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Beneficial Effects of the β-Secretase Inhibitor GRL-8234 in 5XFAD Alzheimer's Transgenic Mice Lessen during Disease Progression

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Abstract

The β-secretase enzyme BACE1, which initiates the cleavage of amyloid precursor protein (APP) into the amyloid-β (Aβ) peptide, is a prime therapeutic target for Alzheimer's disease (AD). However, recent investigations using genetic animal models raise concern that therapeutic BACE1 inhibition may encounter the dramatic reduction of efficacy in ameliorating AD-like pathology and memory deficits during disease progression. Here, we compared the effects of the potent and selective small-molecule BACE1 inhibitor GRL-8234 in different pathological stages of AD mouse model. Specifically, we administered GRL-8234 (33.4 mg/kg, i.p.) once daily for 2 months to 5XFAD transgenic mice, which showed modest (4 months) and massive (10 months of age) Aβ plaque deposition at starting points. Chronic treatments with GRL-8234 reversed memory impairments, as tested by the spontaneous alternation Y-maze task, in the younger 5XFAD group concomitant with significant reductions in cerebral Aβ42 levels. In contrast, only marginal reductions of Aβ42 were observed in 12-month-old 5XFAD mice treated with GRL-8234 and their memory function remained impaired. We found that not only BACE1 but also full-length APP expression was significantly elevated with progressive Aβ accumulation in 5XFAD mice, while GRL-8234 failed to affect these detrimental mechanisms that further accelerate plaque growth in brains of older 5XFAD mice. Therefore, our results provide important insights into the mechanisms by which Aβ accumulation and related memory impairments become less responsive to rescue by BACE1 inhibition during the course of AD development.

Keywords

Alzheimer's disease; BACE1 inhibitor; GRL-8234; amyloid-β; C99; APP; learning and memory; 5XFAD

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INTRODUCTION

β-Site amyloid precursor protein-cleaving enzyme 1 (BACE1 or β-secretase) was identified as an aspartyl ptotease that cleaves amyloid precursor protein (APP) and is highly expressed in the brain [1]. BACE1 is a crucial therapeutic target for Alzheimer's disease (AD) [2–6], since the BACE1-meditaed cleavage of APP is the first and rate-limiting step in the production of neurotoxic amyloid-β (Aβ) that triggers a pathogenic cascade ultimately leading to neuron death and memory deficits [7, 8]. In support of this view, work from our group and others has demonstrated that BACE1 gene deletion ($BACE1^{-/-}$) blocks Aβ generation and prevents the development of AD-like pathologies, neuronal loss and memory impairments in different lines of APP transgenic mice [9–12]. Furthermore, BACE1 haploinsufficiency ($BACE1^{+/-}$; i.e., 50% reduction) has been used as a therapeutic relevant model to evaluate the efficacy of partial inhibition of this enzyme [13–19]. In these studies, $BACE1^{+/-}$ reduction is sufficient to lower brain Aβ concentrations and prevent AD-like phenotypes such as amyloid plaque and tau pathologies, cholinergic neuronal death, mitochondrial dysfunction, hippocampal CA1 synaptic failure, and memory deficits in APP mice. However, it should be noted that the beneficial effects of BACE1 haploinsufficiency in AD transgenic mice dramatically decline with age or the progression of disease [13, 16, 17, 19]. Therefore, these gene-based findings suggest that the therapeutic intervention with BACE1-inhibiting approaches may be more efficacious if targeted on earlier stages of AD.

Since BACE1 has a large catalytic site, it has been challenging to find small-molecule BACE1 inhibitors that can efficiently cross the blood-brain barrier and are still large enough to block the substrate-binding site of this enzyme [20–23]. Remarkably, a recent progress in medicinal chemistry has led to the development of selective and bioavailable BACE1 inhibitors, some of which have been advancing to phase II/III clinical trials in mild-to-moderate AD [5, 6, 24]. Of particular importance, preclinical investigations of BACE1 inhibitor drugs (e.g., GRL-8234 and TAK-070) successfully reveal mnemonic improvements concomitant with significant cerebral Aβ reduction in the Tg2576 mouse model of AD following systemic administration if the treatment is started during the relatively earlier or prepathological phase [25, 26]. In this study, we chose 5XFAD transgenic mice to more rigorously evaluate the efficacy of the β-secretase inhibitor GRL-8234 [26, 27], since this rapid-onset and aggressive amyloid model with multiple FAD mutations can recapitulate some important AD-like phenotypes (e.g., robust BACE1 elevation and neuronal loss during later stages) lacking in most other APP mice [11, 16, 28–30]. We compared the effects of GRL-8234 in 5XFAD mice at different disease phases with modest and robust Aβ plaque pathology, providing important insights into the mechanisms by which pharmacological suppression of BACE1 may suffer from diminished therapeutic benefits during the progression of AD.

MATERIALS AND METHODS

Animals

We used 5XFAD mice (Tg6799 line) that co-overexpress familial AD (FAD) mutant forms of human APP (Swedish mutation: K670N, M671L; Florida mutation: I716V; London mutation: V717I) and presenilin 1 (PS1) (M146L; L286V) transgenes under transcriptional

control of the neuron-specific mouse Thy-1 promoter [10, 11, 28]. 5XFAD transgenic line was maintained by crossing hemizygous transgenic mice to C57Bl/6 breeders (Taconic, Hudson, NY, USA). 5XFAD hemizygotes were tested with non-transgenic wild-type littermate mice served as controls. Genotyping was performed by PCR analysis of tail DNA and all experiments were done blind with respect to the genotype of mice. Our previous study shows that there is no sex difference in cerebral Aβ levels in 5XFAD mice except for the younger age (≤3 months) [28]; therefore, both males and females were used to examine the effects of GRL-8234 in this model during different pathological stages showing modest (4 months) and robust (10 months of age) Aβ deposition. All animal procedures were approved by the Nathan Kline Institute Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines.

Drug Treatments

The potent and selective BACE1 inhibitor GRL-8234 (synthesized at OrgSyn Laboratory, Chicago, IL, USA) was dissolved in vehicle solvent constituted of 1:1 mixture of 0.5 % polyethylene glycol 300 and 5% glucose, as described previously [26, 27]. 5XFAD transgenic mice at 4 and 10 months of age received chronic intraperitoneal injections of 33.4 mg/kg GRL-8234 once daily for 2 months. Age-matched wild-type mice were treated with vehicle as controls. The dosage of 33.4 mg/kg GRL-8234 was chosen on the basis of previous *in vivo* experiments [26], which successfully demonstrated that systemic GRL-8234 enters brain (16% over a 24-h period) and a single daily dose is sufficient to maintain significant inhibition of Aβ production (~50% reduction in basal interstitial Aβ levels) in 3-month-old Tg2576 mice.

Spontaneous Alternation Y-Maze Test

Spontaneous alternation performance was tested using a symmetrical Y-maze, as described previously [9, 14, 31]. Each mouse was placed in the center of the Y-maze and was allowed to explore freely through the maze during an 8-min session. The sequence and total number of arms entered were recorded. Arm entry was considered to be complete when the hind paws of the mouse had been completely placed in the arm. Percentage alternation is the number of triads containing entries into all three arms divided by the maximum possible alternations (the total number of arms entered minus 2) × 100. After behavioral testing, mice were sacrificed for ELISA and immunoblotting experiments.

Immunoblot Analysis

Hemibrain samples were taken from the mice under deep isoflurane anesthesia and were snap-frozen for biochemical assays. For western blot analysis, each sample was homogenized in 8X volumes of homogenization medium containing 70 mM sucrose, 210 mM mannitol, 2 mM HEPES, 0.1 mM EDTA and protease inhibitor cocktail (Calbiochem, La Jolla, CA, USA), and centrifuged at 10,000 g for 10 min to remove any insoluble material. Protein concentrations were determined by a BCA protein assay kit (Pierce, Rockford, IL, USA), and 10–50 μg of protein was run on NuPAGE 4–12% or 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane. After blocking, membranes were probed with the following primary antibodies: anti-BACE1

(1:1,000, B0681, Sigma-Aldrich, St. Louis, MO, USA), an antibody that recognizes C-terminal epitope in APP (1:1,000, C1/6.1, kindly provided by Dr. Paul Mathews, Nathan Kline Institute) to detect full-length APP/C-terminal fragments, an antibody specific for the β-secretase-cleaved soluble ectodomain of APP (sAPPβ) (1:1,000, SIG-39138, Covance, Emeryville, CA, USA), anti-sAPPα (1:500, 11088, Immuno-Biological Laboratories, Minneapolis, MN, USA), and anti-β-actin (1:15,000, AC-15, Sigma-Aldrich). They were then incubated with horseradish peroxidase-conjugated secondary IgG. Immunoblot signals were visualized by an ECL chemiluminescence substrate reagent kit (Pierce), and were quantified by densitometric scanning and image analysis using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Aβ42 ELISA

Sandwich Aβ ELISA was performed as described previously [14, 16, 31]. Briefly, each hemibrain sample was extracted in 8X cold 5 M guanidine HCl plus 50 mM Tris HCl (pH 8.0) buffer, and centrifuged at 20,000 g for 1 h at 4°C to remove insoluble material. Final guanidine HCl concentrations were below 0.1 M. Protein concentrations were determined by a BCA protein assay kit (Pierce). To quantitate total levels of cerebral Aβ42, supernatant fractions were analyzed by a well-established human Aβ42 ELISA kit (KHB3441, Invitrogen) according to the protocol of the manufacturer. Optical densities at 450 nm of each well were read on a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA), and sample Aβ42 concentrations were determined by comparison with the standard curve. Aβ42 concentration values were normalized to total brain protein concentrations and expressed in nanograms per milligram of total protein.

Data Analysis

The significance of differences between the groups was determined by a one-way or two-way ANOVA and *post-hoc* Fisher's PLSD tests were performed when appropriate. Data were presented as mean ± SEM and the level of significance was set for p value less than 0.05.

RESULTS

Age-Dependent Effects of GRL-8234 on Memory Impairments in 5XFAD Mice

We used 5XFAD APP/PS1 transgenic mice that represent a rapid-onset and aggressive amyloid model based on a combination of five FAD mutations and the consequent acceleration of neurotoxic Aβ42 production [10, 11, 28]. 5XFAD mice begin to develop visible Aβ deposition as early as 2 months of age and exhibit memory declines on a battery of hippocampus-dependent tasks around 6 months concomitant with synaptic dysfunction at the Schaffer collateral-CA1 pathway [10, 15, 28, 32–38]. We first compared the effects of the BACE1 inhibitor GR-8234 on memory deficits in this AD model at 6 and 12 months of age (endpoints following chronic administration of 33.4 mg/kg, i.p., once daily for 2 months), using the spontaneous alternation Y-maze paradigm (Fig. 1A, B). The spontaneous alternation performance relies on inherent tendency of mice to enter a less recently visited arm compared to the other arm and has been widely used for assessing hippocampus-dependent spatial working memory function during the successive arm entries [9, 31, 39].

Levels of spontaneous alternation in vehicle-treated 5XFAD mice at 6 months and 12 months of age were around ~50% corresponding to the random performance level in this behavioral assay (Fig. 1A), and were significantly lower than those of the respective age-matched wild-type controls ($p < 0.05$) that showed stable alternation performances (~64%).

Notably, chronic administration of GRL-8234 significantly increased spontaneous alternation in 5XFAD mice at 6 months of age ($p < 0.05$), restoring the impaired spatial working memory completely back to wild-type levels (Fig. 1A). In contrast, levels of spontaneous alternation were indistinguishable between GRL-8234- and vehicle-treated 5XFAD mice at 12 months of age, indicating that GRL-8234 was no longer able to improve spatial working memory deficits. Meanwhile, the total number of arm entries during Y-maze testing was not significantly different between the three groups of mice tested at either 6 months or 12 months of age (Fig. 1B), showing that changes were memory-specific and GRL-8234 did not affect exploratory activities. Together, the results indicate that chronic treatments with GRL-8234 resulted in improved spatial working memory performance in 5XFAD mice at 6 months but not at 12 months of age.

Age-Dependent Effects of GRL-8234 on Cerebral Aβ Levels in 5XFAD Mice

To investigate the association between age-dependent cognitive benefits and brain Aβ levels, we next performed ELISA measurements. Since Aβ42 is more toxic and prone to aggregation than Aβ40 and represents the major Aβ species in 5XFAD model mice [10, 28], the effects of GRL-8234 on total Aβ42 levels in guanidine-solubilized brains were compared between 6 and 12 months of age (Fig. 1C). Chronic administration of GRL-8234 resulted in a significant reduction of total Aβ42 concentration in 5XFAD mice at 6 months of age ($p < 0.05$; ~62% relative to vehicle-treated 5XFAD controls). Importantly, Aβ42 levels were dramatically elevated in 5XFAD mice from 6 to 12 months of age ($p < 0.05$). The increase rate of Aβ42 over this period in 5XFAD mice was much greater than that of a previous report [28] most likely due to different genetic backgrounds, since 5XFAD line was maintained on the C57Bl/6 strain (the present study) or the B6/SJL hybrid background (the previous study). While GRL-8234 was slightly but still significantly able to reduce total Aβ42 in the 5XFAD model with advanced age ($p < 0.05$), residual levels of Aβ42 in GRL-8234-treated 12-month-old 5XFAD mice were much higher than those of vehicle-treated 6-month-old 5XFAD mice ($p < 0.05$), which exhibited poor spontaneous alternation performance (Fig. 1A). Therefore, the results suggest that chronic administration of GRL-8234 was no longer able to ameliorate memory deficits due to its limited impacts on neurotoxic Aβ42 accumulation in 5XFAD mice with advanced age.

Effects of GRL-8234 on β-Amyloidogenic Processing of APP in 5XFAD Mice at Different Disease Stages

To address the mechanisms by which memory impairments as well as Aβ concentrations became less receptive to GRL-8234 treatments in 5XFAD mice with aging, we compared changes in APP processing with immunoblot analyses of brain samples (Fig. 2). GRL-8234 treatments significantly reduced levels of the β-secretase-cleaved C-terminal fragment of APP (β-CTF or C99) in 5XFAD mice at 6 months of age ($p < 0.05$; ~57% relative to vehicle-treated 5XFAD controls) (Fig. 2A, B) correlating with Aβ42 reductions (Fig. 1C).

Importantly, C99 levels dramatically increased in 5XFAD mice at 12 months of age (~290%) as compared to 6-month-old 5XFAD controls ($p < 0.05$). Although GRL-8234 was still able to significantly reduce C99 in 5XFAD mice with advanced age ($p < 0.05$), high levels of C99, which were equivalent to 6-month-old 5XFAD controls (117%), remained in their brains. Intriguingly, we also found that levels of the α-CTF C83 increased with aging in 5XFAD mouse brains and were reduced by GRL-8234 treatments in both age groups (Fig. 2A); however, the minor β-CTF C89 produced by cleavage at Glu11 of Aβ was not detected in 5XFAD mice. Notably, we observed only a single faint C83 band in wild-type controls (data not shown), whereas C83 signals were solid and accompanied by more extensive C99 signals in 5XFAD mice. Given previous reports demonstrating the α-secretase-mediated proteolytic conversion of C99 to C83 [40, 41], it seems most likely that the decreased C83 levels in GRL-8234-treated 5XFAD mice may not result from direct inhibition of the α-cleavage of full-length APP but reflect the changes secondary to C99 reductions produced by the β-secretase inhibitor.

To further address this problem, we compared the effects of GRL-8234 on the other β- and α-cleavage metabolites, soluble ectodomains of APP (sAPPβ and sAPPα, respectively), in 5XFAD brains (Fig. 2C). Since the Covance anti-sAPPβ used in this study (SIG-39138) recognizes the C-terminal neo-epitope generated by the β-cleavage of human/rodent wild-type APP, the immunoblot signals correspond to mouse endogenous sAPPβ but not human APP trangene-derived sAPPβ that contains the Swedish mutation at its C-terminus. Therefore, the sAPPβ band in 5XFAD mice may represent only a minor component of sAPPβ, but it provides a useful measure to directly address alterations in the β-secretase-mediated processing of APP. In fact, we observed that sAPPβ was also age-dependently elevated in 5XFAD mice ($p < 0.05$) consistent with the changes in C99. Moreover, GRL-8234 significantly lowered sAPPβ levels regardless of age ($p < 0.05$), whereas residual levels of sAPPβ in GRL-8234-treated 12-month-old 5XFAD mice were also as high as those of 6-month-old 5XFAD controls (93%) (Fig. 2D). In contrast, levels of sAPPα, a direct α-product of APP, in 5XFAD mice increased with age ($p < 0.05$) but were not affected by GRL-8234 treatments (Fig. 2E). Collectively, these results provide evidence that GRL-8234 specifically inhibits β- but not α-secretase-dependent processing of APP in 5XFAD mice and support the idea that the reduction of C83 observed in GRL-8234-treated 5XFAD mice (Fig. 2A) may not be accounted for by inhibition of the α-cleavage of full-length APP.

Effects of GRL-8234 on Full-Length APP and BACE1 Expression in 5XFAD Mice at Different Disease Stages

To understand the mechanisms underlying high residual levels of the β-cleaved metabolites of APP following GRL-8234 treatments in 12-month-old 5XFAD mice, we further compared expression levels of full-length APP and BACE1 (Fig. 3). Interestingly, both BACE1 and its substrate APP were age-dependently elevated in 5XFAD mice ($p < 0.05$) as compared with wild-type controls (Fig. 3A–C), accounting for dramatic increases in C99 and sAPPβ in 12-month-old 5XFAD mice. Moreover, chronic administration of GRL-8234 did not affect the upregulation of full-length APP or BACE1 expression in older 5XFAD mice (Fig. 3D–F). Taken together, these findings suggest that while GRL-8234 inhibited BACE1 activities, as assessed by reductions of C99 and sAPPβ (direct β-cleavage

metabolites of APP), in 5XFAD mice irrespective of age, it did not affect the BACE1/APP elevation-associated acceleration of β-amyloidogenic processing of APP and thus had limited impacts on further Aβ accumulation in 12-month-old 5XFAD mice.

DISCUSSION

Previous work from our laboratory and others has shown that partial suppression of the β-secretase BACE1 gene (e.g., haploinsufficiency and siRNA) mitigates AD-like pathologies and memory deficits in transgenic mouse models of AD including 5XFAD [13–19, 42]; however, some studies suggest the decreased therapeutic efficacies with progression of disease [13, 16, 17, 19]. To examine the practical translational value of these gene-based observations, we herein compared the effects of GRL-8234, a selective and bioavailable small-molecule BACE1 inhibitor, in 5XFAD mice during two different disease stages. We found that chronic administration of GRL-8234 to 5XFAD mice (once daily for 2 months), which started at 4 months of age showing only modest Aβ pathology and no apparent hippocampal cognitive/synaptic failure [32, 33], successfully reduced cerebral Aβ42 levels and rescued memory declines in the spontaneous alternation Y-maze task. Similarly, it has been reported that long-term treatments of Tg2576 mice with GRL-8234 [26] or another BACE1 inhibitor TAK-070 [25], initiated during the prepathological stages (5.5–10 months of age), suppress plaque formation, reduce Aβ concentrations and prevent spatial learning impairments in the Morris water maze. Therefore, investigations using different animal models and BACE1 inhibitors seem to consistently demonstrate that Aβ-reducing approaches with β-secretase inhibitors applied in preventive settings are beneficial against memory declines associated with AD. Importantly, we also found that GRL-8234 with the same dosage and 2-month regimen had only limited impacts on total Aβ42 levels and failed to improve memory deficits in 5XFAD mice when the onset of treatments was delayed to 10 months of age already suffering from substantial Aβ accumulation. Therefore, the present study points to the possibility that the timing of drug treatments is a key determinant for the success of BACE1-inhibiting strategies, which may better work if applied in a preventive rather than therapeutic mode.

To confirm BACE1-inhibiting actions of GRL-8234 in 5XFAD mice at two different pathological stages, we monitored changes in APP processing as compared with age-matched 5XFAD controls that received vehicle treatments. GRL-8234 treatments significantly reduced levels of two direct β-cleavage metabolites of APP (C99 and sAPPβ) in both age groups of 5XFAD mice, indicating the inhibition of BACE1 activities regardless of disease progression in this model. However, we also found that C83, an α-cleavage product, was unexpectedly reduced in GRL-8234-treated 5XFAD mouse brains. In this regard, it is important to note previous studies reporting that the α-secretase-mediated proteolytic conversion of C99 to C83 may occur in AD conditions [40, 41]. It seems most likely that some component of C83 could derive from the α-cleavage of C99 in 5XFAD mice that overexpress human APP with the Swedish mutation, and that the C83 reduction found in GRL-8234-treated 5XFAD mice may reflect a secondary change that occurs as a consequence of prior C99 reduction caused by β-secretase inhibition. This is supported by the observation that GRL-8234 did not affect levels of sAPPα, a direct α-metabolite of APP, in 5XFAD mice regardless of age. In accordance with our pharmacological data, previous

gene-based findings also showed that partial BACE1$^{+/-}$ deletion reduces not only C99 but also C83 levels without changing sAPPα levels in the Tg2576 transgenic mouse model of AD [43].

We next explored the mechanisms by which the beneficial effects of GRL-8234 in 5XFAD model mice became weaker during disease progression. Our results revealed that 5XFAD mice show age-dependent elevations in expression levels of both BACE1 and its substrate full-length APP in brain. Furthermore, these mechanisms accelerating β-amyloidogenic processing of APP are not corrected by chronic GRL-8234 treatments in 5XFAD mice with advanced age, dampening the inhibitory effect of the BACE1 inhibitor on *de novo* Aβ production. This view is supported by the observations that whereas GRL-8234 inhibits BACE1 activities and consequently reduces the direct β-cleavage metabolites of APP (C99 and sAPPβ) in 5XFAD mouse brains regardless of age, residual levels of both β-products in GRL-8234-treated 12-month-old 5XFAD mice are as high as those of vehicle-treated 6-month-old 5XFAD controls. What mechanisms may account for the persistent upregulation of BACE1 and APP? Although the overexpression of APP transgene is under transcriptional control of the neuron-specific Thy-1 promoter in the 5XFAD model [28], it appears that additional posttranscriptional mechanisms leading to APP as well as BACE1 elevations are implicated in further accelerating β-amyloidogenesis after Aβ accumulation reaches a certain level [16, 29, 30, 44]. Interestingly, recent evidence demonstrates that both BACE1 and APP become elevated in neurons surrounding amyloid plaques in 5XFAD mice [45], in brains of $A\beta_{25–35}$ injected animals [46] and in primary cultured neurons following exposure to Aβ42 [47], indicative of a detrimental feed-forward link between Aβ accumulation and BACE1/APP elevations underlying plaque growth in AD. Given that genetically halting *de novo* Aβ synthesis in inducible APP transgenic mice does not affect preexisting Aβ burden [48], it is reasonable that Aβ-reducing strategies with the BACE1 inhibitor GRL-8234, which was administered to 5XFAD mice during 10–12 months of age with established plaque pathology, had limited effects on total Aβ42 levels. Consequently, BACE1-inhibiting approaches cannot reverse BACE1 or APP elevations associated with already existing Aβ plaques during advanced stages of AD, encountering their lesser efficacy in suppressing further plaque growth or ameliorating memory declines. Clinical relevance of this scenario is supported by the observation that expression of BACE1 and/or APP is significantly increased in postmortem AD brains [49–52], although further investigation is needed to firmly establish it.

In conclusion, the results presented here demonstrate that chronic administration of the BACE1 inhibitor GRL-8234 has beneficial effects on β-amyloidosis and memory deficits in 5XFAD mice if the treatment is initiated during earlier but not advanced stages of Aβ accumulation. Although increasing the dose of the BACE1 inhibitor dependent on stages of AD progression might overcome limited efficacy, it should be kept in mind that chronic over-inhibition of β-secretase activities may induce potentially mechanism-based adverse effects given a variety of known substrates beside APP [3–6]. In recent clinical trial failure of β-secretase inhibitor, it is pointed out that the observed liver toxicity may not necessarily be due to “off-target” (i.e., “off-BACE1) side effects but reflect “off-site” effects (i.e., BACE1 inhibition on β-galactoside α-2,6-sialyltransferase I in liver) that could be masked in

animal models [53]. Our results suggest that a combination of the BACE1 inhibitor and agents that block BACE1-elevating pathways [30, 54, 55], lower APP expression [56, 57] or remove Aβ plaques (e.g., passive immunization) [58] may provide a more efficacious and safe strategy to treat memory deficits in AD with established amyloid pathology.

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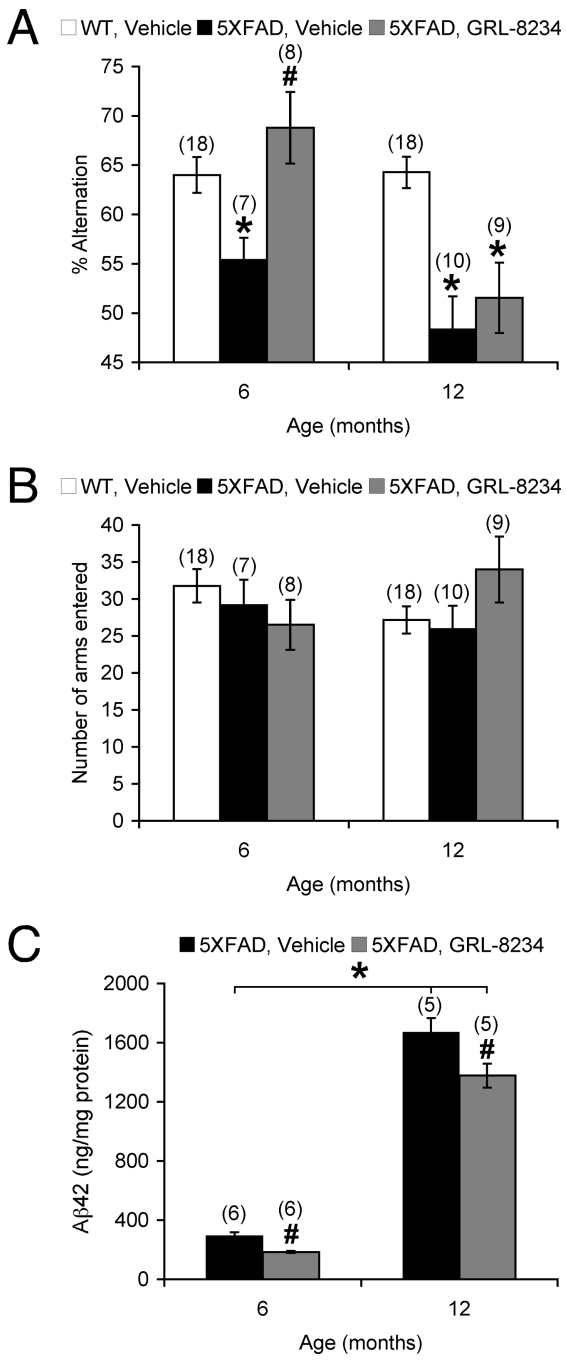

Fig. (1).

Effects of the BACE1 inhibitor GRL-8234 on memory deficits and β-amyloidosis in 5XFAD mice. (A and B) Following chronic administration of GRL-8234 or vehicle once daily for 2 months, the mice at 6 and 12 months of age were tested for the spontaneous alternation Y-maze task. Spatial working memory is significantly impaired in 5XFAD mice regardless of age, as compared to wild-type controls (* $p < 0.05$). Note that GRL-8324-treated 5XFAD mice are rescued completely back to wild-type levels of alternation performance at 6 months but not at 12 months of age ($^{\#}$ $p < 0.05$ versus vehicle-treated 5XFAD controls). Total

number of arm entries reflecting exploratory activities of mice in the Y-maze was indistinguishable between three groups irrespective of age. (C) After behavioral testing, total Aβ42 levels were quantified by sandwich ELISA of guanidine extracts of hemibrain samples and expressed in nanograms per milligram of total protein. Note that GRL-8234 significantly lowers Aβ42 in 5XFAD mice at 6 months of age ($^{\#}p < 0.05$), while Aβ42 levels are dramatically elevated in 12-month-old 5XFAD mice compared with 6-month-old 5XFAD controls ($^{*}p < 0.05$) and less responsive to reduction by GRL-8234 treatments. All data are presented as mean ± SEM and the number of animals used is indicated on top of each column.

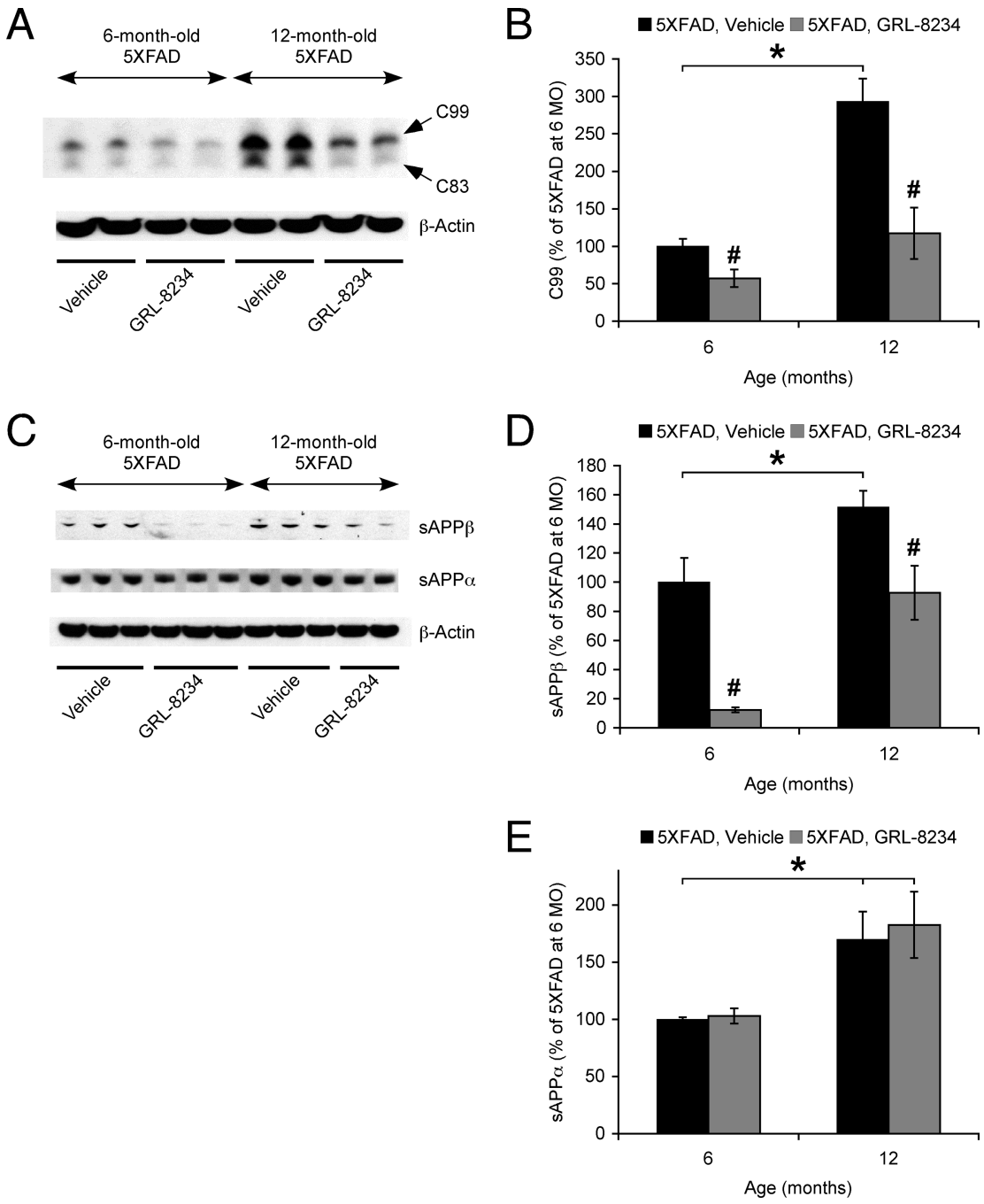

Fig. (2).

Effects of the BACE1 inhibitor GRL-8234 on β-amyloidogenic processing of APP in 5XFAD mice. (A and C) Representative immunoblots of protein extracts from hemibrain homogenates of mice. (B, D and E) Immunoreactive bands were quantified and expressed as the percentage of 6-month-old 5XFAD controls (6-month/vehicle, $n = 6$; 6-month/GRL-8234, $n = 6$; 12-month/vehicle, $n = 5$; 12-month/GRL-8234, $n = 5$). Whereas GRL-8234 significantly reduces C99 and sAPPβ levels in 5XFAD mice irrespective of age compared with vehicle-treated 5XFAD controls ($^{\#}p < 0.05$), both β-cleavage metabolites are

elevated in 5XFAD mice with age (* $p < 0.05$ versus 6-month-old 5XFAD controls) and their remaining levels in GRL-8234-treated 12-month-old 5XFAD mice are equivalent to those of 6-month-old 5XFAD controls. All data are presented as mean ± SEM.

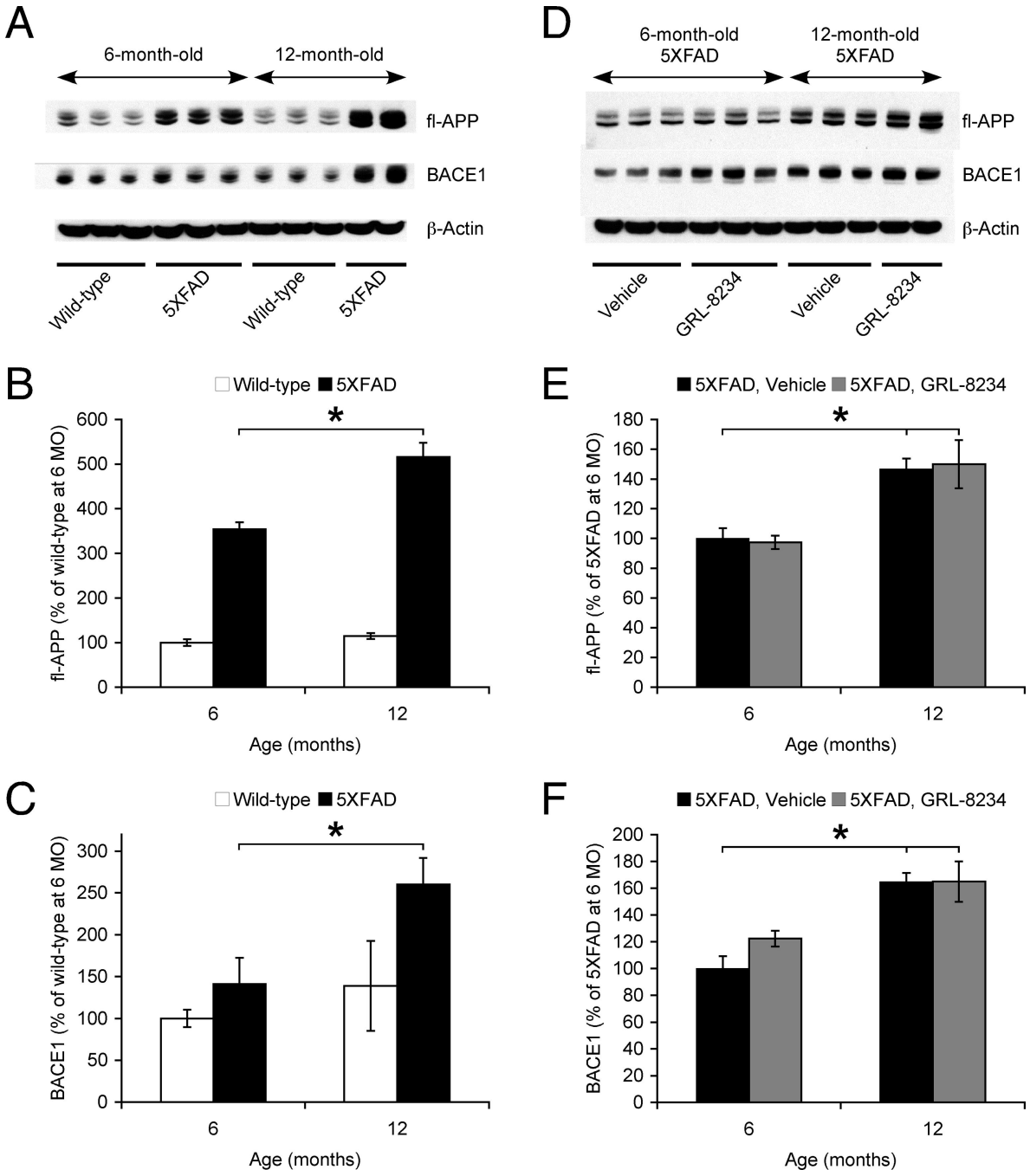

Fig. (3).

Effects of the BACE1 inhibitor GRL-8234 on full-length APP and BACE1 expression in 5XFAD mice. (A and D) Representative immunoblots of protein extracts from hemibrain homogenates of mice. (B and C) Immunoreactive bands were quantified and expressed as the percentage of 6-month-old wild-type controls (6-month-old wild-type, *n* = 6; 6-month-old 5XFAD, *n* = 6; 12-month-old wild-type, *n* = 5; 12-month-old 5XFAD, *n* = 5). Both full-length APP and BACE1 levels are age-dependently elevated in 5XFAD mice. (E and F) Immunoreactive bands were quantified and expressed as the percentage of 6-month-old

5XFAD controls (6-month/vehicle, $n = 6$; 6-month/GRL-8234, $n = 6$; 12-month/vehicle, $n = 5$; 12-month/GRL-8234, $n = 5$). Note that GRL-8234 has no effect on age-dependent increases in full-length APP or BACE1 expression in 5XFAD mice. * $p < 0.05$ (versus 6-month-old 5XFAD controls). All data are presented as mean ± SEM.