HONR 399H-XX: Undergraduate Honors Thesis Research

Title: Roles of Astrocyte-Derived Estrogen in the Brain

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Abstract

The steroid hormone, 17β-estradiol (E2) is an important hormone that regulates many functions in the body. Traditionally, E2 was believed to be produced primarily by the ovaries in females, but a number of studies have shown that brain cells such as neurons and astrocytes can also make significant quantities of E2. The study presented in this thesis examined the role of astrocyte-derived E2 in exerting neuroprotection in the CA1 region of the hippocampus, as well as its ability to regulate two specific pathways implicated in neuroprotection - the LIF and STAT3 pathways. Since the hippocampal CA1 region is known to be highly vulnerable to global cerebral ischemia (GCI), such as occurs after cardiac arrest, we used a mouse GCI model to examine the neuroprotective role of astrocyte-derived E2 in the hippocampal CA1 region. The results of the study indicate that mice that lack the enzyme aromatase in astrocytes and were unable to produce astrocyte-derived E2, have decreased reactive astrocyte activation after GCI, greater neuronal deficits after GCI in both genders, and they have significantly decreased LIF-STAT3 signaling in the hippocampus.

Acknowledgements

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helped me gain confidence to present to work at conferences. I am truly thankful for this irreplaceable and unforgettable experience.

Introduction

Estrogens are steroid hormones that are generated and synthesized primarily by the ovaries in females, and traditionally observed to regulate reproductive function. Estrogens are released by the ovaries into the bloodstream, where they circulate to regulate various target organs in the body. There are three types of estrogen; estrone, estriol, and 17β-estradiol (estradiol). The most potent of these estrogens is estradiol (E2) [1]. Intriguingly, recent studies in a variety of species showed that E2 is not only produced in the ovaries but also produced in the male and female brain [2]. The term "brain-derived E2" has been coined to describe E2 generated in the brain rather than the ovaries. Previous studies have indicated that brain-derived E2 has neuroprotective functions that protects neurons from damage in response to ischemic injury [2]. A key enzyme that synthesizes E2 from androgen precursors is called aromatase. Aromatase is primarily detected in neurons of the brain but it is highly upregulated in astrocytes following a brain injury [3]. Astrocytes are glial cells abundant in the brain that are located throughout the central nervous system (CNS). Astrocytes can become highly activated or hypertrophic (enlarged) in the hippocampus after brain injuries such as Global Cerebral Ischemia (GCI) - an insufficient blood flow to the brain. In contrast, in the basal non-ischemic state, astrocytes are in a less active state, but still exert important neurotrophic functions [4]. Previous work has suggested that astrocyte-derived E2 may have a neuroprotective effect after brain ischemia, but the mechanisms is poorly understood. The Leukemia Inhibitory Factor (LIF) pathway and the Signal Transducer and Activator of Transcription 3 (STAT3) pathway; also known as LIF/STAT3 pathway has been suggested to regulate astrogliosis – activation of astrocytes. According to prior RNA sequencing analysis conducted in Dr. Darrell Brann's lab, LIF/STAT3 is suggested to be among the top regulated pathways by astrocyte-derived E2 after

GCI [4]. Therefore, this research will focus on the potential role of astrocyte-derived E2 in regulating neuroprotection, neuroinflammation and astrocyte activation after GCI, and whether this regulatory function is linked to the LIF/STAT3 pathway.

Hypothesis

Astrocytes-derived estrogen in the brain regulates the activation of astrocytes in the hippocampus after Global Cerebral Ischemia and results in neuroprotective function through Leukemia inhibitory factor (LIF) and Signal Transducer and Activator of Transcription 3 (STAT3) signaling pathways.

Ovarian-derived E2

Ovarian-derived E2, which contributes significantly to peripheral circulating estrogen levels in the blood, has been extensively studied and suggested to exert neuroprotective functions against a variety of neurodegenerative disorders, including strokes, Alzheimer's disease (AD), and Parkinson's disease (PD) [5]. Thus, better understanding of the mechanisms underlying beneficial estrogen actions in the brain could lead to new insights for neurological diseases therapy [5]. It is well established that gender differences exist in the onset and the severity of neurodegenerative diseases. Premenopausal women have high circulating E2 levels and have been shown to be "protected" against stroke, at least until the time of menopause [6]. Further, past literatures showed a parallel correlation between a higher risk of stroke in postmenopausal women and the decrease of circulating E2 levels [6]. Therefore, peripheral estrogen production emphasizes the importance of neuroprotective functions. In other words, the estrogen produced in female ovaries has been classically viewed to play an important role in the neuroprotective functions in the brain, such as protection against neurodegenerative diseases.

Aromatase in male and female brain

While there is a considerable literature on the neuroprotective actions of ovarian-derived circulating E2 in females, comparatively less is known on the role of E2 produced in the brain. Prior data collected from Dr. Darrell Brann's lab showed that aromatase is significantly expressed in the mouse hippocampus, and enzyme-linked immunosorbent assays (ELISA) for E2 revealed high levels of E2 (1 nM) that are being produced locally in the brain – several folds higher than E2 levels in the blood. Intriguingly, both the male and female brain exhibited high levels of aromatase and E2 in rodents, and in almost all species examined to date, including humans [6]. Therefore, it is essential to study the role of brain-derived E2 and how it is related to neuroprotective functions, which may be especially crucial in males and postmenopausal females.

The Hippocampus

The brain is perhaps the most complex organ in the human body. It is composed of many structural parts and brain cells that synchronizes together to carry out body functions. Miscommunication or damage of those neurons can potentially be fatal. The hippocampus, is a key part of the forebrain that is mainly associated with storage of long-term memories. Since the hippocampus is the most vulnerable brain site to GCI, this research will focus upon the hippocampus. Furthermore, it has been reported that cardiac arrest associated with GCI is a major cause of death and long-term disability in the global population. Even though the chances of survival after cardiac arrest in the U.S. population is 10.6%, the chances that those people survive with a good neurological function is approximately 8.3% [7]. In stroke patients, when the circulation to the brain is cut off, the brain cells lack oxygen necessary to perform metabolic functions and begin to die. Similarity, in the GCI model, there's a disruption of an ionic balance,

hypoxia, oxidative stress, cell swelling, inflammation, excitotoxicity, and apoptosis that leads to the loss of brain activities [7]. Thus, the GCI model is commonly used to understand the impairment in vital brain activities after ischemia.

Generating Aromatase Knockout Mice

To address whether astrocytes-derived E2 regulates astrocytes activation after GCI, astrocyte-specific aromatase knockout mice were generated to study the role of astrocyte-derived E2 in the brain. In other words, mice that lack the expression of the aromatase gene in their astrocytes was created. To do this, Dr. Darrell Brann and Dr. Ratna Vadlamudi, co-principal investigators of the study, first utilized a company called Gen-Way to generate ARO-Floxed mouse.

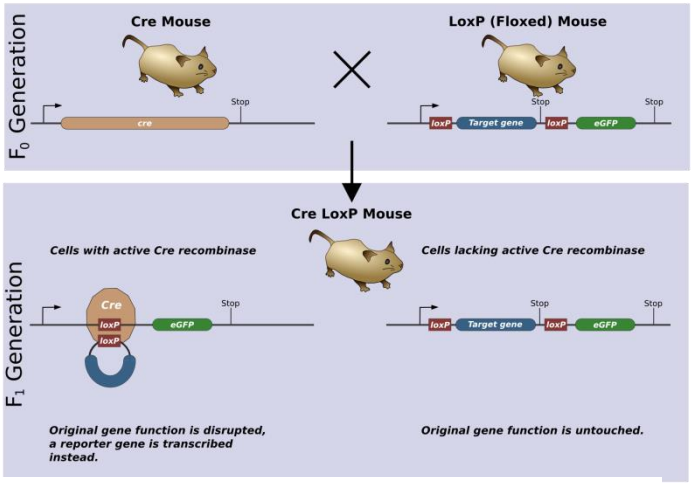

Fig. 1. Generation of Cre/LoxP Mouse [8].

The Flox (loxP) mouse has a loxP site that specifically marks the target gene (shown at top right in Fig. 1). In this case, the target gene is the aromatase allele (Cyp19a1). Then, in order to generate KO mouse there must be an enzyme that comes in and "cuts" the target gene where the loxP sites were marked. This is done by crossing Flox (loxP) mouse and Cre mouse together in F_0 generation (Fig.1). This action will conditionally delete locally synthesized aromatase in the astrocytes [8].

The Cre mouse has an enzyme that will cut the target gene on both sides where loxP sites are located. As a result, Cyp19a1 Cre/LoxP conditional KO mice is generated, under the control of the Glial Fibrillary Acidic Protein (GFAP) promoter that expresses Cre exclusively in brain astrocytes. Thus, this protein can be used to observe astrocyte activity levels in the hippocampus

after GCI. Moreover, we will look at the neuron's protein activity as well. This is done by co-staining neurons with an antibody to NeuN, which is a protein commonly used as a biomarker for neurons [15].

The GFAP cre is an astrocyte specific promoter used to target deletion of part of the coding sequence of the aromatase gene specifically in astrocytes [9]. This leads to loss of E2 production in astrocytes. Once generated, the GFAP-ARO-KO mice and FLOX mice were subjected to GCI to elucidate the role of astrocyte-derived E2 in the brain. The preliminary studies showed that there is a significant increase of neuronal damage in both the male and female KO hippocampus at day 3 after GCI, as well as an enhanced activation of another type of inflammatory glial cell, called microglia. Thus, this suggests that the astrocyte-derived E2 in the brain will exhibit a potential neuroprotective role after a brain injury in this study.

Sham mice will be used as controls. The sham mice will undergo the same surgery procedure as the GCI mice, but the carotid arteries will not be clipped in sham mice. Thus, the mice do not experience GCI and oxygen delivery to the brain is not impaired. The sham mice serve as an important control to make sure that effects seen in GCI mice the study are not due to surgical stress that is part of the GCI procedure, but rather due to ischemia.

Pathways Regulation

Analysis of the RNA-sequencing data will be used to examine differential gene expression that is affected after GCI in both GFAP-ARO-KO mice and FLOX mice. One

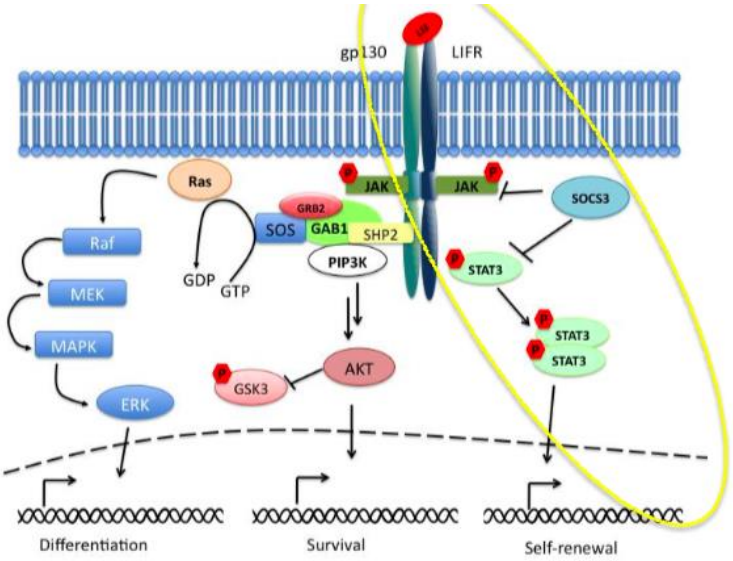

Fig. 2. Schematic representation of the LIF-pathway [10].

of the top regulated pathways in the RNA-sequence analysis was the LIF/STAT3 pathway (Fig.2). The top genes in this pathway are shown in Table 1. In the current study, we will use several techniques to confirm changes in key LIF/STAT3 pathway factors in the hippocampus of both GFAP-ARO-KO mice and FLOX mice after GCI. The techniques that will be used include 1) ELISA (Enzyme-Linked Immunosorbent Assay) test, 2) Immunofluorescent staining, and 3) Western blot analysis.

Table 1. Genes/proteins effected by the leukemia inhibitory factor (LIF)—pathway [10].

LIF	gp130	LIFRβ	STAT3	SOCS3	JAK1	JAK2	Shp2

Materials and Methods

The study in this thesis project regarding the animals and GCI were conducted on samples collected previously in Dr. Brann's lab. The samples were collected from the hippocampus of adult 3-month-old male and female FLOX and GFAP-ARO-KO mice 3 days after GCI. SHAM mice were included as a control. To thoroughly examine the genetic markers of the mice, several tests were performed. For collection of protein samples for use in ELISA and Western blot tests, hippocampus tissue of each mouse was dissected, protein extracted and then stored as described previously by Brann lab (2,4,5). For immunohistochemistry analysis, brain sections were collected and stored until use as described previously (4,5). The group of mice used in the study are described in Table 2.

Table 2. Modified mice categories and its meaning

FLOX-SHAM	Mice with aromatase, no GCI
KO-SHAM	Mice without aromatase, no GCI
FLOX-G3D	Mice with aromatase, 3 days after GCI
GFAP-ARO-KO	Mice without aromatase in astrocytes, 3 days after GCI

Immunofluorescent Staining:

The immunofluorescent staining process visualizes and detects cells that express a certain protein of interest that can be viewed using confocal microscopy and captured into images [4].

Hippocampal brain sections from the groups listed above were washed 3 times for the duration of 10 minutes in a PBS solution. The sections were then incubated in PBS solution that has a nuclear staining solution for 30 minutes in room temperature. The sections were later blocked at room temperature in a blocking solution of PBS with 5% donkey serum for 1 hour. This is to avoid unspecific binding of the antibody to the samples. The samples were then incubated with a primary antibody against the protein of interest overnight at 4°C. The next day, those sections were washed 3 times again for 10 minutes using the PBS solution. Then the fluorophore-conjugated secondary antibody was applied to the slide that is diluted in PBS with 1% serum. This step is done in the dark to avoid photobleaching. After one hour of incubation in room temperature, those sections were later mounted using compatible mounting medium and a coverslip is added on the slides. The slides were then viewed using a confocal laser microscope so as to capture images of cells that express the protein of interest. The captured images were then analyzed using the lab's imaging software.

ELISA (Enzyme-Linked Immunosorbent Assay):

The ELISA procedure measures the overall LIF protein levels in hippocampal samples [13]. The ELISA procedure allows a more quantitative analysis than immunofluorescent staining of hippocampal sections for LIF.

Seven tubes were prepared individually with labels (700 pg/mL, 350 pg/mL, 175 pg/mL, 87. 5 pg/mL, 43.8 pg/mL, 21.9 pg/mL, and 11 pg/mL). Then 200 μL of the Mouse LIF Standard

was added into the prepared tubes, and serial dilutions were performed. Then, 50 μL of Assay Diluent were added into each well. This is to assist in matrix equalization in problematic serum, plasma or cell samples [16]. Then 50 μL of standard, control, and sample were added into each well. Aspirate each well and wash with the wash buffer for total of 5 washes. Then, 100 μL of Mouse LIF conjugate was added into the wells, and the plate was sealed with an adhesive strip and incubated for 1 hour and 30 minutes. Then 100 μL of substrate solution was added. This is the color reagents that should be protected from light. The final step is addition of 100 μL of stop solution to each well. The color turns from blue to yellow and absorbance (optical density) at 450 nm can be read using a microplate reader and calculated to the standard curve to compare the changes in KO and FLOX mice.

Western Blotting:

The Western blot test is a technique used to visualize and detect a specific protein in a sample of tissue or cells [4]. It allows separation of proteins by their molecular weight and semi-quantitative analysis of the levels of the proteins. Prior to performing the Western blot, protein assays were done beforehand in order to make sure we have equal amounts of protein in each well. The Western blot analysis is done by assembling the cassette into the Mini-PROTEAN Tetra Cell machine [14]. Running buffer is added to the inner and outer chambers to avoid overheating from the electricity. Then a syringe is used to transfer loading samples (KO-SHAM, FLOX-SHAM, FLOX-G3D, etc.) into the gel within the cassette. The gel is run for 1 hour and 40 minutes at 110 volts and stopped once the dye front reaches the reference line. The gel is then placed on top of the membrane and run again for 2 hours at 170 volts. The purpose of a membrane is for supporting the gel. Without it, the gel will melt and the proteins will be degraded due to the high voltage that generates heat. Once the timer goes off, the membrane is

taken out and blocking solution is added and incubated for 1 hour. Then, primary antibodies (goat serum) are added and incubated in 4 °C freezer overnight. The next day, the samples are washed 3 times with a wash buffer, and secondary antibody (anti-goat serum) is added and incubated for 1 hour. After another round of washing, the bands are scanned and visualized under the imaging system. The band densities for each protein are compared to the loading controls [4].

Results

Table 3. Symbol Keys Locate in the Graphs

#	P-value less than 0.05 ($p < 0.05$) on FLOX mice
##	P-value less than 0.001 ($p < 0.01$) on FLOX mice
*	P value less than 0.05 ($p < 0.05$) on KO mice
**	P-value less than 0.001 ($p < 0.01$) on KO mice

Note: Smaller p-value suggested as a stronger evidence in favor of the hypothesis, thus, suggesting that there are significant statistical differences. P-value below 0.05 is considered significant.

We first looked at the GFAP and NeuN immunoprotein expression in the hippocampus using immunofluorescent staining. This was done to visualize and detect reactive astrocytes and neurons, since GFAP is a marker of reactive astrocytes, and NeuN is a marker of neurons (Fig. 3). All of the results were taken of the mice 3 days after GCI.

In Fig. 3, the photomicrographs in the first column provides an overview picture of the whole hippocampus and the CA1 area. NeuN protein was immuno-stained in green color and GFAP protein was immuno-stained in red color. The top two rows in Fig. 3A region, showed FLOX-SHAM and KO-SHAM as controls. When compared together in the merged column, there were no significant difference in GFAP and NeuN staining in the hippocampal CA1 region of FLOX and KO sham mice. Moreover, the quantitative data in Fig. 3B and Fig. 3C for SHAM mice did not show significant changes. This indicates that the surgery to expose the carotid

arteries (which were not clamped in sham animals) did not affect NeuN or GFAP immunoreactive levels in the hippocampus.

We next examined the cell GFAP and NeuN immunoreactive protein expression in the hippocampal CA1 region after GCI on FLOX and KO mice. The last two rows of Fig. 3A showed that GFAP immunoreactive protein expression was highly induced in the FLOX mice after GCI, which indicates robust reactive astrocyte induction after GCI. In contrast, GFAP immunoreactive expression after GCI was significantly diminished in the hippocampal CA1 region of KO mice (Fig 3 A, B), indicating impaired reactive astrocyte induction in KO mice after GCI. Moreover, morphologically, GFAP – positive astrocytes in the FLOX group after GCI appeared very hypertrophic (increased volume) as compared to KO mice. In addition to confirm the significance of this volume differences, statistical analysis on the p-values were done on all of the graphs and can be reference on Table 3. The volume increase of astrocytes in FLOX mice are in linear with the neuron numbers in the hippocampus CA1 region after experiencing brain ischemia. The p-values for both astrocytes volume and neuronal counts suggested that there is a significance volume increase in the FLOX group after GCI and not in KO mice. Since astrocyte hypertrophy is a hallmark of reactive astrocytes, this further indicates that KO mice have impaired induction of reactive astrocytes after GCI. Finally, as shown in Fig 3A, C, NeuN immunostaining was significantly decreased in the hippocampal CA1 region of KO mice after GCI, as compared to FLOX mice. This indicates that there was greater neuronal damage in the hippocampal CA1 region in the KO mice after GCI, as compared to FLOX mice.

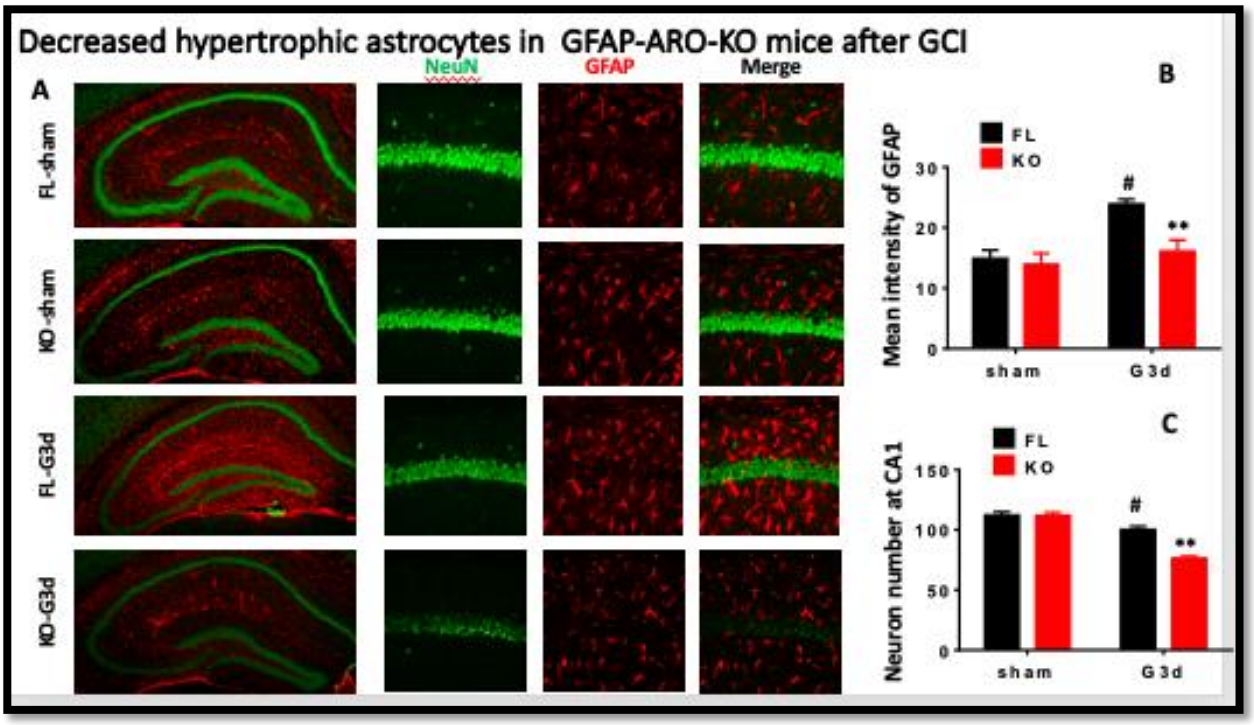

Fig. 3. Decreased reactive astrocyte GFAP expression in the hippocampus CA1 region of KO mice after GCI. (A) Immunofluorescence staining of GFAP (red), a marker of astrocyte reactivity, and NeuN (green) a marker of neurons. Note that there is a robust elevation of GFAP immunoreactive protein expression in the hippocampus in the FLOX mice but not in the KO mice 3 days after GCI. There is also a decrease in NeuN immunostaining in the hippocampal CA1 region of KO mice at 3 days after GCI, which indicates greater neuronal damage in the KO mice after GCI, as compared to FLOX mice. (B) Quantitative analysis of mean intensity of GFAP immunostaining in FLOX and KO mice. (C) Quantitative analysis of the number of NeuN-positive surviving neurons after GCI. Both quantitative data showed no significant changes of the NeuN or GFAP immunoreactive levels in sham mice controls. The significance level was analyzed by the p-values listed as # and ** can be reference in table 3.

Western blot test was performed to further confirm the GFAP findings presented in Fig. 3. Glyceraldehyde -3-Phosphate Dehydrogenase (GAPDH), a housekeeping protein, was used as the loading control, making sure that the protein loads were the same in all wells. The protein sizes for GFAP and GAPDH are indicated in Kilo-Dalton (KD) units in the figure (Fig. 4). In Fig. 4A, the Western blot test shows results of GFAP protein expression in hippocampal samples from FLOX and KO shams. In Fig. 4B, the Western blot test shows results of GFAP protein expression in hippocampal samples from FLOX and KO mice at day 3 after GCI. The results showed a clear decrease of the GFAP band density in the KO mice as compared to the FLOX

mice at day 3 after GCI. Quantification of the Western blot results using intensity analysis further confirmed that there was a significant decrease in GFAP expression in hippocampal samples from KO mice as compared to FLOX mice at 3 days after GCI (Fig. 4C). Note that there was no significant difference in GFAP levels between the sham groups (Fig. 4C). Also, the results of band density analysis can be considered reliable because we can the samples in triplicates and they all display the same results across all three wells for SHAM, FLOX, and KO mice.

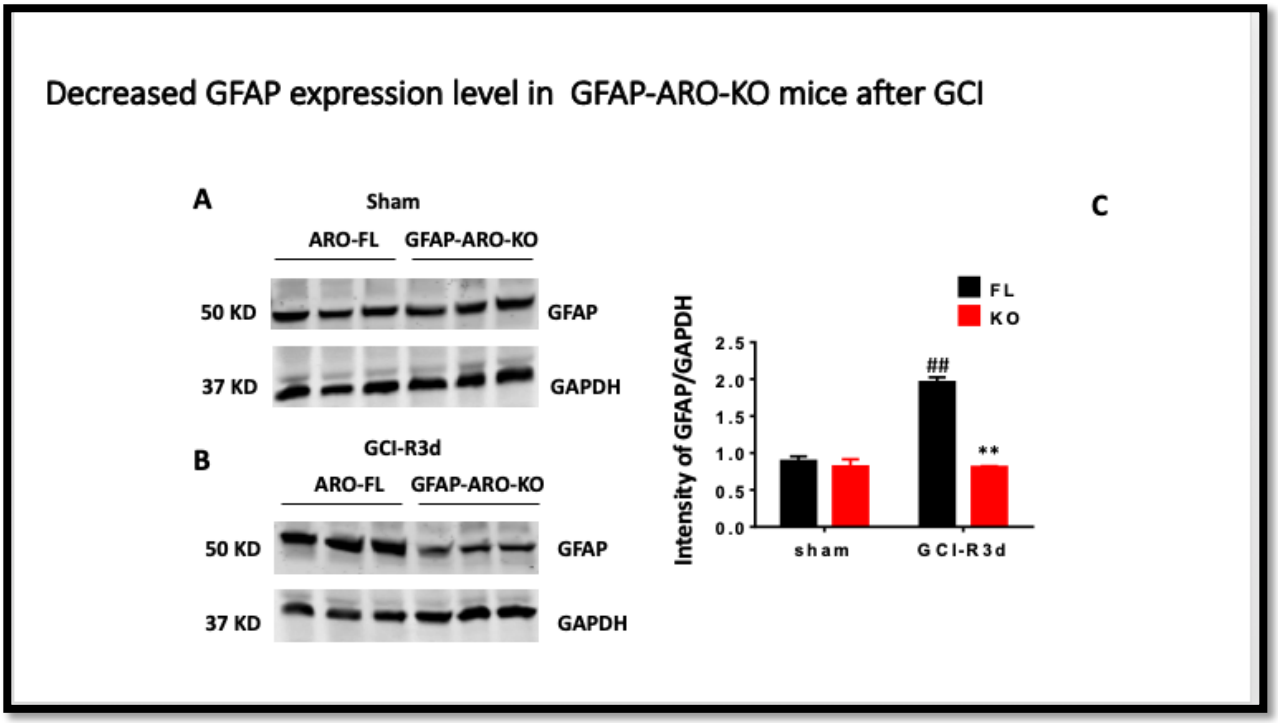

Fig. 4. Western Blotting of GFAP levels in hippocampal samples from FLOX and KO mice after GCI, and in SHAM controls. (A) GFAP expression in sham controls from FLOX and GFAP-ARO-KO hippocampal tissue samples. (b) Note smaller protein band density in KO mice than FLOX mice after GCI. (C) Quantitative analysis on relative intensity of GFAP over GAPDH on western blots of sham and GCI-R3d (3 days after GCI) samples from FLOX and KO mice. The significance level was analyzed by the p-values listed as ## and ** can be reference in table 3.

Fig. 5 focused on the STAT3 pathway, one of the major pathways that is suggested from RNA-sequence results from the Brann laboratory to be among the top pathways regulated by astrocytes-derived E2. In this figure, we are looking at the GFAP protein and phosphorelated-STAT3 (p-STAT3) protein in FLOX and KO mice 3 days after GCI. The fourth column in Fig. 5A showed a zoom in of z-stack which is a third dimension of the p-STAT3 in the astrocytes indicating in yellow color. P-STAT3 protein can be in many cell types not just in astrocytes, thus, the yellow color is when red color (GFAP) and green color (p-STAT3) merged together indicating how much p-STAT3 protein are specifically expressed in the astrocytes of FLOX and KO mice. Quantitative analysis in Fig. 5B showed that FLOX mice has a higher number of GFAP and p-STAT3-positive cells than KO mice 3 days after GCI. To further confirm the results, we used Western blot analysis in Fig 5C. The Western blot also showed a strong decrease in band intensity for GFAP in the KO mice, as compared to the FLOX mice after GCI. The intensity results of these proteins are shown in Fig 5D, which showed a significant decrease of the overall GFAP levels in the KO mice, as compared to the FLOX mice. Since pSTAT3 is a well-known marker of neuroprotective astrocyte induction after ischemia, the results indicate that activation of the STAT3 pathway is severely compromised in KO astrocytes after GCI, leading to less neuroprotective astrocyte activation in KO mice.

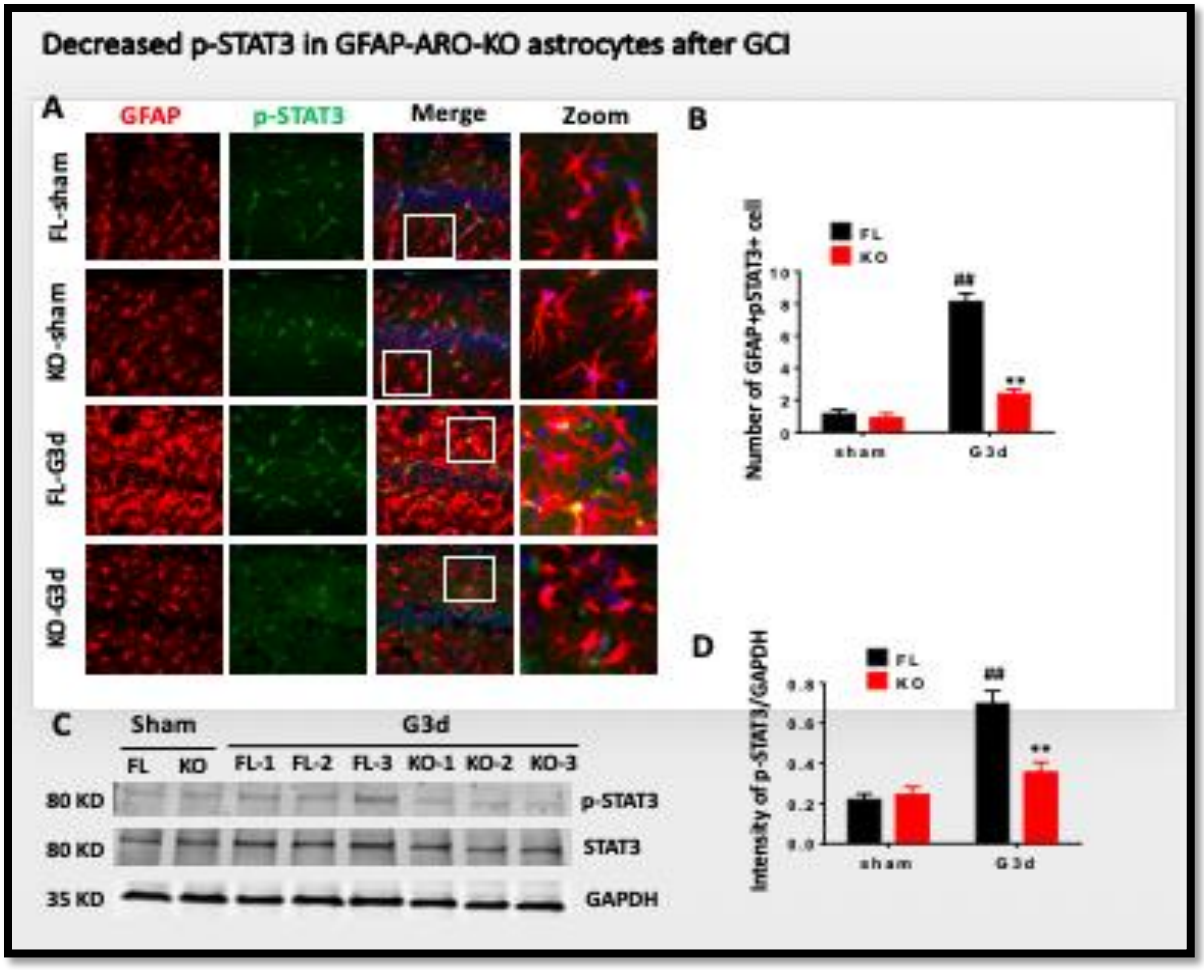

Fig. 5. GFAP and p-STAT3 protein expression in the hippocampal CA1 region of FLOX and KO mice after GCI, and in sham animals. (A) Immunofluorescence staining of the expression of p-STAT3 in astrocytes showed a decrease in p-STAT3 levels in KO as compared to FLOX mice. (B) Quantitative counting of the number of p-STAT3 and GFAP-positive cells (yellow cells in Fig. 5A) indicates that p-STAT3 protein expression in astrocytes is severely compromised in KO mice after GCI, as compared to FLOX mice after GCI. (C) Western blot test to analyze the level of the p-STAT3 protein expression in the hippocampal tissue samples. (D) Quantitative analysis of the relative intensity of pSTAT3 over GAPDH on the Western blots. This figure indicates a decrease in overall p-STAT3 levels in the KO mice than FLOX mice after GCI. The significance level was analyzed by the p-values listed as # and ** can be reference in table 3.

Fig. 6 focused on the other top regulated pathway in the Brann laboratory's RNA-sequence results, the LIF pathway. LIF is an upstream pathway of STAT3. The same methods were performed but mainly focused on expression and colocalization of the GFAP protein of the astrocytes and the LIF protein. Our results showed that the GFAP (red color) and LIF (green color) were highly colocalized in the FLOX group after GCI, as indicated by the yellow colored cells (Fig 6A). In contrast, KO mice after GCI showed little to no yellow cells, indicating that LIF was not induced in the astrocytes in KO mice after GCI, as it was in astrocytes in FLOX mice after GCI. To confirm the results using a more quantitative method, we decided to use an ELISA assay for LIF. In this study, we used both male and ovariectomized female mice to determine if there were gender differences in the regulation of LIF by astrocytes-derived E2. The ELISA assay revealed that LIF immunoreactive levels were significantly decreased in hippocampal samples from both male and female KO mice after GCI, as compared to samples from FLOX mice after GCI. The decrease of LIF showed greater statistical difference in male mice.

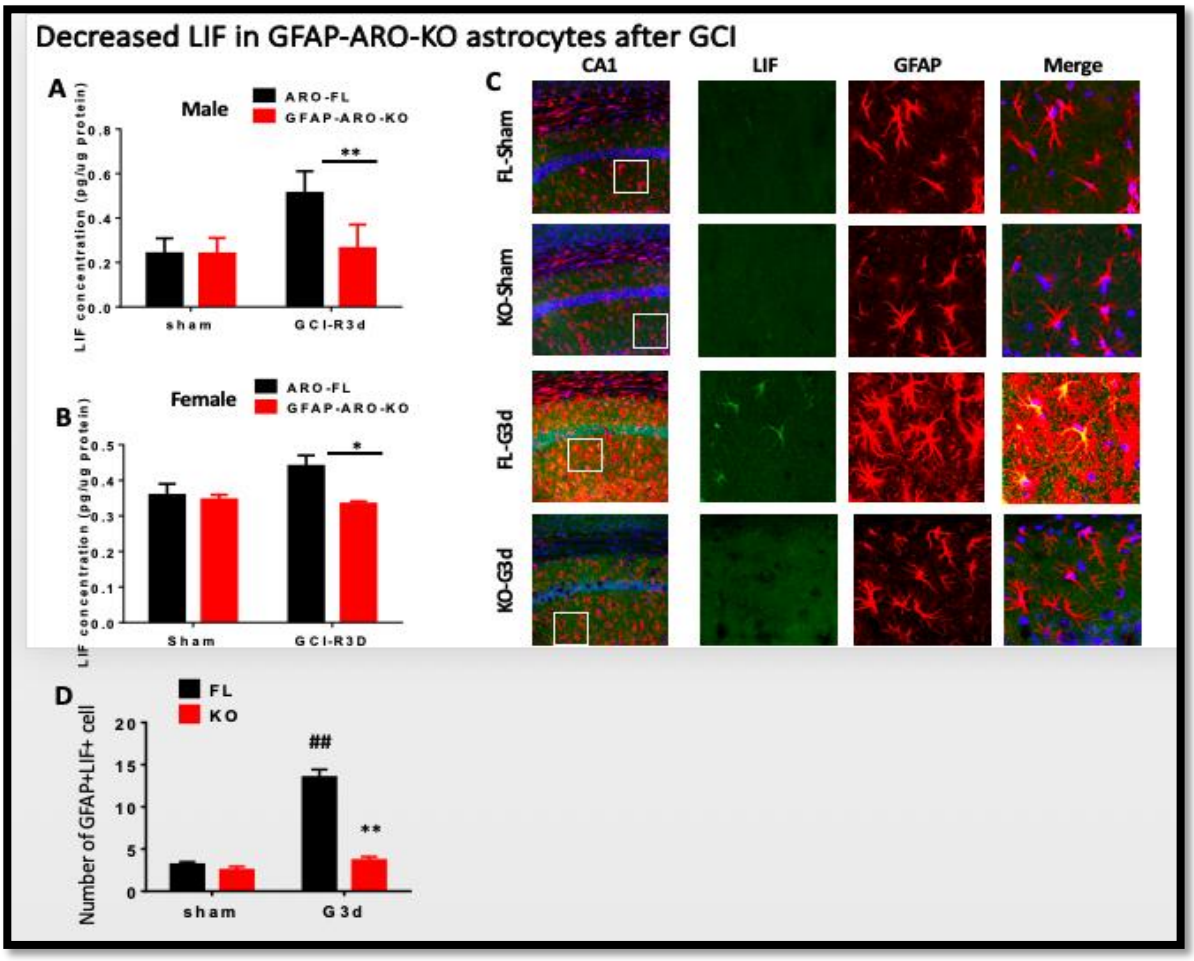

Fig 6. A and B. Induction of LIF is compromised in male and female KO mice at 3 days after GCI. (A and B) ELISA result for LIF levels in hippocampal CA1 region in GFAP-ARO-KO and SHAM mice at 3 days after GCI, as compared to FLOX mice for male and female. (C) Representative confocal images showing LIF colocalization in GFAP astrocytes in FLOX and KO mice. (D) Quantitative data on number of GFAP +LIF-double positive (yellow) cells in FLOX and KO mice in sham and after GCI. The significance level was analyzed by the p-values listed as ## and * and ** can be reference in table 3.

Discussions

In conclusion, the results of our study demonstrate a significant increase in neuronal damage and decrease of astrocyte activation in both male and female GFAP-Aro-KO hippocampus at 3 day after global cerebral ischemia, suggesting that astrocyte-derived E2 has a role in promoting astrocyte activation and is neuroprotective after brain ischemia.

We further hypothesize that astrocytes-derived E2 in the brain regulates the activation of astrocytes in the hippocampus after global cerebral ischemia and results in neuroprotective function through modulation of the LIF and STAT3 signalizing pathways. In our studies, we used several techniques to test our hypothesis, immunohistochemistry, western blot, and ELISA.

Our goal to study estradiol was fueled by previous studies that indicated that the estradiol produced in female ovaries has been classically viewed to play an important role in neuroprotection against neurogenerative disorders such as strokes, AD, and Parkinson's disease. Recent studies found that estradiol was also produced in male and female brain. Thus, we took these findings and looked into specific pathways that helps us further understand the roles of astrocyte-derived estrogen in the brain.

We generated mice that lack expression of the aromatase gene in astrocytes and used the GCI model to study the role of astrocyte-derived E2 in the brain. We focused on the hippocampus area because it's the most vulnerable site of injury after GCI. An important finding from this study was that astrocyte-derived estrogen in both male and ovariectomized female mice exhibits a neuroprotective function from ischemia injury.

Furthermore, LIF and STAT3 singling pathways, which have been implicated in neuroprotection, were tested and shown to be regulated by astrocyte-derived E2 in both the male

and ovariectomized female brain. This suggested that LIF-STAT3 signaling may mediate, at least in part, astrocyte derived E2 neuroprotective functions after GCI in male and female mice.

We tried to measure the LIF expression in hippocampus tissue by western blot, but were unsuccessful in getting a clear band. This result may be due to western blot not being sensitive enough to detect LIF, which is generally expressed at low levels. Thus, we measured LIF level by ELISA. ELISA has a higher sensitivity and can detect cytokine proteins – signaling molecules that mediate and regulate immunity, inflammation, and hematopoiesis [17]. In addition, the expression of LIF in astrocytes was measured by co-immunostaining of hippocampal sections, using antibodies to LIF and the astrocyte marker, GFAP. The use of multiple approaches/procedures enhances confidence in the data and strengthens conclusions drawn from the results.

Future studies

Collectively, the findings in this study suggested that mice that lack the enzyme aromatase in astrocytes and were unable to produce astrocyte-derived estrogen, have greater neuronal deficits after cerebral ischemia in both genders, which may be due to a defect in LIF-STAT3 signaling in the hippocampus. Thus, these findings can open more doors to future studies to further understand the extent of how these deficits can affect behavioral patterns, thinking patterns, or survival chances in a longer time span.

Previous studies that analyzed differential gene expression in the mouse astrocyte-specific aromatase knockout mouse hippocampus via RNA-sequence analysis found that knockout mice failed to upregulate a group of genes that represents the "A2" astrocyte phenotype, a type of neuroprotective astrocyte induced in response to brain injury. In addition, KO mice exhibited an upregulation of genes implicated in the "A1" astrocyte phenotype, a type of astrocyte implicated to be pro-inflammatory and neurotoxic in the brain. Thus, future studies could focus specifically on the significance of the A1 astrocytes to help us have a better understanding of astrocyte function in brain ischemia, and the role of astrocyte-derived estrogen.

Moreover, future studies could focus on the long-term effects of astrocytes-derived E2 deficient, such as examining weeks and months after cerebral ischemia. Whether the astrocytes activity remain excited over time or whether the overall neuron functions progressively degraded over time are some questions that can be looked into further. By increasing knowledge and understanding in this area, a clinical application could be indicated to which could target regulation of the brain E2 pathways, with the goal to slow down the progression of neuronal damage, and any secondary injuries to the brain.

Glossary

A1	Astrocyte implicated to be pro-inflammatory and neurotoxic in the brain
A2	A type of neuroprotective astrocyte induced in response to brain injury
AD	Alzheimer's disease. Progressive mental deterioration
Aromatase	Key enzyme that synthesis E2 from androgen precursors
Aromatase activity assay	Measuring aromatase activity from a biological sample
Astrocytes	Glial cells abundant in the brain known to produce E2 after GCI
Astrocyte-derived E2	Estrogen produced in astrocytes
Astrogliosis	Activation of astrocytes
Brain-derived E2	Estrogen that is produced in the brain
CNS	Central Nervous System. Consist of brain and spinal cord
E2	17β-estradiol. Most potent estrogen
ELISA	Enzyme- linked immunosorbent assay. Making use of an enzyme bonded to a particular antibody or antigen.
Estrogen	Steroid hormones produced by a series of biosynthetic enzymes from cholesterol.
Estrone	A type of estrogen. A steroid hormone
Estriol	A type of estrogen. A steroid hormone
Estradiol	(E2) A type of estrogen. A steroid hormone. Most potent
FLOX	Still have expression of aromatase gene.
GCI	Global Cerebral Ischemia. A type of brain injury that cuts oxygen circulation to the brain
GFAP-ARO-KO	Mouse that lack the expression of aromatase gene in the astrocytes
GFAP Cre	A specific protein used to target deletion of part of the coding sequence of the aromatase gene specifically in astrocytes
Hippocampus	Structural part of the brain associated with long-term memory

Immunofluorescent Staining	process visualizes and detects cells that express a certain protein of interest
KO	Knock-Out mouse, it lacks the expression of aromatase gene. Therefore, it's unable to produce brain-derived estrogen.
LIF	Leukemia Inhibitory Factor. A type of signaling pathway of astrocytes- derived E2
Microglia	Inflammatory glial cells
Ovarian-derived E2	Estrogen produced in ovaries
Osteoporosis	Brittle and fragile of the bones form loss of tissue
PBS solution	Phosphate-Buffered Saline solution. A buffer solution which helps maintaining a constant pH.
PD	Parkinson's disease. Progressive disease marked by tremor, muscular rigidity, and imprecise movement
Phenotype	The descriptive characteristic of a cell
STAT3	Signal Transducer and Activator of Transcription 3. A transcription factors. It is a member of the STAT protein family

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