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ORIGINAL ARTICLE

IGF1R signaling regulates astrocyte-mediated neurovascular coupling in mice: implications for brain aging

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Abstract Aging is associated with a significant deficiency in circulating insulin-like growth factor-1 (IGF-1), which has an important role in the pathogenesis of age-related vascular cognitive impairment (VCI). Impairment of moment-to-moment adjustment of regional cerebral blood flow via neurovascular coupling (NVC) importantly contributes to VCI. Previous studies established a causal link between circulating IGF-1 deficiency and neurovascular dysfunction. Release of vasodilator mediators from activated astrocytes plays a key role in NVC. To determine the impact of impaired IGF-1 signaling on astrocytic function, astrocyte-mediated

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NVC responses were studied in a novel mouse model of astrocyte-specific knockout of IGF1R (*GFAP-Cre^{ERT2}/Igf1r$^{f/f}$*) and accelerated neurovascular aging. We found that mice with disrupted astrocytic IGF1R signaling exhibit impaired NVC responses, decreased stimulated release of the vasodilator gliotransmitter epoxyeicosatrienoic acids (EETs), and upregulation of soluble epoxy hydrolase (sEH), which metabolizes and inactivates EETs. Collectively, our findings provide additional evidence that IGF-1 promotes astrocyte health and maintains normal NVC, protecting cognitive health.

Keywords Insulin-like growth factor 1 · IGF-1 · Vascular cognitive impairment · VCI · Functional hyperemia · Astrocyte · Arachidonic acid metabolites · Neurovascular uncoupling · Cerebrovascular · Neurovascular aging

Introduction

Vascular cognitive impairment (VCI) in the aging population has emerged as one of the major public health challenges in the Western world [1–3]. In addition to pathological alterations of the larger cerebral arteries (e.g., atherosclerosis), functional impairment of the cerebral microcirculation also contributes significantly to the pathogenesis of VCI [4]. In recent years, potentially reversible functional alterations of the aging neurovascular unit have garnered much attention as they have the potential to impair local regulation of cerebral blood flow (CBF) and have been causally linked to neuronal dysfunction [2, 4]. The neurovascular unit is a complex functional and anatomical structure, which consists of perivascular astrocytes (the most abundant glial cells in the brain, which outnumber neurons by fivefold), endothelial cells, vascular smooth muscle cells and pericytes, microglia, and neurons. One area of particular interest when it comes to understanding the contribution of the neurovascular unit to the pathogenesis of VCI is the role of impairment of neurovascular coupling (NVC), a critical homeostatic mechanism responsible for adjusting regional CBF to local neural activity [5]. Upon neuronal activation, the resulting functional hyperemia contributes to the maintenance of an optimal local microenvironment in the active brain region, ensuring adequate delivery of oxygen and glucose, and effective removal of potentially harmful metabolic by-products. Aging results in marked impairment of NVC, which likely contributes to cognitive dysfunction in elderly patients and aged laboratory animals [6–12].

The cellular mechanisms underlying NVC include astrocyte activation induced by neurotransmitters released from firing neurons and consequential astrocytic release of vasodilator mediators that induce prompt vasodilation in arterioles supplying the active brain region [13–16]. Gliotransmitters involved in mediation of NVC responses include vasodilator metabolites of arachidonic acid, including epoxyeicosatrienoic acids (EETs) produced by cytochrome p450 enzymes [17–19]. EETs released from astrocytes elicit dilation of resistance arterioles by activating K^+ channels in the arteriolar smooth muscle cells [20]. Additional mechanisms that contribute to dilation of resistance arterioles induced by astrocyte activation include purinergic mechanisms, release of prostaglandins, and K^+ channel activation [13, 16, 21–30]. Despite the critical role of astrocytes in mediation of functional hyperemia, the impact of shared molecular and cellular mechanisms of aging on astrocyte-mediated NVC responses is not yet fully understood.

There is growing evidence in support of the concept that cell non-autonomous mechanisms of aging play a critical role in brain and neurovascular aging [31–37]. Insulin-like growth factor-1 (IGF-1) is an anabolic hormone produced by the liver and, locally, by diverse cells within the CNS, which exerts multifaceted neuroprotective, vasoprotective, and anti-geronic effects [5, 23, 31, 38–62]. Both circulating and brain concentrations of free IGF-1 decrease significantly with age in humans and in laboratory animals due to an age-related decline in GH production/release [31, 62–65]. Astrocytes abundantly express IGF1R, the receptor for IGF-1, and there is strong evidence that disruption of IGF1R signaling results in marked changes in astrocyte phenotype, which associate with cognitive impairment [50]. Previous studies also demonstrate that circulating IGF-1 deficiency also leads to impaired NVC responses [23]. Despite these advances, the specific role of IGF1R signaling in

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regulation of astrocytic mediation of NVC responses remains elusive.

The present study was designed to test the hypotheses that IGF1R signaling regulates astrocyte-mediated NVC responses and synthesis/release of vasodilator eicosanoid gliotransmitters in the brain and that disruption of IGF1R signaling in astrocytes impair gliovascular coupling, mimicking aspects of the aging phenotype. To test our hypotheses, we used a novel mouse model with adult-onset, astrocyte-specific disruption of IGF1R signaling using Cre-lox technology (*GFAP-Cre^{ERT2}/Igf1r$^{f/f}$*) [50]. NVC, synthesis of eicosanoid gliotransmitters, and expression of soluble epoxy hydrolase, which degrades EETs, were tested.

Methods

Animals

Igf1r$^{f/f}$ (B6;129-Igf1rtm2Arge/J; *loxP* sites flanking exon 3) and *GFAP-Cre ^{ERT2}* (B6.Cg-TgGFAP-cre/ERT2/505Fmv/J) mice were obtained from Jackson laboratories. Mice were housed (3–4 per cage) in Allentown XJ cages with Anderson's Enrich-o-cob bedding (Maumee, OH). *Igf1r$^{f/f}$* mice were bred in house to generate experimental cohorts. Animals were housed under specific pathogen-free (including helicobacter and parvovirus free) barrier conditions in the Rodent Barrier Facility at University of Oklahoma Health Sciences Center. Mice were bred on a 14-h light/10-h dark cycle and weaned mice were maintained in a 12-h light/12-h dark cycle at 21 °C and were given access to standard irradiated bacteria-free rodent chow (5053 Pico Lab, Purina Mills, Richmond, IN) and reverse osmosis filtered water ad libitum. *GFAP-Cre^{ERT2}* (males) mice were bred with *Igf1r$^{f/f}$* (females) to generate *GFAP-Cre^{ERT2}/Igf1r$^{+/-}$* males, which were bred with *Igf1r$^{f/f}$* female mice to obtain the founder colony of *Cre+/Igf1r* homozygous floxed mice as previously described [50]. These mice were allowed to breed with *Igf1r$^{f/f}$* mice to generate experimental cohorts of *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* and *Cre-/Igf1r$^{f/f}$* control mice. Mice were injected intraperitoneally with tamoxifen (75 mg tamoxifen/kg body weight) dissolved in corn oil or sham (corn oil) only for 5 days at 3 months. Mice were allowed to recover for 2 months before initiation of experiments. All procedures were approved by the Institutional Animal Use and Care Committee of the University of Oklahoma Health Sciences Center.

Measurement of neurovascular coupling responses

On the day of experimentation, mice in each group were anesthetized with isoflurane (4% induction and 1% maintenance), endotracheally intubated, and ventilated (MousVent G500; Kent Scientific Co, Torrington, CT). A thermostatic heating pad (Kent Scientific Co, Torrington, CT) was used to maintain rectal temperature at 37°C [12]. End-tidal CO_2 was controlled between 3.2 and 3.7% to keep blood gas values within the physiological range, as described [23, 66]. The right femoral artery was canulated for arterial blood pressure measurement (Living Systems Instrumentations, Burlington, VT) [12]. The blood pressure was within the physiological range throughout the experiments (90–110 mmHg). Mice were immobilized and placed on a stereotaxic frame (Leica Microsystems, Buffalo Grove, IL), the scalp and periosteum were pulled aside, and the skull was gently thinned using a dental drill while cooled with dripping buffer. A laser speckle contrast imager (Perimed, Järfälla, Sweden) was placed 10 cm above the thinned skull, and to achieve the highest CBF response, the right whiskers were stimulated for 30 s at 10 Hz from side to side as described [67, 68]. Differential perfusion maps of the brain surface were captured. Changes in CBF were assessed above the left barrel cortex in six trials in each group, separated by 5–10-min intervals. To assess the role of EETs in mediation of NVC, CBF responses to whisker stimulation were repeated after administrating the epoxigenase inhibitor MSSPOH (N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide; Cayman Chemicals; 20 mg/kg, dissolved in DMSO and diluted to final concentration with 45% cyclodextrin, Cayman Chemicals) which inhibits EET production [23, 69]. MS-PPOH is a selective inhibitor of the epoxygenation reactions catalyzed by specific CYP450 isozymes [70]. MS-PPOH inhibits the formation of arachidonate 11,12-epoxides by CYP4A2 and CYP4A3 enzymes with an IC50 value of 13 μM, but has no effect on the formation of 20-HETE, the ω-hydroxylation product of CYP4A1 [71]. Changes in CBF were averaged and expressed as percent (%) increase from the baseline value [72]. Experiments lasted <1 h/mouse, which permitted stable physiological parameters to be obtained. In each study, the experimenter was blinded to the treatment of the

animals. At the end of the experiments, the animals (with the exception of those assigned to brain lipidomics studies) were transcardially perfused with ice-cold PBS and decapitated. Animals assigned to brain lipidomics studies were decapitated without perfusion to avoid wash-out of lipid mediators. The brains were immediately removed and samples were collected for subsequent studies. All reagents used in this study were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise indicated.

Measurement of glutamate-induced release of EETs from acute hippocampal slices

To determine how disruption of IGF1R signaling affects synthesis of eicosanoid gliotransmitters, horizontal hippocampal slices of 325 μm thickness from mice in each cohort were prepared using a HM650V vibrating microtome (Thermo Scientific) in ice cold solution containing (in mmol/L) sucrose 110, NaCl 60, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 28, sodium ascorbate acid 0.6, glucose 5, $MgCl_2$ 7, and $CaCl_2$ 0.5 as reported [73]. Slices were then transferred to a holding chamber containing oxygenated artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.25, $MgCl_2$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10, pyruvic acid 2, ascorbic acid 0.4. Slices were left to recover for at least 60 min at room temperature prior to experimentation, then were transferred to a 24-well plate containing oxygenated aCSF, 2 slices per plate. Five minutes later, 500uL of aCSF was removed, mixed with 1mL of LC-MS grade methanol (ThermoFisher, A456-1), snap frozen, and used for control purposes. To activate astrocytes, glutamate (3×10^{-4} mol/L, for 5 min) was added to the chamber. Then, the aCSF was removed, mixed with 1mL of LC-MS grade methanol, and snap-frozen for analyses. Brain slices were snap frozen for protein concentration analyses to normalize the lipidomics data. Identification and quantification of EET gliotransmitters involved in NVC responses (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET and their degradation products, 5,6-DiHET [5,6-dihydroxy-eicosatrienoic acid], 8,9-DiHET, 11,12-DiHET, and 14,15-DiHET), by LC-MS/MS was performed by the Schwartzman laboratory using a Shimadzu Triple Quadrupole Mass Spectrometer LCMS-8050 and multiple reaction monitoring mode [74]. Protein concentrations from frozen brain slices were used to normalize the data.

Western blotting

Cortex samples were homogenized in RIPA buffer containing HALT protease and phosphatase inhibitors. The tissue homogenate was centrifuged at 12,000rpm for 10 min and the supernatant was isolated for protein estimation by Thermoscientific BCA assay. Equal amounts of protein (45μg) were resolved on NuPAGE 10% Bis-Tris Midi gel and then transferred on to a PVDF membrane using semi-dry transfer method (BioRad). The membranes were blocked using 5% BSA in Tris-buffered saline-Tween 20 (TBST) for 1h at RT followed by overnight incubation with the following primary antibodies at 4°C: IGFR1β (1:1000, Cell Signaling); sEH (1:200, Cayman Chemicals); and β-actin (1:5000, Abcam). The membranes were washed 3X with TBST and then incubated with the respective HRP-conjugated antibodies (Abcam, 1:10000) for 1 h at RT. The membranes were washed again 3X with TBST and developed using SuperSignal West Pico or SuperSignal West Femto chemiluminescent substrate solutions (Thermo Fisher Scientific). Digital images were obtained using myECL imaging system (Thermoscientific) and the densitometric analysis was performed using Fiji software.

Statistical analysis

Statistical analysis was carried out by unpaired *t* test or one-way ANOVA followed by Bonferroni multiple comparison test, as appropriate, using Prism 5.0 for Windows (Graphpad Software, La Jolla, CA). A *p* value less than 0.05 was considered statistically significant. Data are expressed as mean ± S.E.M.

Results

Astrocyte-specific disruption of IGF-1/IGF1R signaling impairs neurovascular coupling

Changes in CBF in the whisker barrel cortex in response to contralateral whisker stimulation were significantly attenuated in *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice (Fig. 1a–c), indicating that astrocyte-specific disruption of IGF1R signaling leads to neurovascular uncoupling.

Upon activation by neuronal-derived glutamate, astrocytes were shown to convert arachidonic acid by P450 epoxygenase to vasodilator EETs [16, 23].

Consistent with this concept, we found that in control animals, administration of the P450 epoxygenase inhibitor MS-PPOH (Fig. 1b–c) significantly decreased CBF responses in the barrel cortex elicited by contralateral whisker stimulation. In *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice, the effects of MS-PPOH (Fig. 1b–c) were significantly decreased, suggesting that astrocyte-specific disruption of IGF1R signaling impairs mediation of NVC by EETs.

LC/MS/MS measurements demonstrated that astrocyte-specific disruption of IGF1R signaling resulted in a diminished cerebral production of EETs in response to glutamate stimulation of brain slices (Fig. 2).

Astrocyte-specific disruption of IGF1R signaling results in upregulation of sEH

Western blot analysis showed that in whole brain samples derived from *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$*, mice expression of IGF1R was significantly decreased, as compared to control mice, consistent with a successful knockdown of IGF1R in the astrocyte compartment but not in other cellular compartments (Fig. 3a, b). Protein expression of sEH was significantly increased in *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice as compared to control mice (Fig. 3c).

Discussion

In astrocytes, upon neuronal activation and glutamate release from the synapses, a calcium wave is initiated, leading to activation of the synthesis/release of vasodilator metabolites of arachidonic acid, including EETs that have an important role in mediation of NVC responses [16, 24, 75]. The present study provides critical evidence that cell-specific disruption of IGF1R signaling in astrocytes alters their phenotype, impairing EET-mediated gliovascular coupling responses. These findings are consistent with the results of previous studies showing that circulating IGF-1 deficiency, which decreases cerebral IGF-1 levels, also impairs the astrocyte-mediated NVC [23]. The mechanisms by which disruption of astrocytic IGF-1/IGF1R signaling impairs EET-mediated gliovascular coupling may include upregulation of sEH and altered expression of cytochrome P-450 enzymes [23]. Further studies are warranted to

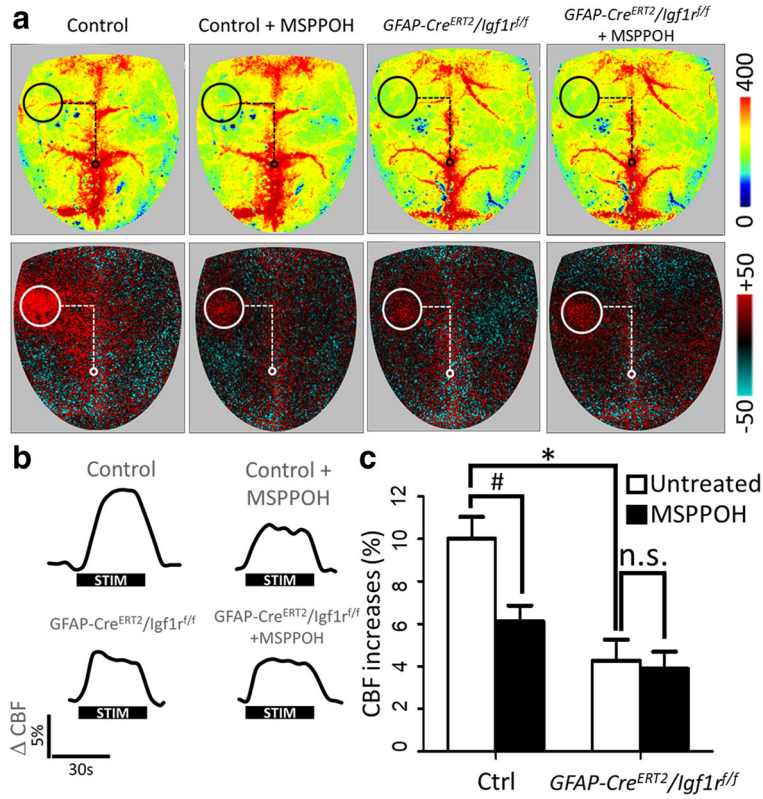

Fig. 1 Astrocyte-specific disruption of IGF1R signaling impairs neurovascular coupling responses. **a** Representative pseudocolour laser speckle flowmetry maps of baseline CBF (upper row; shown for orientation purposes) and CBF changes in the whisker barrel field relative to baseline during contralateral whisker stimulation (bottom row, right oval, 30 s, 5 Hz) in control and *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice before and after administration of the P450 epoxygenase inhibitor MS-PPOH. Panel **b** shows the time-course of CBF changes after the start of contralateral whisker stimulation (horizontal bars). Summary data are shown in panel **c**. Data are mean ± S.E.M. (n=6–10 in each group), *P<0.05 vs. control; $^{\#}P$<0.05 vs. untreated. n.s. not significant

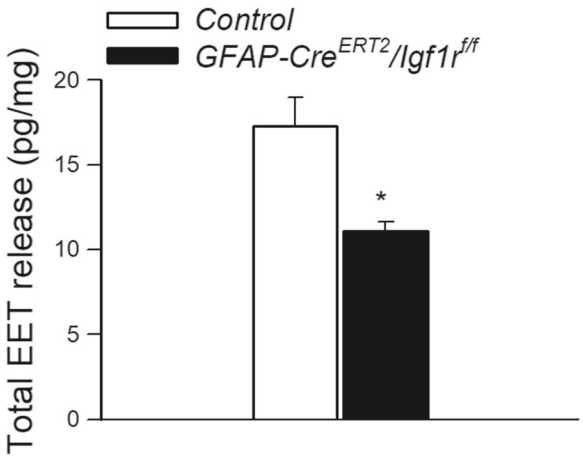

Fig. 2 Astrocyte-specific disruption of IGF1R signaling impairs glutamate-induced release of eicosanoid gliotransmitters. Shown is production of EETs in glutamate-activated brain slices from control and *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice, as measured by liquid chromatography/mass spectrometry (LC/MS). Data are mean ± S.E.M. (n=6–10 in each group), *P<0.05 vs. control (see the "Methods" section)

investigate transcriptional changes in detail in the astrocytic cellular compartment in *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice. Future studies should also determine the effects of pharmacological inhibitors of sEH on NVC responses and EET release in *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice.

On the basis of the available evidence [66, 76], we propose that neurovascular dysfunction associated with disruption of IGF-1/IGF1R signaling in astrocytes may contribute to the cognitive defects observed in *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice [50]. Previous studies showed that in addition to regulating gliovascular coupling mechanisms, IGF-1/IGF1R signaling also regulates many other important aspects of astrocyte function, including mitochondrial energy metabolism [50, 77], ROS metabolism [50, 78, 79], glucose uptake [80–82], and glutamate transport [49, 83]. Perivascular astrocytic endfeet contain a large number of mitochondria [84], and based on previous research [50], disruption of energy metabolism and increased oxidative stress from IGF-1/IGF1R signaling deficiency in astrocytes could impair calcium buffering necessary for NVC. Thus, disruption of IGF-1/IGF1R signaling may also impact these aspects of astrocyte function, which are also likely to contribute to cognitive impairment in *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice. IGF-1 can enter the brain from the circulation by transcytosis across the blood brain barrier [85]. On the basis of the aforementioned observations, one may expect that interventions that increase circulating IGF-1 will also increase IGF-1 levels in the aged brain, which would positively impact astrocyte function and may improve NVC and cognition. In humans, IGF-1 deficiency leads to cognitive dysfunction that can be ameliorated by interventions increasing circulating IGF-1 levels [31]. Aged rodents exhibit a similar decline in circulating IGF-1 levels and treatment of aged rats with IGF-1 was shown to partially rescue cognitive

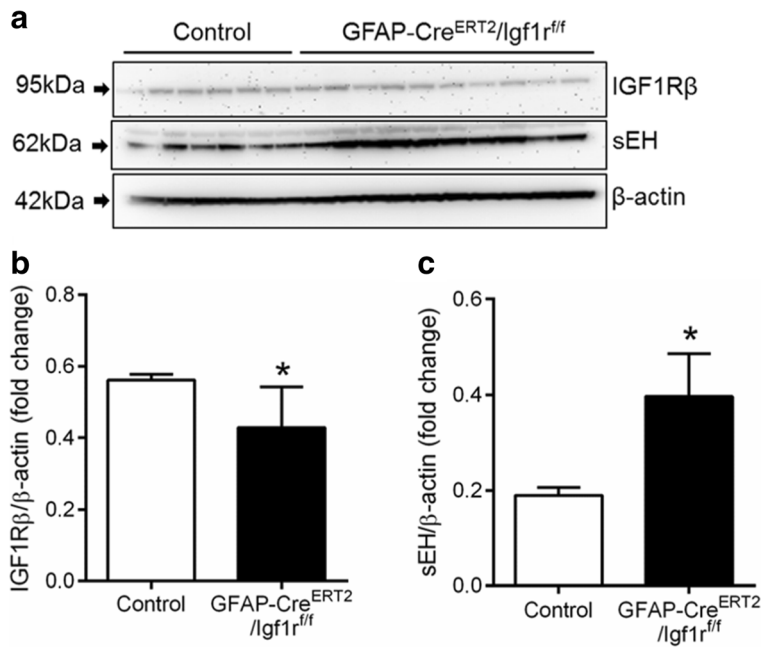

Fig. 3 Upregulation of sEH in mice with astrocyte-specific disruption of IGF1R signaling. **a** Representative Western blot showing upregulation of soluble epoxide hydrolase (sEH), a key enzyme in the metabolism of vasodilatory epoxyeicosatrienoic acids, and decreased expression of IGF1Rβ in cortical samples derived from tamoxifen-treated *GFAP-Cre^{ERT2}/Igf1r$^{f/f}$* mice, as compared to control mice. **b**–**c** Bar graphs are summary densitometric data showing expression of IGF1Rβ (**a**) and sEH (**b**), normalized to β-actin expression. Data are normalized to control mean values and are expressed as fold changes. Data are mean ± S.E.M. (n=6–10 in each group), *P<0.05 vs. control

impairment [31]. Future studies are warranted to determine how astrocytic NVC responses are impacted in the aforementioned experimental settings. The effects of strategies aimed at selectively increasing central IGF-1 action on neurovascular and cognitive outcomes have been discordant. Specifically, studies on an inducible, brain-specific (*TRE-IGF-1* × *Camk2a-tTA*) IGF-1 overexpression mouse model showed that increases in central IGF-1 in aging can improve various domains of cognition without significantly affecting gliovascular coupling responses [39]. This further adds to the complexity of IGF-1 actions that may be cell-type and region specific within the brain. IGF-1 has been shown to have pleiotropic, sex-, and tissue-specific effects [86, 87]. In that regard, it should be noted that the effects of age-related circulating IGF-1 deficiency on the cellular mechanisms involved in NVC are likely multifaceted. Cerebromicrovascular endothelial cells are directly exposed to circulating IGF-1 and are known to abundantly express IGF-1 receptors [32]. Ample evidence supports the view that endothelial cells also play a key role in mediation of functional hyperemia [22] and that aging critically impairs endothelium-mediated NVC responses [11, 12, 67, 68]. Because previous studies demonstrate that mouse models of circulating IGF-1 deficiency also exhibit cerebromicrovascular endothelial dysfunction and impaired endothelium-mediated NVC responses [23], in future studies, the effects of endothelium-specific disruption of IGFR-1 signaling on functional hyperemia should also be determined.

Taken together, our findings add to the growing evidence that deficient IGF-1 input to astrocytes compromises their function, impairing gliovascular coupling responses and likely multiple other aspects of brain health. The findings that disruption of IGF-1/IGF1R signaling results in functional and phenotypic alterations in astrocytes have important clinical relevance for cognitive impairment associated both with advanced age and genetic IGF-1 deficiency (e.g., growth hormone releasing hormone-receptor [GHRH-R] defect; isolated GH deficiency; GH receptor gene defects [Laron syndrome]). Furthermore, expression of IGF1R and downstream signaling transcripts are decreased in astrocytes in human brain with progression of Alzheimer's neuropathology [88]. Of note, multiple IGF1R mutations have been diagnosed in children born *small for gestational age* (SGA) [89, 90], who have lower IQ scores than age-matched control subjects [91]. Future studies determining how IGF1R mutations in humans affect gliovascular coupling and regulation of CBF should be quite revealing. Our present findings, taken together with the results of previous studies [31], point to potential multifaceted benefits of interventions improving IGF-1 input to the brain in aging.

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Declarations

Conflict of interest The authors declare no competing interests.

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