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Retro-inversal of Intracellular Selected β-Amyloid-Interacting Peptides: Implications for a Novel Alzheimer's Disease Treatment

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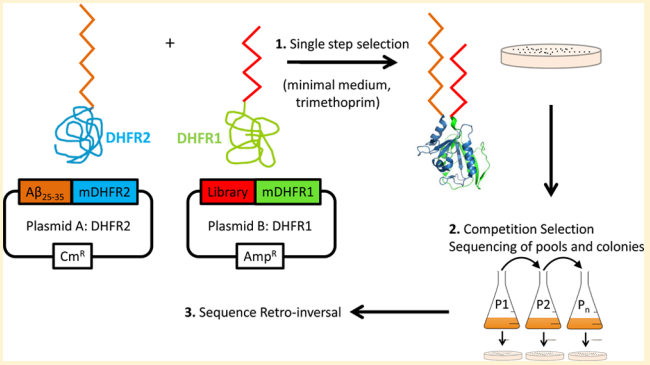
ABSTRACT: The aggregation of β-amyloid (Aβ) into toxic oligomers is a hallmark of Alzheimer's disease pathology. Here we present a novel approach for the development of peptides capable of preventing amyloid aggregation based upon the previous selection of natural all-L peptides that bind $Aβ_{1-42}$. Using an intracellular selection system, successful library members were further screened via competition selection to identify the most effective peptides capable of reducing amyloid levels. To circumvent potential issues arising from stability and protease action for these structures, we have replaced all L residues with D residues and inverted the sequence. These retro-inverso (RI) peptide analogues therefore encompass reversed sequences that maintain the overall topological order of the native peptides. Our results demonstrate that efficacy in blocking and reversing amyloid formation is maintained while introducing desirable properties to the peptides. Thioflavin-T assays, circular dichroism, and oblique angle fluorescence microscopy collectively indicate that RI peptides can reduce amyloid load, while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays demonstrate modest reductions in cell toxicity. These conclusions are reinforced using *Drosophila melanogaster* studies to monitor pupal hatching rates and fly locomotor activity in the presence of RI peptides delivered via RI−trans-activating transcriptional activator peptide fusions. We demonstrate that the RI−protein fragment complementation assay approach can be used as a generalized method for deriving Aβ-interacting peptides. This approach has subsequently led to several peptide candidates being further explored as potential treatments for Alzheimer's disease.



A vast body of evidence implicates Aβ as a major player in Alzheimer's disease (AD),[1] with genetic studies revealing abnormal production in cell culture and animal models. Aβ aggregation is implicated in neuronal death and impaired memory,[2] with a wealth of evidence suggesting that amyloid load in AD sufferers does not correlate with disease severity.[2,3] The insoluble fibrils that are characteristic of the disease may serve as reservoirs that sequester a number of more toxic and soluble oligomeric species. Consequently, targeting Aβ via therapeutic intervention has led to numerous inhibition strategies. Rare familial mutations that increase the Aβ oligomer concentration (e.g., the E22G Arctic mutation[4]) have been shown to accelerate the onset of AD as a result.[4] Many β-sheet breaker (BSB) molecules that block or break down amyloid fibers have had limited success and in certain cases have been counteractive, because of either an increased level of oligomer production or a failure to accelerate their removal.[5−11] Therefore, because oligomers as small as dimers have been shown to exhibit cytotoxicity,[12,13] molecules effective at lowering amyloid levels may need to either sequester fibrils in the insoluble state or preferably bind Aβ as a small but ultimately nontoxic oligomer, possibly even the monomer.[14]

We have previously used intracellular library screening and selection to identify Aβ-interacting peptides.[15] In this approach, residues 25−35 of Aβ are used as a design scaffold. This sequence, along with residues 15−20, is known to form amyloid in isolation[5,6] and is thought to be responsible for instigating Aβ self-association in the parental protein. Many amyloid inhibitors have therefore been based upon these regions[5−11] (see also ref 16 and references therein), with many strategies focused on simple modifications such that they retain the ability to bind Aβ but prevent amyloid formation by introducing blocking or charged groups into these short sequences. For example, Tjernberg et al. demonstrated that $Aβ_{16-20}$, despite forming fibrils itself, binds residues 25−35 of Aβ and prevents fibril formation.[5] Soto and co-workers rationally designed proline-containing peptides based on $Aβ_{17-21}$.[10,11] It was subsequently shown that clearance of large amyloid fibrils can lead to the population of smaller more cytotoxic intermediates.[17] Therefore, the search for BSB peptides has been hampered by the fact that successful

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molecules must be capable of preventing the population of amyloid oligomers while avoiding the generation of cytotoxic species. In the approach described here we use an intracellular protein fragment complementation assay (PCA) selection, which works by recombining a split enzyme that is essential for cell survival.[15,18−20] We apply a variation on this system whereby the Aβ target is fused at the genetic level to half of the essential enzyme murine dihydrofolate reductase (mDHFR), with a peptide library fused to the other half. Following protein expression in the cytoplasm, binding of a library member to Aβ reconstitutes mDHFR, leading to bacterial cell growth and colony formation under selective conditions. Because the entire process is intracellular, no assumptions are made with regard to the mechanism of antagonist action or which amyloid states become populated during selection. The only prerequisite for success of any given library member is therefore that it must (i) bind to Aβ and reconstitute mDHFR and (ii) prevent Aβ aggregation that has been shown to slow cell growth, with *Escherichia coli* rescued from the toxic effects of aggregation. In addition, the PCA approach is predicted to select peptides that are resistant to degradation by bacterial proteases, to be soluble in solution, and to be target specific in the presence of other cytoplasmic proteins. Using PCA with an 8000-member library based on Aβ_{29-35} led to an initial interacting sequence. A second library of 160000 members using this first hit as a design scaffold yielded two further novel interacting sequences.[15] These peptides consequently shared no homology with the original Aβ_{29-35} design template. All selected peptides were found to be capable of binding Aβ, inhibiting amyloid formation, and breaking down preformed fibrils.

A potential limitation of this previous study was the use of natural unmodified L peptides that are susceptible to degradation by mammalian proteases. This was partially addressed by undertaking library selection in the cytoplasm of *E. coli*. However, while bacterial growth experiments indicated that toxicity was lowered, cytotoxicity experiments using mammalian cells suggested that protease protection was not observed when they were transferred to the PC12 cell environment.[15] In a continuation of the study described above, we present data on retro-inverse (RI) analogues of these previously selected peptides whereby we have substituted the L amino acids for their D counterparts and reversed the sequence. The D amino acid sequences give a mirror conformation, while the retro-peptides, consisting of the same sequence of L amino acids, leads to a reversal of order. Sequence retro-inversal therefore leads to a mimic of the original peptide because of inversion of the peptide bonds.[21,22] Using this approach, peptides have been shown to retain the same inhibitory aggregation qualities while displaying vastly increased protease resistance.[23] For example, rationally designed RI peptide inhibitors of Aβ aggregation have previously been derived by appending arginine residues to the Aβ_{16-20} (KLVFF) sequence.[24,25] These peptides were also shown to be serum-stable and with the addition of a RI cell-penetrating peptide fusion were also able to cross the blood−brain barrier and display significant activity in transgenic mouse models.[26] In addition, although not an RI approach, mirror image phage display has been used to screen a 12-mer library against an immobilized D enantiomer of the Aβ_{1-42} peptide, with the most successful peptide binder replaced by D amino acids to yield a protease-stable peptide capable of binding to the natural L form of Aβ_{1-42}.[27] The success of these studies further highlights the potential for peptide-based therapeutic strategies.

Here we report a novel approach that uses intracellular library screening and selection followed by sequence retro-inversal (RI-PCA) as a generalized strategy for creating stable peptides capable of antagonizing protein−protein interactions (PPIs). We have tested the effectiveness of peptides using a range of *in vitro* techniques that report on the amount of fibril present. These experiments have been coupled with PC12 neuronal cell-based assays that report on the overall effect of toxicity in the presence of various peptides, as well as *in vivo* studies using *Drosophila melanogaster*, to verify peptide efficacy in the context of a whole cell Alzheimer's disease model organism.

MATERIALS AND METHODS

PCA and Expression Vector Cloning. PCA has been extensively used to derive PPI antagonists of activator protein-1.[18,19,28,29] More recently, this has been extended to Aβ-interacting peptides, where a full description of the methods can be found.[15] Briefly, mDHFR was split and one half fused to an Aβ_{25-35} target peptide and the other half to the library.[15] Only target binding library members bring two halves of mDHFR into the proximity of each other, render it active, and lead to colony formation on M9 selective plates. Trimethoprim is used to selectively inhibit bacterial DHFR, thereby ensuring that colonies can arise only as the result of an interaction between Aβ and a peptide library member. The Aβ_{25-35} gene was synthesized using overlap extension polymerase chain reaction and cloned into the pES300d-DHFR2 vector system using NheI and AscI restriction sites.

PCA Library Construction. Library construction and cloning have been described previously.[15,18] Briefly, in the first library, positions 31−33 of Aβ_{29-35} were completely randomized using degenerate oligonucleotides containing NNK codons. NNK was used to encode all 20 residues while removing two of three stop codons[30] to create an 8000-member library. The second library was designed using the first PCA winner ("KAT") as a design scaffold. In this case, residues 29, 30, 34, and 35 of KAT were randomized, again using the NNK codon, this time to generate a library of 160000 members. Thus, in starting with Aβ_{29-35} as an initial design scaffold, we subsequently derived a completely unrelated sequence. Competition selection during PCA means that only the most effective one or two sequences are isolated from the 50−100 Aβ binders that are initially identified during single-step selection.

Aβ Peptide Preparation. Aβ_{1-42} was purchased as a pure recombinant peptide from rPeptide (Stratech) and used for all experiments described. Prior to being used, the peptide was treated to three rounds of dissolution in hexofluoro-2-propanol (HFIP), sonication, drying, and dissolution in trifluoroacetic acid (TFA), followed by sonication and drying, according to the Zagorski protocol.[31] The peptide was then aliquoted into appropriately sized batches for subsequent assays and dried via lyophilization before being dissolved in 10 mM potassium phosphate buffer (pH 7.4) to generate a final concentration of 50 μM. Rounds of TFA/HFIP treatment were used to ensure that amyloid growth always proceeded from the same monomeric state, thus reducing errors in amyloid growth and consequent assay measurements. For the second library winners, two versions of each peptide were synthesized: (i) the parental sequence including selected residues (L2P1a-RI/L2P2a-RI) and (ii) the parental sequence including additional amino acids from restriction sites during cloning into the

pES230d vector (L2P1b-RI/L2P2b-RI).[15] See Table 1 for all peptide sequences.

Table 1. PCA-Derived Retro-inversed Sequences and Related Sequences with Retro-inversed TAT Fusions for Promoting Intracellular Uptake in *D. melanogaster*

Name	Sequence
KAT-RI	mltakag-NH2
L2P1a-RI	nstaksf-NH2
L2P1b-RI	pagnstaksfsa-NH2
L2P2a-RI	attakvp-NH2
L2P2b-RI	pagattakvpsa-NH2
Tat-KAT-RI	rrrqrrkkrmltakag-NH2
Tat-L2P1-RI	rrrqrrkkrnstaksf-NH2
Tat-L2P2-RI	rrrqrrkkrattakvp-NH2
TAT-dummy-RI	rrrqrrkkrGGGGGGG-NH2
iAβ5	LPFFD-NH_2

Peptide Preparation. KAT-RI, L2P1a-RI, L2P1b-RI, L2P2a-RI, L2P2b-RI, and both a positive control from the literature, iAβ5,[10] and a negative control peptide, TAT-dummy-RI, were obtained by Peptide Protein Research (Fareham, U.K.) as pure lyophilized peptides. In addition, for *D. melanogaster* experiments, TAT-KAT-RI, TAT-L2P1-RI, and TAT-L2P2-RI were also used. Peptides were weighed using an analytical balance, and 1 mM stock solutions were subsequently dissolved in ultrapure water. Prior to the assay, peptides were either aliquoted and lyophilized or diluted from the stock as required.

Thioflavin T Assays. ThT inhibition assays were performed using 50 μM Zagorski-treated[31] monomeric Aβ_{1-42} in 100 μL of 10 mM potassium phosphate buffer (pH 7.4) with or without each peptide at a concentration of 5 μM (for a 1:0.1 molar ratio), 50 μM (for a 1:1 molar ratio), 100 μM (for a 1:2 molar ratio) and 200 μM (for a 1:4 molar ratio). In addition, for substoichiometric experiments, concentrations of 0.5 μM (1:0.01 molar ratio), 50 nM (1:0.001 molar ratio), and 5 nM (1:0.0001 molar ratio) were also included to demonstrate that the activity progressively decreases as the peptide dose is decreased, thus demonstrating a trend of dose dependency. To achieve this, sufficient Aβ_{1-42} was lyophilized, dissolved, and thoroughly vortexed as one single batch (for immediate use in all Aβ_{1-42} target/peptide mixtures) to a concentration of 50 μM in potassium phosphate buffer. Each inhibitor was lyophilized and redissolved in an Eppendorf tube to concentrations of 5 μM (1:0.1), 50 μM (1:1), 100 μM (1:2), and 200 μM (1:4). Finally, a 100 μL aliquot of the target solution was added to each inhibitor to give a total assay volume of 100 μL containing 50 μM target and the appropriate inhibitor. The assay mixture was vortexed and stored at 37 °C for 3 days to induce aggregation in the presence of each inhibitor. The ThT assay solution was prepared from a 25× stock containing 500 μM ThT. The stock was aliquoted and kept frozen until it was required. It was then allowed to thaw at room temperature for 10 min before being diluted in 10 mM Tris buffer (pH 7.4), giving the required freshly prepared ThT assay solution containing 20 μM ThT in 10 mM Tris and buffer at pH 7.4. A total of 197.1 μL of the ThT assay solution was then added to 2.9 μL of each inhibition/reversal assay mixture, and the mixture was thoroughly vortexed and transferred into an appropriate well of the multiplate. The fluorescence of amyloid-bound ThT was measured by fluorescence spectroscopy using a Cary Eclipse fluorescence spectrophotometer; bound ThT exhibits a new excitation maximum at 450 nm and an enhanced emission maximum at 482 nm.[32] For the inhibition assays, the Aβ_{1-42} target/peptide mixtures were incubated together on day 0 at 37 °C, and single ThT readings were taken on day 3. For the reversal assays, the target was incubated alone at 37 °C for 3 days before the addition of 100 μL to each lyophilized inhibitor. The vortexed Aβ_{1-42} target/peptide solutions were then incubated at 37 °C for a further 3 days, at which point single ThT readings were taken.

Circular Dichroism (CD). Far-UV circular dichroism (CD) spectra were recorded on an Applied Photophysics Chirascan CD spectrometer at 20 °C. Peptide [10 μM in 10 mM potassium phosphate buffer (pH 7.4)] was added to a 1 mm CD cell (Hellma), and spectra were recorded over the range of 200−300 nm at a scan rate of 10 nm/min with step size of 1 nm. Spectra were recorded as the average of two scans as raw ellipticity. Spectra for RI peptides alone were subtracted from spectra of Aβ_{1-42} target/peptide solutions, to leave normalized CD spectra accounting for the effect of the peptide upon Aβ_{1-42}.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Cell Toxicity Assay. MTT experiments were undertaken using rat pheochromocytoma (PC12) cells to assess the effect of the toxicity of Aβ_{1-42}. PC12 cells are known to be particularly sensitive, and their use in this assay is well-established.[33] The MTT Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) was used to measure the conversion of the water-soluble MTT dye to formazan, which is then solubilized, and the concentration determined by a color change monitored via absorbance measurement at 570 nm. The change in absorbance can then be converted to a percentage MTT reduction that can be used as an indicator of PC12 cell health in the assay. The assay was performed with 10 μM Aβ_{1-42} and varying peptide molar ratios of 1:0.1 (0.1 μM), 1:1 (10 μM), 1:2 (20 μM), and 1:4 (40 μM). PC12 cells were maintained in RPMI 1640 with 2 mM glutamine medium mixed with 10% horse serum and 5% fetal bovine serum, supplemented with 20 mg/mL gentamycin. Cells were transferred to a sterile 96-well plate at a density of 30000 cells per well and experiments performed in triplicate. Briefly, different concentrations of peptides were screened in the presence of 10 μM Aβ_{1-42}. The required volume from peptide and target stock solutions was freeze-dried overnight. The freeze-dried peptide and Aβ_{1-42} target were resuspended in 100% dimethyl sulfoxide (DMSO), each at a 100× stock concentration (i.e., 1, 2, 4, or 10 mM). For example, for a 1:1 molar ratio, a total of 5 μL from each of the resuspended peptide/DMSO and target/DMSO solutions was mixed in a well of a 96-well preparation plate, thus giving 10 μL of 1 mM peptide/1 mM Aβ_{1-42} target mixture in 100% DMSO. A total of 90 μL of RPMI medium was added to the 10 μL peptide/Aβ_{1-42} target mixture (100 μM peptide/100 μM Aβ_{1-42} target mixture in 10% DMSO). A total of 10 μL of the 50 μM peptide/50 μM Aβ_{1-42} target mixture in 10% DMSO was then dispensed in 90 μL of media and PC12 cells, at final peptide and Aβ_{1-42} target concentrations of 10 μM. These were incubated for 24 h at 37 °C and 5% CO_2 prior to the addition of the MTT dye. A total of 10 μL of the day was added to each well and incubated for a further 4 h at 37 °C and 5% CO_2. A total of 100 μL of the DMSO (stop/solubilization solution) was then added to each well and allowed to stand for 10 min. The absorbance was measured at 570 nm using a 96-well Versamax tunable microplate reader.

Oblique Angle Fluorescence Microscopy Experiments. Samples were imaged on a custom-built oblique

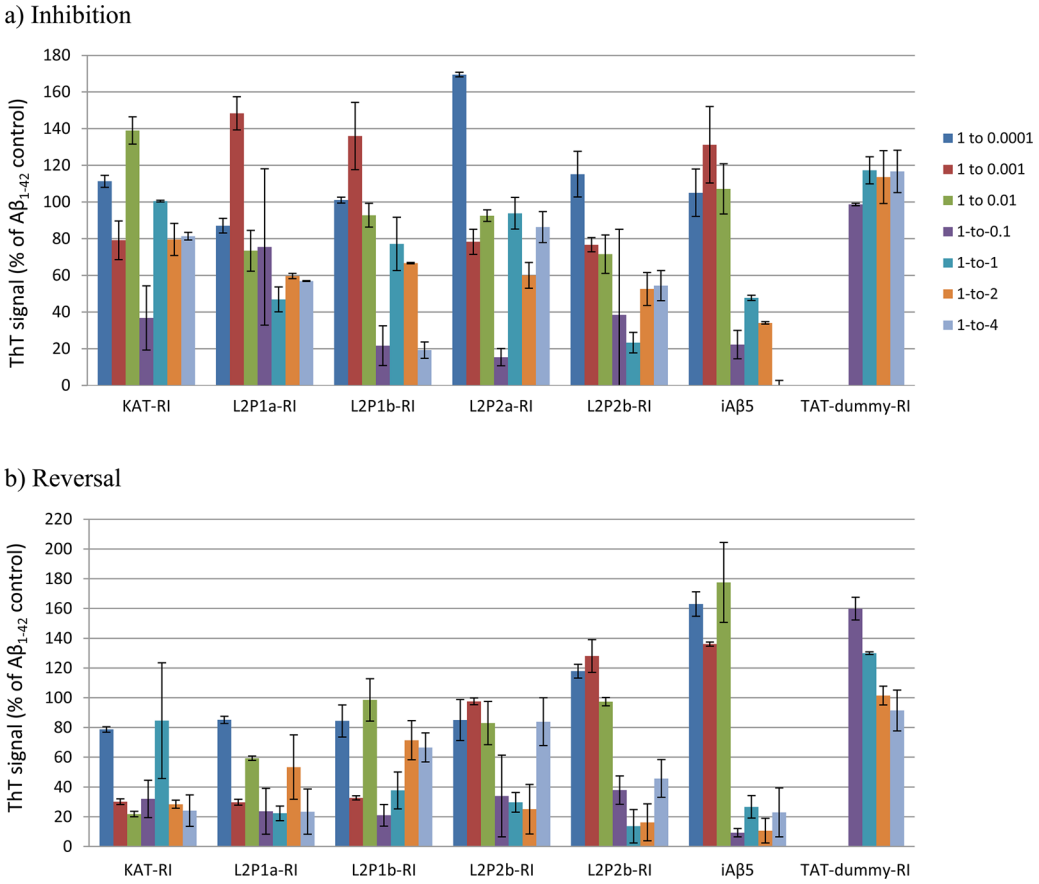

Figure 1. ThT inhibition and reversal data. The data in panels a and b show the effects of different stoichiometries of peptides KAT-RI, L2P1a-RI, L2P1b-RI, L2P2a-RI, and L2P2b-RI on the aggregation of 50 μM Aβ_{1-42} (on day 3 for the inhibition assay and day 6 for the reversal assay). Molar ratios (Aβ:peptide) of 1:0.0001, 1:0.001, 1:0.01, 1:0.1, 1:1, 1:2, and 1:4 were used for each peptide. iAβ5 and TAT-dummy-RI are included as positive and negative controls, respectively. Errors are given as the standard deviation of all errors at each molar ratio. The data show that for the three lowest molar ratios (all substoichiometric; 1:0.01, 1:0.001, and 1:0.0001) the average decrease in the amount of ThT bound was minimal (106% for inhibition and 89% for reversal). In contrast, at the three highest molar ratios, the decrease in the amount of ThT bound was significantly greater (58% for inhibition and 38% for reversal). The most effective average molar ratio for peptides was 1:0.1, which displayed ThT bound values of 35 and 26% for inhibition and reversal, respectively, which were approximately 71 and 63% lower than the averages of the three lowest stoichiometries, respectively.

angle fluorescence (OAF) system as described previously.[15,34] The excitation and emission wavelengths were 488 and 500–605 nm, respectively. Although both the excitation and emission wavelengths were off peak for ThT, the image quality and photobleaching characteristics were excellent. All samples were prestained with 10 μM ThT, pipetted onto a clean glass slide, air-dried, and then imaged in KPP buffer supplemented with 100 mM DTT to further minimize photobleaching. For the sake of consistency and cross-correlation, the same samples were used for inhibition and reversal imaging as those in ThT and CD experiments. In addition, all samples provided for OAF imaging were supplied blind.

***D. melanogaster* Assays.** The effect of RI peptides upon *D. melanogaster* was assayed by fusing sequences to the (also retro-inversed) nine-residue cell-penetrating peptide (CPP), TAT.[35−37] In these experiments, flies expressing Aβ_{1-42} were fed nutrient containing either RI peptides or RI-TAT-peptide fusions (see Table 1) and two key effects monitored: (i) the speed of pupae hatching in which the cumulative hatching fractions for each treatment were monitored relative to a negative control group and (ii) fly motility using an automated fly tracking system to monitor the walking speed of the transgenic flies. In the control group, flies typically become immobile as they age over the first 10−15 days of life.

RESULTS

We have previously undertaken PCA screening on libraries to derive peptides capable of clearing Aβ_{1-42} aggregates and generating significant enhancements in bacterial growth rates.[15] To produce peptides that retain the ability to bind Aβ_{1-42} while adding additional properties to the molecule such as stability and protease resistance,[23,38] we have now retro-inversed these sequences. The RI-PCA-derived peptides (Table 1) have been synthesized and characterized using a number of methods, including ThT dye fluorescence to report on amyloid formation, CD to measure global changes in β-sheet content associated with fibril formation, and direct imaging using OAF microscopy. These techniques have demonstrated that RI peptides retain the ability of the PCA-selected parental peptides to prevent aggregation and remove preformed fibrils. In addition, MTT cytotoxicity assays undertaken using a neuronal PC12 cell line were used to demonstrate that Aβ_{1-42} toxicity is lowered when bacterially selected peptides (which were not effective in MTT experiments[15]) are retro-inversed in sequence. To explore cell-based experiments further, we have

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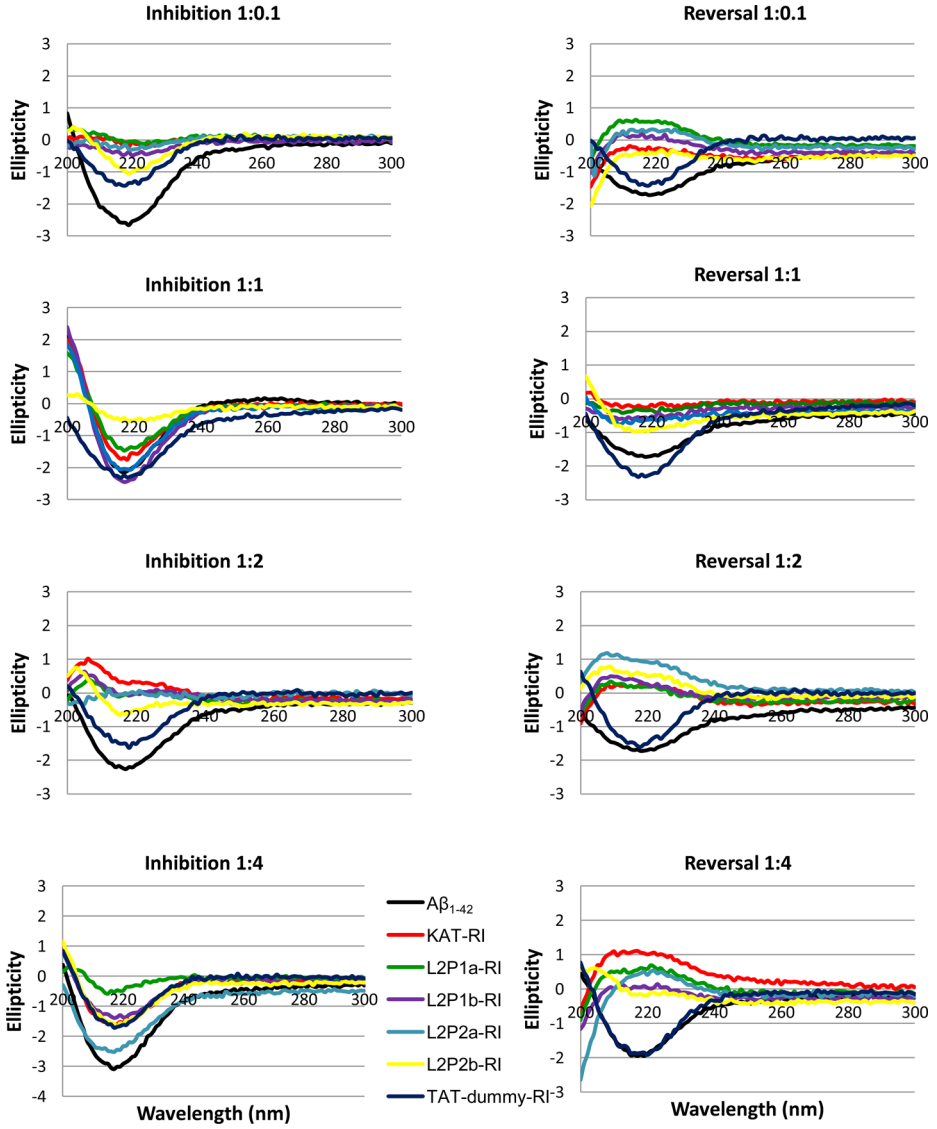

Figure 2. Circular dichroism spectra show the β-sheet content of (i) RI peptides with Aβ and (ii) RI peptides alone and (iii) the β-sheet content of RI peptides subtracted from the combined signal (i.e., ii − i). This is shown at all molar molar ratios studied for inhibition and reversal conditions. Both experiments were undertaken 3 days after mixing and performed at a $A\beta_{1-42}$ concentration of 10 μM.

undertaken studies in which flies (*D. melanogaster*) expressing wild-type $A\beta_{1-42}$ were fed RI peptides as well as RI-TAT-peptide fusions to bring cell permeability to these $A\beta_{1-42}$-interacting compounds.

ThT Binding Indicates a Reduced Fibril Load. To determine the ability of PCA-derived peptides to either prevent fibril assembly (inhibition) or break down preformed fibrils (reversal), ThT was used as an indicator of the degree to which $A\beta_{1-42}$ had aggregated into amyloid fibrils. In this assay, $A\beta_{1-42}$ was rendered monomeric[31] and redissolved at a concentration of 50 μM before being aggregated by incubation without agitation at 37 °C. For the inhibition assay, peptides were added on day 0, whereas for the reversal assay, the peptides were added after $A\beta_{1-42}$ fibril growth for 3 days. Once they had been incubated together, $A\beta_{1-42}$ target/peptide solutions were tested after 3 days. In addition to the five RI-PCA derived peptides, the positive control L peptide i$A\beta$5 was also included as it is known to perform well in ThT assays and lead to a reduction in fibril load.[11] Finally, a negative control peptide TAT-dummy-RI was included to demonstrate no effect on ThT binding and therefore specificity for the PCA-derived peptides. Figure 1 shows the results of these experiments at a number of $A\beta_{1-42}$:peptide ratios for each peptide. Experiments were undertaken at stoichiometries of 1:0.0001, 1:0.001, 1:0.01, 1:0.1, 1:1, 1:2, and 1:4.

Inhibition experiments demonstrate that peptides are able to prevent aggregation (Figure 1a) with reductions in the amount of ThT bound relative to the $A\beta_{1-42}$ control of up to 80%. During these experiments, a concentration dependence was observed as the Aβ:peptide ratio was lowered (Figure 1). At increasingly higher stoichiometries, this trend was less apparent. For example, L2P1a-RI decreased the amount of bound ThT by ∼30−40% at Aβ:peptide stoichiometries of ≥1:0.01. Similarly, L2P2b-RI was also able to reduce the amount of bound ThT by >40%. RI peptides incubated in the absence of Aβ did not bind ThT, displayed weakly helical CD spectra, and were soluble to high concentrations in stock solutions. The lack of concentration dependence for ThT assays at higher molar ratios is likely to be due to signal-to-noise difficulties, as is shown by consistently large errors that are typical of this assay. In

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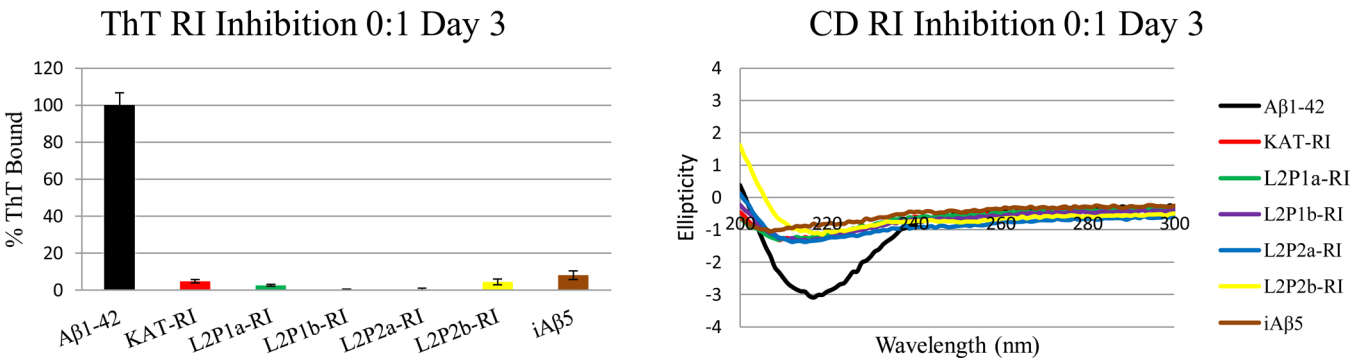

Figure 3. Circular dichroism spectroscopy and ThT experiments undertaken on RI peptides in isolation that have been incubated at 50 μM for 3 days under conditions identical to those of aggregation assays using Aβ_{1-42}.

addition, ThT is known to exhibit different fluorescence levels according to the oligomeric state populated.[39] Given that there are a number of potential intermediates in the growth of Aβ, different fluorescence intensities could result. Reassuringly, the all-L residue positive control peptide, iAβ5,[11] performed well, providing a decrease of 60−80%, which was lost in a dose-dependent manner at lower Aβ:iAβ5 ratios. In addition, the negative control TAT-dummy-RI peptide had no effect over a range of molar ratios, again demonstrating specificity for the PCA-derived sequences. RI peptides performed favorably relative to their PCA-derived L peptide counterparts.

In the case of reversal experiments (Figure 1b), as observed previously for the L peptide counterparts, more pronounced decreases are observed relative to inhibition experiments, >60% for all peptides tested in all but six instances. At molar ratios of 1:0.1, decreases of >60% were observed for all peptides. Increasing the molar ratio did not lead to improvements in the reversal of amyloid in most cases, indicating that a 10-fold substoichiometric concentration of peptide may be sufficient to reverse fibril formation. As previously reported for parental L peptides, a progressively reduced activity was observed as the peptide dose tended toward zero, demonstrating a dose dependency.[15] Experiments with RI peptides in isolation indicate that peptides do not aggregate into fibrils. They do not bind ThT and do not generate CD spectra consistent with a β-sheet structure. Again, as predicted from the literature, iAβ5 performed well at higher molar ratios, and this effect is lost at lower ratios. These results compare favorably with those of PCA-derived L peptide counterparts in being able to reduce the amount of ThT bound by ∼60%.[15] Again, the negative control TAT-dummy-RI peptide also had no effect over a range of molar ratios. Errors associated with ThT experiments preclude more detailed interpretations.

CD Studies Indicate Reduced β-Sheet Content. Because amyloid fibrils are predominantly β-sheet, we have used CD as a measure of the global signal upon incubation with peptides in inhibition and reversal assays. In these experiments, the same aggregating samples that were used in ThT experiments have been measured to allow for direct comparison between experiments. However, CD experiments in which the aggregating all-L residue Aβ_{1-42} target is mixed with D residue-containing RI peptides must be approached with caution; upon incubation of the peptides together with Aβ_{1-42}, it was unknown if a signal at 218 nm caused by the RI peptides might obscure the signal at 218 nm arising from the Aβ_{1-42} target. Therefore, the CD signals of Aβ_{1-42} and RI peptides were both measured in isolation. The CD spectra arising from these controls could then be accounted for [i.e., CD spectra of Aβ_{1-42} against [Aβ_{1-42} + RI peptide] − [RI peptide alone] (Figure 2)], allowing the overall loss or gain of the β-sheet signal exerted by the action of the RI peptide upon Aβ aggregation to be established. In this assay, we observe impressive decreases in β-sheet content for all peptides at various molar ratios, supporting the ThT data by demonstrating that RI peptides reduce the global β-sheet content of the sample and therefore the amyloid content. In agreement with the ThT data, the negative control peptide TAT-dummy-RI had little effect on the CD signal, demonstrating Aβ specificity for the PCA-derived sequences.

ThT and CD Experiments Demonstrate That RI Peptides Do Not Aggregate in Isolation. ThT experiments and CD spectroscopy experiments undertaken on RI peptides in isolation that have been incubated at 50 μM for 3 days under conditions identical to those used in aggregation assays using Aβ_{1-42} demonstrate that peptides do not bind significant amounts of ThT, and that the CD signal for all peptides (at 0:1) is consistent with that of a random coil or weakly helical conformation (Figure 3). They therefore indicate along with computational aggregation prediction programs on the L residue parent peptides (e.g., Waltz,[40] Amylpred,[41] Pasta,[42] Zyggregator,[43] and Tango[44]) that peptides do not form amyloid in isolation.

MTT Experiments Indicate a Reduced Amyloid Toxicity to Cells. MTT cell toxicity experiments were performed using rat pheochromocytoma (PC12) neuronal-like cells to assess the toxicity of Aβ_{1-42} and the preventative effects of the peptides generated in this study. MTT assays (Figure 4) were preformed across Aβ_{1-42} target:RI peptide ratios and normalized relative to cells in isolation (normalized as 0% death) and cells incubated with Aβ_{1-42} alone (normalized as 100% death). Peptides did not improve cell viability upon being incubated at 1:0.1. At an increased ratio of 1:1, a decrease in toxicity of ∼30% was observed for all RI peptides studied. At increased molar ratios of 1:2 and 1:4, a decrease in toxicity of approximately 10−20% was observed for all RI peptides studied. This experiment demonstrates a modest but significant decrease in Aβ_{1-42}-induced toxicity in the presence of RI peptides. This was more promising than for the PCA-derived L peptide parent molecules[15] and more impressive than for the iAβ5 peptide, which has previously been shown to perform poorly in MTT cytotoxicity experiments using the PC12 cell line.[7] Reassuringly, the TAT-dummy-RI peptide had a minimal effect of the viability of PC12 cells incubated with Aβ.

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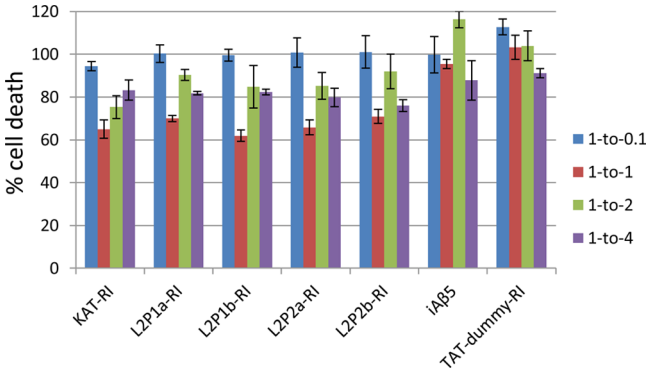

Figure 4. MTT toxicity assay using Aβ_{1-42} and selected RI peptides using different molar ratios after incubation for 24 h. The assay was performed with 10 μM Aβ_{1-42} and different concentrations of inhibitor, for example, 1:0.1 (1 μM), 1:1 (10 μM), 1:2 (20 μM), and 1:4 (40 μM).

Oblique Angle Fluorescence (OAF) Microscopy Indicates a Decrease in Amyloid Levels. To allow direct comparison, samples used in ThT and CD experiments were also imaged using OAF microscopy[34] for both inhibition and reversal experiments (Figure 5). To prevent bias toward any one sample, each experiment was performed blind. This technique allows for surface-associated and stacked aggregates of amyloid fibers to be imaged directly. OAF also permits assessment of the amount of protein deposited as amyloid and its morphology. To further quantify the amount of amyloid deposited, we analyzed the mean fluorescence value for each condition using ImageJ (National Institutes of Health, Bethesda, MD) over a randomly chosen 160 pixel × 160 pixel area and found a similar correlation. In studying the effects of the peptides on reversal, we found KAT-RI, L2P1b-RI, and L2P2a-RI were the most potent, resulting in the fewest observed fibrils on the surface. Also consistent with the

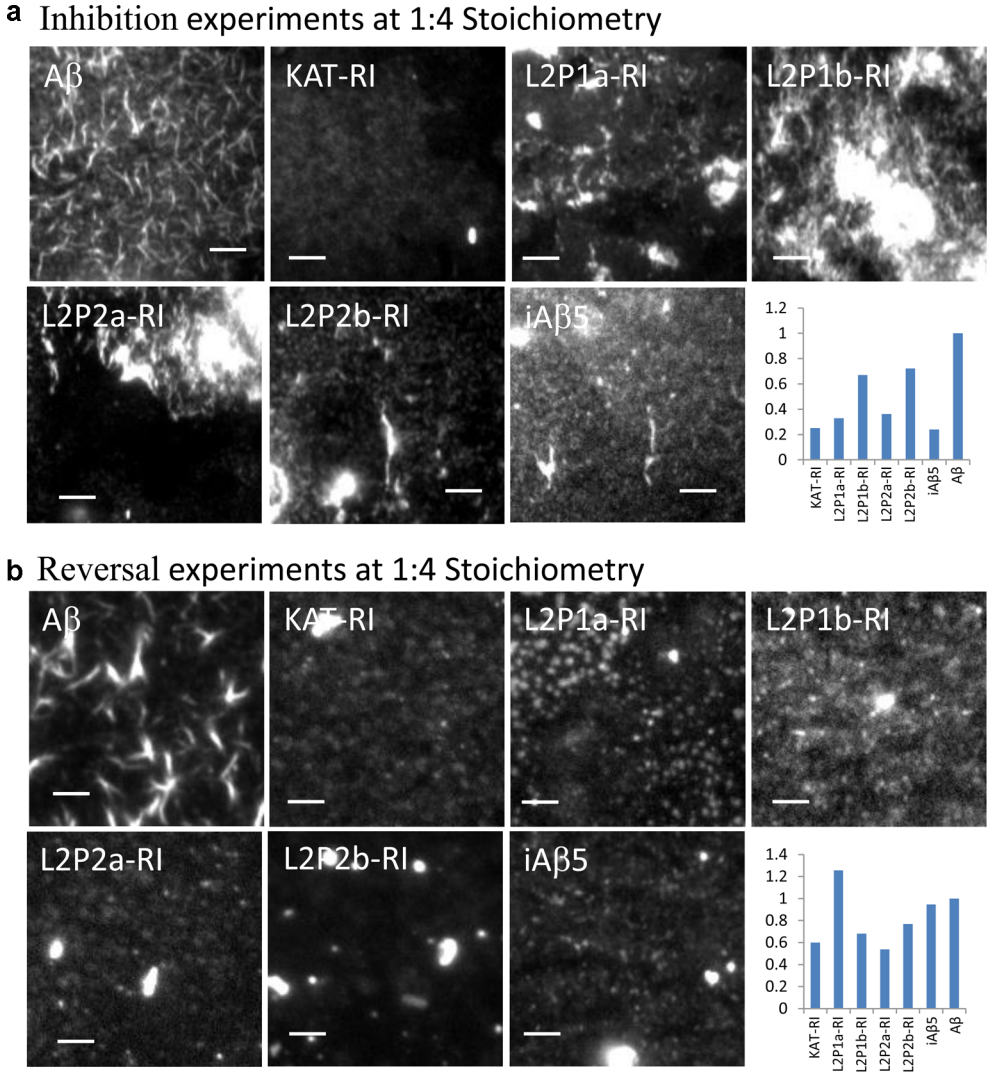

Figure 5. Oblique angle fluorescence (OAF) microscopy data. During reversal experiments, Aβ_{1-42} was grown alone for 3 days, after which the RI peptide was added at a stoichiometry of 1:4 followed by a further 3 day incubation to assay for peptide-induced reversal of amyloid deposition. Each sample was then imaged by fluorescence microscopy, and panels show representative images. To quantify amyloid deposition, the mean gray value over a 160 pixel × 160 pixel area randomly chosen for five separate images is plotted as fluorescence intensity. Each data point is normalized to the control iAβ5 peptide by subtraction. Shown are (a) inhibition data and (b) reversal data. It can be clearly seen that both KAT and L2P1B are strongly inhibitory for this reversal assay. The scale bars represent a distance of 2 μm. Each data point was then scaled to overcome the "background noise" by taking Aβ (1:0) to be the maximum.

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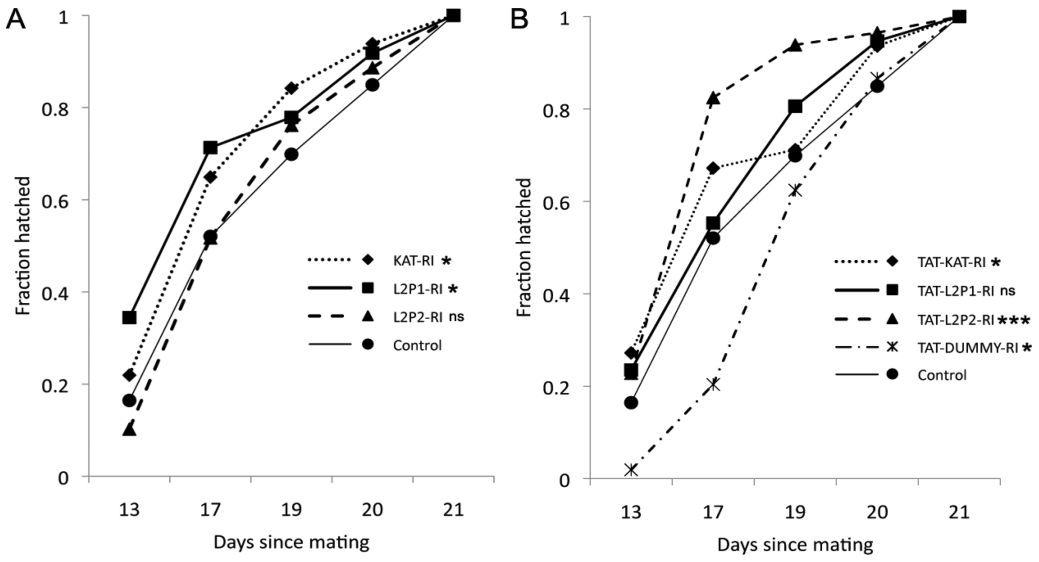

Figure 6. Oral administration of RI peptides suppresses the delayed eclosion associated with pan-neuronal expression of Aβ_{42}. (A) Compared to Aβ_{42}-expressing flies treated with water (control), those flies treated with KAT-RI and L2P1-RI showed a small but significant suppression of developmental delay ($p < 0.05$). (B) Upon fusion with the TAT peptide, the potency of the RI peptides increased with TAT-KAT-RI ($p < 0.05$) and TAT-L2P2-RI ($p < 0.001$), exhibiting significant suppression of delayed eclosion as compared to water (control). Oral administration of a dummy RI peptide resulted in a significant increase in the delay in eclosion ($p < 0.05$). Significance determined by the log-rank test.

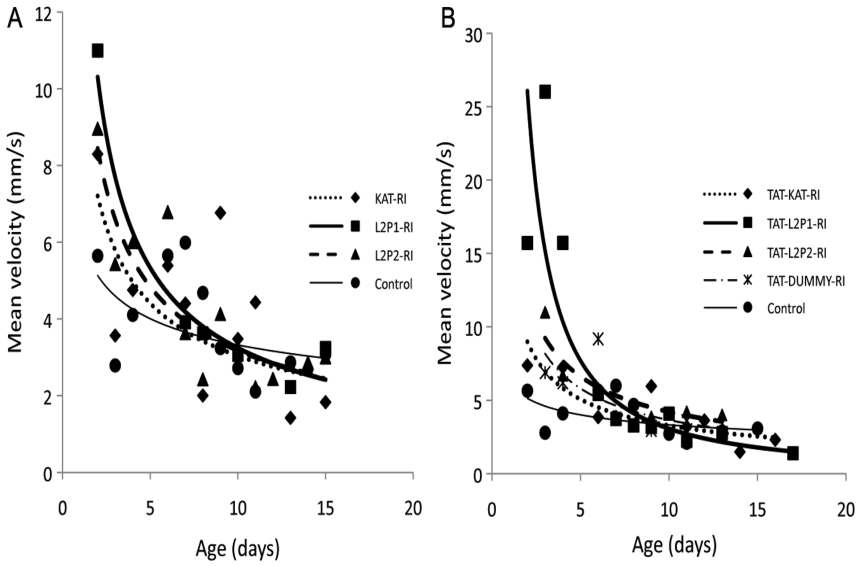

Figure 7. Oral administration of RI peptides suppresses the locomotor deficits associated with pan-neuronal expression of Aβ_{42}. (A) Compared to flies treated with water (control), flies receiving RI peptides exhibited higher walking velocities in the first days of adult life: L2P1 > L2P2 > KAT > control. (B) Upon fusion with the TAT peptide, the potency of the TAT-L2P1-RI peptide was particularly enhanced. The rank order of the velocities was similar: L2P1 > L2P2 > (dummy>) KAT > control.

decrease in the amount of ThT bound, L2P1a-RI and L2P1b-RI appear to have removed the vast majority of fibrils present in the solution. Finally, L2P2b-RI displays a number of deposits that are smaller and have a morphology different from that of the Aβ_{1-42} sample.

OAF microscopy data for inhibition experiments demonstrated that fibrils are present in iAβ5, L2P1a-RI, L2P1b-RI, L2P2a-RI, and L2P2b-RI samples. However, the average intensity of these fibrils was reduced relative to that of Aβ_{1-42}. KAT-RI stood out as the one sample with reduced fluorescence intensity and no appearance of fibrils on the surface, indicating that KAT-RI reduced the rate of deposition of amyloid. Collectively, these data suggest that KAT-RI is most effective overall, while L2P1b-RI and L2P2a-RI are also effective at reversal.

Retro-inverso Peptides Rescue Developmental Delay in a *Drosophila* Model of Aβ Toxicity. Our initial experiments used retinal expression of the Arctic (E22G) variant of Aβ_{1-42} to screen for amelioration of the expected rough eye phenotype. The flies were treated with RI peptides in their food throughout larval development. Upon eclosion, the severity of the phenotype was scored by investigators blind to the treatment history. No differences in the rough eye phenotype, as compared to untreated flies, could be detected for any of the peptides (data not shown). We did, however, notice that some treated flies appeared to hatch either earlier or

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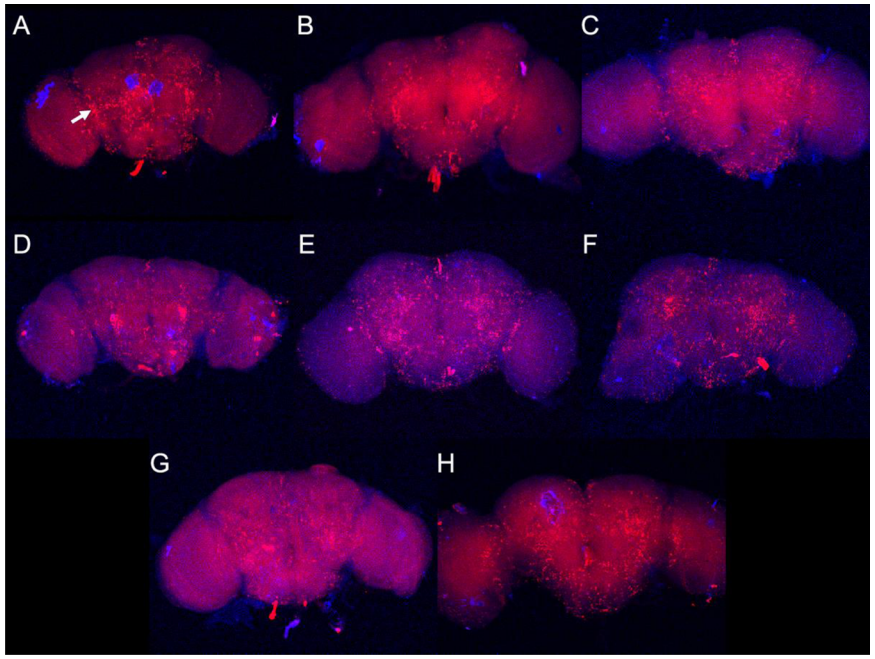

Figure 8. Oral administration of RI peptides did not alter the pattern of $A\beta_{42}$ deposits in the *Drosophila* brain. Flies treated with KAT-RI (A), L2P1-RI (B), L2P2-RI (C), TAT-KAT-RI (D), TAT-L2P1-RI (E), TAT-L2P2-RI (F), TAT-dummy-RI (G), and water (H) all exhibited similar deposits of Aβ-reactive material (arrow, stained with the 6E10 antibody).

later than normal. When we formally assessed the hatching times of flies, expressing wild-type $A\beta_{1-42}$, we found that two RI peptides lacking a TAT sequence led to an increase in the hatching time compared to that of untreated control flies expressing $A\beta_{1-42}$ (Figure 6A, left panel). In addition, we saw three significant differences for TAT-containing peptides. Considering the $A\beta_{1-42}$-expressing flies, we found that those treated with the TAT-L2P2-RI peptide hatched significantly earlier than those treated with TAT-KAT-RI and TAT-L2P1-RI, which were comparable to water-fed controls. Surprisingly, treatment with a poly-Gly dummy peptide linked to the TAT sequence resulted in a more severe developmental delay compared to untreated flies (Figure 6B, right panel). This sequence reassuringly demonstrates that the effect of improved hatching is sequence specific, with this control sequence having the opposite effect. As an additional control, nontransgenic flies were treated with TAT-L2P2-RI and TAT-dummy-RI peptide; these flies hatched at the same time as control flies treated with water (data not shown), suggesting that the effects on development are Aβ specific.

Retro-inverso Peptides Promote Locomotor Activity in a *Drosophila* Model of Aβ Toxicity. The expression of Aβ peptides in the nervous system of the fly is known to reduce the walking velocity of flies with an increase in age. To determine whether $A\beta_{1-42}$-expressing flies treated with RI peptides might retain their locomotor function for longer periods of time. As shown in the left panel of Figure 7A, when we treated flies with RI peptides lacking a TAT sequence, there were only small effects on the walking velocity, particularly for <5-day-old flies. Those peptides that carry a TAT sequence (Figure 7B, right panel) also increased the walking velocity of young $A\beta_{1-42}$-expressing flies. In particular, flies treated with the TAT-L2P1-RI peptide exhibited markedly increased walking velocities.

Retro-inversed Peptides Have a Minimal Effect on the Presence of Plaques. Oral administration of RI peptides did not alter the pattern of $A\beta_{42}$ deposits in the *Drosophila* brain (Figure 8). Flies treated with KAT-RI, L2P1-RI, L2P2-RI, TAT-KAT-RI, TAT-L2P1-RI, TAT-L2P2-RI, TAT-dummy-RI, and water all appeared to exhibit similar deposits of Aβ-reactive material (arrow, stained with the 6E10 antibody), suggesting that although amyloid deposits recognized by this antibody have not been abolished, the production of toxic species has been modulated.

DISCUSSION

The PPI field has long been considered undruggable using conventional small molecules, while peptide-based approaches have gained considerable traction in recent years.[23,38] This is due to the fact that PPIs feature extended interacting surfaces with many points of contact that are too shallow to accommodate traditional small molecule inhibitors, making peptides and their mimetics promising candidates for intervention. Peptides often form highly specific interactions with their target, and many associated barriers (e.g., cell permeability and bioavailability) can now be addressed via a number of modification options. To develop peptide inhibitors of Aβ amyloidosis, we have combined a protein fragment complementation assay (PCA) approach with semirational library design, and PCA screening. This has been followed by retro-inversal of the selected peptide sequences to identify molecules capable of binding $A\beta_{1-42}$ and lowering toxicity. The process has been iterative; the initial library search used the $A\beta_{29-35}$ sequence as a design scaffold, and the second library design was based on the initial winner, to yield a completely unrelated sequence to the parent template. By retro-inversing these PCA library-selected sequences, we aimed to increase peptide bioavailability while retaining desirable inhibitory properties. Our results demonstrate that the inhibitory properties found in the L peptide templates are retained in the RI sequences. For both inhibition and reversal ThT experiments, we observed a decrease in the amount of ThT bound to $A\beta_{1-42}$ upon incubation with RI sequences.

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Reassuringly, the decrease in the amount of ThT bound is smaller at increasingly substoichiometric peptide concentrations, demonstrating a dose dependency. ThT data are supported by a large decrease in the magnitude of the global β-sheet CD signal upon examination of the same samples. These experiments therefore suggest that RI peptides can bind $A\beta_{1-42}$ and exert their effect by reducing the amount of protein in the amyloid form. The decrease in the magnitude of the CD signal was more pronounced than for ThT, reflecting the fact that the assays measure different outputs. Thus, while fibrils are being broken down into smaller structures with significantly lower β-sheet content, the species that become populated are still able to bind ThT, although to a lesser extent than for $A\beta_{1-42}$ in isolation. In addition, as observed for L peptides, the decrease in the amount of ThT bound is more pronounced for reversal experiments, suggesting that RI peptides are more effective at reversing preformed fibrils than preventing amyloid assembly. RI peptides in isolation displayed either random coil or weakly helical spectra, perhaps indicating a mechanism of binding that involves a structural change in the target protein. MTT cytotoxicity data additionally indicate that although at substoichiometric ratios RI peptides are effective at reducing β-sheet content and the level of ThT binding, they are ineffective at causing a decrease in $A\beta_{1-42}$ toxicity. For this to be achieved, molar ratios of $\geq$1:1 are required. This decrease in toxicity is modest (~30% at most), suggesting that although populated amyloid species are not toxic to the bacteria in which they were selected, the decrease in toxicity is reduced when the sample is transferred to the context of a mammalian line. Because bacterial cells harboring parental L peptide−DHFR2 fusions have demonstrated an improvement in DHFR activity as well as increased bacterial doubling rates, this suggests that toxicity reduction is successful within the confines of the selection system. One possibility for circumventing this issue and improving outputs from the MTT assay would be to use libraries based on existing sequences while transferring the PCA selection to a therapeutically relevant neuronal cell line.[20] However, testing these RI compounds in *D. melanogaster* has demonstrated that several sequences can go on to improve either hatching rates or locomotor activity, with TAT-L2P2-RI being the most striking for the former and TAT-L2P1-RI for the latter. Lastly, the presence of comparable brain plaque levels for each of the peptides suggests that amyloid deposits recognized by this antibody have not been abolished. Interpreting this result alongside fly hatching times and locomotor activity data suggests that the production of the toxic Aβ species has been modulated. These results compare favorably with results of ThT, CD, OAF microscopy, and MTT studies in demonstrating that peptides function by modulating amyloid levels and consequently reducing the associated cytotoxicity. The RI-PCA approach therefore offers the potential to derive protease resistant Aβ-interacting peptides that are capable of lowering the toxicity associated with amyloid, which may in turn serve as potential precursors for an Alzheimer's disease treatment.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

$A\beta_{1-42}$, β-amyloid 1−42 variant; CD, circular dichroism; PPI, protein−protein interaction; PCA, protein fragment complementation assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ThT, thioflavin-T; HFIP, hexafluoro-2-propanol; TFA, trifluoroacetic acid; CPP, cell-penetrating peptide; TAT, trans-activating transcriptional activator.

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