Disorders of the Nervous System

Angiotensin Converting Enzyme Inhibitors and Angiotensin Receptor Blockers Rescue Memory Defects in Drosophila-Expressing Alzheimer’s Disease-Related Transgenes Independently of the Canonical Renin Angiotensin System

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Abstract

Alzheimer’s disease (AD) is a degenerative disorder that causes progressive memory and cognitive decline. Recently, studies have reported that inhibitors of the mammalian renin angiotensin system (RAS) result in a significant reduction in the incidence and progression of AD by unknown mechanisms. Here, we used a genetic and pharmacological approach to evaluate the beneficial effects of angiotensin converting enzyme inhibitors (ACE-Is) and angiotensin receptor blockers (ARBs) in Drosophila expressing AD-related transgenes. Importantly, while ACE orthologs have been identified in Drosophila, other RAS components are not conserved. We show that captopril, an ACE-I, and losartan, an ARB, can suppress a rough eye phenotype and brain cell death in flies expressing a mutant human C99 transgene. Captopril also significantly rescues memory defects in these flies. Similarly, both drugs reduce cell death in Drosophila expressing human Ab42 and losartan significantly rescues memory deficits. However, neither drug affects production, accumulation or clearance of Ab42. Importantly, neither drug rescued brain cell death in Drosophila expressing human Tau, suggesting that RAS inhibitors specifically target the amyloid pathway. Of note, we also observed reduced cell death and a complete rescue of memory deficits when we crossed a null mutation in Drosophila Acer into each transgenic line demonstrating that the target of captopril in Drosophila is Acer. Together, these studies demonstrate that captopril and losartan are able to modulate AD related phenotypes in the absence of the canonical RAS pathway and suggest that both drugs have additional targets that can be identified in Drosophila.

Key words: Alzheimer’s disease; amyloid; Drosophila; memory; presenilin; renin angiotensin system

Significance Statement

Alzheimer’s disease (AD) is a devastating neurodegenerative disorder for which there is no cure. Recently, studies have reported a significant reduction in the incidence of AD and dementia among patients taking angiotensin converting enzyme inhibitors (ACE-Is) and angiotensin receptor blockers (ARBs). Given the enormous and immediate potential of ACE-Is and ARBs for AD therapeutics, it is imperative that we understand how they function and why they are beneficial in some patients but not others. Here, we show that captopril, an ACE-I, and losartan, an ARB, can restore memory defects in flies expressing human AD transgenes in the absence of the canonical renin angiotensin system (RAS) pathway. These studies provide us with a unique opportunity to identify novel targets of ACE-Is and ARBs and evaluate their therapeutic effectiveness in robust models of AD.
Introduction

Alzheimer’s disease (AD) is a degenerative disorder of the central nervous system that causes progressive memory and cognitive decline during mid to late adult life. Mutations in three genes, APP, presenilin 1, and presenilin 2 (PS1 and PS2), cause early-onset autosomal dominant AD, which accounts for <5% of AD cases (Goate et al., 1991). APP encodes a single-pass transmembrane protein that is cleaved by two proteases, β-secretase and γ-secretase, to generate amyloid peptides. PSs encode the catalytic component of γ-secretase (Wolfe et al., 1999), which cleaves the C-terminal fragment of APP (APP-CTF, C99) to produce Aβ peptides. Generally, longer Aβ peptides (Aβ42) are prone to self-aggregation and are concentrated in amyloid plaques, which are associated with brain atrophy, regional hypometabolism, network dysfunction, inflammation, and oxidative stress (Holtzman et al., 2011). Therefore, Aβ42 and plaques are often used as a diagnostic tool for AD prognosis and progression (Hansson et al., 2007; Lewczuk et al., 2015).

Recently, biochemical studies have shown that additional proteins can associate with PS and γ-secretase to modulate its assembly and/or interaction with specific targets (Bursavich et al., 2016; Tan et al., 2016). Proteins that modulate γ-secretase assembly would provide valuable insight into the function of this important complex during development and disease. Similarly, proteins that modulate the interaction of γ-secretase with specific targets such as APP, or affect the production of Aβ peptides or their clearance, might allow for the development of new therapeutic targets for AD. Although extremely promising, only a few PS and γ-secretase modulators have been identified and their mechanism of action remains largely unknown.

Using a genetic approach in Drosophila, we previously identified Acer and Ance–S, two orthologs of human angiotensin converting enzyme (ACE), as modifiers of PS and C99 (van de Hoef et al., 2009). ACE is a metallopro tease that cleaves angiotensin 1, a major component of the renin angiotensin system (RAS) that regulates blood pressure in humans. Importantly, while ACE orthologs have been identified in Drosophila, other components of the RAS are not conserved. Interestingly, several studies have established a link between RAS-targeting anti-hypertensive drugs, such as angiotensin converting enzyme inhibitors (ACE-Is) and angiotensin receptor blockers (ARBs), and AD (Ohru et al., 2004; Davies et al., 2011; Abdalla et al., 2013; Qiu et al., 2013; Yasar et al., 2013; de Oliveira et al., 2014; Wharton et al., 2015). For example, both ACE-Is and ARBs have been shown to delay the onset of cognitive impairment and neurodegeneration in mouse models of AD and in some patients, although the mechanism of action remains unclear (Alvarez et al., 1999; Ohru et al., 2004; Hajjar et al., 2005; Edwards et al., 2009; Miners et al., 2009; Belbin et al., 2011; Qiu et al., 2013; Soto et al., 2013; Yasar et al., 2013; de Oliveira et al., 2014; Kauwe et al., 2014; O’Caoimh et al., 2014; Wharton et al., 2015; Ho et al., 2017).

Here, we have examined the effects of ACE-Is and ARBs in Drosophila that express human AD-related transgenes. We show that captopril, an ACE-I and losartan, an ARB, suppress a rough eye phenotype and cell death in the brains of flies expressing a human C99 transgene carrying a London mutation. Moreover, captopril significantly rescues memory deficits in these flies. Similarly, both drugs reduce cell death and losartan significantly rescues memory deficits in Drosophila expressing human Aβ42. Importantly, neither drug affects the levels or clearance of Aβ42. We also observed no effects of either drug on degenerative phenotypes observed in Drosophila expressing human Tau, suggesting that the beneficial effects are specific to APP-CTF and Aβ42 expressing flies. Importantly, we found that an Acer null mutant was able to rescue cell death and memory deficits in Drosophila expressing Aβ42 consistent with Acer being the target of captopril in Drosophila. However, since the downstream targets of Acer including angiotensin and the angiotensin receptor are not conserved, we could not use a similar approach to identify the target/s of losartan. Together, these studies demonstrate that captopril and losartan are able to modulate AD related phenotypes in Drosophila. Moreover, since these beneficial effects are observed in the absence of the canonical RAS, these studies suggest that captopril and losartan may have additional targets that can be identified in Drosophila.

Materials and Methods

Drosophila stocks

 Stocks and crosses were maintained on standard media with or without drug treatment at 28°C for eye models and at 25°C for CNS models with 65% relative humidity and a 12/12 h light/dark cycle. gmr-GAL4;UAS-mCD8-GFP/SM5CyO recombinant line was generated as described (Burr et al., 2014; referred to as gmr-GAL4-UAS-GFP). UAS-APP39,40, UAS-APPc99J6 (referred to as UAS-C99H), and UAS-APPc99J6V717I London mutation (referred to as UAS-C99V717I) have been previously described (Finelli et al., 2004). elav-GAL4/CyO (8765), elav-GAL4C155 (458), UAS-APPΔβ2.2 (33769; referred to as
UAS-\(A\beta42\), UAS-Tau\(^{41T,13}\) (51362; expresses the 2N4R isoform of human Tau referred to as UAS-Tau), \(w^{1118}\) and Canton-S (referred to as wt) were obtained from the Bloomington Stock Center. The Acer null allele (Acer\(^{A168}\)) was obtained from (Carhan et al., 2011) and crossed to elav-GAL4\(^{C155}\), UAS-APPC\(^{C99V,717I}\) and UAS-A\(\beta42\) flies to generate fly lines expressing AD-related transgenes with an Acer null mutation. elav-GAL4\(^{C155}\) driver was used instead of elav-GAL4/Cyo for Acer null-related experiments for the purpose of generating a homozygous Acer null mutation.

Drug treatments

All adult flies were maintained on standard media with or without addition of either captopril (5 mm; Sigma-Aldrich) or losartan (1 mm; US Pharmacopreal Convention) from the first day after eclosion (DAE = 0).

GFP and REP imaging

Heads from 7-d-old adults were removed using spring scissors and slide mounted using double-sided tape. Heads were imaged at room temperature using a confocal Leica TCS SP5 microscope (Leica Microsystems Inc.), with 20\(\times\) objective and standard GFP filters with Leica Application Suite (LAS X) software (Leica Microsystems Inc.). Images were processed using ImageJ (Rasband, W. S., ImageJ, NIH; http://imagej.nih.gov/ij/, 1997–2016). GFP expression was analyzed using corrected total cell fluorescence (CTCF) calculations (based on Burgess et al., 2010). Rough eye phenotype images were captured with a 4\(\times\) objective using a Nikon SMZ-2T light microscope and an OpticCam Summit K2 microscope camera with ToupView software (by ToupTek Photonics).

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) labeling

Brains from 28-d-old adults were dissected in cold PBS with 0.5\% Triton X-100 and fixed in 4\% paraformaldehyde at room temperature for 30 min. Brains were then rinsed twice in PBS with 0.5\% Triton X-100 for 10 min each and washed once in \(H_2O\) plus 0.5\% Triton X-100 and 0.1\% sodium citrate solution for 15 min at 4\(^\circ\)C followed by two washes in PBS with 0.5\% Triton X-100 for 10 min each. TUNEL staining was performed according to the manufacturer instructions (Roche, in situ cell death detection kit, catalog #11684795910). Images were captured as a Z-stack and compressed into a single image using a Nikon A1R confocal microscope. Cell death was manually counted for statistical analysis.

Courtship conditioning assay

All experiments and analyses were performed double-blind as previously described (Kamyshhev et al., 1999). Experimental flies were collected within 6 h after eclosion and kept individually in culture vials on standard media with or without drugs (captopril or losartan) for 28 d until the experiment was performed. One day before the experiment, Canton-S virgin females were mated with same age males. Mated females were then used for training and testing. All behavioral experiments were performed within a 3-h time window (between 4 and 7 P.M.) in an environmental control room. Male courtship behavior was observed in a custom-made Perspex chamber (15-mm diameters, 5 mm high) with a sliding opaque partition that divided the chamber into two halves, with two lateral entries (3-mm diameter) with stoppers. Before training or testing, each chamber was cleaned with 50\% ethanol and dried. For training, a naïve male (with no sexual experience) was placed into an experimental chamber together with a 5-d-old mated Canton-S female. After several minutes to recover from the transfer the divider was withdrawn and the flies were left together for 1 h. After training, an experimental male was isolated for 30 min and then tested for short-term memory (STM) performance with a mated female during 10 min. Courtship behavior during the test session was video recorded using a color camera (EverFocus EQ.610, Polistar II) that was fitted with a CCTV lens (Computar, VariFocal TG4Z2813 FCS-IR) and fixed on a mounting bracket ~50 cm above the chamber. The distance of the camera to the object as well as the zoom, focus and iris aperture were optimized for video recording. Subsequent video analysis of time spent performing courtship behavior and all statistical comparisons were done using computer software (Drosophila Courtship Lite 1.4, developed by N. G. Kamyshhev, Russian Academy of Science). Courtship index (CI) was defined as the percentage of time spent performing courtship behavior during the observation period. Memory index (Ml) was calculated as: [100 (1 – CI with training/mean of CI without training)] (Kamyshhev et al., 1999; Lim et al., 2018).

Western blottings

Ten heads (five male, five female) from 7- and 28-d-old adults were lysed in 2\(\times\) tricine sample buffer (Bio-Rad catalog #1610739), boiled for 5 min, and run on 16.5\% Tris-tricine gels (Bio-Rad catalog #4563066) with 1\(\times\) SDS/Tris/tricine running buffer (Bio-Rad catalog #1610744). Protein was transferred onto 0.2-\(\mu\)m nitrocellulose membranes (Bio-Rad catalog #1620168) using standard transfer buffer. Membranes were boiled 3 min in 1\(\times\) PBS then blocked for 1 h using 1\(\times\) TBST with 5\% skim milk. Primary antibody detection was done overnight at 4\(^\circ\)C using A\(\beta\)-6E10 (1:500; Biolegend catalog #803001) and anti-\(\alpha\)-tubulin (1:1000) or anti-A\(\beta\)-actin (1:1000) in 1\(\times\) TBST 5\% skim milk. Membranes were washed 3\times in 1\(\times\) TBST for 10 min each. Secondary antibody detection was done using anti-mouse-horseradish peroxidase for 2 h at 4\(^\circ\)C (1:10,000). Membranes were then washed 3\times in 1\(\times\) TBST for 10 min each. Signal was detected using chemiluminescence substrates (Bio-Rad catalog #1705060) and membranes were imaged using LI-COR Odyssey Fc imager.

ELISA assays

A\(\beta42\) peptide levels were determined using human A\(\beta\) specific ELISA kits (Invitrogen, catalog #3441) as per manufacturer’s instructions. Forty heads from 28-d-old
maintained at 25°C were lysed in 1× RIPA buffer with a complete protease inhibitor (Roche) containing 50 mM Tris, 150 mM NaCl, 1% SDS, 1% NP-40, and 0.5% sodium deoxycholate, pH 8.0. The homogenates were diluted twofold before loading onto the plate. The signals were measured at 450 nm using a microplate reader. The whole experiment was performed as described previously (Van de Hoef et al., 2009).

Plaque staining

Flies expressing Aβ42 in the CNS were maintained on standard media with or without drugs (captopril or losartan) for 28 d after eclosion and subjected to plaque staining using the amyloid specific luminescent conjugated oligo-hiophene (LCO), p-FTAA, as previously described (Jonson et al., 2018). Fly brains were dissected in cold PBS and fixed in 96% ethanol for 10 min. Samples were then rehydrated following a step wash with 70%, 50%, 0% ethanol, then washed with PBS and stained with p-FTAA diluted 1:1000 in PBS for 30 min. After incubation with p-FTAA, samples were washed in PBS and mounted using DAKO mounting medium. Z-stack images of whole brains were acquired using a Sp8 confocal microscope and images were analyzed using Velocity Software. Levels of amyloid deposits were determined by measuring total pixel count over set threshold across z-stacks.

Statistics

Statistical analyses were done using GraphPad Prism or SPSS. Two-tailed Student’s t test was used to analyze differences between two groups. One-way ANOVA with Bonferroni post hoc analysis was used for multiple comparisons. Kruskal–Wallis ANOVA followed by Dunn’s multiple comparisons post hoc test was used for non-parametric analyses. Data are graphically reported as mean ± SEM. Kruskal–Wallis ANOVA test followed by Dunn’s multiple comparisons test and Mann–Whitney U test were used for statistical comparisons for the courtship-conditioning assay. Data are graphically reported as mean/median, and the box-and-whisker plots for CIs show 10th, 25th, 75th, and 90th percentiles. MIs are shown as mean ± SEM.

Results

Characterization of C99wt, C99V717I, and Aβ42 phenotypes

To determine whether pharmacological inhibition of the RAS pathway using ACE-Is and ARBs can exert any beneficial effects in fly models of AD, we used the GAL4-UAS system to target expression of human AD-related transgenes in the compound eye and CNS of Drosophila (Brand and Perrimon, 1993). Previous studies have shown that expression of these transgenes in the compound eye results in a rough eye phenotype, characterized by changes in the size of the eye that can be because of changes in photoreceptor neurons, loss of interomatalial bristles and pigmentation, and necrotic tissue (Prüßing et al., 2013; Iyer et al., 2016). Expression of AD-related transgenes in the CNS has also been shown to lead to Aβ aggregation, plaque formation, neurodegeneration, shortened lifespan, and deficits in learning and memory (Ye and Fortini, 1999; Finelli et al., 2004; Greeve et al., 2004; Iijima et al., 2004, 2008; Chakraborty et al., 2011; Prüßing et al., 2013).

To quantitate the rough eye phenotype generated by expression of human AD-related transgenes, we crossed each UAS-transgenic line with flies expressing membrane bound UAS-GFP to a gmr-GAL4 driver that targets expression in the developing eye. In previous studies, GFP intensity has been shown to be negatively correlated with retinal cell death (Burr et al., 2014). We found that expression of both gmr–C99V717I and gmr–Aβ42 resulted in a significant decrease in mean GFP intensity (46.67 ± 2.96% and 40.32 ± 3.39%, respectively) compared with a driver-control (97.82 ± 4.22% ; Fig. 1), while expression of gmr–C99wt showed intermediate levels of GFP intensity (73.01 ± 4.15%) compared with controls (Fig. 1).

We also examined the pathologic effects associated with expression of human AD transgenes in the CNS using the pan-neuronal elav–GAL4 driver (Fig. 2). We first examined brain cell death using TUNEL analysis and found that expression of elav–C99V717I or elav–Aβ42 resulted in a significant increase in cell death within the adult brain (11.5 ± 1.6 and 11.8 ± 0.7, respectively) compared with that observed in flies expressing elav–C99wt or wt (2.3 ± 0.7 and 0.6 ± 0.4, respectively; Figs. 2A, B). These results are consistent with previously reported data (Finelli et al., 2004; Iijima et al., 2004, 2008; Chakraborty et al., 2011; Prüßing et al., 2013). We also examined memory performance using a conditioned courtship suppression paradigm (Siegel and Hall, 1979; Kamyshev et al., 2011; Prüßing et al., 2013). Since all tests for trained males were done in the span of 30 min after a 1-h training session, it can be defined as a test for STM performance (Kamyshhev et al., 1999; McBride et al., 2005). The difference between CIs of trained and naive males was equal. Both elav–C99wt and elav–C99V717I as well as elav–Aβ42 males showed no significant decrease in courtship activity compared with their naive counterparts (elav–C99wt CI naïve = 33.133 vs CI trained = 17.194 U = 196.5, p = 0.0891; elav–C99V717I CI naïve = 32.650 vs CI trained = 14.189, U = 175, p = 0.0504; elav–AB42 CI naïve = 38.889 vs CI trained = 29.487 U = 333.5, p = 0.1252), while elav–w1118 driver-control males showed a significant decrease in courtship activity (elav–w1118 CI naïve = 33.340 vs CI trained = 3.704, U = 130, p = 0.0001; Fig. 2C).

Since all tests for trained males were done in the span of 30 min after a 1-h training session, it can be defined as a test for STM performance (Kamyshhev et al., 1999; McBride et al., 2005). The difference between CIs of trained and naive males can be represented as a MI (Kamyshhev et al., 1999; Lim et al., 2018). Kruskal–Wallis ANOVA test revealed significant differences in memory performance between driver control line and transgenic lines [H: (3, N = 107) = 19.09, p < 0.001]. We found that males expressing elav–C99V717I and elav–Aβ42 transgenes showed a...
significant loss in STM compared with elav driver control line (p < 0.05 and p < 0.001, respectively). However, it has to be noted that males expressing wild-type C99 also exhibited a reduction in STM performance, although this difference was not statistically significant (Fig. 2D).

Together, these data suggest that expression of Aβ42 either in fly eyes (gmr-GAL4) or pan-neuronally (elav-GAL4) produced the most pathologic phenotypes while expression of the London mutation C99V717I generally produced more severe phenotypes compared with wild-type C99. Thus, our results support previous findings (Finelli et al., 2004; Iijima et al., 2004, 2008; Chakraborty et al., 2011; Prüßing et al., 2013) and provide us with models to evaluate the effect of RAS inhibitors on the development of AD-related phenotypes.

Figure 1. gmr-GAL4 Drosophila model of AD. Confocal GFP (top row) and light microscope (bottom row) images of 7-d-old gmr-GAL4-UAS-GFP>W1118, gmr-GAL4-UAS-GFP>UAS-C99wt, gmr-GAL4-UAS-GFP>UAS-C99V717I, and gmr-GAL4-UAS-GFP>UAS-Aβ42 fly heads as labeled. Kruskal–Wallis ANOVA analysis of GFP quantification showed significant differences between transgenes (p < 0.0001). Multiple comparison analysis using Dunn’s corrected multiple comparison test showed flies expressing C99wt (N = 41), C99V717I (N = 56), and Aβ42 (N = 30) have a significant decrease in GFP expression compared with wt (N = 88; p = 0.0388, p < 0.0001, and p < 0.0001, respectively). Data are shown as mean ± SEM; *p < 0.05, ****p < 0.0001.
Captopril and losartan suppress degenerative phenotypes observed in mutant C99V717I and Ab42 flies

To determine whether captopril or losartan could suppress the rough eye phenotype observed in Drosophila expressing AD-related transgenes, we raised flies on medium with and without drugs and examined GFP intensity as described (Fig. 1). We did not observe any effect of either drug on GFP intensity in flies expressing C99wt or Ab42 (Fig. 3). In contrast, gmr.C99V717I flies exhibited significant increases in retinal GFP expression (26% and 41%, respectively) after administration of either captopril or losartan. Similarly, both drugs significantly reduced the number of TUNEL-labeled brain cells in four-week-old elav.C99V717I flies (Fig. 4). Moreover, a similar effect was observed in elav.Aβ42 flies that were fed with losartan for 28 d, whereas elav.C99wt flies showed no differences in TUNEL-labeled brain cells regardless of drug condition (Fig. 4).

Together, these data demonstrate that known inhibitors of the RAS signaling pathway in humans (captopril and losartan) can suppress toxic phenotypes observed in the eye and CNS of flies expressing AD-related transgenes.

Captopril and losartan selectively rescue STM in mutant C99V717I and Ab42 flies

To determine whether captopril or losartan could restore cognitive function in our AD models we examined STM using the courtship conditioning paradigm described in Figure 2 (Siegel and Hall, 1979; Kamyshev et al., 1999). Since lack of courtship activity in naive males may significantly skew the results of courtship conditioning, we first analyzed the potential differences in male sexual activity among naive males of different genotypes and drug conditions. A two-way ANOVA did not reveal any significant effects for genotype (F(3,272) = 0.624, p = 0.599), drug condition (F(2,272) = 0.577, p = 0.563), or their interaction (F(6,272) = 0.668, p = 0.596). Courtship and memory indices for all comparable groups are shown (Fig. 5; note that we have also included the data from Fig. 2 for “no drug” condition for comparative purposes). We found that administration of either drug (captopril or losartan) did not significantly change 30-min STM in the elav.w1118 control flies (Fig. 5), whereas for the transgenic lines these drugs exert a selective effect. Administration of both drugs in these flies resulted in an increased MI, similar to that observed in elav.w1118 controls. However,
because of large variance within the \textit{elav}>C99\textsuperscript{wt} expressing flies, the multiple comparison test revealed statistical significance only for losartan. Opposite effect was observed in flies expressing \textit{elav}\textsuperscript{C99V717I}. Captopril shows a significant memory improvement while losartan does not. \textit{elav}>\textalpha{42} flies showed obvious increase of MI in response to both drugs, although only for losartan the effect was statistically significant (Fig. 5). Overall, these data demonstrate that known inhibitors of the RAS pathway in humans, can significantly improve memory performance in \textit{Drosophila} expressing AD-related transgenes.

\textbf{Captopril and losartan do not suppress degenerative phenotypes observed in \textalpha{42} flies}

To determine whether captopril and losartan exert beneficial effects in other forms of AD, we examined their ability to suppress brain cell death in flies expressing human Tau protein. Previous studies have shown expression of human Tau in animal models leads to several neurodegenerative phenotypes similar to human AD cases including an increase in cell death, shrinkage in brain size and defects in cognitive ability (Wittmann et al., 2001; Gistelinck et al., 2012). We found that neither drug affected the number of TUNEL-labeled brain cells when maintained on either captopril or losartan for 28 d in \textit{elav}>\textalpha{42} flies (Fig. 6), suggesting that the beneficial effects of RAS inhibitors are specific to APP-CTF and \textalpha{42} expressing flies.

\textbf{Captopril and losartan do not affect APP-CTF or \textalpha{42}}

Previous studies have suggested that ACE-Is may be beneficial in AD by regulating the production, degradation, conversion and/or clearance of \textalpha{42} peptides. Whether ARBs have similar effects is unknown. To determine whether the beneficial effects of RAS inhibitors on
brain cell neurodegeneration and STM in our AD-related transgenic flies occur through similar mechanisms we first used Western blotting to quantitate the levels of C99 in the presence or absence of drugs. We found that administration of either captopril or losartan throughout the adult lifespan of both C99wt and mutant C99V717I flies had no effects on the levels of C99 in either fly eyes (gmr-GAL4 driver) or in the central nervous system (elav-GAL4 driver; Fig. 7A,B, respectively).

We then asked whether captopril or losartan affect the levels of Aβ peptides by measuring the soluble Aβ42 levels from lysates of adult fly heads using Western blotting and ELISA. We found that administration of either captopril or losartan throughout the adult lifespan of gmr-C99V717I and gmr-Aβ42 flies had no effect on the levels of Aβ42 at 7 d after eclosion (Fig. 8A). Similarly, neither drug had significant effect on the levels of Aβ42 in elav-Aβ42 flies at 28 d after eclosion (Fig. 8B); Aβ42 was undetected in both elav-C99wt and mutant elav-C99V717I regardless of drug treatment. To examine the effects of both drugs on insoluble Aβ42, we measured and compared Aβ aggregates in the brains of

Figure 4. Captopril and losartan reduce TUNEL-labeled brain cell death in flies expressing AD London Mutation, C99V717I and Aβ42. Confocal microscope images of 28-d elav-GAL4>UAS-C99wt, elav-GAL4>UAS-C99V717I, and elav-GAL4>UAS-Aβ42 fly brains with or without drug treatments are shown as labeled. Kruskal–Wallis ANOVA analysis showed that flies expressing C99wt (N ≥ 7 per condition) had no significant difference in the number of cell death when compared between no drug versus drugs (p = 0.768). However, Kruskal–Wallis analysis with Dunn’s multiple comparisons test showed that flies expressing C99V717I (N ≥ 6 per condition) had significant lower number of cell death in drug-treated flies when compared between captopril to no drug and losartan to no drug (p = 0.0343 and p = 0.0035, respectively). Similarly, for flies expressing Aβ42 (N ≥ 8 per condition), a significant lower number of cell death was observed in losartan-treated flies when compared with no drug (p = 0.0066). Data are shown as mean ± SEM; *p < 0.05, **p < 0.01; n.s., not significant.
elav
Aβ42 flies with or without drug treatment using the amyloid-specific LCO, p-FTAA stain, to detect Aβ plaques at 28 days after eclosion. Comparison across different conditions revealed no significant changes (Fig. 9).

Together, these results suggest that the beneficial effects of captopril and losartan are independent of APP-CTF processing or accumulation/clearance of Aβ42.

A null mutation in Drosophila Acer recapitulates the beneficial effects of captopril in C99717I and Aβ42 flies

To determine whether components of RAS underlie the beneficial effects of captopril (ACE-I) in our Drosophila AD models, we obtained an Acer null mutant (Carhan et al., 2011) and recombined it with our AD transgenic lines

elav\textsuperscript{C155}\textgreater UAS-C99\textsuperscript{V717I} or elav\textsuperscript{C155}\textgreater Aβ42, elav\textsuperscript{C155}\textgreater Acer driver was used instead of elav\textsuperscript{C155}\textgreater CyO for genetic recombination purposes and generated flies expressing C99\textsuperscript{V717I} or Aβ42 in a homozygous Acer null background. Since elav\textsuperscript{C155} endogenously drives expression of GAL4 at higher levels, the phenotypes observed in our transgenic lines were more severe than those previously observed using elav\textsuperscript{C155}\textgreater CyO, which expresses GAL4 at lower levels. Of note, although there are several ACE homologs in Drosophila, we focused on Acer since previous studies have shown that it contains the N-terminal catalytic site observed in human ACE and can be inhibited by captopril in vitro (Houard et al., 1998). We found that a null mutation in Acer significantly reduced brain cell death in both four-week-old elav\textsuperscript{C155}\textgreater C99717I.
Aβ42 flies similar to what we observed after captopril treatment (Fig. 10A, B). Similarly, an Acer null mutation also rescued STM in both four-week-old elav\(^{C155}\)C99\(^{V717I}\) and elav\(^{C155}\)Aβ42 flies (p < 0.0001, p = 0.0001, respectively, compared with no drug treatment; Fig. 11). Importantly, we did not observe any additive effects when the same flies were fed captopril for 28 d after eclosion (Figs. 10A, B, 11). Interestingly, we also observed that flies heterozygous for the Acer null mutation also suppressed brain cell death in four-week-old elav\(^{C155}\)Aβ42 flies similar to captopril treatment and no additive effects were found when fed with either captopril or losartan (Fig. 10C). Together, these data are consistent with Acer being the target of captopril that mediates the beneficial effects observed in our transgenic lines expressing AD-related transgenes. Whether losartan acts in the same downstream pathway remains to be determined and requires further targets to be discovered.

**Discussion**

Recent studies have shown that administration of antihypertensive medications such as ACE-Is and ARBs is associated with reduced onset and progression of AD. However, the mechanisms by which these drugs lead to beneficial effects in AD are unclear. Here, we examined the effects of captopril (ACE-I) and losartan (ARB) in *Drosophila* that express human AD-related transgenes in the eye and CNS. We found that administration of either drug significantly reduced cell death within the brain and improved STM. We also found that the beneficial effects were most pronounced in flies expressing Aβ42 peptides although neither drug affected the production, accumulation or clearance of Aβ42. We also observed no effects of either drug on degenerative phenotypes in *Drosophila* expressing human Tau, suggesting that the beneficial effects are specific to APP-CTF and Aβ42 expressing flies. Finally, we found that the beneficial effects observed on captopril treatment could be completely recapitulated by introducing an Acer null mutation into our AD fly models consistent with Acer being the target of captopril in *Drosophila*. Interestingly, while ACE orthologs have been identified in *Drosophila* the RAS, which includes downstream effectors of ACE, including angiotensin I/II and the angiotensin receptor, are not conserved. This suggests that the beneficial effects of ACE-Is and ARBs in *Drosophila* may involve mechanisms that are distinct from those mediated by the canonical RAS.

Several studies have shown that use of ACE-Is and ARBs correlates with decreased incidence and improved cognitive outcomes in AD patients (Ohri et al., 2004; Davies et al., 2011; Qiu et al., 2013; Yasar et al., 2013; de
Figure 7. Captopril and losartan do not change C99 levels in either gmr or elav model of C99 expressing flies. A, Western blottings using samples from gmr-GAL4-UAS-GFP>UAS-C99wt and gmr-GAL4-UAS-GFP>UAS-C99Val heads with or without drug treatments are shown as labeled. Each condition was tested with two technical replicates each time with a total of three biological replicates (N=3, n=2). Kruskal–Wallis ANOVA analysis showed that both captopril and losartan had no significant effects on the levels of C99 in both gmr-C99wt and gmr-C99Val flies at 7 d (p = 0.9929 and p = 0.5429, respectively). B, Western blottings using samples from elav-GAL4>UAS-C99wt and elav-GAL4>UAS-C99Val heads with or without drug treatments are shown as labeled. Each condition was tested with two technical replicates each time with a total of three biological replicates (N=3, n=2). Kruskal–Wallis ANOVA analysis showed that both captopril and losartan had no significant effects on the levels of C99 in both elav-C99wt and elav-C99Val flies at 28 d (p = 0.8786 and p = 0.7214, respectively). Data are shown as mean ± SEM; n.s., not significant.
Figure 8. Captopril and losartan do not change soluble Aβ42 levels in flies expressing Aβ42 using a gmr or elav driver. A. Western blottings using samples from gmr-GAL4-UAS-GFP>UAS-C99V717I and gmr-GAL4-UAS-GFP>UAS-Aβ42 heads with or without drug treatments are shown as labeled. Each condition was tested with three biological replicates (N=3). Kruskal–Wallis ANOVA analysis showed that both captopril and losartan had no significant effects on the levels of soluble Aβ42 in both gmr-C99V717I and gmr-Aβ42 flies at 7 d (p = 0.6286 and p = 0.2964, respectively). B. Levels of Aβ42 in elav-GAL4>UAS-Aβ42 heads at 28 d after eclosion were measured using human Aβ42 ELISA. The two-tailed unpaired t test showed that captopril had no significant effect on Aβ42 levels when compared with no drug condition (p = 0.31). A similar result was observed in elav-GAL4>UAS-Aβ42 flies treated with losartan (p = 0.5182). Each condition was tested with three technical replicates and two biological replicates in total (N=2, n = 3). Data are shown as mean ± SEM; n.s., not significant.
Importantly, only brain-penetrating ACE-Is and ARBs have been shown to delay the onset of cognitive impairment and neurodegeneration in mice models and humans, demonstrating that their beneficial effects are independent of their role in regulating blood pressure (Alvarez et al., 1999; Braszko et al., 2003; Ohrui et al., 2004; Hajjar et al., 2005; Edwards et al., 2009; Miners et al., 2009; Belbin et al., 2011; Davies et al., 2011; Gao et al., 2013; Qiu et al., 2013; Soto et al., 2013; Yasar et al., 2013; de Oliveira et al., 2014; Kauwe et al., 2014; O’Caoimh et al., 2014; Wharton et al., 2015; Ho et al., 2017). Several in vitro studies have suggested that ACE may be involved in Aβ degradation, conversion, and clearance (Kehoe et al., 1999; Hemming and Selkoe, 2005; Liu et al., 2014). In vivo studies, however, are controversial with some studies demonstrating that ACE-Is promote Aβ42 deposition (Zou et al., 2007; Bernstein et al., 2014), have little to no effect on Aβ42 peptide levels or plaque deposition (Hemming et al., 2007; Dong et al., 2011), and reduce Aβ deposits in the hippocampus (Abdalla et al., 2013). Despite this conflicting evidence, ACE-Is have consistently demonstrated improved cognitive outcomes in mice models of AD and in patients (Ohrui et al., 2004; Hajjar et al., 2005; El Sayed et al., 2009; Yamada et al., 2010; Dong et al., 2011; AbdAlla et al., 2013; Soto et al., 2013; de Oliveira et al., 2014; O’Caoimh et al., 2014). Similarly, ARBs have also been reported to improve cognitive function in rodent models (Takeda et al., 2009; Tsukuda et al., 2009; Shindo et al., 2012; Bild et al., 2013; Singh et al., 2013; Royea et al., 2017) but do not appear to alter Aβ levels (Ongali et al., 2014) or aggregation (Ferrington et al., 2011).

Given the known role of ACE-Is and ARBs in modulating RAS, several in vivo studies have examined the effect of regulating specific components of RAS on AD related phenotypes. These studies demonstrated toxic effects associated with Ang II/AT1R signaling in the brain resulting in an increase in the levels and deposition of Aβ42 (Faraco et al., 2016), increased oxidative stress and enhanced cognitive defects (Bild et al., 2013; Royea et al., 2017). On the other hand, protective effects including a decrease in neuronal degeneration and improved cognitive function, were observed with enhanced Ang II/AT2R and Ang IV/AT4R signaling (Bild et al., 2013; Royea et al., 2017). In line with these findings, studies have also shown beneficial roles of ACE-Is and ARBs in animal models of AD whereby the drugs prevented Ang II production and inhibited Ang II/AT1R signaling (Tsukuda et al., 2009; AbdAlla et al., 2013; Royea et al., 2017). Together, these studies suggest that the protective effects of ACE-Is and ARBs in AD may be associated with inhibition of Ang II/AT1R signaling, however, the role of RAS in AD pathology is still unclear.

We first identified two ACE-like factors in Drosophila, Acer and Ance-5, in a genetic screen for PS and C99 modifiers (van de Hoef et al., 2009). Interestingly, although Drosophila have ACE orthologs, the canonical RAS that includes angiotensin I/II and the angiotensin receptor is not conserved. Importantly, only Acer and Ance-5 were identified in our screen and, of these, Acer shares greater amino acid similarity and identity to human ACE and also retains the ACE active site and enzyme activity (Coates et al., 2000). In addition, ACE inhibitors are significantly more potent toward Acer (Cornell et al., 1995; Houard et al., 1998). Indeed, we found that ACE-Is can significantly reduce cell death within the brain and improve STM in...
Figure 10. A homozygous Acer null mutant reduces brain cell death in flies expressing C99V717I and Aβ42. Confocal microscope images of 28-d (A) elav-GAL4C155>UAS-C99V717I and (B) elav-GAL4C155>UAS-Aβ42 fly brains in the presence or absence of captopril and an Acer null mutation are shown as labeled. Mann–Whitney analysis showed that C99V717I flies (N ≥ 6 per condition) treated with captopril as well as those carrying an Acer null mutant +/− captopril had significantly lower numbers of cell death than compared with control flies on no drug (p < 0.0001, p < 0.0001, and p = 0.0031, respectively). A similar effect was observed in Aβ42 flies (N ≥ 7 per condition) treated with captopril or in flies carrying an Acer null mutations +/− captopril (p = 0.003, p = 0.0001, and...
Drosophila expressing AD-related transgenes except Tau. Moreover, we observed similar beneficial effects in Drosophila treated with an ARB, although the angiotensin receptor is not conserved. At present, the mechanism by which ACE-Is and ARBs function in Drosophila is unclear. Both captopril and losartan consistently suppress AD-related phenotypes in flies expressing either human C99 carrying a London mutation or Aβ42 however, these beneficial effects are not associated with any changes in the production, accumulation or clearance of Aβ42. This finding is consistent with previous in vivo studies in mice and humans demonstrating that ACE-Is and ARBs improved cognitive function without affecting Aβ levels (Hemming et al., 2007; Wharton et al., 2012) but contrasts with in vitro studies, demonstrating that ACE-Is lead to increased Aβ42 production and aggregation (Kehoe et al., 1999; Hemming and Selkoe, 2005; Zou et al., 2007; Liu et al., 2014). Therefore, based on our findings, it is unlikely that these drugs are modulating AD-related phenotypes through γ-secretase cleavage of C99. It is also unlikely that the ability of ACE-Is and ARBs to rescue cell death and cognitive dysfunction in Drosophila is because of effects on Angiotensin receptors since, other than ACE, the canonical RAS is not conserved in Drosophila.

Figure 11. A homozygous Acer null mutant rescues STM in flies expressing C99V717I and Aβ42. Percentage of CIs and MIs are shown as labeled for (A) elav-GAL4C155/UAS-C99V717I and (B) elav-GAL4C155/UAS-Aβ42 flies. CIs were calculated by dividing the time a male spent in courtship to a total given time. Trainer and tester females: –, none; m, mated female. Box-and-whisker plots for CI show 10th, 25th, 75th, and 90th percentiles and mean (+). MIs were calculated as [100 \times \frac{1}{100}] - CI with training/mean of CI without training]). Kruskal–Wallis test followed by Dunn’s multiple comparisons test was used for statistical comparisons (N ≥ 20 per genotype per condition). elav-GAL4C155/UAS-C99V717I flies treated with captopril as well as those carrying an Acer null mutant +/- captopril had significantly higher MIs when compared with no drug condition (p = 0.0005, p < 0.0001, and p < 0.0001, respectively). A similar effect was observed in Aβ42 flies treated with captopril or in flies carrying an Acer null mutant +/- captopril (p = 0.0001, p < 0.0001, and p = 0.0001, respectively). Data are shown as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; n.s., not significant.
the function of Acer in Drosophila is not fully understood. Some ACE-like factors have been shown to be affected by ACE-Is including Acer and Ance (Williams et al., 1996; Houard et al., 1998), however, the targets of either protein have yet to be identified. Acer null mutants have also been shown to exhibit disruptions in night-time sleep and sleep fragmentation (Carhan et al., 2011) as well as altered behavioral and metabolic responses to diet (Glover et al., 2019). However, these flies develop normally to adulthood, suggesting that major developmental or signaling pathways have not been affected. Flies lacking Ance have also been shown to develop normally without any obvious physiological defects (Kim et al., 2017). Similarly, the target for ARBs in Drosophila is currently unknown as no homolog of ATR has been discovered. Together, our data demonstrate that ACE-Is and ARBs can alleviate toxic phenotypes in Drosophila expressing human AD transgenes. Since these beneficial effects are observed in the absence of the canonical RAS this also suggests that captopril and losartan may be acting on a more ancestral function of this pathway and have additional targets that can be identified in Drosophila.

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