



Published in final edited form as:

Mol Cell Neurosci. 2017 July ; 82: 118–125. doi:10.1016/j.mcn.2017.05.004.

Inhibition of miR-181a Protects Female Mice from Transient Focal Cerebral Ischemia by Targeting Astrocyte Estrogen Receptor- α

Creed M. Stary^{1,*}, Lijun Xu^{1,*}, Le Li^{1,2}, Xiaoyun Sun¹, Yi-Bing Ouyang¹, Xiaoxing Xiong^{1,3}, Jing Zhao^{1,4}, and Rona G. Giffard¹

¹Dept. of Anesthesiology, Perioperative and Pain Medicine, Stanford University School of Medicine, Stanford, CA 94305-5117, USA

²Department of Anesthesiology, Zhujiang Hospital, Southern Medical University, 253 Industrial Road, Guangzhou, 510280, China

³Department of Neurosurgery, Renmin Hospital of Wuhan University, Wuhan, Hubei, 430060, China

⁴Department of Pathophysiology, Chongqing Medical University, Chongqing, 400016, China

Abstract

Whether the effect of miR-181a is sexually dimorphic in stroke is unknown. Prior work showed protection of male mice with miR-181a inhibition. Estrogen receptor- α (ER α) is an identified target of miR181 in endometrium. Therefore we investigated the separate and joint effects of miR-181a inhibition and 17 β -estradiol (E2) replacement after ovariectomy. Adult female mice were ovariectomized and implanted with an E2- or vehicle-containing capsule for 14d prior to 1h middle cerebral artery occlusion (MCAO). Each group received either miR-181a antagomir or mismatch control by intracerebroventricular injection 24h before MCAO. After MCAO neurologic deficit and infarct volume were assessed. Primary male and female astrocyte cultures were subjected to glucose deprivation with miR-181a inhibitor or transfection control, and E2 or vehicle control, with/without ESR α knockdown with small interfering RNA. Cell death was assessed by propidium iodide staining, and lactate dehydrogenase assay. A miR-181a/ER α target site blocker (TSB), with/without miR-181a mimic, was used to confirm targeting of ER α by miR-181a in astrocytes. Individually, miR-181a inhibition or E2 decreased infarct volume and improved neurologic score in female mice, and protected male and female astrocyte cultures. Combined miR-181a inhibition plus E2 afforded greater protection of female mice and female astrocyte

Corresponding Authors: Creed M Stary, M.D., Ph.D., Stanford University, Department of Anesthesiology Perioperative and Pain Medicine, 300 Pasteur Drive, Stanford, California 94305-5117, cstary@stanford.edu, Telephone: 650-725-5875, Fax: 650-725-8052. Rona G. Giffard, Ph.D., M.D., Stanford University, Department of Anesthesiology Perioperative and Pain Medicine, 300 Pasteur Drive, Stanford, California 94305-5117, rona.giffard@stanford.edu, Telephone: 650-498-4759, Fax: 650-725-8052.

*These authors contributed equally to this work

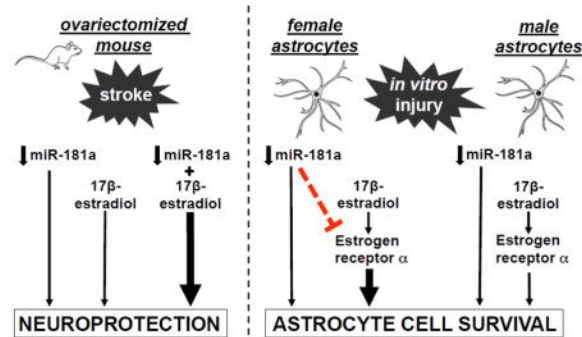
Disclosures

None

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cultures, but not in male astrocyte cultures. MiR-181a inhibition only increased ER α levels *in vivo* and in female cultures, while ER α knockdown with siRNA increased cell death in both sexes. Treatment with ER α TSB was strongly protective in both sexes. In conclusion, the results of the present study suggest miR-181a inhibition enhances E2-mediated stroke protection in females in part by augmenting ER α production, a mechanism detected in female mice and female astrocytes. Sex differences were observed with combined miR-181a inhibition/E2 treatment, and miR-181a targeting of ER α .

Graphical Abstract



Keywords

microRNA; astrocyte; ischemia-reperfusion injury; MCAO; stroke; estrogen

2. Introduction

Stroke remains a major source of mortality and morbidity worldwide, including in the United States (Mozaffarian *et al.*, 2015). Despite advances in gene therapy techniques and promising results in pre-clinical animal models, treatment for the most common form of stroke (thrombotic/non-hemorrhagic) remains limited to early reperfusion with thrombolytics or clot retrieval. In the human population, sexual dimorphism in thrombotic stroke risk and outcome is well documented. The Framingham Heart Study demonstrated women overall have a lower risk of stroke than men (Petrea *et al.*, 2009), however in old age, women have a higher incidence of stroke and poorer functional outcome (Towfighi *et al.*, 2007; Petrea *et al.*, 2009; Mozaffarian *et al.*, 2015). This age-dependent sexual dimorphism in stroke risk and outcome may hold clues to the translation barrier that persists in the development of new stroke therapies. Importantly, a similar sexual dimorphism has been identified for injury outcome in rodent models of stroke. Overall, adult female rodents have smaller infarct volumes and improved neurobehavioral outcomes following transient cerebral ischemia than their age-matched male counterparts (Alkayed *et al.*, 1998; Selvamani *et al.*, 2014; Xiong *et al.*, 2011), however this effect is reversed in aged female rodents (Manwani *et al.*, 2014). Delineating the mechanisms that regulate the differences between males and females in risk and outcome following stroke may yield new insight and more effective therapeutics.

Although a full understanding of the mechanisms underlying sex differences in stroke remains to be determined, the ovarian hormone estrogen is known to play a role in ischemic protection (Manwani *et al.*, 2015; Simpkins *et al.*, 1997). Lower circulating levels of estrogen that occur with physiologic estrous cycling in female rats and mice are associated with a greater degree of injury from cerebral ischemia (Xiong *et al.*, 2015; Liao *et al.*, 2001). Estrogen exerts a protective effect *via* several mechanisms including antioxidant and immune modulation, as well as activation of cell survival signaling pathways (for review, Ritzel *et al.*, 2013). The sex difference in infarct volume observed between intact adult males and females is abolished with ovariectomy (OVX), which depletes estrogen, resulting in larger infarcts in adult females (Alkayed *et al.*, 1998). This effect is in part reversed by exogenous replacement of 17 β -estradiol (E2, Dubai *et al.*, 1998; Rusa *et al.*, 1999) improving sensorimotor and spatial memory deficits in ovariectomized females (Li *et al.*, 2004; Gulinello *et al.*, 2006).

MicroRNAs (miRs) are short (19–22 nucleotide), non-coding RNAs that modulate endogenous gene expression in all tissues during both normal physiological functioning and in response to stress. miR expression patterns are organ- and cell type-specific, and in the brain miRs play a central role in the response to cerebral ischemia (for review(Ouyang *et al.*, 2013). MiR expression patterns have also been observed to exhibit both sex- and age-related dysregulation in response to cerebral ischemia (Selvamani *et al.*, 2014). MiRs most commonly function by binding the 3' untranslated region (UTR) of target messenger RNAs (mRNAs) to inhibit protein translation. We have previously demonstrated a protective effect of miR-181a-5p inhibition in male rodent models of stroke (Ouyang *et al.*, 2012) and forebrain ischemia (Moon *et al.*, 2013) by targeting a stress protein and regulators of apoptosis. However, whether sex differences exist in the role of miR-181a-5p (hereafter miR-181a) in stroke has not been previously investigated. In endometrial cells, the miR-181 family has been shown to target estrogen receptor- α (ER α , Su *et al.*, 2014), a transcription factor that activates pro-survival pathways (Yu *et al.*, 2012), suggesting that sex differences in downstream targets of miR-181a may exist. Therefore, in the present study we examined the roles of miR-181a, E2, and ER α in female mice subjected to OVX with or without replacement of E2, and subjected to cerebral ischemia two weeks later.

Astrocytes are principle regulators of neuronal homeostasis and survival following injury and express greater levels of miR-181a than neurons (Hutchison *et al.*, 2013). We have previously demonstrated that local astrocyte dysfunction precedes and contributes to neuronal cell death following forebrain ischemia (Ouyang *et al.*, 2007), and that targeting astrocytes can be an effective therapeutic approach for protection (Xu *et al.*, 2010). In focal ischemia as used here, astrocyte cell death is part of the core injury. We previously demonstrated that reducing levels of miR-181a in astrocytes helps preserve mitochondrial function and protect against astrocyte cell death from *in vitro* ischemia (Ouyang *et al.*, 2012). Substantial previous work supports a role for E2 and estrogen receptors in astrocytes (see Ritzel *et al.*, 2013 for review). Therefore, in the present study we followed up stroke studies in female mice with studies in female and male astrocyte cultures evaluating differences in the effect of miR-181a, E2 and ER α in the response to cell stress.

3. Materials and Methods

3.1 *In vivo* experimental protocols

All experimental protocols using animals were approved by the Stanford University Animal Care and Use Committee, and performed in accordance with NIH guidelines. Adult female C57/B6 mice (age 8–10 weeks, Charles River, Wilmington, MA) were surgically ovariectomized (OVX, described below) and randomly assigned by coin flip to subcutaneous placement of a capsule with or without E2. These two groups were further randomly subdivided (Figure 1A) into groups receiving intracerebroventricular injection (ICV) of either miR-181a antagomir, or mismatch-control sequence, on post-OVX day 13. Animals were either sacrificed for assessment of miR-181a levels and protein expression, or subjected to 1 hr middle cerebral artery occlusion (MCAO) 24 hr following ICV treatment. A total of 140 mice underwent OVX, of those 78 also underwent MCAO.

3.2 Ovariectomy (OVX) and E2 replacement

Surgery was performed as previously described (Sisk & Meek, 2001; McCullough *et al.*, 2003). Mice were anesthetized with isoflurane, and an incision bisecting the angle formed by the ribs and spinal column was made. The ovary, fat pad, and uterine horn were exposed, exteriorized, the uterine horn ligated with surgical silk and the ovary excised. The ligated uterine horn was fed back into the body cavity, the wound closed and the procedure repeated on the opposite side. Prior to closure and dressing of the second incision, a capsule containing 30 µg E2 (E8875, Sigma-Aldrich, St. Louis, MO) in sesame oil, or a capsule with sesame oil alone, was placed subcutaneously. Concentration of serum E2 was assessed prior to OVX and in OVX mice with and without E2 replacement at post-OVX days 7 and 14 by enzyme-linked immunosorbent assay (ELISA, ES180S-100, Calbiochem, San Diego, CA).

3.3 Intracerebroventricular (ICV) injection

Mice were anesthetized with 2% isoflurane, placed in a stereotaxic frame, and a 26-gauge brain infusion needle was placed stereotaxically into the left lateral ventricle (bregma: – 0.58 mm; dorsoventral: 2.1 mm; lateral: 1.2 mm) as previously described (Ouyang *et al.*, 2012). MiR-181a antagomir, 3 pmol/g body weight, or mismatch-control (details in Supplemental Table SI) was mixed with the cationic lipid DOTAP (6µl total volume; Roche, Basel, Switzerland) and infused over 20 minutes as previously (Ouyang *et al.*, 2012).

3.4 Transient focal cerebral ischemia, infarct and neurologic assessment

Mice were anesthetized with isoflurane in oxygen and 1h transient ischemia was produced by occluding the middle cerebral artery (MCAO) with a monofilament followed by 24 hr of reperfusion as described previously (Xiong *et al.*, 2015; Ouyang *et al.*, 2012). The neuroscore was assessed 24 hr after MCAO: a score of 0 (no observable neurological deficit), 1 (failure to extend right forepaw), 2 (circling to the right), 3 (falling to the right) or 4 (unable to walk spontaneously) was assigned (Yang *et al.*, 1994). Brains were harvested to assess infarct volume after transcardiac perfusion with saline, then 4% paraformaldehyde. Infarct volume was quantified by a blinded observer on 4 sections/brain using 50 µm coronal sections stained with Cresyl Violet (EMD-Millipore Chemicals, Hayward, CA) and

corrected for edema using Image J software (v1.46, NIH, Bethesda, MD) as described (Ouyang *et al.*, 2012). Mice with no evidence of acute neurological deficit (n=2), that died < 24 hr following surgery (n=3), or with evidence of significant bleeding (n=1) were excluded from analysis. Treatment groups did not differ in numbers of excluded animals.

3.5 Cell cultures

Primary astrocyte cultures were prepared from postnatal day 1–3 mice as previously described (Dugan *et al.*, 1995). Experimental treatment protocol is outlined in Figure 1B. Briefly, following euthanasia pups were sexed according to visual inspection of genitalia (Greenham & Greenham, 1977). Correct sex of pups was confirmed using RT-qPCR on tail biopsies for SRY, the male sex determining region on the Y chromosome. C6 glioma cells (originally from a male rat) were purchased from American Type Culture Collection (CCL-107) and grown in DMEM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% FBS and 100 µg/ml penicillin/streptomycin. Cell cultures were transfected when near-confluent (days-in-vitro 14–17) with 30 pmol miR-181a inhibitor (see Supplemental Table S2 for details) or mismatch-control, and treated with either 20 nM E2 (in 1:5000 DMSO) or 1:5000 DMSO alone (Figure 1B). Primary astrocyte cultures were treated with 30 pmol miR-181a mimic, a dose previously (Xiong *et al.*, 2015; Ouyang *et al.*, 2012) shown to increase miR-181a levels by > 50 fold, and/or a custom target site blocker (Exiqon, Vedbaek, Denmark) to competitively inhibit the mmu-miR-181a-5p binding site on the mmu-ER1 mRNA 3'UTR (2000 5' -UUGAAUGU-3'). To verify transfection, cultures were assessed for 6-FAMTM fluorescence (the reporter on the TSB) 24 hr after transfection, following fixation as previously described (Stary *et al.*, 2016).

3.6 Cell Injury and Assessment

Cells were subjected to injury 24 hr following transfection (Figure 1B). Injury was induced by glucose deprivation (GD) for 48 hr, this longer duration was needed to induce injury in these relatively young cultures (Papadopoulos *et al.*, 1998a). We chose GD as a cell stress because it reliably induces mitochondrial dysfunction as seen in ischemia (both substrate deprivation and oxidative stress) that progresses to cell death. We quantified cell death because it can be reliably quantified by parallel measures, and is the common final end-point for cellular dysfunction. Cell death was assessed by staining with Hoechst 33342 (5 µM, Sigma-Aldrich) and propidium iodide (PI, 5 µM, Sigma-Aldrich) and quantitated by measuring lactate dehydrogenase (LDH) released from dead cells, as previously described (Ouyang *et al.*, 2012). In all cell culture experiments non-injury wash control resulted in <5% cell death.

3.7 Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA from tissue and cell culture was isolated with TRIzol® (ThermoFisher Scientific). Reverse transcription was performed as previously described (Ouyang *et al.*, 2012). PCR was performed with predesigned primer/probes for mmu-miR-181a-5p, sex-determining region Y (SRY), GAPDH, and U6 (see Supplemental Table S3 for reagent details). qPCR reactions were conducted as previously (Ouyang *et al.*, 2012). Ct values for SRY we normalized to GAPDH, miR-181a was normalized to U6 (Ct). Comparisons were calculated as the inverse log of the Ct (Livak & Schmittgen, 2001).

3.8 Immunoblotting

Immunoblotting was performed using 30 µg of protein/sample as previously described (Ouyang *et al.*, 2012), using primary antibodies to ER α , GRP78, XIAP, Bcl2 and β -actin. Briefly, cells were harvested 24 hr after transfection or brain protein was isolated after perfusion with iced saline, then 30 µg of protein/sample was separated on a 4–10% Bis-Tris mini-gel (NP0304BOX, ThermoFisher Scientific), and electro-transferred to Immobilon polyvinylidene fluoride membrane (IPVH00010, Millipore EMD Corp.). Membranes were blocked and incubated with the appropriate primary antibody and dilution (see Supplemental Table S4) overnight at 4°C. Membranes were then washed (and incubated with 1:15,000 the appropriate conjugated secondary antibody (see Table S4). Immunoreactive bands were visualized using the LICOR Odyssey infrared imaging system. Densitometric analysis was performed using Image J software (v1.46, National Institutes of Health) by an observer blinded to treatment group. Target band intensity was normalized to β -actin. See Table S4 for reagent details and antibody information.

3.9 Fluorescence detection for 6-FAMTM

To verify transfection, cultures were assessed for 6-FAMTM fluorescence (the reporter on the TSB) 24 hr after transfection, following fixation in 24-well plates as previously described (Stary *et al.*, 2016). Primary astrocyte cell cultures were fixed in 4% paraformaldehyde for 30 min at room temperature. Nonspecific binding was blocked with 5% normal goat serum (S-1000, Vector) and 0.3% Triton X-100 (X-100, Sigma Aldrich) in PBS for 1 hr. Cells were counterstained with the nuclear dye DAPI (4',6'-diamidino-2-phenylindole, 0.5 µg/ml; D9542, Sigma-Aldrich). To verify transfection of ER α TSB, cells were assessed for 6-FAMTM fluorescence with a Zeiss (Carl Zeiss AG, Jena, Germany) Axiovert 200M epifluorescence microscope and AxioVision software (v4.8.1) by an observer blinded to treatment groups.

3.10 Statistical Analysis

All cell culture data represent experiments repeated 3X with cells from 3 different dissections. All data shown are mean \pm SE. Statistical analysis was two-way analysis of variance with repeated measures for assessment of serum E2 levels, and one-way analysis of variance with Bonferroni post-hoc test for all other comparisons. A p-value of <0.05 was considered significant.

4. Results

When measured before OVX, serum E2 levels were equivalent, while 7 and 13 days after OVX, E2 was significantly lower in animals replaced with vehicle, versus E2 replacement (Figure 2A). Treatment with either miR-181a antagomir or E2 decreased MCAO infarct volume (Figures 2B,C), while combined treatment resulted in significantly greater protection than either treatment alone. Similarly, miR-181a antagomir or E2 significantly improved neurological deficit score (Figures 2D), with combined treatment resulting in better neurological outcome than either treatment alone. Treatment with either miR-181a inhibitor or E2 alone resulted in significant protection of C6 glioma cells from GD (Figures 3A,B). However, in contrast to *in vivo* observations in female mice (Figure 2C), combined treatment

did not induce additional protection. To further investigate, primary cultures of astrocytes isolated from male or female pups were compared. Treatment with either miR-181a inhibitor or E2 resulted in significant protection (Figures 3C,D). Male cultures, like C6 cells (from a male rat), did not show greater protection with combined treatment, while female cultures did, matching the female *in vivo* observations. Co-treatment with ER α siRNA abolished the protective effects of both miR-181a inhibitor and E2 in both male (Figure 3C) and female (Figure 3D) primary astrocyte cultures, suggesting that ER α is important for protection by both miR-181a inhibition and E2 treatment in cultures of both sexes.

Brain levels of miR-181a and ER α (example of ER α immunoblot is shown in Supplemental Figure S1) were assessed 24 hr after ICV injection of miR-181a antagomir in OVX mice (Figures 4A,B). ICV injection with miR-181a antagomir significantly decreased miR-181a levels to $64\% \pm 7$ (relative to ICV injection with MM-control oligonucleotide) and significantly increased ER α protein expression by $48 \pm 13\%$. In contrast, no change in ER α protein expression was observed following miR-181a inhibition in male primary astrocyte cultures (Figure 4C). However, in female primary astrocyte cultures, an increase in ER α was observed in miR-181a inhibitor treated groups (Figure 4D), similar to observations *in vivo*. Male and female cultures without treatment had similar levels of ER α . Three other targets previously identified in male rodents, GRP78, Bcl2 and XIAP were also assessed. Protein levels of Bcl2 and XIAP were not altered in female brain (Table S5). Grp78 levels were increased about 30% both by E2 and miR-181a antagomir, as well as combined treatment (Table S5).

To confirm that targeting of ER α by mir-181a contributes to cell death, we co-transfected primary astrocyte cultures with miR-181a mimic or miR-181a mimic/ER α TSB. Transfection efficiency of ER α TSB assessed with a covalent 6-FAMTM fluorescent reporter (Figure 5A), was $85 \pm 10\%$. In both female and male cultures, transfection with miR-181a mimic, which increases levels of miR-181a, caused a trend to increased cell death from glucose deprivation (Figure 5B). In astrocyte cultures of both sexes co-transfection with mir-181a mimic and ER α TSB led to marked reduction in GD injury to levels below control injury, suggesting endogenous levels of miR-181a were already increasing cell death.

5. Discussion

The present study examined the efficacy of miR-181a antagomir in female stroke, with and without E2. We observed significant protection, similar in magnitude to the protection seen in male mice (about 48% reduction in infarct volume, (Ouyang *et al.*, 2012) only with combined E2 and miR-181a antagomir (53% reduction), while antagomir alone provided a significant reduction of infarct volume in females of 24%. We identified ER α as a target for miR-181a in female brain and astrocyte culture, but not in male cultures. Other targets investigated included Grp78, Bcl2 and XIAP. Of these only Grp78 showed a trend to increase with antagomir in female brain of about 30%, while prior observations in males showed a greater than 50% increase (Ouyang *et al.*, 2012). Here, E2 treatment also led to increased Grp78 levels, consistent with the reported ability of E2 to reduce levels of miR-181a, previously observed in breast cancer cells (Maillot *et al.*, 2009; Kastrati *et al.*, 2015). However, we did not observe a decrease in miR-181a levels with E2 alone, which

suggests there are additional mechanisms by which E2 can influence Grp78 levels. Thus targets and magnitudes of change differed between male and female brains, as well as the magnitude of protection.

E2 has been shown to rapidly activate pro-survival signaling pathways in astrocytes, including MAPK (Ivanova *et al.*, 2001; Zhang *et al.*, 2002) and phosphatidylinositol 3-kinase (PI3K)/Akt (Dhandapani *et al.*, 2005). E2 also regulates the expression of neuroprotective proteins in astrocytes, including heat shock proteins (Lu *et al.*, 2002), glutamate transporters (Cimarosti *et al.*, 2005; Lee *et al.*, 2009; Pawlak *et al.*, 2005), and growth factors (Galbiati *et al.*, 2002; Melcangi *et al.*, 2001). E2 signaling occurs *via* ligand-mediated activation of the two known estrogen receptors, ER α and ER β , the non-canonical G-protein coupled estrogen receptor (GPER/GPR30), as well as by non-genomic effects. In the present study, we identified ER α as a physiological target of miR-181a in female mice and astrocytes. This is consistent with the presence of a complementary sequence in the 3' UTR of ER α , but not in the 3' UTR of ER β or GPER (TargetsCan.org 7.1). Despite parallel signaling pathways, substantial differences between ER α and ER β exist. The importance of each receptor in ischemic protection is controversial with different findings reported. Dubal *et al.* (Dubal *et al.*, 2001) demonstrated that genetic deletion of ER α but not ER β abolished E2 protection in a model of distal MCAO. Antisense knockdown also suggested a more critical role for ER α versus ER β in E2 protection from cerebral ischemia (Zhang *et al.*, 2009). Selective ER α activation but not ER β was neuroprotective (Dai *et al.*, 2007). However, a role for ER β in neuroprotection was seen in some injury models, including no change in infarct in ER α knockout mice, protection seen with an ER β agonist, or protection *via* both receptors (Miller *et al.*, 2005; Sampei *et al.*, 2000; Carswell *et al.*, 2004).

Interestingly, in breast tissue (Kastrati *et al.*, 2015) and endometrium (Gao *et al.*, 2016) E2 downregulates expression of miR-181a, providing evidence for a positive feedback loop for ER α activation. E2-mediated activation of ERs also regulates pro-survival gene expression *via* non-classical/ER-independent co-activation of critical transcription factors, most notably cAMP-response element binding protein (CREB, Coleman *et al.*, 2003) and c-fos/jun (Kalaitzidis & Gilmore, 2005). Interestingly, c-fos (Gao *et al.*, 2016) and CREB (Liu *et al.*, 2013) are both negatively regulated by miR-181a in other tissues, which may have also contributed to our observations in the present study of a combined neuroprotective effect of E2 plus miR-181a inhibition.

The present study is the first to demonstrate sex differences in the effect of miR-181a on astrocyte survival. Although astrocytes from both males and females express ER α (Garcia-Ovejero *et al.*, 2002; Bondar *et al.*, 2009) sex differences in the effect of E2 in astrocytes have been described. Zhang *et al.* demonstrated sexual dimorphism in activation and downstream signaling of MAPK (Zhang *et al.*, 2002). Kuo *et al.* (Kuo *et al.*, 2010) demonstrated in female but not male astrocytes that E2 treatment increased insertion of ER α into the cell membrane and enhanced progesterone synthesis. Astrocyte estrogen receptors appear to be altered by the hormone loss that occurs during aging (Arimoto *et al.*, 2013), and an estrous-related response in the number of tyrosine kinase A receptor (TrkA)-immunoreactive astrocytes has been described (McCarthy *et al.*, 2002).

One caveat to the present study is that astrocytes cultured from neonatal mice may differ substantially from astrocytes in the adult brain. In the present study we did not observe any differences in baseline levels of ER α expression between male and female cultures, or in susceptibility to injury in vehicle-treated (control) cultures. We previously observed in sex-mixed astrocyte cultures that susceptibility to oxidative stress increases with age (Papadopoulos *et al.*, 1998b), but this effect has not yet been investigated in single sex cultures. A future approach to further study sexual dimorphism in the effects of miR-181a and E2 on astrocytes in the injured adult brain would be to investigate the effects of pre-treatment in astrocytes isolated from the adult brain following injury.

A final important consideration is that other cell types in the brain express ERs and likely play a role in the effect of E2 and miR-181a on injury outcomes in the brain. Resident microglia, central to the inflammatory response to injury and relevant to neuronal survival, express ERs and respond to E2 (Ritzel *et al.*, 2013). Other resident brain cell types that express ERs include neurons, dendritic cells and oligodendrocytes, as well as migrating ER-expressing immune cells (monocytes, macrophages and neutrophils) all of which may participate in the response to E2 and miR-181a levels. Future studies investigating the effects of E2 and miR-181a in other cell types will further define the relevance of miR-181a inhibition as a potential therapy for stroke.

6. Summary

This report is the first to demonstrate that decreasing brain levels of miR-181a in female mice is protective, and enhances the neuroprotective effects of E2. This combined effect was recapitulated in female primary astrocyte cultures but not male cultures. We identified ER α as a novel target of miR-181a in female cortical astrocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support

This study was funded in part by NIH grants NS084396 and NS080177 to RGG, American Heart Association grant FTF-19970029 to CMS.

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Highlights

- Inhibition of microRNA-181a in the brain is protective against middle cerebral artery occlusion in female mice.
- Estrogen receptor- α is identified as a novel target of microRNA-181a in female cortical astrocytes.
- Sex differences are observed in primary cortical astrocyte cultures in the effects of estradiol and microRNA-181a.

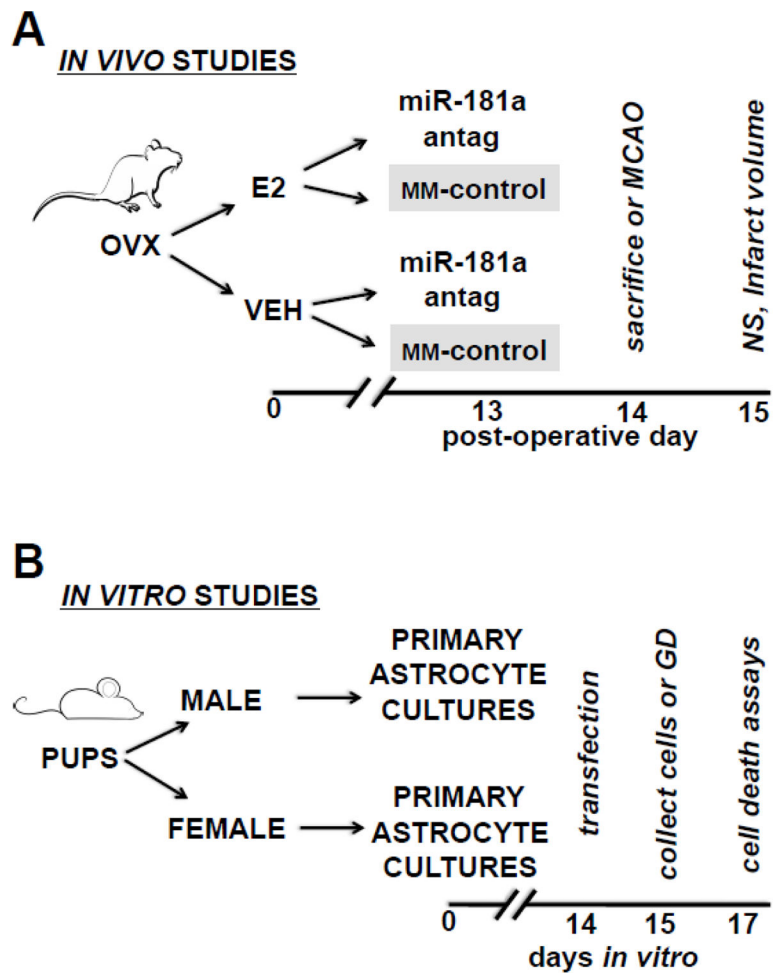


Figure 1. Experimental protocols for *in vivo* (A) and primary astrocyte (B) studies. Antag = antagomir; E2 = 17 β -estradiol; GD = glucose deprivation; MCAO = 1 hr middle cerebral artery occlusion; MM-control = mismatch control; NS = neurological deficit score; OVX = ovariectomy; V = control vehicle.

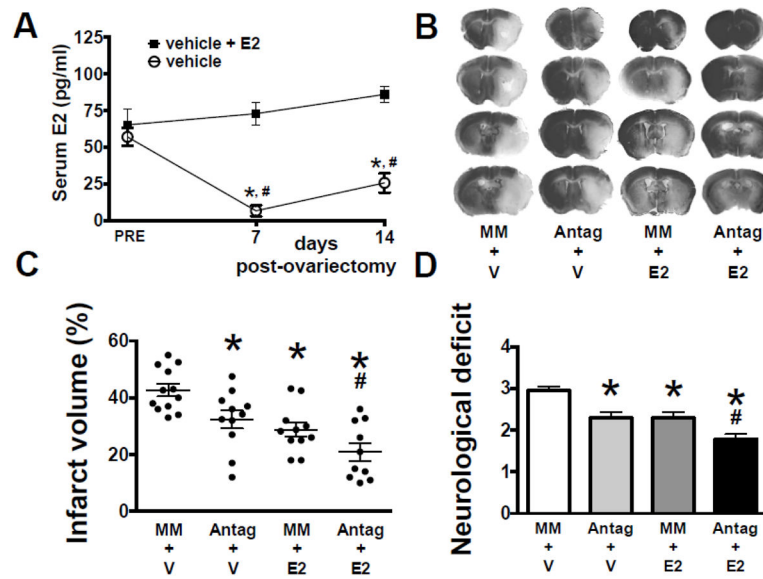


Figure 2. Effect of miR-181a antagonist and E2 on injury from middle cerebral artery occlusion (MCAO)

(A) Serum E2 levels in female mice prior to and following ovariectomy (OVX), with and without 17 β -estradiol (E2) replacement. (B) Coronal brain sections stained with Cresyl Violet to assess infarct volume in OVX mice treated with mismatch control (MM) or miR-181a antagonist (Antag), and E2 or vehicle (V), 24 hr following 1 hr MCAO; infarcted areas are lighter in color. Quantification of infarct volume (C) and neurological deficit (D) 24 hr following MCAO. All graphs show mean \pm SE, * = $p < 0.05$ versus pre or control injury; # = $p < 0.05$ versus Antag or E2 alone. N = 8–9 OVX animals per group. E2 = 17 β -estradiol.

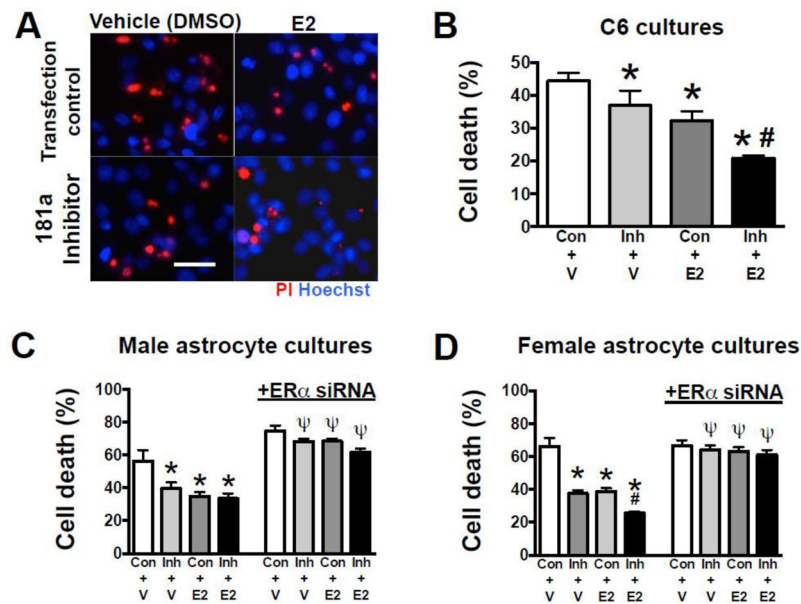


Figure 3. Effect of miR-181a inhibitor and E2 on *in vitro* glucose deprivation (GD) astrocyte injury

(A) Representative micrographs of cell death assessed by propidium iodide (PI, red) staining following GD injury in C6 cells treated with control scramble sequence (Con) or miR-181a inhibitor, and E2 or vehicle (V). All cell nuclei are stained blue with Hoechst. Quantification of cell death by LDH release from C6 cells (B), male primary astrocyte cultures (C), and female primary astrocyte cultures (D) treated with miR-181a inhibitor, E2, or both. Blocking ER α using small interfering RNA (siRNA) abolished the protection seen with miR-181a inhibitor and E2 in both male and female cultures (C, D). N = 8 cultures per experiment. All graphs = mean \pm SE, * = p<0.05 versus control; # = p<0.05 versus E2 or inhibitor alone; Ψ = p<0.05 versus same condition without siRNA. E2 = 17 β -estradiol Inh = inhibitor. Bar = 15 μ m.

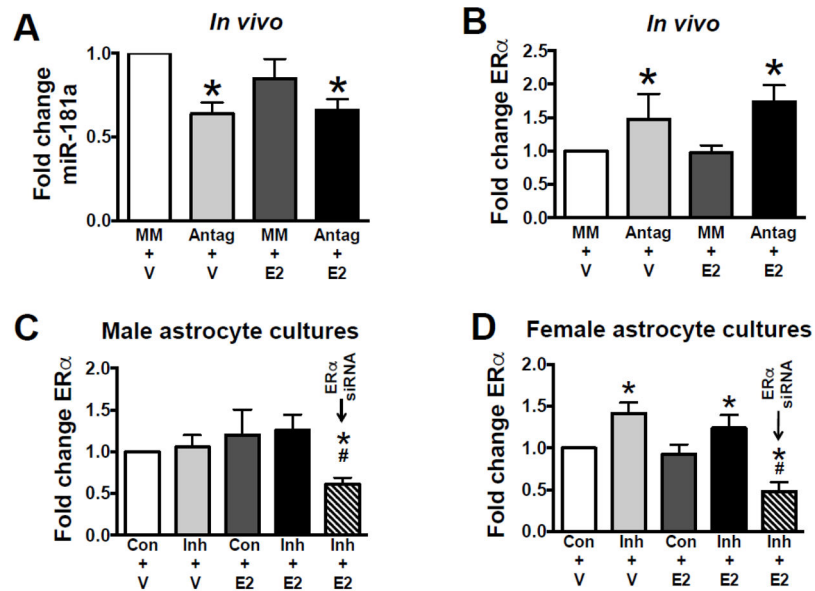


Figure 4. *In vivo* and *in vitro* ER α protein expression

(A) Quantification of miR-181a levels from brains of OVX mice. Quantification of ER α protein from brains of OVX mice (B), male (C) and female (D) primary astrocyte cultures. ER α levels were significantly ($p < 0.05$) lower in cultures co-treated with ER α siRNA (C, D). N = 8 cultures per experiment. All graphs = mean \pm SE, * = $p < 0.05$ versus control; # = $p < 0.05$ versus all other conditions. Antag = miR-181a antagomir, Con = transfection control (scrambled sequence), E2 = 17 β -estradiol, Inh = miR-181a inhibitor, V = vehicle.

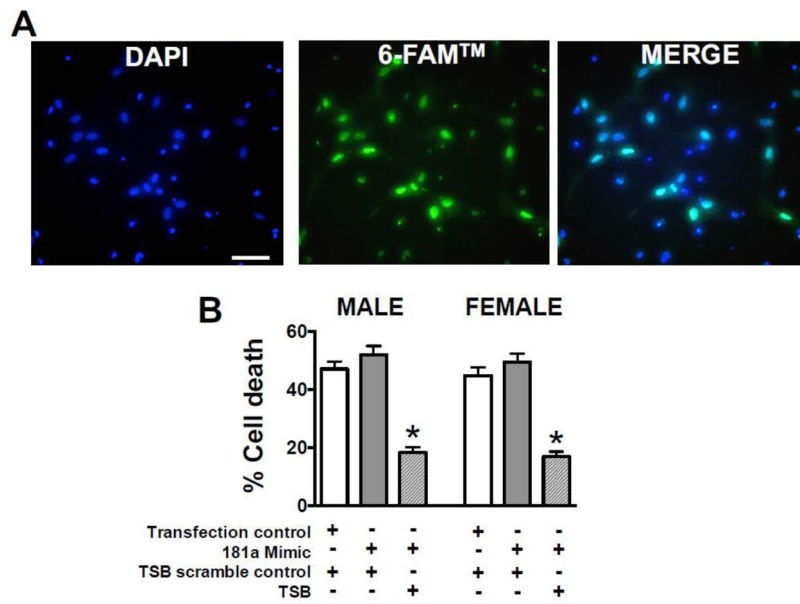


Figure 5. Cell death following miR-181a/ER α binding inhibition in astrocytes

(A) Primary astrocytes stained with the nuclear dye DAPI (4',6'-diamidino-2-phenylindole, blue, left panel) display 6-FAM™ reporter fluorescence (green, middle panel) when transfected with miR-181a/ER α target site blocker (TSB). Transfection efficiency is quantified by blue/green co-localization (right panel). Bars = 15 μ m. (B) Quantification of cell death from GD injury of primary male and female astrocytes treated with control transfection or miR-181a mimic and TSB scramble control sequence or miR-181a/ESR α TSB. N = 8 cultures per experiment, mean \pm SE, * = p<0.05 versus control.