Heliyon 6 (2020) e03919

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Epidermal growth factor treatment of female mice that express *APOE4* at an age of advanced pathology mitigates behavioral and cerebrovascular dysfunction

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ARTICLE INFO

Keywords:
Biochemistry
Cell biology
Neuroscience
Pathology
Pathophysiology
APOE4
EGF
Cerebrovascular function

ABSTRACT

APOE4 is a major genetic risk factor for Alzheimer's disease and high amyloid-β (Aβ) levels in the brain are a pathological hallmark of the disease. However, the contribution of specific *APOE*-modulated Aβ-dependent and Aβ-independent functions to cognitive decline remain unclear. Increasing evidence supports a role of *APOE* in modulating cerebrovascular function, however whether ameliorating this dysfunction can improve behavioral function is still under debate. We have previously demonstrated that systemic epidermal growth factor (EGF) treatment, which is important for vascular function, at early stages of pathology (treatment from 6 to 8 months) is beneficial for recognition and spatial memory and cerebrovascular function in female mice that express *APOE4*. These data raise the important question of whether EGF can improve *APOE4*-associated cerebrovascular and behavioral dysfunction when treatment is initiated at an age of advanced pathology. Positive findings would support the development of therapies that target cerebrovascular dysfunction associated with *APOE4* in aging and AD in individuals with advanced cognitive impairment. Therefore, in this study female mice that express *APOE4* in the absence (E4FAD- mice) or presence (E4FAD+ mice) of Aβ overproduction were treated from 8 to 10 months of age systemically with EGF. EGF treatment mitigated behavioral dysfunction in recognition memory and spatial learning and improved hippocampal neuronal function in both E4FAD+ and E4FAD- mice, suggesting that EGF treatment improves Aβ-independent *APOE4*-associated deficits. The beneficial effects of EGF treatment on behavior occurred in tandem with improved markers of cerebrovascular function, including lower levels of fibrinogen, lower permeability when assessed by MRI and higher percent area coverage of laminin and CD31 in the hippocampus. These data suggest a mechanistic link among EGF signaling, cerebrovascular function and *APOE4*-associated behavioral deficits in mice with advanced AD-relevant pathology.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and a major healthcare problem facing modern science. AD presents as progressive cognitive impairment and high amyloid-β (Aβ) levels in the brain are a pathological hallmark of the disease. *APOE* genotype is a major genetic risk factor for AD, with *APOE4* increasing AD risk up to 12-fold compared to *APOE3* [1, 2, 3, 4, 5, 6, 7, 8], an effect that is greater in females [9, 10, 11]. As *APOE* modulates a number of Aβ-dependent and Aβ-independent [12, 13, 14, 15, 16, 17, 18, 19] functions in the brain, one challenge is deciphering the contribution of specific *APOE* modulated functions to cognitive decline. Increasing evidence supports a role of *APOE* in modulating cerebrovascular function [20]. With *APOE4* in both aging and AD there are higher levels of plasma proteins in the brain, which is indicative of disrupted cerebrovascular barrier function, and in AD patients there is also lower cerebrovascular coverage that suggests

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https://doi.org/10.1016/j.heliyon.2020.e03919
Received 19 June 2019; Received in revised form 18 November 2019; Accepted 30 April 2020

vessel degeneration [20, 21, 22, 23, 24, 25, 26]. These human data are recapitulated in female mice, where there is greater cerebrovascular dysfunction with *APOE4* compared to *APOE3* [27, 28, 29]. Given the importance of cerebrovascular function in neuronal homeostasis, identifying the contribution of *APOE*-modulated cerebrovascular dysfunction to behavioral impairments may reveal novel insight into *APOE4*-induced AD risk.

Epidermal growth factor (EGF) plays an important role in modulating the function of epithelial and endothelial cells in a number of organs (reviewed in [30]). Evidence suggests that the effects of EGF also extend to cerebrovascular function [30, 31, 32, 33, 34, 35], either acting directly on brain endothelial cells [36] or on support cells [31, 32, 37]. Since *APOE4* is associated with cerebrovascular dysfunction and cellular changes that may be modulated by EGF, one approach to assess the contribution of cerebrovascular dysfunction to behavioral dysfunction is evaluating the activity of EGF treatment in mice that express *APOE4*. Indeed, after treatment, EGF could directly improve brain endothelial cell function through signaling from the blood and also via crossing into the brain and targeting cells in the central nervous system [38]. We recently demonstrated that systemic EGF treatment at early stages of pathology (treatment from 6 to 8 months) is beneficial for recognition and spatial memory and cerebrovascular function in female mice that express *APOE4* in the absence [29] and presence of Aβ overproduction [27]. These data raise the important question of whether EGF can improve *APOE4* associated cerebrovascular and behavioral dysfunction when treatment is initiated at an age of advanced pathology. Addressing this question could provide important information on whether advanced *APOE4*-associated cerebrovascular dysfunction can be improved with EGF treatment and the contribution of this dysfunction to altered neuronal and behavioral function. Positive findings would support the development of therapies that target cerebrovascular dysfunction associated with *APOE4* in aging and AD in individuals with advanced cognitive impairment. Therefore, the goal of this current study was to determine whether EGF is beneficial at later stages of *APOE4*-associated pathology. We treated female E4FAD mice that express *APOE4* in the absence (E4FAD-) or presence (E4FAD+) of Aβ overproduction systemically with EGF in a reversal paradigm. This model was selected because it exhibits well characterized *APOE4*-relavent changes in cerebrovascular impairment [39]. We utilized behavioral, electrophysiological, histochemical, biochemical and magnetic resonance imaging techniques to evaluate the impact of EGF treatment on behavior, neuronal and cerebrovascular function.

2. Methods and methods

2.1. Experimental design and treatments

All experiments were conducted with ethical approval from UIC Institutional Animal Care and Use Committee protocols. Breeding and colony maintenance were conducted as described in [27, 29, 40]. E4FAD mice were produced by crossing mice that express 5 Familial Alzheimer's disease (FAD) mutations (APP K670N/M671L + I716V + V717I and PS1 M146L + L286V) with *APOE*-targeted replacement mice [41]. E4FAD carriers are $APOE4^{+/+}$/5xFAD$^{+/-}$ (E4FAD+) and non-carrier mice are $APOE4^{+/+}$ 5xFAD$^{-/-}$ (E4FAD-). Female E4FAD- and E4FAD+ mice were utilized for this study as identified by genotyping of tail samples. Female E4FAD+ and E4FAD- mice were treated from 8 to 10 months of age with recombinant mouse EGF (Shenandoah, 300 μg/kg, once per week) or vehicle (water) by intraperitoneal injection (i.p.). Within a cage, mice were randomly assigned a treatment so that in every cage there was a vehicle- and EGF-treated mouse. Investigators were blinded for treatment throughout the study. Four different treatment cohorts were conducted (Figure 1), with treatment frequency and duration identical up until 10 months. In the first cohort, mice were tested longitudinally for behavior using open field, novel object recognition and novel arm entry (Y-maze) at 6, 8, 9 and 10 months of age. Subsequently, cerebrovascular leakiness was assessed by magnetic resonance imaging (MRI) analysis. 30 minutes prior to tissue harvest, mice were given a final injection of EGF or vehicle and tissue was processed for biochemical and immunohistochemical analysis in order to evaluate the extent that EGF crossed into the brain. In cohort 2, mice were tested for behavior at 10 months of age and neuronal hippocampal function was assessed using electrophysiological recordings. In cohort 3, mice were tested using the Morris water maze (MWM) task, and then cerebrovascular leakiness was determined by MRI analysis. Thus, the final *n* for MRI analysis was derived from both cohort 1 and cohort 3, minus images that were unusable due to issues related to motion artifacts.

2.2. Behavioral analyses

Mice were housed in a 12-hour light, 12-hour dark cycle (lights off at 11 am and on at 11 pm). As mice are nocturnal, behavioral tests were conducted in the mouse active (dark) cycle at ~ 1 pm and analyzed using the ANY-Maze software as described in [27, 29, 40]. All behavior tests were timed to minimize the acute effects of injections, either due to stress

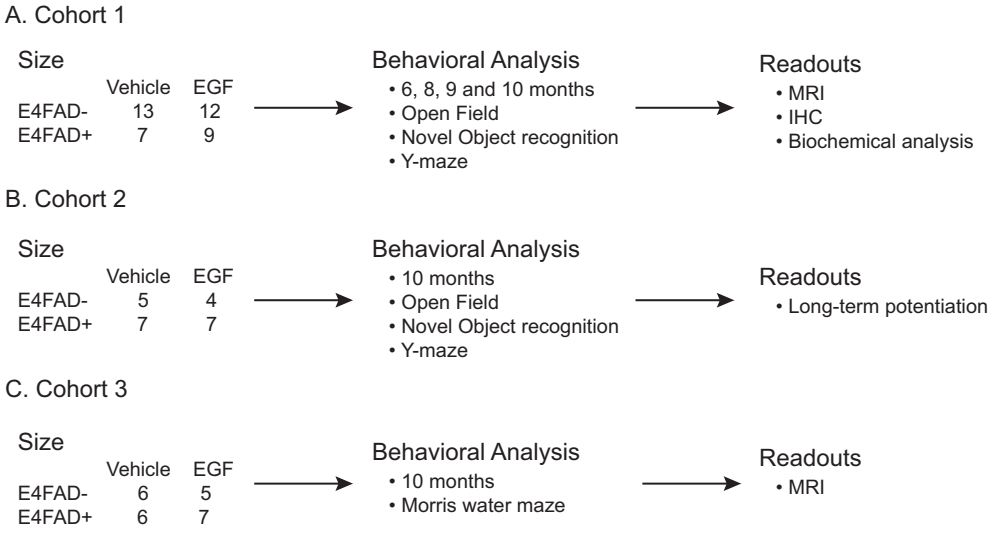

Figure 1. Study design. Female mice that express human *APOE4* in the absence (E4FAD-) and presence (E4FAD+) of FAD mutations were treated from 8 to 10 months of age with EGF (300 μg/kg) or vehicle, i.p. once a week in three cohorts. A. In cohort 1, mice were tested longitudinally for behavior using open field, Y-maze and novel object recognition at 6, 8, 9 and 10 months of age. Cerebrovascular leakiness was then assessed by MRI and tissue was processed for biochemical and immunohistochemical analysis. B. In cohort 2, mice were tested for behavior at 10 months of age and long term potentiation analysis conducted in the hippocampus. C. In cohort 3, mice were tested using Morris water maze test then cerebrovascular leakiness was determined by MRI analysis.

or EGF, on behavioral performance. In cohorts 1 and 2, 72 h after EGF or vehicle injection mice were tested sequentially (24 h break between tests) by open field, novel object recognition followed and then Y-maze. Mice in cohort 3 were tested in the Morris water maze, which was initiated 24hr after treatment so that no treatments were administered during the testing period.

2.2.1. Open field

Mice were placed in the center of a white box (38.5 × 30 × 30 cm) for 10 min and the distance traveled was measured [27, 29, 40].

2.2.2. Novel object recognition

Open field was conducted one day prior to novel object recognition and therefore served as the habituation phase. Mice were placed in a white box (38.5 × 30 × 30 cm) and introduced to two identical objects for 7 min and, 1 h later, with a familiar and novel object for 7 min. The preference index (ratio of time spent with the novel object divided by total investigation time for both objects) was calculated [27, 29, 40]. In longitudinal testing the same objects were used for each test.

2.2.3. Novel arm entry (Y-maze)

Mice were placed into the maze with one of the arms blocked for 10 min, returned to the home cage for 60 min, and placed back in the maze with access to all three arms for 5 min. The time spent in the novel arm was calculated [27, 29, 40]. In longitudinal testing, the same visual cues were used for each test.

2.2.4. MWM

We utilized the MWM test to assess the effect of EGF on learning and memory behavior [27, 29, 40]. The MWM consisted of a circular pool (approx. 120cm in diameter x 50cm tall) containing a circular escape platform submerged below the water line. The water was made opaque white using non-toxic tempera paint. High contrast visual cues, consisting of different black and white poster board patterns, were placed throughout the testing room for spatial orientation. In the acquisition phase, mice were tested for 5 days (60 s trial time, 4 trials each day with a 20-minute ITI) for the time to locate the hidden platform. The entry quadrant varied, but the platform location remained constant. The average of each day's trials was plotted. 24 hours later a single probe trial was performed for 60 s, with the platform removed. One hour after the probe trial, a visual acuity trial (60 s) was conducted by placing a flag on the platform in a different quadrant to that used in the rest of the MWM test; all mice found the platform within 60 s (observational data).

2.3. Synaptic plasticity

For mice in cohort 3, one week after the MWM test, long-term potentiation (LTP) analysis was conducted on slices from the hippocampus as described in [42, 43, 44, 45, 46]. Briefly, mice were anesthetized (Isoflurane inhaled anesthetic), brains removed, hippocampi dissected, sliced (300μm) and allowed to recover in PBS at 32 °C with constant oxygenation. Slices were then placed in a continuously perfused interface recording chamber, a stimulating electrode placed over the Schaffer Collateral Commissural Pathway and recording electrodes placed in the middle molecular layer. Basal synaptic transmission was recorded with single stimuli at 50% of the threshold for population spike initiation every 30 s until stable values for 20 min were obtained. LTP was induced by theta burst stimulation (10 bursts at 1 Hz of 4 at 100 Hz stimuli at test intensity) and recorded for a further 60 min. The magnitude of LTP was calculated from the initial slope of the low frequency evoked field excitatory postsynaptic potentials (fEPSPs) normalized to baseline.

2.4. Tissue harvest

Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine (i.p.), blood drawn by cardiac puncture, and transcardial perfusion performed with PBS as described in [27, 29, 40] prior to processing for biochemical or IHC analysis. Dissected left hemi-brains were frozen in O.C.T. and stored at -80 °C until IHC analysis and the dissected hippocampus from the right hemi-brain was flash frozen in liquid nitrogen and stored at -80 °C until processing for biochemical analysis.

2.5. Western blot and ELISA analysis

Hippocampal samples were weighed and homogenized using a plastic pestle in SDS lysis buffer (1% SDS + 10mM NaF + 2mM Na_3VO_4 in HEPES; pH = 7.4) at 5.5 μl/mg of brain tissue. Homogenates were then incubated in a water bath (90 °C for 2 min), sonicated (20% amplification, 6 cycles) and centrifuged (100,000 × *g* for 30 min at 4 °C). Aliquots of the supernatants were flash frozen in liquid nitrogen and stored at -80 °C. The pellet was then re-suspended in 70% formic acid (FA), incubated with gentle rotation at room temperature for 2 h with occasional vortexing, and centrifuged (100,000 × *g* for 1 h at 4 °C). The FA-soluble supernatant was neutralized with 20 volumes of 1M Tris base, aliquoted, and snap frozen in liquid nitrogen. Total protein in SDS extracts was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher) and in the neutralized FA extract with Bradford Reagent (Bio-Rad).

2.5.1. Western blot analysis

Samples were loaded to enable comparisons of EGF and vehicle treatment within E4FAD- or E4FAD+ mice (i.e. all vehicle and EGF treated E4FAD- mice samples loaded on one gel and all E4FAD+ mice samples loaded on another gel). 20 μg of protein were separated on 26 well, 4–12% Bis-Tris Midi gels (Invitrogen), transferred onto low-fluorescence PVDF membranes, blocked with 5% milk in 0.1% Tween-20 in TBS (TBS-T), and probed with primary antibodies (see Table 1) in 1% bovine serum albumin (BSA) in TBS with 0.02% sodium azide overnight at 4 °C. After washing (3 × 5 min, TBS-T), membranes were incubated for 45 min in the appropriate fluorescent secondary antibodies in 1% milk in TBST and 0.01% SDS (LI-COR). Proteins were imaged and quantified using the Odyssey ® Fc Imaging System and normalized to GAPDH. Normalized protein levels (to GAPDH) were expressed as a ratio of vehicle treated mice for within genotypes (i.e. within E4FAD- or E4FAD+ mice). GAPDH levels were not different between vehicle-treated and EGF-treated E4FAD- mice (($t(23) = 0.409$, $p = 0.687$) or E4FAD+ mice (($t(14) = 0.1940$, $p = 0.849$), supporting this method of normalization.

2.5.2. ELISA analysis

ApoE and Aβ40 and Aβ42 were measured by ELISA in the SDS and FA extracts (different plates for each extract) of E4FAD+ mice. The apoE ELISA was performed using anti-apoE (1:2,000, Millipore) and biotinylated anti-apoE (1:5000, Meridian) for capture and detection antibodies, respectively, as described in [47]. Aβ40, Aβ42 (Life Technologies) and EGF (R&D systems) were measured by a commercially available ELISA kit following the manufacturer instructions. ApoE, Aβ40, Aβ42 and EGF levels were normalized to total protein levels in each extract.

2.6. Immunohistochemical (IHC) analysis

IHC analysis for fibrinogen extravasation, laminin and CD31 coverage was conducted as described in [27, 29, 40] (see Table 1 for antibodies). Briefly, sagittal sections were taken beginning at the stereotaxic coordinate of ML 3.72 mm through 0 mm in order to encompass the entire

Table 1. Antibodies utilized in this study.

Target	Vendor	cat#	Application	Dilution
Ms α-PSD-95	Cell Signaling	36233	Wester blot	1:3,000
Ms α-GAPDH	Sigma	G8795	Western blot	1:3,000
Rb α-GAD1(GAD67)	Protein Tech	10408-1-AP	Wester blot	1:500
IRDye 680RD Gt α-mouse IgG	LI-COR	925–68070	Western blot	1:10,000
IRDye 680RD Gt α-Rb IgG	LI-COR	926–32211	Western blot	1:10,000
Rt α-CD31	BD Biosciences	550274	IHC	1:10
Rb α-Laminin	Abcam	Ab11575	IHC	1:200
Rb α-Fibrinogen	Agilent Dako	A0080	IHC	1:200
Gt α-Rt Alexafluor 488	Thermoscientific	A11006	IHC	1:200
Gt α-Rb Alexafluor 594	Thermoscientific	A-11012	IHC	1:200
Gt α-Rb Alexafluor 488	Thermoscientific	A-11034	IHC	1:200

hippocampus. Nine nonadjacent, 12 μm frozen sections (192 μm apart) per animal were fixed with 10% Neutral Buffered Formalin (Sigma), permeabilized with TBS containing 0.25% triton X-100 (TBSX, 3 × 5 min), blocked with 5% BSA (2 h), incubated with primary antibodies (4 °C, overnight), washed (3 × 5 min in TBSX), incubated with secondary antibodies (2 h), washed with TBSX (3 × 5 min) followed by TBS (1 × 5 min), and mounted. Mosaic images were obtained at 10x magnification on a Keyence BZ-X microscope or Molecular devices ImageXpress Micro 4 with equal exposure settings. The boundaries of each individual brain section were set using the mosaic function, sequential images were obtained that encompassed the entire area of the tissue boundary (between ~10-20 images per section), and all the individual images were then stitched together to produce a single composite image. Converted images were thresholded equally using ImageJ. The hippocampus was then traced and fibrinogen, laminin or CD31 levels were quantified using the "Analyze Particles" feature in image J, focusing on the punctate staining.

2.7. MRI analysis of cerebrovascular permeability

To assess cerebrovascular permeability, diffusion weighted arterial spin labeling (DW-ASL) datasets were acquired of each mouse at 10 months using a diffusion weighted prepared spin echo Signal Targeting with Alternating Radio frequency (STAR) [48] sequence on a 9.4T small-animal scanner (Varian/Agilent) (TR/TE 2000/9.6 ms, post labeling delay 500 ms, FOV 25 × 25 mm^2, slice thickness 1 mm, matrix 64 × 64, tagging gradient = 0.4 G/cm, number of averages = 1, tagging gaps of -15,-1,-1,0,1,2,3,4,5,6,7,8,9,10,11,12,15 mm, b-values of 0 and 50 s/mm^2). Images were processed using the TADZZ method to remove ΔB_0 bias; similar to the ΔB_0 bias correction used in chemical exchange saturation transfer (CEST) [49] albeit applied to ASL. The leakage maps were generated as the ratio of the rCBF map at b = 0 and the rCBF map at b = 50, rCBF(b = 50)/rCBF(b = 0) [50].

2.8. Statistical analysis

All data are presented as mean +/- S.E.M or in the case of LTP +/- variance and were analyzed using one-way ANOVA followed by Tukey's post hoc comparisons, or by using Student's t-test with GraphPad Prism version 8, as described in the figure legends. In addition, the variance of the LTP response was assessed by F-test analysis. Since our primary focus was the assessment of EGF compared to control treatment, all statistical analysis was performed for within genotype analysis.

3. Results

To determine whether EGF treatment can improve behavioral and cerebrovascular dysfunction, we utilized E4FAD+ and E4FAD- mice. E4FAD- express human *APOE4* under the mouse endogenous promoter, and E4FAD+ mice express *APOE4* and overproduce Aβ42 via 5xFAD mutations (i.e. a model of high human Aβ levels) [41]. Previous data have demonstrated that memory-relevant behavior, and cerebrovascular impairment in 8-month-old female mice are greater with *APOE4* than *APOE3* in both EFAD- and EFAD+ mice [27, 28, 29]. In addition, we have demonstrated that EGF treatment from 6 to 8 months of age reduces decline in memory and improves cerebrovascular function in E4FAD- and E4FAD+ female mice [27, 29]. Therefore, in order to evaluate activity at advanced stages of behavioral dysfunction in this study, we treated female E4FAD+ and E4FAD- mice with EGF (300 μg/kg, i.p., weekly) or vehicle from 8 to 10 months of age. 8 months was selected to start treatment at a time of deficits in behavioral tasks (novel object recognition) and advanced cerebrovascular dysfunction in both E4FAD- and E4FAD+ mice. We utilized E4FAD- and E4FAD+ mice to evaluate the activity of EGF in mouse models that express *APOE4* in the absence and presence of FAD mutations that produce high levels of human Aβ. The dose of EGF and treatment frequency was chosen because it does not promote oncogenesis or produce overt signs of toxicity in mice [27, 29, 51] and matched our previous prevention paradigm [27, 29]. We did not observe any changes in bodyweight between EGF-treated and vehicle-treated mice throughout the study (Supplementary Figure 1A). Four different cohorts of mice were utilized to assess all of our read-outs for cognitive, cerebrovascular and neuronal function (Figure 1).

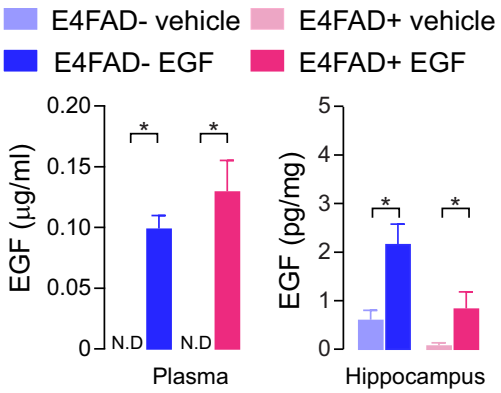

Figure 2. Plasma and hippocampal EGF levels are higher in female E4FAD- and E4FAD+ mice after EGF treatment. 30 minutes prior to tissue harvest, mice in cohort 1 were treated with a final dose of EGF. When assessed by ELISA, plasma and hippocampal EGF levels are higher in E4FAD- (Plasma; $t(23) = 8.023$, $p < 0.0001$. Hippocampus: $t(23) = 3.727$, $p = 0.001$) and E4FAD+ (Plasma; $t(14) = 4.602$, $p = 0.0004$. Hippocampus: $t(14) = 2.011$, $p = 0.033$) mice treated with EGF compared to vehicle treatment. Data expressed as mean +/- SEM. *$p<0.05$ by Student's t-test. n = 13 (vehicle-treated E4FAD- mice), 12 (EGF-treated E4FAD- mice), 7 (vehicle-treated E4FAD+ mice) and 9 (EGF-treated E4FAD+ mice.

3.1. EGF treatment improves learning and memory-relevant behavior in E4FAD- and E4FAD+ mice

In order to determine whether there were altered levels of EGF after treatment we measured plasma and hippocampal EGF levels by ELISA (Figure 2). As expected, in EGF-treated mice plasma EGF levels were higher 30 min after the final injection compared to vehicle-treated E4FAD- and E4FAD+ mice. We also observed higher hippocampal levels of EGF after treatment. The higher levels of EGF in the plasma and brain may modulate functions that are important for behavior in mice that express *APOE4*. Therefore, in one cohort of mice we tracked behavior longitudinally at 6, 8, 9, and 10 months of age using open field,

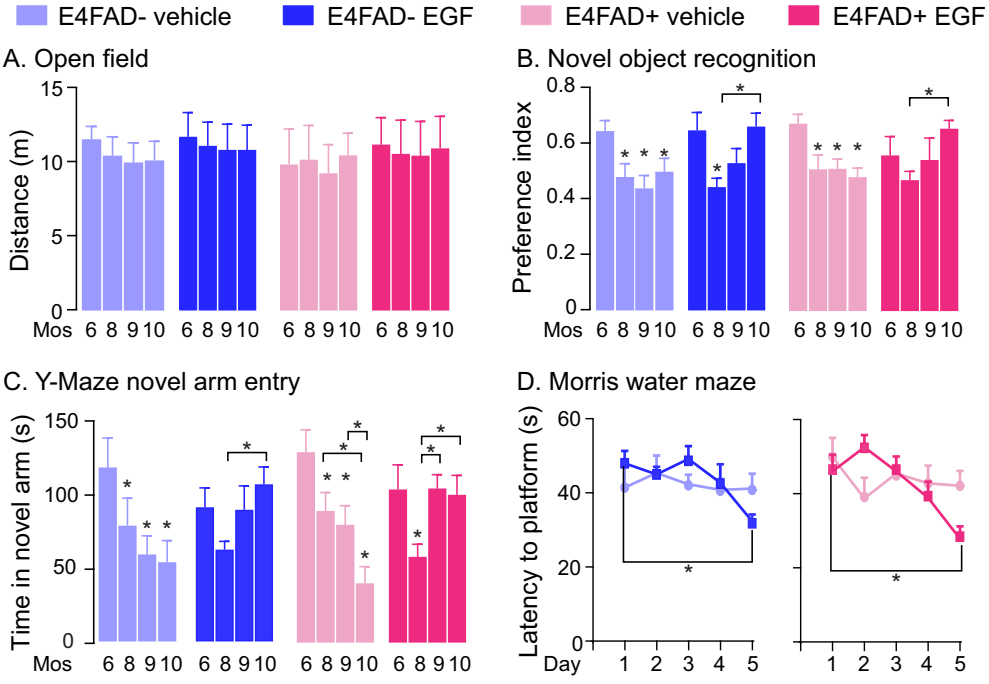

Figure 3. EGF mitigates learning and memory dysfunction in female E4FAD- and E4FAD+ mice. A. There were no changes in the open field task in vehicle- or EGF-treated E4FAD+ or E4FAD- mice during the course of the study (Vehicle E4FAD- mice: $F(51) = 1.715$, $p = 0.196$. EGF treated E4FAD- mice: $F(47) = 0.721$, $p = 0.497$. Vehicle E4FAD+ mice: $F(27) = 0.325$, $p = 0.678$. EGF treated E4FAD+ mice: $F(35) = 0.631$, $p = 0.472$). When assessed longitudinally, vehicle treated E4FAD- and E4FAD+ mice declined in performance from 6 to 10 months when assessed by B. novel object recognition (E4FAD- mice: $F(47) = 4.742$, $p = 0.014$. E4FAD+ mice: $F(27) = 8.305$, $p = 0.006$) and C. novel arm entry (E4FAD- mice: $F(51) = 8.211$, $p = 0.016$. E4FAD+ mice: $F(27) = 14.4$, $p = 0.0015$). Compared to 8 months, performance of EGF-treated E4FAD- (Novel object recognition: $F(47) = 4.784$, $p = 0.015$. Novel arm entry: $F(47) = 3.861$, $p = 0.047$ and E4FAD+ (Novel object recognition: $F(31) = 4.051$, $p = 0.033$. Novel arm entry: $F(35) = 3.485$, $p = 0.027$) was improved by 10 months of age. Data expressed as mean +/- SEM.*$p<0.05$ by repeated measures one-way ANOVA and Tukey's post-hoc analysis for age comparisons within treatments and genotypes. $n = 13$ (vehicle treated E4FAD- mice), 12 (EGF-treated E4FAD- mice), 7 (vehicle-treated E4FAD+ mice) and 9 (EGF-treated E4FAD+ mice). One vehicle-treated E4FAD- mouse, and one EGF-treated E4FAD+ mouse were excluded in novel object recognition, since mice did not investigate either object for a total of 10 s in the familiarization phase at one age. D. In a separate cohort of mice, learning was evaluated in the MWM spatial navigation task. When assessed by repeated measured two-way ANOVA, there was a time ($F(2,44) = 4.319$, $p < 0.005$) and time by treatment interaction ($F(2,44) = 4.148$, $p < 0.0062$) in E4FAD+ mice. Post hoc analysis comparing day 1 to each other day demonstrated that EGF-treated E4FAD+ mice but not vehicle-treated E4FAD+ mice learned the location of the platform by day 5, however there were no treatment effects. In E4FAD- mice, when assessed by two-ANOVA there were no differences. However, when assessed using repeated measured one-way ANOVA and Tukey's post-hoc analysis EGF- ($F(24) = 3.996$, $p = 0.0153$) but not vehicle- treated ($F(29) = 0.628$, $p = 0.68$) mice learned the location of the platform by day 5. Data expressed as mean +/- SEM. *$p<0.05$ by repeated measures two-way ANOVA (E4FAD+ mice) or one-way ANOVA (E4FAD- mice) and Tukey's post-hoc analysis. $n = 6$ (vehicle-treated E4FAD- mice), 5 (EGF-treated E4FAD- mice), 6 (vehicle-treated E4FAD+ mice) and 7 (EGF-treated E4FAD+ mice).

novel object recognition, and novel arm entry in the Y maze (Figure 3). The total distance traveled in open field did not change with age in either the vehicle or EGF treatment groups (Figure 3A), supporting that EGF does not modulate locomotor activity using this treatment regimen in this model. As previously demonstrated [27, 29], memory performance declines from 6 to 8 months age in vehicle-treated E4FAD- mice and E4FAD+ mice, and remains impaired at 10 months when assessed by novel object recognition (Figure 3B) and novel arm entry (Figure 3C). Thus, in female mice there is age-dependent decline in memory with *APOE4* in the absence and presence of high Aβ levels. Importantly, in both E4FAD- and E4FAD+ mice treated with EGF at 8 months of age, the memory impairments were mitigated by 10 months of age. EGF-treated E4FAD- and E4FAD+ mice performed ~30% higher in the novel object recognition test (Figure 3B) and spent 60% longer in the novel arm of the Y maze test (Figure 3C) at 10 months of age compared to 8 months of age. We then assessed whether EGF treatment improved learning in the MWM navigation task after 2 months of treatment. 10-month-old vehicle-treated E4FAD- and E4FAD+ mice failed to learn the location of the platform over the 5 days of testing (Figure 3D). However, EGF-treated E4FAD- and E4FAD+ mice located the platform with a lower latency time on the 5th day compared to the 1st day of testing (Figure 3D). Consistent with these data, EGF-treated E4FAD+ mice crossed the previous platform area a greater number of times than vehicle treated E4FAD+ mice in a probe phase (Supplementary Figure 1B) and a similar trend was observed in E4FAD- mice (p=0.07). Therefore, EGF-treated E4FAD- and E4FAD+ mice learned the location of the platform, whereas vehicle-treated mice did not. Collectively, these data demonstrate that weekly EGF treatment is beneficial for behavior in a reversal paradigm using E4FAD- and E4FAD+ mice.

3.2. EGF treatment improved hippocampal neuronal function

Synaptic plasticity is a critically important neural function and an important read-out that can validate any changes in behavior. LTP is a form of plasticity, in which there is a persistent increase in synaptic strength following high-frequency stimulation. These core synaptic processes are considered a model of the cellular basis of memory, particularly in the hippocampus [52]. We next assessed whether EGF treatment improved hippocampal LTP in E4FAD- and E4FAD+ mice. To validate in this cohort of mice that EGF treatment improved behavior, we conducted novel object recognition and novel arm entry prior to LTP assessment. As observed for longitudinal analysis, EGF treatment resulted in improved performance in both tests compared to vehicle treatment in EFAD- and EFAD+ mice (Figure 4A). Dendritic fEPSPs were recorded in the CA1 molecular layer in response to electrical stimulation of the Schaffer Collateral Commissural Pathway. EPSP test fEPSP amplitudes were measured prior to and after LTP induction by a theta burst protocol [46], in the same mice that were tested for novel object recognition and novel arm entry. EGF treatment had no effects on the resultant mean magnitude of LTP in the hippocampus. In EFAD- mice, theta burst evoked a 125 ± 30% increase with vehicle treatment and a 157 ± 12% increase in EGF-treated mice (n = 6 and 5 respectively, p = 0.15). In E4FAD+ mice,

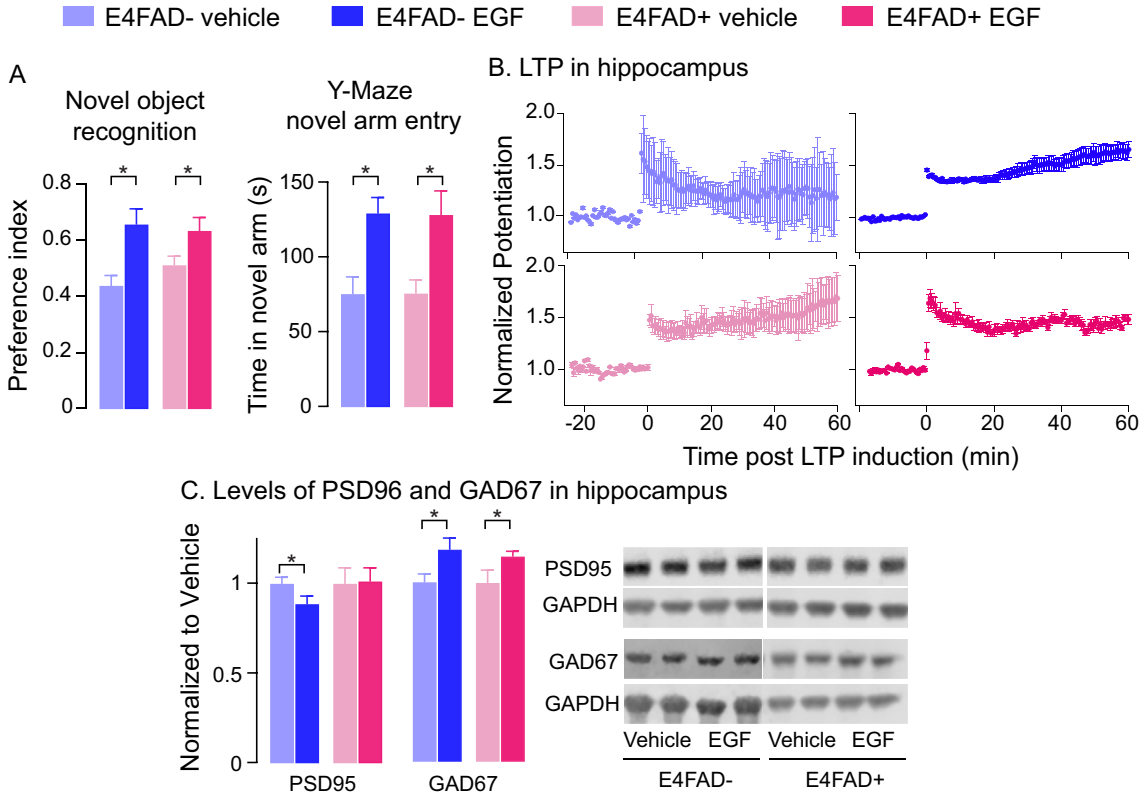

Figure 4. EGF treatment improved hippocampal neuronal function in female E4FAD- and E4FAD+ mice. A. Female E4FAD- and E4FAD+ mice were treated with EGF from 8 to 10 months of age in a separate cohort of mice (cohort 2). At the end of the study, EGF treatment resulted in improved behavior when assessed by novel object recognition (E4FAD- mice: t(7) = 4.837, p = 0.002. E4FAD+ mice: t(12) = 2.772, p = 0.017) and novel arm entry (E4FAD- mice: t(7) = 4.226, p = 0.037. E4FAD+ mice: t(12) = 4.189, p = 0.001) and B. lower variance of the LTP response (when analyzed by F-test statistical comparisons). C. Hippocampal homogenates were assessed for levels of PSD95 and GAD67 by western blot analysis. PSD95 levels were lower in EGF treated E4FAD- mice (t(23) = 2.16, p = 0.0417) but were not altered by treatment in E4FAD+ mice (t(14) = 0.114, p = 0.911). GAD67 levels were higher in EGF treated E4FAD- (t(23) = 2.457, p = 0.022) and E4FAD+ mice (t(14) = 2.258, p = 0.041). Levels of GAD67 or PSD95 were normalized to GAPDH, and then further normalized to the average of the vehicle control group within each genotype. Data expressed as mean +/- SEM (A and C) or +/- variance (B). *p<0.05 by Student's t-test. In A and B, n = 5 (vehicle-treated E4FAD- mice), 4 (EGF-treated E4FAD- mice), 7 (vehicle-treated E4FAD+ mice) and 7 (EGF-treated E4FAD+ mice). In C, n = 13 (vehicle treated E4FAD- mice), 12 (EGF-treated E4FAD- mice), 7 (vehicle-treated E4FAD+ mice) and 9 (EGF-treated E4FAD+ mice).

theta burst evoked a 152 ± 19.2% increase with vehicle treatment compared to a 160 ± 10% increase in EGF-treated treated mice ($n = 14$ and 10 respectively, $p = 0.29$). However, we observed significantly higher variance in the amplitude of evoked LTP response of vehicle-treated compared to EGF-treated mice (Figure 4B). This variance was significantly reduced by EGF treatment in both E4FAD- (F-test, $p = 0.05$, $n = 6$ untreated and 5 treated) and E4FAD+ mice ($p = 0.031$, $n = 14$ untreated and 10 treated mice). These results indicate that EGF-treatment stabilizes LTP responses in female mice that express *APOE4*.

In the hippocampus, *APOE4* is associated with dysfunction of both excitatory glutamatergic [53] and inhibitory GABAergic [54, 55, 56, 57] neurons. Therefore, we further assessed whether EGF treatment modulated levels of the archetypical marker of glutamatergic synapses, PSD95, and glutamate decarboxylase (GAD67), which catalyzes glutamate decarboxylation to GABA and is a marker of GABAergic synapses. Our data demonstrate that levels of GAD67, but not PSD95, are higher in EGF-treated E4FAD- and E4FAD+ mice compared to vehicle-treated mice by ~ 15–20% (Figure 4C, see Supplementary Figure 2 for full images). Thus, EGF treatment-dependent improvements in behavior are accompanied with lower variance in synaptic plasticity and higher GAD67 levels.

3.3. Fibrinogen levels and water permeability are lower and CD31 coverage is higher in the hippocampus of EGF- treated mice

A second major goal of this study was to determine whether EGF treatment at an age of advanced pathology could improve cerebrovascular function. One marker of cerebrovascular dysfunction is higher permeability. In conditions involving large changes in permeability, e.g.,

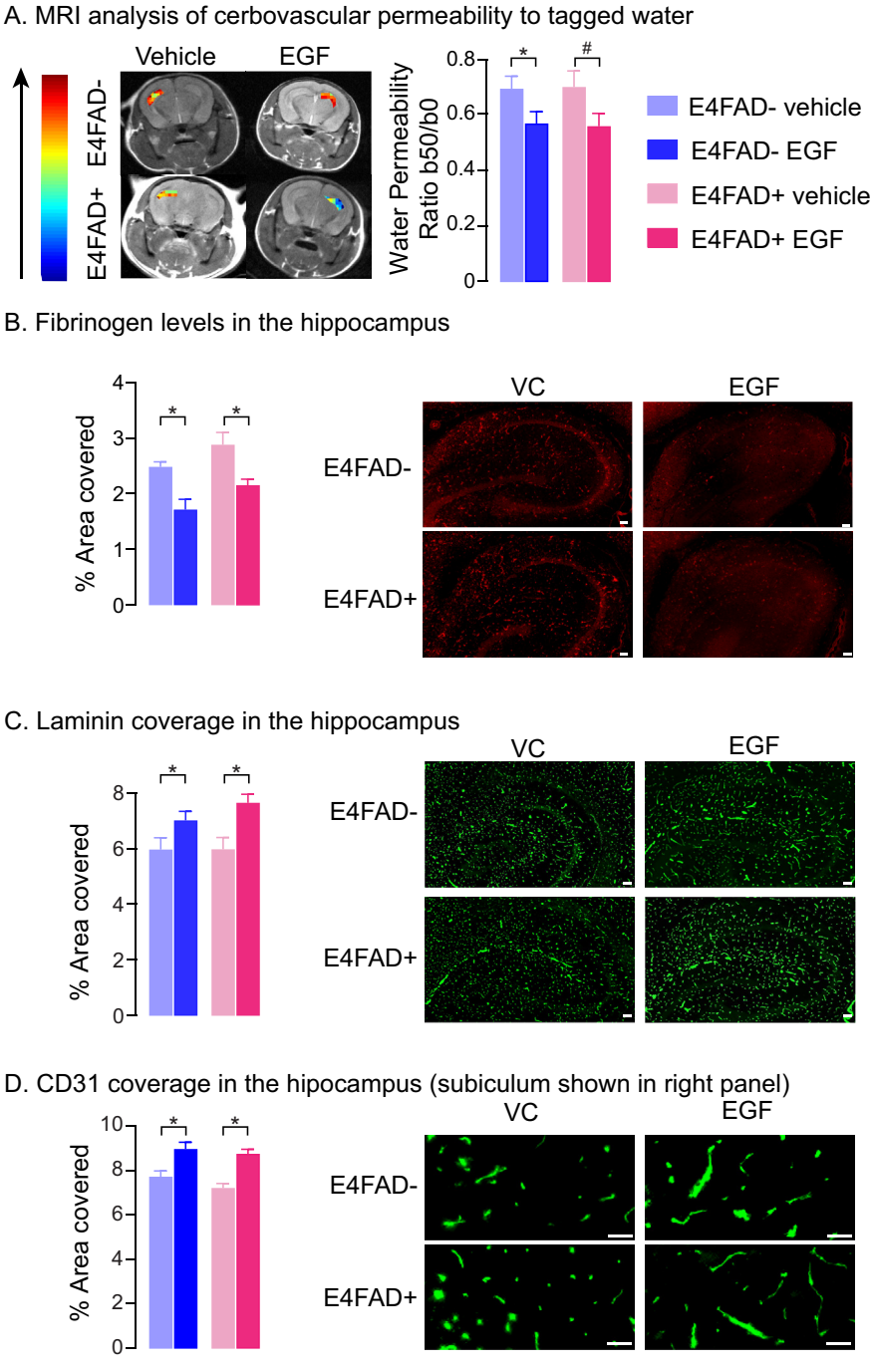

Figure 5. Markers of cerebrovascular permeability are lower and CD31 staining is higher in the hippocampus of EGF-treated female E4FAD- and E4FAD+ mice. Cerebrovascular permeability is lower in EGF-treated E4FAD+ and E4FAD- mice compared to vehicle-treated mice when assessed by A. MRI analysis (E4FAD- mice: $t(25) = 2.12$, $p = 0.044$. E4FAD+ mice: $t(13) = 2.01$, $p = 0.031$) and B. fibrinogen extravasation (E4FAD- mice: $t(23) = 3.915$, $p = 0.007$. E4FAD+ mice: $t(14) = 3.41$, $p = 0.004$). In addition, there is C. higher percentage area immunostained with laminin (E4FAD- mice: $t(23) = 2.1$, $p = 0.049$. E4FAD+ mice: $t(14) = 3.6$, $p = 0.003$) and D. CD31 (E4FAD- mice: $t(23) = 3.42$, $p = 0.003$. E4FAD+ mice: $t(14) = 6.43$, $p =< 0.0001$) in EGF-treated E4FAD- and E4FAD+ mice. Data expressed as mean +/- SEM. * (two tail) # (one tail) $p<0.05$ by Student's t-test. In A, $n = 14$ (vehicle-treated E4FAD- mice), 13 (EGF-treated E4FAD- mice), 7 (vehicle treated E4FAD+ mice and 8 (EGF treated E4FAD+ mice). In B and C, $n = 13$ (vehicle-treated E4FAD- mice), 12 (EGF-treated E4FAD- mice), 7 (vehicle-treated E4FAD+ mice) and 9 (EGF-treated E4FAD+ mice). Scale bars in B and C = 100μm and in D = 20μm.

tumor and stroke, dynamic contrast enhanced MRI is used by measuring the T1 enhancement of gadolinium leaking into the interstitium from an exogenously supplied bolus. Using DCE is challenging in the case of minor leakiness [58], e.g. AD; and, in our experience, application of MRI based on exogenous contrast can be challenging with the use of intravenous tail vein catheters. Therefore, we performed diffusion weighted arterial spin labeling (DW-ASL) MRI [59] in the hippocampus, to correlate with changes in synaptic function. This method allows quantification of cerebrovascular permeability to water without exogenous contrast agents. In the DW-ASL MRI method, water molecules are tagged in the blood and then the amount of water that crosses into the brain is quantified. When assessed by DW-ASL MRI, the permeability of the cerebrovasculature to water molecules was lower with EGF treatment compared to vehicle treatment (Figure 5A). Indeed, there were ~20% lower levels of tagged-water molecules in the hippocampus of EGF-treated E4FAD- and E4FAD+ mice compared to vehicle controls. To validate this finding, we measured endogenous hippocampal levels of the plasma protein fibrinogen (extravasation), which does not cross into the brain in high amounts with an intact cerebrovasculature, using IHC analysis. Consistent with MRI analysis, fibrinogen extravasation was ~40% lower in EGF-treated E4FAD- and E4FAD+ compared to vehicle treatment (Figure 5B).

Changes in the density or length of the cerebrovasculature, which we term vessel coverage, is a second marker of cerebrovascular damage that is associated with *APOE4 in vivo* [27, 28, 29]. Therefore, we measured levels of laminin, a basement membrane protein and a marker of vessel coverage by immunohistochemical staining. EGF-treated E4FAD- and E4FAD+ mice had 20% higher percentage area immunostained with laminin in the hippocampus compared to vehicle-treated mice (Figure 5C). Although laminin is a surrogate for vessel coverage, it was important to evaluate whether EGF treatment specifically modulated levels of the basement proteins, or also resulted in higher brain endothelial cell coverage. Through conducting quantitative immunohistochemical staining for CD31, we found that EGF-treated mice had ~15-20% higher percentage area immunostained with CD31 compared to vehicle-treated EFAD- and E4FAD+ mice (Figure 5D).

Collectively these data demonstrate that EGF-treatment results in improved cerebrovascular function in E4FAD- and E4FAD+ mice.

3.4. EGF treatment did not alter Aβ levels in E4FAD+ mice

EGF treatment improved behavior as well as synaptic, and cerebrovascular function in both E4FAD- and E4FAD+ mice. These data suggest that the beneficial effects of EGF are independent of high Aβ levels. There was also the possibility that EGF treatment modulated apoE levels in the brain to impact neuronal and cerebrovascular function. Therefore, we assessed human Aβ and apoE levels in the hippocampus of E4FAD+ mice by ELISA. There were no differences in Aβ40 (Figure 6A), Aβ42 (Figure 6B) or apoE levels (Figure 6C) in EGF-treated mice compared to vehicle-treated E4FAD+ mice for SDS or formic acid extracts. These data support that the beneficial effects of EGF are not related to modulation of Aβ or apoE levels in E4FAD+ mice.

4. Discussion

This current study demonstrates that peripheral EGF treatment at a time of advanced pathology, mitigates dysfunction in recognition memory and spatial learning and improves neuronal function in female mice that express *APOE4* independent of high human Aβ levels. In addition, results support that the beneficial impact of EGF on behavior may be mediated through improvement of cerebrovascular function.

APOE4 is associated with increased AD risk (reviewed in [60]), and impaired cognitive performance in humans during aging (reviewed in [20]), and *in vivo* [61, 62, 63, 64]. Our results show that EGF treatment likely improves *APOE4*-associated, rather than Aβ-induced, neuronal dysfunction. Interestingly, our behavioral data suggest that there are no changes in recognition memory and spatial learning in E4FAD+ mice compared to E4FAD- mice. That *APOE4* and female sex together are enough to alter learning and memory in mice is consistent with several reports [61, 62, 63, 64]. The lack of an effect of high Aβ pathology due to the 5xFAD mutations on behavior, when comparing E4FAD- and E4FAD+ mice, may relate to the lack of sensitivity of our behavioral tests, and is the focus of our ongoing research. *APOE4* is linked to alterations in neuronal function throughout the brain, however hippocampal function is considered particularly relevant in the context of cognitive decline. Our LTP analysis specifically demonstrates that EGF treatment reduces variance of the LTP response in mice that express *APOE4*. This finding is interesting, since there is a large focus on LTP research assessing the magnitude, rather than the variance of LTP. The neuronal basis of higher variance that is normalized with EGF treatment hypothetically could include instability of glutamatergic neurons, self-potentiation of glutamatergic neurons and disruption of GABAergic interneuron function. The latter disruption might lead to substantial variability in the strength of the LTP induction protocol targeting postsynaptic neurons undergoing LTP. Although there are lower levels of glutamatergic [53] and GABAergic [54, 55, 56, 57] neuronal markers with *APOE4 in vivo*, a growing body of evidence support that *APOE4* associated hippocampal GABAergic defects contribute to behavioral impairment in female mice [54, 55, 57, 65]. Published data have demonstrated in female mice expressing *APOE4* that there are lower numbers of hippocampal hilar GABAergic neurons and that both reducing *APOE4* expression by GABAergic neurons and pharmacological activation of $GABA_A$ receptors, prevent cognitive decline [54, 55, 57, 65]. In addition, neurons derived from induced pluripotent stem cells display GABAergic neuron degeneration with *APOE4* [66]. In our current study, levels of GAD67 in the hippocampus were ~15-20% higher in EGF-treated E4FAD- and E4FAD+ mice compared to vehicle-treated mice. GAD67 is an enzyme that produces GABA and is therefore a marker of the GABAergic neuronal activity. Although the magnitude of this effect may be considered modest, our recent data suggests that such a change in GAD can improve behavior

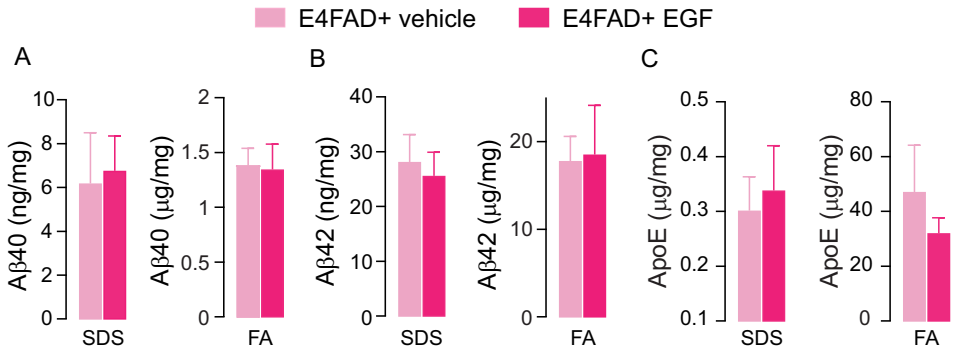

Figure 6. Aβ and ApoE levels are not modulated by EGF treatment in female E4FAD+ mice. There are no differences in hippocampal levels of A. Aβ40 (SDS: $t(14) = 0.212$, $p = 0.835$. FA: $t(14) = 0.123$, $p = 0.904$), B. Aβ42 (SDS: $t(14) = 0.397$, $p = 0.698$. FA: $t(14) = 0.11$, $p = 0.914$) or C. apoE (SDS: $t(14) = 0.315$, $p = 0.734$. FA: $t(14) = 0.945$, $p = 0.361$) levels in EGF-treated E4FAD+ mice compared to vehicle treatment when assessed by ELISA. Data expressed as mean +/- SEM. $p>0.05$ by Student's t-test. $n = 13$ (vehicle-treated E4FAD- mice), 12 (EGF-treated E4FAD- mice), 7 (vehicle-treated E4FAD+ mice) and 9 (EGF-treated E4FAD+ mice).

and neuronal function. In a model of Huntington's disease, EGF treatment resulted in higher levels of GAD65 and GABAergic transmission and also improved behavioral performance [67]. In some ways this parallels our biochemical, electrophysiological and behavioral data obtained here with EGF treatment of E4FAD- and E4FAD+ mice. Further studies may reveal the cellular basis for improved GABAergic function in E4FAD- and E4FAD+ mice. Interestingly, EGF treatment also resulted in lower levels of PSD95 in E4FAD- mice, which is often associated with glutamatergic neuron function. These data raise the possibility that another way that EGF could alter the variance of LTP is reducing glutamatergic activity in addition to increasing GABAergic activity. Overall, our data support that treatments which improve GABAergic function in the hippocampus could provide a novel therapeutic opportunity for *APOE4*-induced AD risk in females.

The beneficial effects of EGF on behavior and neuronal function may be the result of improved cerebrovascular function in E4FAD+ and E4FAD- mice. Multiple reports have demonstrated that *APOE4* is associated with cerebrovascular dysfunction in aging, AD patients, and in mice (reviewed in [20]). Two important markers of cerebrovascular dysfunction observed with *APOE4* are higher levels of plasma proteins and lower levels of CD31 and laminin in the brain, both of which were improved by EGF treatment. The relevance for neuronal dysfunction is that higher cerebrovascular permeability, particularly to plasma proteins, can lead to damage of neurons either directly or through effects on supporting cells, and vessel degeneration limits the supply of essential nutrients to neurons. In addition, higher laminin and CD31 immunostaining in EGF-treated E4FAD- and E4FAD+ mice may have resulted in higher intramural peri-arterial drainage, which could lead to greater clearance of toxic molecules from the neuronal environment [68]. Although we did not observe any lowering of human Aβ levels after EGF-treatment in EFAD+ mice, this does not preclude this hypothesis. Future research could reveal whether GABAergic neurons are particularly sensitive to changes in cerebrovascular function. One potential mechanism through which EGF improved cerebrovascular function is via direct brain endothelial cell signaling. This hypothesis is consistent with previous *in vitro* data that EGF improves brain endothelial cell function [36] and the general protective functions of EGF in epithelial and endothelial cells (reviewed in [30]). From a signaling standpoint, the EGF receptor is a tyrosine kinase that activates a number of signaling pathways [30] and our ongoing studies are focused on identifying the relevance of each pathway for improving *APOE4* associated BEC dysfunction.

The EGF receptor is expressed throughout the brain and in the periphery, and therefore a number of functions may have been modulated by EGF treatment independent of directly improving brain endothelial cell function. Indeed, in this study we observed higher levels of EGF in the brain after EGF treatment, which may be the result of a leaky cerebrovasculature or other transport mechanisms [38]. EGF receptor signaling in astrocytes [31], pericytes [32], and microglia [37] could have improved cerebrovascular function and/or neuronal function. In addition, EGF is reported to play a role in modulating GABAergic neuronal function directly [69]. Genetic approaches would reveal the cell-type specific role of the EGF receptor in modulating cognitive function endogenously, and in response to EGF treatment. A further point to note is that the behavioral improvements in this current reversal paradigm were not as pronounced as in our previous prevention study [27, 29] in E4FAD- and E4FAD+ mice. We propose that a higher treatment frequency will result in more pronounced beneficial effects for behavior in reversal and long-term treatment studies. Alternatively, EGF treatment may have preserved the function of surviving neurons, rather than reversing damage that has already occurred. Critical for addressing all the questions raised above, is conducting further research to understand the pharmacokinetic-pharmacodynamic relationship of EGF after both chronic and acute administrations in EFAD mice. After i.v. and s.c. injections, EGF is cleared rapidly from the plasma in wild-type rodents (low levels 2 h after treatment) [70]. It is important to evaluate whether the pharmacokinetics of EGF are similar in wild-type mice and E4FAD mice during treatment. However, if over the 2-month treatment period the pharmacokinetics of EGF are similar to wild-type mice in both E4FAD+ and E4FAD- mice, then one interpretation is that the protective effects of EGF on the cerebrovascular are due to cellular changes that occur repeatedly due to weekly injections, rather than effects that would occur with sustained plasma levels. One potential mechanism is that the EGF treatment triggered long-lasting (weekly) repeated gene-transcription changes in cells at the cerebrovasculature. Such changes may be observable after an acute injection, although equally as plausible is that EGF was protecting the cerebrovascular from additional disruption that occurs with age, which would only be revealed with longer-term treatment. Similar considerations are important to address for the higher levels of EGF we observed in the hippocampus at the end of the study. For example, it is unclear whether the higher hippocampal EGF levels would have been observed after a single injection, are modulated by age (e.g. if a single injection at 8 but not 6 months resulted in higher levels), occur due to gradual EGF accumulation, and/or represent EGF that was present inside brain endothelial cells. Although we focused our study design and statistical analysis of within-genotype comparisons, there was trend that hippocampal EGF levels reached higher levels in EGF-treated E4FAD- mice compared to E4FAD+ mice. As EGF could to cross into the brain via receptor and receptor mediated pathways, these data raise the intriguing possibility that the transport mechanisms of EGF at the cerebrovasculature are altered by FAD mutations and/or Aβ levels. As discussed above, EGF may modulate cells within the brain to alter cerebrovascular and/or neuronal function. Therefore, assessing the acute effects of EGF on cerebrovascular and neuronal function in EFAD mice is an important question and is the focus of our ongoing studies. Since EGF was beneficial for behavior and cerebrovascular function in E4FAD- and E4FAD+ mice, it is tempting to speculate that inhibiting the EGF receptors would exacerbate behavior and learning and memory dysfunction in E4FAD+ and E4FAD- mice, acutely or chronically. However, blocking EGF receptor tyrosine kinase activity with gefitinib has been reported to improve behavior in a model of AD-like pathology [71]. There are a number of plausible explanations for the apparent contrast in data between the current study and previous study including: mouse models, treatment duration, treatment paradigm and also the potential non-selectivity of gefitinib for the EGF receptor [72]. These data highlight the important of future research aimed at identifying the role of the EGF receptor in modulating cell-type specific function in an AD-relevant context.

In the current study, in the vehicle control groups EGF levels were detectable in the hippocampus but not the plasma. These data raise several important questions for further mechanistic study. The overall question of whether EGF levels and/or EGF receptor activation are disrupted by *APOE* genotype, aging, Aβ levels, or sex could reveal insight into the regulation of EGF in an AD-relevant context. For example, assessing whether endogenous plasma and brain EGF levels are linked, or are independent of one another. Interestingly, although both higher and lower plasma EGF levels have been reported in AD patients compared to controls [73, 74, 75, 76], a more recent study suggested that low EGF levels predict the conversion of amnestic MCI to AD [77]. Lower plasma levels of EGF are also associated in Parkinson's disease, which may imply that changes in plasma EGF are a general feature of neurodegenerative disorders rather than specific to AD. However, changes in plasma EGF levels may also be related to AD-risk factors. For example, a finding that plasma or brain levels of EGF are lower with *APOE4*, may also be linked to whether the beneficial effects of EGF treatment are restricted to female mice that express *APOE4* or are applicable to male and female mice regardless of *APOE* genotype and/or high Aβ levels. Addressing these questions may provide a deeper understanding on the role of EGF signaling in aging and AD.

There are limitations in the translation of findings from this study to cognitive dysfunction observed with *APOE4* in aging and AD. One is the lack of a comparison between EGF-treated E4FAD- and E4FAD+ mice

and either wild-type mice, younger E4FAD mice or mice expressing *APOE3*. Without such a comparison the extent that EGF resulted in lower fibrinogen extravasation and higher vessel coverage is unclear, and requires further study, with the addition of more frequent dosing and higher doses. We would also like to recognize a potential caveat in our longitudinal behavioral analysis. Although there was a reasonable time between repeated behavioral testing (1–2 months) to limit carry over effects, we cannot completely rule out that they did impact results. For example, EGF treatment may have resulted in improvements in rehearsal/repetition-based memory tests. It should be noted however, that mice in cohort 2 were not tested repeatedly, and similar results were obtained as cohort 1, although our *n* size was lower. There are number of additional steps required in order to translate our finding to human *APOE4* carriers including: identifying cell types and pathways that are important for the beneficial effects of EGF in mice: evaluating the extent that they contribute to behavioral deficits in *APOE4* mice throughout the lifespan and the interaction with other known pathologies that are relevant for aging and AD and; determining whether these pathways are also disrupted with *APOE4* during aging and/or in neurodegenerative disorders across all stages of the disease, with the caveat that there is likely high variability in the human conditions and it may not be possible to directly track such changes longitudinally.

5. Summary

Specific functions that contribute to *APOE4*-induced AD risk to remain unclear. Our data demonstrate that two-month EGF treatment improves behavior and neuronal and cerebrovascular function in female mice that express *APOE4* at an age of advanced pathology. These data suggest a mechanistic link between EGF signaling and *APOE4*-induced cognitive decline, and also suggest that therapies that target the cerebrovasculature may be efficacious even if administered at ages of more advanced dysfunction.

Declarations

Author contribution statement

Steve Zaldua, Frederick C. Damen, Kejia Cai, Felecia M Marottoli, Simon Alford, Leon M Tai: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rohan Pisharody, Riya Thomas, Kelly D Fan, Giri K. Ekkurthi, Sarah B. Scheinman, Sami Alahmadi: Performed the experiments.

Funding statement

This work was supported by National Institutes of Health Grants R01AG061114, R01AG061114-01S1 R21AG061715 and R21AG053876 and University of Illinois at Chicago institutional start-up funds (awarded to LMT). We would like to acknowledge that the use of the Keyence microscope was enable by UL1TR002003. KC is supported by R21EB023516 from the Institute of Biomedical Imaging and Bioengineering.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e03919.

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