal handling procedures were carried out in accordance with guidelines set by the Canadian Council on Animal Care and the National Institutes of Health in the USA. All procedures were approved by the Animal Resource Committee of the School of Medicine at McGill University, and are outlined in McGill University Animal Handling Protocol #5057.

fEPSP recordings and Long Term Potentiation. fEPSPs were recorded in the CA1 *stratum oriens* at 30 °C as previously described⁷⁶ with minor modifications. Briefly, a surgical cut was made between CA3 and CA1. Schaffer collateral axons were then stimulated with an insulated platinum–iridium bipolar electrode. The stimulus strength was between 40 μA and 200 μA , to achieve one-half to one-third of the maximum response, and stimulation was provided every 10 s. Data points were then pooled and averaged into five minute intervals for statistical analysis and graphical display in the figures. LTP was induced with a TBS paradigm consisting of bursts of five pulses at 100 Hz; the bursts were applied five times at intervals of 200 ms, and delivered five times every 10 s. Slices were treated with freshly-dissolved 1 μM A β 42 peptide, 20 μM AIP, or 1 μM A β 42 peptide and 20 μM AIP-containing media for 24 hours prior to recordings.

Eye structure analysis of *D. melanogaster*. UAS-A β 42 flies were generated as previously described¹⁶. Briefly, transgenic flies harboring UAS-A β 42 were crossed with flies containing GMR-GAL4 to achieve eye-specific A β 42 expression. Flies were bred on Jazz-Mix Drosophila fly food (Fisher Scientific, USA) containing 5 mM L-AIP or D-AIP, respectively. Five-day-old flies were collected and analyzed. Fly eyes were fixed in 2.5% glutaraldehyde, 2% formaldehyde in 100 mM PB buffer, pH 7.4, post-fixed in 1.5% osmium tetroxide. Samples were embedded in epon and polymerized at 60 °C for at least 48 hours. The sections were counterstained with aqueous uranyl acetate followed by lead citrate. Micrographs were taken using a Philips CM120 electron microscope at 80 kV and a 1 K CCD camera.

Electroretinography. Flies were immobilized inside the end of a plastic pipette tip. The ERGs were recorded using borosilicate pipette microelectrode in contact with the eye, and a reference tungsten electrode inserted into the abdomen. The microelectrode was filled with artificial cerebral-spinal fluid containing: 115 mM NaCl, 5 mM KCl, 23 mM glucose, 26 mM sucrose, 4.2 mM HEPES, 2.5 mM CaCl₂, and 1.3 mM MgCl₂, pH 7.2. Signals were amplified and filtered (1 kHz) using an Axopatch 200B amplifier, sampled at 5 kHz using a Digidata 1440A, and recorded with Clampex 10.3 (Molecular Devices). Blue light from a LUXEON®Star LED with a dominant wavelength of 470 nm was delivered to the fly head via an optical fiber cable 5 cm from the fly head. Light stimulus was driven by a Thor labs LEDD1B controller with stimulus of 1000 ms. Representative traces were low bandpass filtered.

Statistical Analyses. All results are expressed as means \pm SEM (Standard Error of Mean). Statistical comparisons were made by either unpaired Student's t-test, or by One-way ANOVA followed by Tukey-Kramer or Dunnett's *post hoc* test to identify differences between treatments. All data presented are from a minimum of three independent experiments. P values < 0.05 were considered significant: *p < 0.05, **p < 0.01; ***p < 0.001.

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Author Contributions

G.M., R.A.M.C.K. and P.W.H. designed the research, G.M. supervised the project. C.B. carried out most of the experiments. Data analyses were performed by C.B., F.L. and S.H., H.J.B. carried out the molecular modeling studies, P.K.Y.C., S.C., H.S., F.M.C., W.Z., Y.R., P.D. and D.S. assisted with *in vitro* and *in vivo* toxicity studies. M.A.H., A.H., M.G. and R.L. performed biophysical and biochemical analyses and JPR designed and supervised AFM studies. G.M., C.B., F.L. and S.H. wrote the manuscript.

Additional Information

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