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Age-related reduction in brain ACE-2 is not exacerbated by Alzheimer's disease pathology in mouse models of Alzheimer's disease

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ABSTRACT

An imbalance in the circulatory and organ-specific renin-angiotensin system (RAS) pathways is associated with age-related dysfunction and disease including cardiovascular burden and more recently Alzheimer's disease (AD). It is currently unclear whether an age-associated imbalance in components of the RAS within the brain precedes the onset of AD or whether a RAS imbalance is associated with the onset of disease pathology and cognitive decline.

Angiotensin-converting enzyme-1 (ACE-1) and -2 (ACE-2) protein (ELISA) and enzyme activity (FRET assay), markers of the classical and counter-regulatory RAS axis respectively, and Ang-II and Ang-(1–7) peptide levels (ELISA), were measured in the left cortex across four transgenic AD mouse models of amyloid pathology (5xFAD – 2, 6, and 12 months of age; Apd9 – 3-4, 12, and 18 months of age; Tg2576 – 3-4 and 24 months of age; and PDAPP – 3-4, 7, 11, 15, and 18 months of age) and littermate wild-type (WT) controls.

ACE-1 level, and enzyme activity, was unaltered in relation to age in WT mice and across all four models. In contrast, ACE-2 level and enzyme activity, was reduced and Ang-II increased with ageing in both WT animals and disease models. The changes in ACE-2 and Ang-II in AD models mirrored WT mice, except for the 5xFAD model, when the reduction in ACE-2 (and elevated Ang-II) was observed at a younger age.

These data indicate an age-related dysregulation of brain RAS is likely to be driven by a reduction in ACE-2. The reduction in ACE-2 occurs at a young age, coinciding with early pathological changes and the initial deposition of Aβ, and preceding neuronal loss and cognitive decline, in the transgenic AD models. However, the age-related loss was mirrored in WT mice suggesting that the change was independent of pathological Aβ deposition.

Introduction

The circulatory renin-angiotensin system (RAS) regulates blood pressure and fluid homeostasis throughout the body [1]. Chronic long-term overactivation of the systemic classical RAS (cRAS) axis has long been established

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to contribute to hypertension and related cardiovascular diseases [2,3]. The RAS also operates locally within specific organs including the brain [4]. Overactivation of cRAS is observed in Alzheimer's disease (AD) and is related to a loss of activity in the protective regulatory RAS (rRAS) pathways, including the ACE-2/Ang-II/MasR pathway [5] and dysregulation within the Ang-III/Ang-IV/IRAP pathway [6]. An imbalance between cRAS and rRAS pathways is associated with elevated disease pathology in AD [5–8], likely driven by triggering pathophysiological processes including neuroinflammation and oxidative stress (reviewed [9]). Overactivation of the cRAS axis causes inflammation, mitochondrial dysfunction, and generation of reactive oxygen species (ROS) through Ang-II type 1 receptor (AT1R) signalling. Together, the proinflammatory environment and ROS accelerate synaptic loss and neuronal damage, contributing to AD pathogenesis. Rodent studies have shown that infusion of Ang-II promotes Aβ production and tau hyperphosphorylation in Sprague-Dawley rats [10,11] implying that RAS contributes to disease onset in AD.

Circulating Ang-II can interact directly with AT1R expressed in circumventricular organs such as the subfornical organ and area postrema - regions that are typically involved in blood pressure regulation or thirst control and are devoid of an intact blood-brain barrier (BBB) [12,13]. However, in most brain regions, including the frontal and temporal lobes that are associated with AD pathology, a fully formed BBB prevents the influx of circulatory Ang-II into the tissue and therefore presumably relies solely on RAS components synthesised within the brain tissue. There is evidence that most RAS components are expressed locally within the brain and can therefore function independently from circulating RAS although there is still debate as to whether renin, responsible for the conversion of angiotensinogen into angiotensin I, is expressed locally or derived systemically [14].

Angiotensin-converting enzyme-1 (ACE-1) is primarily neuronally expressed [15] and is responsible for cleaving the inactive peptide, Ang-I to Ang-II, which elicits vasoconstrictive, pro-inflammatory, and oxidative stress effects, via activation of AT1R. Angiotensin-converting enzyme-2 (ACE-2), primarily expressed in astrocytes, shares 42 % sequence homology within the catalytic domain of ACE-1. ACE-2 converts Ang-II to angiotensin (1–7) (Ang-(1–7)), which via activation of the Mas receptor (a founding member of the G-protein proto-oncogene family), promotes vasodilatory effects [16], as well as eliciting neuroprotective and pro-cognitive effects within the hippocampus [17].

In animal models of AD, Ang-II signalling lowering drugs (ACE-1 inhibitors (ACEi) and AT1R blockers (ARB)) are protective against cognitive decline associated with a reduction in disease pathology within brain regions such as the hippocampus [18–20]. Evidence from human observational studies also indicate that these same cRAS blocking medications, commonly used to manage hypertension, provide cognitive protection, delay the onset, and reduce disease pathology in AD [21,22]. Clinical trials are underway to test if RAS-altering medication has therapeutic potential in treating Alzheimer's disease. The CALIBREX study investigated the therapeutic potential of candesartan (ARB) and lisinopril (ACEi) in 146 hypertensive individuals with mild cognitive impairment. The results showed improvement to their cognitive performance with either drug, but candesartan gave the most efficacious effect [23]. The RADAR trial investigated the therapeutic potential of an ARB, losartan, by measuring brain atrophy of 211 individuals with mild-moderate AD. It was concluded that losartan did not reduce brain atrophy, but this may have been due to administration of the drug to individuals whose AD pathology was too advanced [24].

Questions remain as to whether the brain RAS is altered in normal ageing and the timing of brain RAS dysregulation in relation to the onset of AD pathology. Evidence suggests that systemic RAS becomes underactive with ageing, with studies in humans showing a reduction in plasma renin activity [25–28]. In contrast, organ-specific cRAS is often reported to be overactivated with age with evidence of concurrent loss of rRAS activity, including reduced ACE-2. Yoon et al. reported increased expression of prorenin receptor, Ang-II, ACE-1 and AT1R and lower expression of ACE-2, Mas receptor (MasR) and Ang-II type 2 receptor (AT2R) with increasing age in the thoracic aorta of 2-, 12- and 24-month old mice [29]. Interestingly, the reduction in ACE-2 expression between 2- and 12-months preceded changes in cRAS, suggesting that loss of rRAS activity may contribute to cRAS overactivation. These changes are consistent with findings reported by Burks et al. showing increased AT1R expression following a reduction in AT2R expression in the cardiac tissue of ageing WT mice [30] and lower endogenous Ang-(1–7) levels in the medulla of older Sprague-Dawley rats without a change in Ang-II levels [31]. Together, these data indicate that age is a contributing factor to systemic and local-organ RAS dysregulation.

The objective of this study was to (i) characterise age-related changes in the expression of markers of cRAS activity (ACE-1 and Ang-II) and rRAS activity (ACE-2 and Ang-(1–7)) in brain tissue in WT littermates and (ii) determine if RAS changes were exacerbated and related to the onset of disease pathology and cognitive decline across four established transgenic mouse models of AD-related amyloid pathology. We studied four AD models that display amyloid-β (Aβ) pathology and cognitive changes at different ages (Fig. 1) allowing for a broader analysis into the potential timing of RAS dysregulation in relation to the onset of disease pathology and cognitive decline. We hypothesised that an age-related imbalance in brain RAS would be accelerated in the mouse models of amyloid pathology compared to WT mice and that the timing of an imbalance in RAS would coincide with the development of Aβ-related disease pathology and cognitive decline. A better understanding of the timing of RAS changes in relation to age and the onset of amyloid pathology and cognitive decline may provide insight into the potential therapeutic window for RAS-acting interventions in clinical trials.

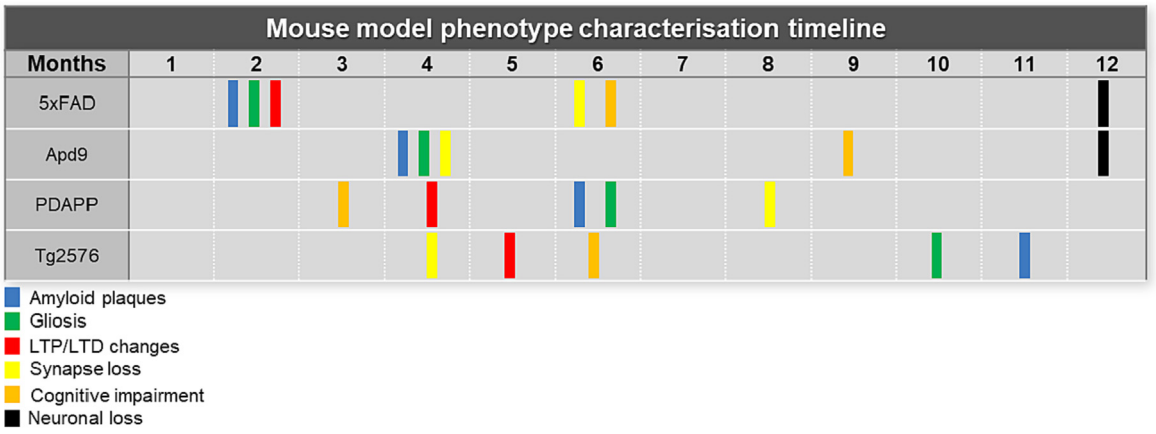

Fig. 1. Summary of the phenotypic characteristics and timeline of disease progression in the four transgenic APP mouse models (information sourced from alzforum.org/research-models).

Methods

Mouse models

Four transgenic AD models and WT littermate controls were included in the study: the APP/PSEN-1 ΔE9 (Apd9) model, the 5xFAD model, the Tg2576 model and the PDAPP model [32–35]. The number of animals and other characteristics in each age group are shown in Table 1. The four AD models display amyloid-β (Aβ) pathology and cognitive changes at different ages (Fig. 1). The age-range studied covers the onset and development of disease pathology and Aβ-related cognitive decline for each model (Fig. 1). The age-related onset of Aβ pathology was confirmed in the 5xFAD and Apd9 mice (Supplementary Fig. 1) and has been established in the Tg2576 and PDAPP mouse models [33,34].

Brain tissue homogenisation

The left hemisphere (after removal of the cerebellum) was weighed and homogenised in 1 % SDS buffer and protease inhibitors in a Precellys 24® homogeniser (using a ratio of 1 ml buffer:200 mg of brain tissue) (Bertin Technologies, Montigny-le-Bretonneux, France) as previously described [7]. The homogenates were centrifuged at 12,000 g at 4 °C and the supernatant aliquoted and stored at −80 °C.

Table 1
Breakdown of the ages and numbers of each mouse model used in the study. (n) refers to number of animals in each age-group.

		Age-at-death (Months)									Total (n)
		2	3–4	6	7	11	12	15	18	24	
5xFAD	Tg (n)	3		3			3				19
	WT (n)	3		3			4				
Apd9	Tg (n)		3				3		3		19
	WT (n)		4				3		3		
PDAPP	Tg (n)		5		7	4		7	6		60
	WT (n)		5		7	6		7	6		
Tg2576	Tg (n)		5							5	21
	WT (n)		5							6	

ACE-1 protein levels

ACE-1 protein level was measured in mouse brain tissue homogenates using a commercially available mouse ACE-1 ELISA duoset following manufacturer's guidelines (R&D Systems, Abington, Oxford, UK). In brief, a NUNC maxisorp 96-well plate (R&D Systems, Abington, Oxford, UK) was coated with a capture antibody (100μl) that was diluted in PBS (1600 ng/ml) and incubated overnight at room temperature. After washing (x5) and blocking for 1 h at room temperature with 1 % BSA:PBS, brain homogenates diluted in 1 % BSA:PBS (ACE-1: 1/80), a serial dilution of ACE-1 (125–8000 pg/ml), and 1 % BSA:PBS blanks were added to respective wells for 2 h at room temperature. After further washing (x5), the detection antibody diluted in 1 % BSA:PBS (400 ng/ml) was incubated for 2 h at room temperature. The plate was washed (x5) and streptavidin horseradish peroxidase (1/200 in PBS:0.01 % Tween-20) was added for 20 min at room temperature in the dark. After a final wash step, TMB substrate (R&D Systems, Abington, Oxford, UK) was added to each well for 30 min at room temperature and 2 N sulphuric acid was added to stop the reaction. Absorbance was read at 450 nm for each well using a microplate reader (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK). ACE-1 concentration was interpolated from the serial dilution of recombinant mouse ACE-1. The average value for each sample was obtained from duplicates.

ACE-1 enzyme activity assay

ACE-1 activity was measured using an ACE-1 fluorogenic peptide, Abz-FRK(Dnp)-P, as previously described for human brain tissue samples [7,8]. Mouse brain tissue homogenates (50μl) (in triplicate) were diluted 1:10 in HEPES buffer (50 μM HEPES, pH 6.5) and loaded into a black Fluoronunc 96-well plate (Fisher Scientific, Loughborough, UK). Captopril (10μl at 10 μM) or distilled water

(10μl) were added to uninhibited wells (in duplicate) or inhibited wells, respectively, and incubated at 26 °C for 10 min. A serial dilution of recombinant human ACE-1 (R&D systems, Oxford, U.K), was diluted twofold in HEPES buffer to generate a standard curve (450–28,750 pg/ml) and was used as a reference between plates. HEPES buffer alone (50μl) was used as a blank. Fluorogenic substrate (50μl) (Abz-FRK(Dnp)-P) (Enzo Life Sciences, Exeter, UK), diluted 1 in 200 in assay buffer, was then added to all wells and incubated for 2.5 h at 26 °C in the dark. The level of fluorescence was read using a microplate reader (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK) at excitation 320 nm and emission 405 nm. ACE-1 activity was calculated for each sample by subtracting the inhibited activity from the mean uninhibited activity and was expressed as relative fluorescence units (r.f.u).

ACE-2 protein level

ACE-2 protein level was measured in brain tissue homogenates using a commercially available mouse ACE-2 ELISA duoset (R&D Systems, Abington, Oxford, UK) following manufacturer's guidelines with minor modifications. Capture antibody was diluted at 4 μg/ml in PBS (2-fold higher than recommended) in a NUNC maxisorp 96-well plate (R&D Systems, Abington, Oxford, UK) and coated overnight at room temperature. After a wash and block step, brain tissue homogenates diluted 1 in 10 in PBS:1% BSA, a serial dilution of recombinant ACE-2, or a blank, was added to the wells in duplicate for 2 h at room temperature. Following a wash step, detection antibody at 200 ng/ml (2-fold higher than recommended) was added to the plate for 2 h at room temperature. Following a further wash step, streptavidin horse-radish peroxidase at 1 in 20 (2-fold higher than recommended) was incubated for 20 min. TMB substrate (R&D Systems, Abington, Oxford, UK) was added to each well for 30 min at room temperature and 2 N sulphuric acid was added to stop the reaction. Absorbance was read at 450 nm for each well using a microplate reader (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK). The concentration of ACE-2 was interpolated from the standard curve. Each sample was measured in duplicate and the average calculated.

ACE-2 activity assay

ACE-2 activity was measured in mouse brain samples using the fluorogenic peptide, Mca-APK(Dnp), (Enzo Life Sciences, Exeter, UK). Brain homogenates (50μl) diluted 1:10 in Tris assay buffer (75 mM Tris, 1 M NaCl, pH7.5), were added in triplicate (one inhibited and two uninhibited wells) to black Fluoronunc 96-well plate (Fisher Scientific, Loughborough, UK). A serial dilution of recombinant human ACE-2 (R&D Systems, Abington, Oxford, UK), diluted twofold in Tris assay buffer, was to generate a standard curve (19.5–1250 pg/ml) and used as a reference standard between plates. HEPES buffer was used as a blank. ACE-2 inhibitor (MLN-4760) (Millipore, Darmstadt, Germany) diluted to 2 μg/ml in distilled water, or distilled water alone, was added to the inhibited or uninhibited wells, respectively and incubated for 10 min at 37 °C prior to the addition of fluorogenic substrate (50μl), diluted to 10 μg/ml in assay buffer. After incubation at 3 h at 37 °C, fluorescence was read using a microplate reader (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK) at excitation 330 nm and emission 390 nm. ACE-2 activity was calculated for each sample by subtracting the inhibited activity from the mean uninhibited activity and was expressed as relative fluorescence units (r.f.u).

Ang-II level

Angiotensin II (Ang-II) concentration was measured by direct ELISA using an in-house assay as previously described [36,37]. In brief, brain homogenates diluted in PBS (1:20), a serial dilution of recombinant angiotensin II (Abcam, Cambridge, UK) (78–5000 ng/ml), and PBS alone as a blank, were all incubated in a NUNC maxisorp 96-well plate (R&D Systems, Abington, Oxford, UK) for 2 h with shaking at room temperature. The plate was washed with 0.5 % Tween-20:PBS (x5) and blocked with 1 % BSA:PBS for 1 h at room temperature. The plate was then incubated with an Ang-II biotinylated detection antibody (1 μg/ml) (Cloud-Clone, Wuhan, China) for 2 h. After a further wash step, streptavidin horse-radish peroxidase (1:200, R&D Systems, Abington, Oxford, UK) was added for 20 min at room temperature, the plate was further washed, and TMB substrate (R&D Systems, Abington, Oxford, UK) was added for 30 min at room temperature in the dark. 2 N sulphuric acid was added to stop the colour change and absorbance was read using a microplate reader (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK) at 450 nm for each well. Concentrations of Ang-II were interpolated from the standard curve. Each sample was measured in duplicate and the average calculated.

Ang-(1–7) direct ELISA

Ang-(1–7) concentration was measured by direct ELISA using an in-house assay as previously described [5]. In brief, brain homogenates diluted in PBS (1:1000), a serial dilution of known recombinant Ang-(1–7) (Abcam, Cambridge, UK) (78–5000 ng/ml), or a PBS blank was incubated for 2 h with shaking at room temperature in a NUNC maxisorp 96-well plate (R&D Systems, Abington, Oxford, UK). After washing in 0.5 % Tween-20:PBS (x5), the plate was blocked with 1 % BSA:PBS for 1 h at room temperature. After further washing, Ang-(1–7) biotinylated detection antibody (1 μg/ml) (Cloud-Clone, Wuhan, China) was incubated for 2 h. After a further wash step, streptavidin horseradish peroxidase (1:200, R&D Systems, Abington, Oxford, UK) was added for 20 min at room temperature, the plate was further washed, and TMB substrate (R&D Systems, Abington, Oxford, UK) was added for 20 min at room temperature. 2 N sulphuric acid was added and absorbance was read using a microplate reader (FLUOstar OPTIMA; BMG Labtech) at 450 nm for each well. The concentration of Ang-(1–7) were interpolated from the standard curve. Each sample was measured in duplicate and the average calculated.

Statistical analysis

Unpaired *t* tests, ANOVA with Tukey's *post hoc* analysis or Kruskal Wallis analysis was used for comparisons between grouped data and Spearman's rank order correlation was used (as appropriate) with GraphPad Prism version 8. P-values <0.05 were classed statistically significant.

Results

ACE-1 protein level and enzyme activity was unaltered in relation to age and onset of disease pathology in wild-type and APP models

ACE-1 protein level did not change in relation to age in wild-type or APP transgenic mouse models and was unchanged between WT and model mice at all ages (5xFAD: age p = 0.61, WT v transgenic model p = 0.18; Apd9: age p = 0.78, WT v transgenic model p = 0.16; Tg2576: age p = 0.99, WT v transgenic model p = 0.56; PDAPP: age p = 0.75, WT v transgenic model p = 0.18) (Fig. 2A–D).

ACE-1 enzyme activity was similarly unchanged in relation to age in wild-type and APP-expressing mouse models and did not differ between model and WT mice at any age (5xFAD: age p = 0.69, pathology p = 0.54; Apd9: age p = 0.85, pathology p = 0.65; Tg2576: age p = 0.84, pathology p = 0.71; PDAPP: age p = 0.45, pathology p = 0.27) (Fig. 3A–D).

ACE-2 level and enzyme activity declines with age in both wild-type and APP transgenic mice

ACE-2 level was significantly reduced in 6- and 12-month old 5xFAD mice compared to 2-month old mice (both p < 0.05) (Fig. 4A). No corresponding age-related changes were observed in WT mice. Age-related changes in ACE-2 protein level were not observed in Apd9 transgenic mice, however, ACE-2 level was lower in the WT mice at 12 months of age compared to 3–4 month old mice (p < 0.05) (Fig. 4B). No significant age-related change was

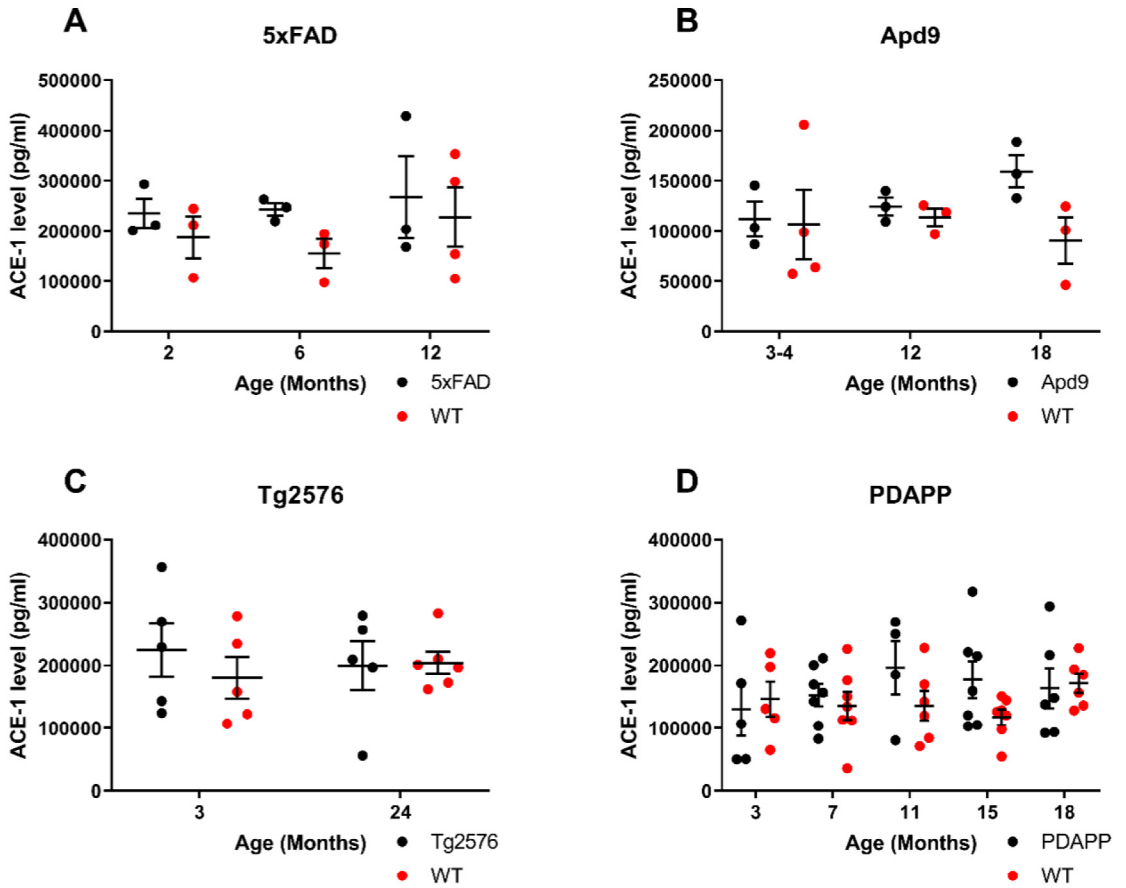

Fig. 2. ACE-1 protein level was unaltered in relation to age in mouse models of AD and WT controls. ACE-1 protein level was unchanged in relation to age across all four human APP (5xFAD, Apd9, Tg2576 and PDAPP) and wild-type mice. Scatterplots show ACE-1 protein level (mean ± SEM) measured in the left cortex (each dot represents the mean of duplicate measures from a single mouse). No significant differences were observed between WT and model mice in any age-group.

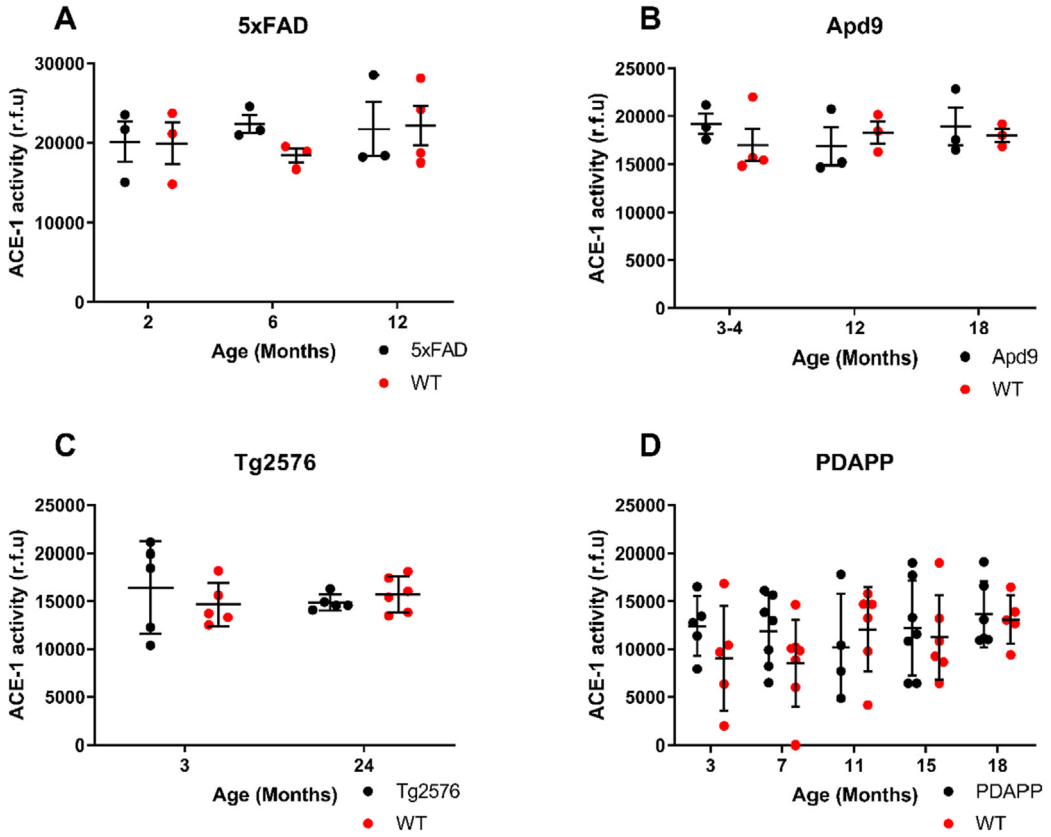

Fig. 3. ACE-1 enzyme activity was unchanged in relation to age in mouse models of AD and WT controls. Scatterplots show brain ACE-1 enzyme activity (mean ± SEM) in the left cortex at different ages in four transgenic human APP expressing mouse models (5xFAD, Apd9, Tg2576 and PDAPP mice) and wild-type controls (each dot represents the mean of duplicate measures from a single mouse). ACE-1 activity did not change with age and was unaltered in the disease models in all age-groups.

observed for ACE-2 level in Tg2576 mice and WT mice, although a similar non-significant trend ($p < 0.07$) was observed (Fig. 4C). ACE-2 level was significantly lower ($p < 0.05$) in 15-month-old mice compared to 3-month-old mice in both PDAPP mice and WT controls (Fig. 4D). No significant differences were observed in ACE-2 level between the WT and APP transgenic mice at any age (Fig. 4).

ACE-2 enzyme activity was reduced with age in all four transgenic models and WT mice (Fig. 5). ACE-2 was reduced in 5xFAD transgenic mice between 2 and 6 months of age ($p < 0.05$) whereas in WT mice, ACE-2 activity was significantly lower at 12 months compared to 6 months in WT mice ($p < 0.05$) (Fig. 5A). ACE-2 enzyme activity was significantly reduced in both Apd9 transgenic and WT mice at 12 months compared to 3–4 months of age ($p < 0.0001$ and $p < 0.0001$, respectively), but no further decrease was observed at 18 months of age in either the model or WT mice (Fig. 5B). ACE-2 activity was significantly lower in Tg2576 ($p < 0.01$) and WT mice ($p < 0.01$) at 24 months of age compared to 3 months (Fig. 5C). In PDAPP mice, ACE-2 activity was significantly lower in 7-month-old mice compared to 3 months of age ($p < 0.01$) (and was also lower at 11, 15 and 18 months compared to 3-month old mice). At 15 months of age, ACE-2 activity was at its lowest in both WT and PDAPP mice, and was significantly lower at 15 and 18 months of age compared to 7-month-old mice ($p < 0.05$ for both) (Fig. 5D). No significant differences were seen in ACE-2 activity between the WT and APP transgenic mice at any age (Fig. 5).

A detailed breakdown of post-hoc analysis for ACE-2 protein and enzyme activity in relation to age in 5xFAD mice is shown in supplementary Table 1.

Ang-II level increased with age

Ang-II levels were generally raised with age across all four models and WT controls (Fig. 6). In 5xFAD transgenic mice, Ang-II level was significantly increased at 6 ($p < 0.001$) and 12 months ($p < 0.01$) compared to 2 months of age (Fig. 6A) – the same trend was not apparent in WT mice ($p > 0.39$). Despite reaching significance by ANOVA ($p < 0.05$), no significant group differences were observed by *post hoc* analysis for the Apd9 and their WT littermates (Fig. 6B). No significant differences were observed in Ang-II levels in relation to age in the Tg2576 model or their counterpart WT controls (Fig. 6C). In the PDAPP and WT mice, Ang-II protein was significantly raised ($p < 0.05$, $p < 0.01$, respectively) at 15 months of age compared to

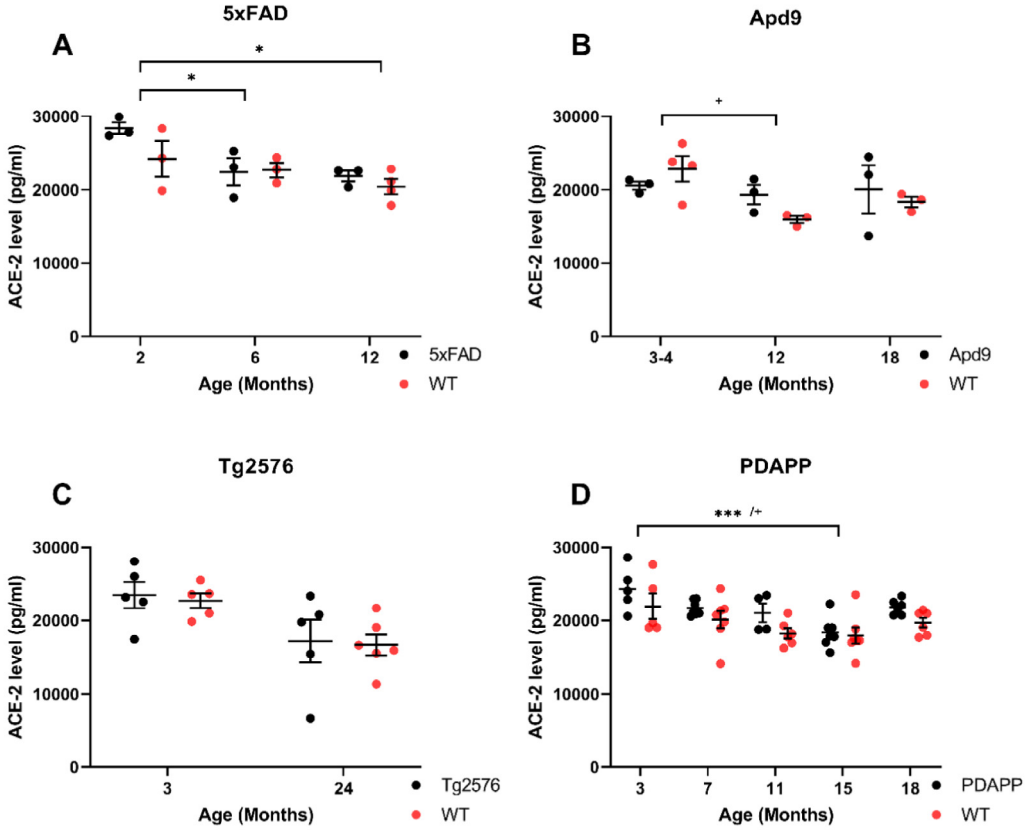

Fig. 4. ACE-2 protein level was reduced in relation to age in both wild-type mice and APP-transgenic models. Scatterplots show age-related changes in brain ACE-2 protein level (mean ± SEM) in the left cortex across four transgenic human APP expressing mouse models (5xFAD, Apd9, Tg2576 and PDAPP mice) and wild-type controls. ACE-2 level was reduced with age in both WT and APP models. No significant differences were observed between WT and model mice at any time point across all four models. Each dot represents the mean of duplicate measures from a single mouse. * and + $p < 0.05$, *** $p < 0.001$. * = significant difference between ages in transgenic model, + = significant difference between ages in WT.

3-month-old mice and in 7-month old PDAPP mice ($p < 0.01$) (Fig. 6D). Interestingly, Ang-II level was significantly lower at 18 months compared to 15-month-old PDAPP mice ($p < 0.01$) (Fig. 6D). No significant differences were observed in Ang-II level between the WT and transgenic mice at any age (Fig. 6).

Angiotensin (1–7) levels varied with age across APP models

Ang-(1–7) protein level was significantly raised in 6- and 12-month-old 5xFAD and WT mice compared to 2-month old mice ($p < 0.05$ for both) (Fig. 7A). Ang-(1–7) level was significantly higher in 18-month compared to 3-month-old WT (Apd9) mice ($p < 0.05$) (Fig. 7B). Ang-(1–7) was unaltered in Tg2576 and WT mice at 3 and 24 months (Fig. 7C). In PDAPP WT mice, Ang-(1–7) levels were reduced at 7 months compared to 3 months ($p < 0.05$) (Fig. 7D). A significant reduction in Ang-(1–7) was also observed in PDAPP mice at 15 months compared to 3 months ($p < 0.01$). Ang-(1–7) level was elevated in 18-month PDAPP mince compared to 15-month-old mice ($p < 0.001$) (Fig. 7D). No significant differences were seen in Ang-(1–7) levels between the WT and APP transgenic mice at any age (Fig. 7).

Discussion

We have investigated age-related changes in markers of classical RAS (ACE-1/Ang-II) and regulatory RAS activity (ACE-2/Ang-(1–7)) within the left cerebral cortex from four models of AD and their WT counterparts that present with disease pathology and Aβ-related cognitive decline at different ages to give us a broader insight into the potential timing of the changes that occur in the RAS. We show an imbalance in brain RAS in relation to age in both WT and AD mice that is driven by a reduction in ACE-2 protein expression and enzyme activity. The reduction in ACE-2 in AD models was detected at a young age, coinciding with the dysregulation of LTP, synaptic dysfunction and initial Aβ deposition, and generally preceding neuronal loss and the onset of cognitive decline. Surprisingly, however, the age-related changes in RAS in transgenic APP mice were mirrored closely in WT mice, except for 5xFAD mice, when ACE-2 declined and Ang-II increased at a younger age,

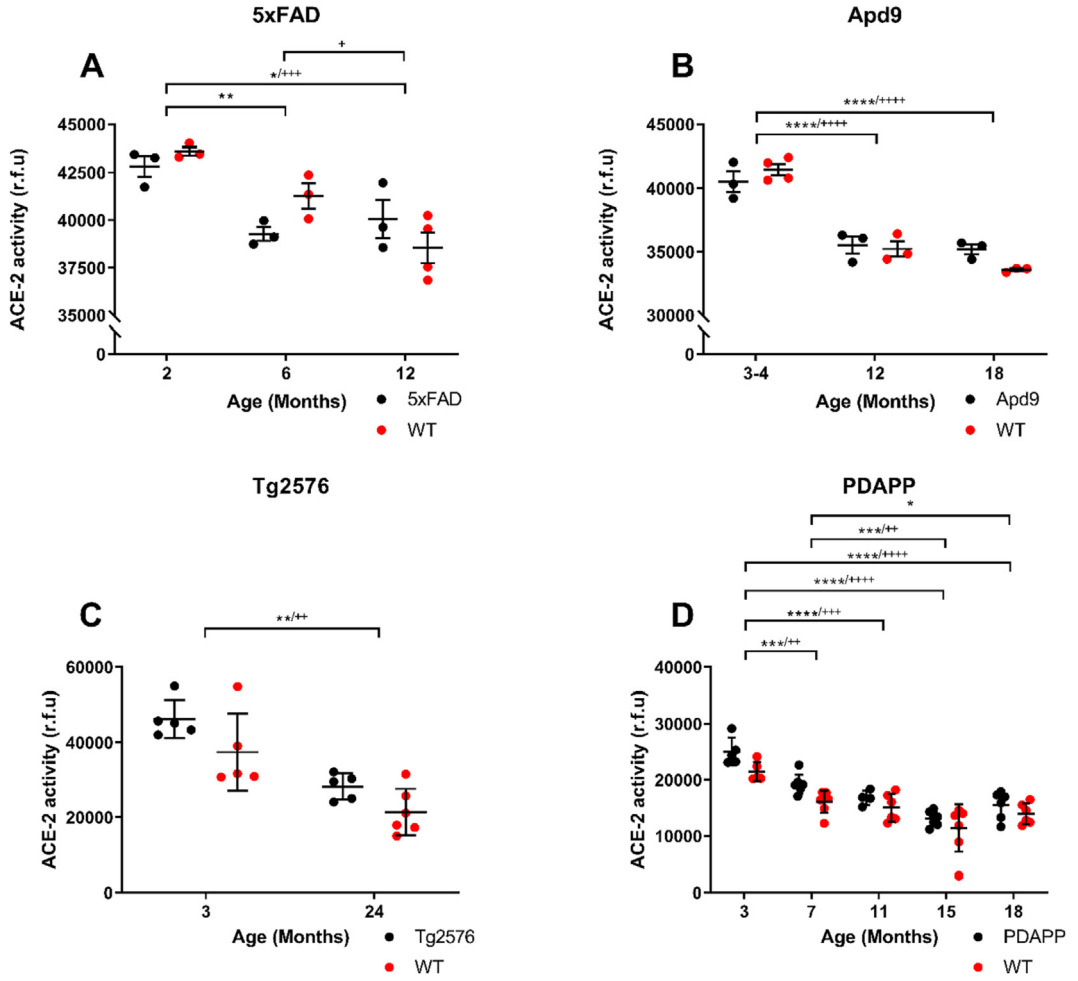

Fig. 5. ACE-2 enzyme activity was reduced in relation to age in both APP-transgenic and wild-type mice. Scatterplots show brain ACE-2 enzyme activity (mean ± SEM) in the left cortex at different ages in four transgenic human APP expressing mouse models (5xFAD, Apd9, Tg2576 and PDAPP mice) and wild-type controls. No significant differences were observed between WT and model mice at any time point across all four models Each dot represents the mean of duplicate measures from a single mouse. */+ $p < 0.05$, **/++ $p < 0.01$, ***/+++ $p < 0.001$, ****/++++ $p < 0.0001$. * = significant difference in model, + = significant difference in WT.

perhaps indicating that an imbalance in RAS is accelerated in this model of AD. The 5xFAD model was the most aggressive model studied, with amyloid deposition, gliosis, and changes to long term potentiation and depression reported from 2 months of age, perhaps accounting for accelerated reduction in ACE-2. Together, our data demonstrate a clear age-related imbalance in brain RAS, due to reduced ACE-2, that was not generally exacerbated by the presence of APP and or presenilin mutations.

Age is a widely accepted risk factor for cardiovascular disease and dementia, but we know little about the impact of age-related changes of RAS in aging and mouse models of AD [2,5–8]. Most studies to-date indicate that local organ-specific cRAS within the periphery becomes overactive, and rRAS underactive, in relation to ageing [29–31]. Here we show a similar imbalance in RAS with age within the brain, although it is not possible to discriminate if this imbalance is a result of local brain or vascular RAS change. Notably, ACE-2 showed a marked decline whilst ACE-1 remained relatively unchanged. The reduction in ACE-2 occurred at a young age in both APP models of AD and WT mice and remained stable in older animals. These data suggest that an early loss of ACE-2 may precede changes in ACE-1, as has been shown in other tissue [29], that occur at older ages, beyond the ages examined in this study. Despite ACE-1 remaining stable, levels of Ang-II (produced predominantly by ACE-1) were elevated with ageing. This might be explained by a reduction in Ang-II metabolism due to loss of ACE-2. If ACE-2 reduction was a contributor to the elevated Ang-II, we would have also expected a concurrent reduction in Ang-(1–7), however this was only observed in one of the four AD models tested, whereas an increase in Ang-(1–7) was observed for the other models.

These findings in mouse tissues contrast with our recent study in human post-mortem brain tissue that also revealed an age-related imbalance in RAS [38]. In human tissue, ACE-1 protein and Ang-II level were *both* elevated, indicative of cRAS overactivation, in normal ageing. In contrast, ACE-1 enzyme activity was reduced in normal ageing

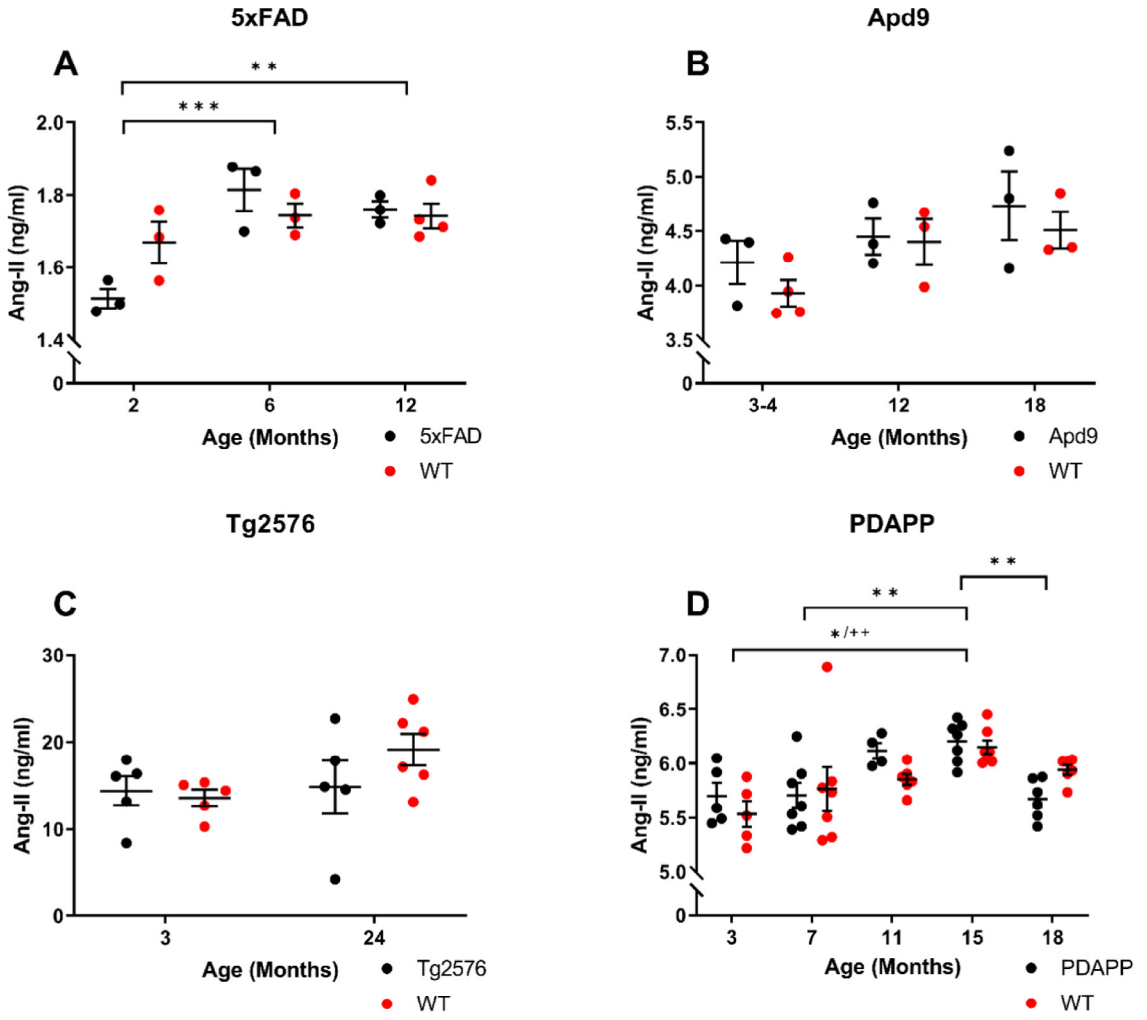

Fig. 6. Ang-II level was increased in relation to age in APP-transgenic and wild-type mice. Scatterplots showing brain Ang-II protein level (mean ± SEM) in the left cortex at different ages in four transgenic human APP expressing mouse models (5xFAD, Apd9, Tg2576 and PDAPP mice) and wild-type controls. No significant differences were observed between WT and model mice at any time point across all four models Each dot represents the mean of duplicate measures from a single mouse. * $p < 0.05$, **/++ $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. * = significant difference between ages in transgenic model, + = significant difference between ages in WT.

but was elevated in the early stages of AD (i.e. Braak tangle-stage III-IV). ACE-1 enzyme activity correlated inversely with Ang-II in normal ageing, consistent with previous studies indicating that Ang-II down-regulates ACE-1 activity as a normal protective physiological response [39] and which we suggest may become dysregulated in the early stages of AD. Comparison of whole brain (mouse) vs regional brain sampling (human) may account for these differences, although we can't exclude possible species differences in ageing related changes in RAS. Furthermore, the AD mouse models investigated only harbour Aβ-related pathologies and thus do not capture the full spectrum of other pathological processes (e.g. tau pathologies) that are normally present in human brain tissue and which might also have separate interactions with RAS [10] and elicit a more precipitous decline. Interestingly, a reduction in Ang-II and elevation in Ang-(1–7), indicative of a compensatory response of RAS, was noticed in PDAPP and WT mice at 18-months of age, possibly suggesting that disease pathology in APP mice does not alter RAS in the same way as humans.

The observed age-related changes in brain ACE-2 (reduced) and Ang-II (increased) in the transgenic AD models were closely replicated in WT mice indicating that APP expression and Aβ deposition did not influence age-related changes in RAS. In 5xFAD mice, however, ACE-2 protein and activity were reduced at 6 months (compared to 12 months in WT mice) and Ang-II levels were elevated in 5xFAD mice only. The timing of ACE-2 loss in 5xFAD mice, which was most marked between 2 and 6 months,

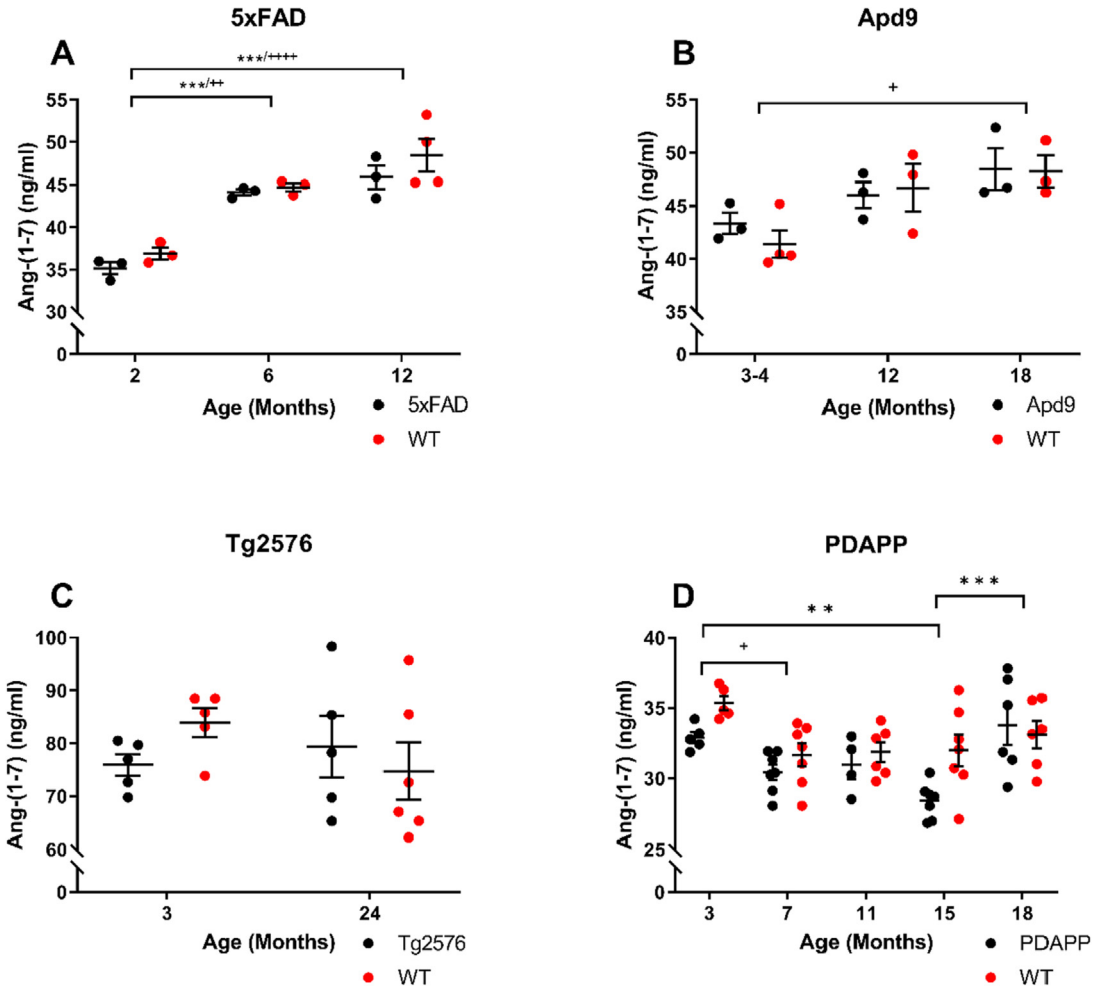

Fig. 7. Age-related changes in brain Ang-(1–7) level in APP-transgenic and wild-type mice. Scatterplots showing brain Ang-(1–7) protein level (mean ± SEM) in the left cortex at different ages across four transgenic human APP expressing mouse models (5xFAD, Apd9, Tg2576 and PDAPP mice) and wild-type controls. Each dot represents the mean of duplicate measures from a single mouse. */+ $p < 0.05$, **/++ $p < 0.01$, *** $p < 0.001$, ****/++++ $p < 0.0001$. * = significant difference between ages in transgenic model, + = significant difference between ages in WT.

coincides with the reported timing of initial Aβ deposition, gliosis, and changes to LTP and LTD and precedes neuronal loss and cognitive decline. We have previously shown in human post-mortem tissue that reduced ACE-2 activity correlated with Aβ deposition in AD [5]. Despite a reduction in ACE-2, Ang-(1–7) protein levels were unexpectedly elevated in older 5xFAD and Apd9 mouse models and WT mice (although a significant decrease was observed in PDAPP and WT mice). This might indicate that other 'compensatory' pathways are responsible for the production of Ang-(1–7) e.g., neprilysin converts Ang-I directly to Ang-(1–7) and has been reported to be elevated in normal ageing [40,41].

In relation to broader health implications, our findings may have some relevance, and thus warrant some brief reflections in relation to the recent emergence of ACE-2 as the primary receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [42] that has caused a global health pandemic since 2020. Infection with SARS-CoV-2 has been shown to downregulate membrane-expressed ACE-2, potentially leading to overactivation of cRAS and elevated production of Ang-II mediated vasoconstriction and inflammation – underpinning pathological processes associated with SARS-CoV-2 infection and COVID-19 disease [42]. Since 2020, numerous studies have described various effects that SARS-CoV2 has upon the brain [43–48] with recent data showing reduced grey matter thickness and increased cognitive decline in people over the age of 51 with infection [49]. An age-related reduction of ACE-2 may contribute to the increased risk of severe acute disease, or of the possibility of delayed and protracted clinical problems, in COVID-19 infected patients. ACE-2 is expressed most strongly in astrocytes and pericytes in the CNS [50–53] – these cells also have important regulatory properties and maintain vascular homeostasis and become dysfunctional in the pathogenesis in AD [54].

The limitations of this pilot study include the small numbers of mice per group and the limited age-range studied for each model. Apart from the 24 month old Tg2576 mice, we did not study mice older than 12 months in the 5xFAD and 18 months in the Apd9 and PDAPP models – thus we may have missed RAS changes associated with advanced age in these models. Another limitation was the limited scope to directly relate RAS changes to cognitive decline and pathological features in the AD models (which were not directly measured in this study). Instead, we relied on the reported timings of pathological and cognitive changes from the literature. A further potential limitation was that we measured RAS markers in brain homogenates from the entire cortex in mice whereas our previous studies in human tissue were undertaken in specific brain regions. RAS signalling differs across brain regions [55] and therefore any regional changes in RAS in relation to age or disease may have been masked in this study. Furthermore, any cell-specific changes in the expression and distribution of RAS receptors [56] may not be detected by the methods employed in this study. Future studies will need to focus on investigating regional and cell-specific changes in RAS in normal ageing aging and AD models. Discriminating the potential effect of sex in mouse models would also be beneficial (this study was restricted to male mice) because of the potential impact of the estrous cycle on RAS expression [57]. Further studies in mice would also benefit from a more comprehensive examination of other RAS markers including RAS receptors and other rRAS pathways (Ang-III/Ang-IV/AT4R) and assessment of RAS changes in relation to age in the recently developed APP knock-in mice and mice that also develop tau pathology. Despite the limitations of this study, we have generated preliminary evidence demonstrating that an age-related loss of counter-regulatory ACE-2 at an early age may be the trigger for age-related imbalance in RAS in the murine brain.

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Author contributions

Brain tissue was dissected by C.E. and S.C. Experiments, data collection and analysis were conducted by R.M. and J. S.M. R.M., J.S.M., M.A.G., S.Y.C., and P.G.K. contributed to early drafts of the manuscript. Final version was written by R.M. and J.S.M.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbas.2022.100062.

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