Published in final edited form as:

Mitochondrion. 2016 September ; 30: 248–254. doi:10.1016/j.mito.2016.08.013.

miR-29a Differentially Regulates Cell Survival in Astrocytes from Cornu Ammonis-1 and Dentate Gyrus by Targeting VDAC1

Creed M. Stary^a, Xiaoyun Sun^a, YiBing Ouyang^a, Le Li^{a,b}, and Rona G. Giffard^a

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

^bDepartment of Anesthesiology, Zhujiang Hospital, Southern Medical University, 253 Industrial Road, Guangzhou, 510280, Guangdong Province, China

Abstract

Neurons in the cornu ammonis 1 (CA1) region of the hippocampus are vulnerable to cerebral ischemia, while dentate gyrus (DG) neurons are more resistant. This effect is mediated by local astrocytes, and may reflect differences in subregional hippocampal expression of miR-29a. We investigated the role of miR-29a on survival of hippocampal astrocytes cultured selectively from CA1 and DG in response to glucose deprivation (GD). CA1 astrocytes exhibited more cell death and a greater decrease in miR-29a than DG astrocytes. A reciprocal change was observed in the mitochondrial voltage dependent cation channel-1 (VDAC1), a regulator of mitochondria and target of miR-29a. In CA1 astrocytes, increasing miR-29a decreased VDAC1 and improved cell survival, while knockdown of VDAC1 improved survival. Finally, the protective effect of miR-29a was eliminated by inhibition of miR-29a/VDAC1 binding. These findings suggest that the selective vulnerability of the CA1 to injury may be due in part to a limited miR-29a response in CA1 astrocytes, allowing a greater increase in VDAC1-mediated cellular dysfunction in CA1 astrocytes.

Keywords

Glia; microRNA; brain; glucose deprivation; ischemia; stroke; hippocampus; mitochondria

INTRODUCTION

Global cerebral ischemia leads to post-resuscitation neurological impairment in survivors. Pyramidal neurons in the cornu ammonis 1 (CA1) region of the hippocampus are selectively sensitive to ischemia, dying in the days following reperfusion. However neurons in the adjacent dentate gyrus (DG) have a relatively higher ischemic resistance and survive (for review, Ouyang *et al.*, 2014). Delayed neuronal death in the CA1 occurs secondary to

Correspondence, Creed M. Stary, MD, PhD, cstary@stanford.edu, 300 Pasteur Drive, MC 5117, Stanford, CA 94305-5117, Tel: 650-725-5875.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

disruption in mitochondrial function (Owens *et al.*, 2015), inducing release of cytochrome c and other pro-apoptotic factors into the cytoplasm (Ouyang *et al.*, 1999; Niizuma *et al.*, 2009). Delineating the mechanisms that determine observed differences between the CA1 and DG hippocampal subregions in the cellular response to injury might provide new avenues in the development of clinical therapies for ischemic brain injury.

Lack of consideration for other cell types in the brain has been a proposed factor in the translational failure of potential neuroprotective strategies (Nedergaard & Dirnagl, 2005). Astrocytes, the most abundant cell type in the brain, play many key roles supporting normal neuronal functioning, including preserving ionic and acid-base balance, modulating neurotransmission, and maintaining neuronal energy stores (Clarke & Barres, 2013; Barreto *et al.*, 2011). Critically, astrocyte homeostasis is tightly coupled to neuronal cell fate following ischemic injury (Nedergaard & Dirnagl, 2005; Barreto *et al.*, 2011; Ouyang *et al.*, 2014). We have previously observed that both neurons and astrocytes isolated from different brain regions show differential sensitivity to injuries (Xu *et al.*, 2001). We further observed that within the hippocampus CA1 astrocytes were more sensitive to ischemic injury, with greater mitochondrial dysfunction compared to DG astrocytes (Ouyang *et al.*, 2007). Moreover we demonstrated that disruption of mitochondrial homeostasis in resident astrocytes contributes to neuronal cell death in CA1 following transient forebrain ischemia (Xu *et al.*, 2010; Ouyang *et al.*, 2013). However, the factors that determine regional hippocampal differences in post-ischemic astrocyte dysfunction, and therefore neuronal cell fate, remain incompletely understood.

Cell function and fate following stress are determined in part by the interface between gene transcription and epigenetic modifiers of gene expression (Mehler, 2008). MicroRNAs (miRs) are a class of endogenously expressed, non-coding RNAs, which modify gene expression by binding the 3' untranslated region (UTR) of target genes and inhibiting translation. Numerous miRs are expressed in a cell-specific manner, and miR-29 is selectively enriched in astrocytes (Smirnova *et al.*, 2005). Expression of miR-29a is suppressed in neurodegenerative disorders, including Alzheimer's disease and Huntington's disease (Roshan *et al.*, 2009), and brain-targeted knockdown of miR-29a in developing animals results in neurological dysfunction, notably region-specific hippocampal neuronal cell death (Roshan *et al.*, 2014). We previously observed in an *in vivo* rodent model of transient global cerebral ischemia that miR-29a increased in the DG, but decreased in the CA1, and that overexpression of miR-29a resulted in protection of CA1 neurons from delayed neuronal death (Ouyang *et al.*, 2013). Further, we observed in cortical astrocyte cultures that increasing levels of miR-29a protected cells from ischemia-like stress, while decreasing levels of miR-29a disrupted mitochondrial homeostasis, resulting in cell death (Ouyang *et al.*, 2013). However, the mechanisms for this effect remain unclear. To further delineate mechanisms of hippocampal regional heterogeneity, which may explain subregion-specific vulnerability, we utilized astrocytes selectively cultured from hippocampal CA1 and DG subregions to investigate the roles of miR-29a, and a mitochondrial target, the voltage-dependent anion channel-1 (VDAC1, (Bargaje *et al.*, 2012), in astrocyte cell death following ischemia-like stress.

METHODS and MATERIALS

Cell cultures and transfection

All experimental protocols using animals were performed according to protocols approved by the Stanford University Animal Care and Use Committee and in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Primary hippocampal astrocyte cultures were prepared from postnatal (days 3–4) Swiss Webster mice (Simonsen, Gilroy, CA) as previously described (Xu *et al.*, 2001). Briefly, the left and right hippocampi were identified morphologically and by anatomical location, and dissected free in their entirety, while maintaining the anatomical orientation (Hagihara *et al.*, 2009). The dorsal region of the hippocampus containing primarily CA1 was dissected free of the remainder of the hippocampus. The ventral hippocampus (containing DG) was further dissected with removal of the CA3 region. CA1 and DG hippocampal regions from individual animals were pooled, treated with 0.05% trypsin/EDTA (Life Technologies, Carlsbad, CA, USA), and plated in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) with 10% equine serum (ES, HyClone, GE Healthcare Life Sciences, Logan, Utah), 10% fetal bovine serum (FBS, Hyclone) and 10 ng/ml epidermal growth factor (Sigma Chemicals, St Louis, MO, USA). Cultures were maintained at 37°C in a 5% CO₂ incubator. Verification of successful hippocampal subregional separation was confirmed by quantification of desmoplakin in cultures (Lein *et al.*, 2004) by reverse transcription quantitative polymerase chain reaction (RT-qPCR, see below).

Primary hippocampal astrocyte cultures were transfected on days 8–9 *in vitro* with mmu-miR-29a-3p mimic or negative control sequence (30 pmol/well cat. #MC10518 and #4464076 respectively, ThermoFisher Scientific, Waltham, MA) using Lipofectamine-2000™ reagent (Invitrogen, Foster City, CA) according to the manufacturer's instructions (Stary *et al.*, 2015a). Relatively younger cultures were used in the present study to achieve a higher efficiency of transfection. Overexpression of miR-29a was confirmed by RT-qPCR, below. Selective inhibition of miR-29a/VDAC1 binding was achieved by transfection with a custom target site blocker (Power TSB™, #480003-00, Exiqon, Woburn, MA) synthesized to competitively inhibit the mmu-miR-29a-3p binding site on the hsa-VDAC1 3'UTR (409 5'-UCACACCCU-3'). miR-29a/VDAC1 TSB was synthesized with an integrated 5' 6-FAM™ green fluorescent reporter, and transfection of TSB was assessed by fluorescence immunocytochemistry and immunoblotting (below). VDAC1 knockdown was achieved by transfection with small interfering RNA (siRNA) targeted against VDAC1 mRNA (50 pmol/well, Silencer Select cat. #S75920, ThermoFisher Scientific) or negative control (cat. #4390843, ThermoFisher Scientific). Knockdown of VDAC1 protein expression was confirmed by immunoblotting.

Injury paradigms and assessment of cell injury

Glucose deprivation (GD) injury was selected as an ischemia-like stress (Papadopoulos *et al.*, 1997) and was performed as we have described previously (Ouyang *et al.*, 2011; Ouyang *et al.*, 2006) with the following modification: cells were washed twice with medium lacking glucose separated by a 15 min equilibration period in the incubator to remove as much substrate as possible. Because vulnerability of primary astrocyte cultures to ischemic injury

increases with age (Papadopoulos *et al.*, 1998), an extended duration (48 hrs) of GD was necessary to induce an adequate level of cell death in these relatively younger (DIV 9–10) cultures. Assessment of cell viability and cell counting were performed after staining with Hoechst 33342 (5 μ M, Sigma Chemicals) and propidium iodide (PI, 5 μ M, Sigma Chemicals). PI stains dead cells while Hoechst is a cell-permeant nucleic acid stain that labels nuclei of both live and dead cells. PI-positive cells were manually counted by a blinded investigator; numbers of Hoechst-positive cells were calculated using an automated macro (Image J, v1.49b, National Institutes of Health, USA). PI-positive and Hoechst-positive cells were counted in 3 microscopic fields per well at 200X magnification. The number of PI-positive cells was expressed as a percent of the total number of cells. The degree of injury was also quantitated by measuring lactate dehydrogenase (LDH) released into the medium in some experiments, as previously described (Xu & Giffard, 1997). Samples obtained at the end of the experiments were compared to values in medium after cells were frozen/thawed for maximum LDH release. The percent death (% of LDH release) was calculated by dividing the experimental time point by the maximum values $\times$ 100.

Reverse Transcription Quantitative real-time Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated with TRIzol® (ThermoFisher Scientific) from cultures 24 hrs following transfection as previously described (Ouyang *et al.*, 2013). Reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription Kit for total RNA (ThermoFisher Scientific). Predesigned primer/probes for polymerase chain reaction were obtained from Life Technologies for miR-29a (#002112), desmoplakin mRNA (#Mm01351876) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #Mm99999915) mRNA as internal gene standard. Real time quantitative polymerase chain reaction was conducted using the TaqMan® Assay Kit (ThermoFisher Scientific). Measurements for miR-29a were normalized to U6 (Ct) and comparisons calculated as the inverse log of the Ct to provide relative fold change (Livak & Schmittgen, 2001). Liu *et al.* have validated U6 as not changing in cerebral ischemia (Liu *et al.*, 2010). Measurements for desmoplakin mRNA were normalized to within-sample glyceraldehyde 3-phosphate dehydrogenase from controls and comparisons calculated as the inverse log of the Ct. All PCR experiments were repeated 3 times, each using separate sets of samples.

Immunoblotting

Immunoblotting of 20 μ g protein from astrocyte cultures was performed as described previously (Ouyang *et al.*, 2007) with VDAC1 mouse monoclonal antibody (# ab14734 Abcam, Cambridge, UK, 1:500 dilution), and actin rabbit monoclonal antibody (# 926-42210, Li-Cor Biosciences, Lincoln, NE, 1:3000). Immunoreactive bands were visualized using the Li-Cor Odyssey infrared imaging system (Li-Cor Biosciences) as described previously (Sary *et al.*, 2015b). Densitometric quantifications were performed using ImageJ software (v1.46, National Institutes of Health, Bethesda, MD) and band intensities were normalized to β -actin.

Fluorescence Immunocytochemistry

Fluorescence immunocytochemistry was performed on cell cultures in 24-well plates as described previously (Ouyang *et al.*, 2013). Cultures were fixed in 4% paraformaldehyde for

30 min at room temperature. Nonspecific binding was blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 1 hr. To verify transfection of miR-29a/VDAC1 TSB, cells were assessed for 6-FAM fluorescence with a Zeiss (Carl Zeiss AG, Jena, Germany) Axiovert 200M epifluorescence microscope and AxioVision software (v4.8.1). To verify localization of VDAC1, cells were incubated with mouse monoclonal primary antibody to VDAC1 (# ab14734, Abcam, 1:200 dilution) and the mitochondrial marker ATP5a (# ab176569, Abcam, 1:200) overnight at 4°C. Cells were then washed and incubated with Alexa Fluor 488 nm- or 594 nm-conjugated secondary antibody (1:500; Invitrogen, Grand Island, NY) for 1 hr. Cells were counterstained with the nuclear dye DAPI (4',6'-diamidino-2-phenylindole, 0.5 µg/ml; Sigma-Aldrich, St Louis, MO, USA) and imaged on the epifluorescence microscope.

Statistics

All data reported represent at least 3 independent experiments for n=6 cultures in each experiment. Data reported are means ± SD. Statistical difference was determined using T-test for comparison of two groups or ANOVA followed by Newman Keuls post test for experiments with >2 groups. In all analyses p<0.05 was considered significant.

RESULTS

In order to verify successful separation of CA1 and DG hippocampal subregions we analyzed astrocyte cultures for relative expression of desmoplakin mRNA, which is selectively expressed in the DG (Lein *et al.*, 2004). Levels of desmoplakin mRNA were 4.0 ± 0.2 fold higher in astrocytes cultured from the DG versus from CA1. No difference was observed in baseline levels of miR-29a expression between CA1 and DG astrocytes ($DG = 1.19 \pm 0.78$ fold versus CA1). Subjecting cultures to GD injury for 48 hrs resulted in a significantly greater reduction in miR-29a expression in astrocytes from CA1 versus DG (Fig. 1A). Transfection of cultures with miR29a mimic resulted in significantly greater expression of miR-29a in both CA1 (355 ± 157 fold) and DG (335 ± 56 fold) astrocyte cultures, which then decreased with injury but remained significantly elevated versus control (87 ± 13 fold and 164 ± 6 fold in CA1 and DG respectively). Astrocyte cultures were assessed for cell death with PI staining following 48 hrs of GD (Fig. 1B). This ischemia-like stress resulted in significant injury in cultures from both CA1 and DG, with a greater degree of injury observed in CA1 astrocytes (Figs. 1B, C). Artificially increasing levels of miR-29a by pre-treatment with miR-29a mimic decreased cell death from GD injury in both DG and CA1 astrocytes (Figs. 1B, C). Similar results were observed with cell death assessment by LDH activity assay (data not shown).

VDAC1 plays a critical role in mitochondria-mediated cell death (Shoshan-Barmatz & Ben-Hail, 2012) and is a target of miR-29a-3p in other cell types (Bargaje *et al.*, 2012). In the present study, we assessed the time course of cell death and VDAC1 expression in response to 8, 24, and 48 hrs GD injury (Fig. 2A). We observed that regions with higher levels of VDAC1 expression tended to have higher regional cell death (Fig. 2B). When quantified, cell death (Fig. 2C) and VDAC1 expression (Fig. 2D) significantly increased in both CA1 and DG cultures by 24 hrs of GD, with no differences between subregions. However by 48

hrs of GD, cell death and VDAC1 expression were significantly higher in CA1 astrocytes relative to DG. When hippocampal astrocytes were assessed for subcellular localization of VDAC1, VDAC1 co-localized with the mitochondrial marker ATP5a (Fig. 2E). Pre-treatment of CA1 and DG astrocytes with either 30 or 50 pmol siRNA targeted to VDAC1 resulted in significant knockdown of VDAC1 expression in both CA1 and DG astrocytes following 48 hrs of GD (Figs. 3A, B). This coincided with a significant decrease in cell death in astrocytes from both subregions (Figs. 4A, C). In CA1 astrocytes, treatment with VDAC1 siRNA significantly attenuated the increase in VDAC1 in response to GD (Fig. 3D), while DG showed a similar trend to reduced expression. Knockdown of VDAC1 with siRNA pretreatment in both DG and CA1 astrocytes resulted in a significant reduction in cell death from GD (Fig. 3E).

Transfection with miR-29a mimic attenuated the larger increase in VDAC1 expression from 48 hrs of GD in CA1 astrocytes when assessed by immunocytochemistry (Fig. 4A) or immunoblot (Figs. 4B, C). Targeted inhibition of the miR-29a/VDAC1 interaction was achieved by transfection with TSB. Transfection efficiency of miR29a/VDAC1 TSB (assessed by 6-FAM^{TM} reporter fluorescence, Fig 5A) was $65 \pm 26\%$ in CA1 astrocytes and $61 \pm 14\%$ in DG astrocytes (mean $\pm$ SD). Because reversing miR-29a suppression of VDAC1 translation with TSB should result in an increase in VDAC1 translation, we assessed the effect of TSB transfection on VDAC1 expression as an additional measure of TSB transfection: cells positive for TSB 6-FAM^{TM} reporter fluorescence displayed significantly higher levels of VDAC1 immunofluorescence (Figs. 5B, C) relative to cells with low or absent levels of reporter. When assayed for cell death, transfection with TSB alone or in conjunction with miR-29a increased pre-injury levels in both CA1 and DG astrocytes (Fig. 5D). Following GD injury, co-transfection with miR-29a mimic plus miR29a/VDAC1 TSB resulted in a greater degree of cell death in both CA1 and DG astrocytes versus transfection with miR-29a mimic alone. This observation suggests that in these hippocampal astrocytes the protective effect of miR-29a from GD injury occurred via targeted inhibition of VDAC1 translation.

DISCUSSION

The results of the present study are the first to demonstrate a differential response in miR-29a to stress between astrocytes cultured from the more ischemia-sensitive CA1 hippocampal subregion and astrocytes from the DG. We have previously observed that increasing levels of miR-29a in cortical astrocytes preserved mitochondrial homeostasis following ischemia-like stress (Ouyang *et al.*, 2013). In the present study, overexpression of miR-29a in both CA1 and DG subregion astrocytes augmented survival in response to extended GD injury. Here we demonstrate for the first time in astrocytes that the protective effect of miR-29a to ischemic injury occurs via targeted inhibition of VDAC1 expression. A functional relationship between miR-29a and VDAC1 in astrocytes is supported by two key observations from the present study: first, reciprocal changes in expression were observed between miR-29a and VDAC1 in hippocampal astrocyte cultures following stress, consistent with previous observations in other cell types (Bargaje *et al.*, 2012; Roshan *et al.*, 2014). Second, co-transfection of miR-29a mimic and miR-29a/VDAC1 TSB (which inhibited the

binding of miR-29a to the VDAC1 mRNA) eliminated the protective effect of miR-29a following ischemia-like stress.

Located in the outer mitochondrial membrane, VDACs mediate inter-compartmental transport of anions, cations, and ATP between the cytosol and mitochondria (for review, Shoshan-Barmatz & Ben-Hail, 2012). Of the three known isoforms, VDAC1 is the most abundantly expressed, and is thought to participate in the mitochondrial response to cell stress (Huang *et al.*, 2014). VDAC1 has also been shown to induce apoptosis via release of apoptogenic proteins (such as cytochrome c) from mitochondria (Lee *et al.*, 2004). Mitochondria-mediated cell death is dependent on formation of the mitochondrial permeability transition pore (MPTP), a multiprotein complex that forms a channel between the inner and outer mitochondrial membranes (for review, Sims & Muyderman, 2010). Although VDAC1 has been considered to be a constituent of the MPTP, the role of VDAC1 in the formation of the MPTP subsequent to ischemic injury remains controversial (McCommis & Baines, 2012). No differences were observed in the mitochondrial response to stress or MPTP formation in cardiac cells from VDAC1 double knockout mice versus cells from wild type mice (Baines *et al.*, 2007). However, pharmacological inhibition of VDAC1 in cardiac myocytes prevented MPTP formation and improved cell survival following ischemia reperfusion injury (Liao *et al.*, 2015). VDAC1 may also play a role in ischemia-reperfusion injury by contributing to the formation of the mitochondrial associated membrane (MAM) macromolecular complex, together with the chaperone glucose-related protein 75 (Grp75/Hsp75) and inositol triphosphate receptor-1 (IP3R1). MAM serves to regulate direct Ca^{2+} transfer from endoplasmic reticulum (ER) to mitochondria (Szabadkai *et al.*, 2006), and disruption of MAM function in cardiac myocytes via knockdown of either Grp75 or IP3R1 attenuated accumulation of mitochondrial Ca^{2+} , resulting in protection from hypoxia-reperfusion injury (Paillard *et al.*, 2013). Together these findings suggest that VDAC1 may regulate cell death from ischemia-reperfusion injury.

A functional relationship between miR-29a and VDAC1 was previously observed in the brain by Roshan *et al.* (Roshan *et al.*, 2014), who noted a corresponding increase in hippocampal VDAC1 levels in mice with brain-targeted knockdown of miR-29. The observed increase in hippocampal neuronal cell death that occurred with miR-29 knockdown was partially reversed by co-suppressing VDAC1 expression. These observations support the findings in the present study, in which inhibition of miR-29a/VDAC1 binding by TSB (which increased baseline expression of VDAC1) resulted in an increase in pre-injury levels of astrocyte cell death. The role of VDAC1 in brain injury is less clear. Chaudhuri *et al.* (Chaudhuri *et al.*, 2016) demonstrated in neuroblastoma SH-SY5Y cells that knockdown of VDAC1 decreased ROS production and injury from complex-1 inhibition, indicating that VDAC1 directly contributes to neuronal cell death secondary to depletion of ATP. However Li *et al.* (Li *et al.*, 2014) observed in a rodent model of subarachnoid hemorrhage that *in vivo* suppression of VDAC1 with siRNA exacerbated injury and worsened neurological outcome. This finding is at odds with our *in vitro* observations in astrocytes from both CA1 and DG subregions in response to GD injury: 1) increased expression of VDAC1 corresponded over time with increases in cell death; 2) overexpression of VDAC1 (via TSB-mediated inhibition of miR-29a binding) resulted in increased cell death; and, 3) knockdown of VDAC1 with siRNA resulted in decreased cell death. This discrepancy may be due to differences in the

effect of VDAC1 in different brain cell types and/or differences in the type or duration of ischemic brain injury. For example, while the GD injury model used in the present study has the advantage of a high degree of reliability and repeatability in the level of astrocyte cell death, combined oxygen-glucose deprivation may more closely mimic the stress of cerebral ischemia. Further exploring the role of VDAC1 and miR-29a in alternative models of *in vitro* injury, and *in vivo*, will likely be relevant in the pursuit of novel therapeutics for cerebral ischemia.

Conclusions

Astrocytes cultured from the hippocampal CA1 subregion exhibited a greater decrease in miR-29a and more cell death in response to extended GD injury versus astrocytes from the hippocampal DG subregion. This functional, subregional heterogeneity is neutralized by exogenously elevating miR-29a levels, or by siRNA-mediated knockdown of the miR-29a target VDAC1. Moreover, blocking the miR-29a/VDAC1 interaction results in augmented expression of VDAC1, resulting in an increase in pre-injury levels of cell death, and eliminating the protective effect of miR-29a against GD injury. Together, these findings suggest that VDAC1 plays a downstream role in the miR-29a response to ischemic injury in astrocytes. Future investigations exploring the mechanisms that determine regional differences in hippocampal astrocyte gene expression may reveal new directions for the development of effective therapies for the injured brain.

Acknowledgments

Supported by American Heart Association 14FTF-19970029 to Dr. Stary and by National Institutes of Health grants R01 NS084396, R01 NS053898 and R01 NS 080177 to Dr. Giffard.

References

- Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol.* 2007; 9:550–555. [PubMed: 17417626]
- Bargaje R, Gupta S, Sarkeshik A, Park R, Xu T, Sarkar M, Halimani M, Roy SS, Yates J, Pillai B. Identification of novel targets for miR-29a using miRNA proteomics. *PLoS One.* 2012; 7:e43243. [PubMed: 22952654]
- Barreto G, White RE, Ouyang Y, Xu L, Giffard RG. Astrocytes: targets for neuroprotection in stroke. *Cent Nerv Syst Agents Med Chem.* 2011; 11:164–173. [PubMed: 21521168]
- Chaudhuri AD, Choi DC, Kabaria S, Tran A, Junn E. MicroRNA-7 Regulates the Function of Mitochondrial Permeability Transition Pore by Targeting VDAC1 Expression. *J Biol Chem.* 2016; 291:6483–6493. [PubMed: 26801612]
- Clarke LE, Barres BA. Emerging roles of astrocytes in neural circuit development. *Nat Rev Neurosci.* 2013; 14:311–321. [PubMed: 23595014]
- Hagihara H, Toyama K, Yamasaki N, Miyakawa T. Dissection of hippocampal dentate gyrus from adult mouse. *J Vis Exp.* 2009
- Huang H, Shah K, Bradbury NA, Li C, White C. Mcl-1 promotes lung cancer cell migration by directly interacting with VDAC to increase mitochondrial Ca²⁺ uptake and reactive oxygen species generation. *Cell Death Dis.* 2014; 5:e1482. [PubMed: 25341036]
- Lee MJ, Kim JY, Suk K, Park JH. Identification of the hypoxia-inducible factor 1 alpha-responsive HGTD-P gene as a mediator in the mitochondrial apoptotic pathway. *Mol Cell Biol.* 2004; 24:3918–3927. [PubMed: 15082785]

- Lein ES, Zhao X, Gage FH. Defining a molecular atlas of the hippocampus using DNA microarrays and high-throughput in situ hybridization. *J Neurosci*. 2004; 24:3879–3889. [PubMed: 15084669]
- Li J, Lu J, Mi Y, Shi Z, Chen C, Riley J, Zhou C. Voltage-dependent anion channels (VDACs) promote mitophagy to protect neuron from death in an early brain injury following a subarachnoid hemorrhage in rats. *Brain Res*. 2014; 1573:74–83. [PubMed: 24880016]
- Liao Z, Liu D, Tang L, Yin D, Yin S, Lai S, Yao J, He M. Long-term oral resveratrol intake provides nutritional preconditioning against myocardial ischemia/reperfusion injury: involvement of VDAC1 downregulation. *Mol Nutr Food Res*. 2015; 59:454–464. [PubMed: 25488258]
- Liu DZ, Tian Y, Ander BP, Xu H, Stamova BS, Zhan X, Turner RJ, Jickling G, Sharp FR. Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. *J Cereb Blood Flow Metab*. 2010; 30:92–101. [PubMed: 19724284]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- McCommis KS, Baines CP. The role of VDAC in cell death: friend or foe? *Biochim Biophys Acta*. 2012; 1818:1444–1450. [PubMed: 22062421]
- Mehler MF. Epigenetic principles and mechanisms underlying nervous system functions in health and disease. *Prog Neurobiol*. 2008; 86:305–341. [PubMed: 18940229]
- Nedergaard M, Dirnagl U. Role of glial cells in cerebral ischemia. *Glia*. 2005; 50:281–286. [PubMed: 15846807]
- Niizuma K, Endo H, Nito C, Myer DJ, Chan PH. Potential role of PUMA in delayed death of hippocampal CA1 neurons after transient global cerebral ischemia. *Stroke*. 2009; 40:618–625. [PubMed: 19095966]
- Ouyang YB, Tan Y, Comb M, Liu CL, Martone ME, Siesjo BK, Hu BR. Survival- and death-promoting events after transient cerebral ischemia: phosphorylation of Akt, release of cytochrome C and Activation of caspase-like proteases. *J Cereb Blood Flow Metab*. 1999; 19:1126–1135. [PubMed: 10532637]
- Ouyang YB, Voloboueva LA, Xu LJ, Giffard RG. Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. *J Neurosci*. 2007; 27:4253–4260. [PubMed: 17442809]
- Ouyang YB, Xu L, Liu S, Giffard RG. Role of Astrocytes in Delayed Neuronal Death: GLT-1 and its Novel Regulation by MicroRNAs. *Adv Neurobiol*. 2014; 11:171–188. [PubMed: 25236729]
- Ouyang YB, Xu L, Lu Y, Sun X, Yue S, Xiong XX, Giffard RG. Astrocyte-enriched miR-29a targets PUMA and reduces neuronal vulnerability to forebrain ischemia. *Glia*. 2013; 61:1784–1794. [PubMed: 24038396]
- Ouyang YB, Xu LJ, Emery JF, Lee AS, Giffard RG. Overexpressing GRP78 influences Ca²⁺ handling and function of mitochondria in astrocytes after ischemia-like stress. *Mitochondrion*. 2011; 11:279–286. [PubMed: 21047562]
- Ouyang YB, Xu LJ, Sun YJ, Giffard RG. Overexpression of inducible heat shock protein 70 and its mutants in astrocytes is associated with maintenance of mitochondrial physiology during glucose deprivation stress. *Cell Stress Chaperones*. 2006; 11:180–186. [PubMed: 16817324]
- Owens K, Park JH, Gourley S, Jones H, Kristian T. Mitochondrial dynamics: cell-type and hippocampal region specific changes following global cerebral ischemia. *J Bioenerg Biomembr*. 2015; 47:13–31. [PubMed: 25248415]
- Paillard M, Tubbs E, Thiebaut PA, Gomez L, Fauconnier J, Da Silva CC, Teixeira G, Mewton N, Belaidi E, Durand A, Abrial M, Lacampagne A, Rieusset J, Ovize M. Depressing mitochondria-reticulum interactions protects cardiomyocytes from lethal hypoxia-reoxygenation injury. *Circulation*. 2013; 128:1555–1565. [PubMed: 23983249]
- Papadopoulos MC, Koumenis IL, Dugan LL, Giffard RG. Vulnerability to glucose deprivation injury correlates with glutathione levels in astrocytes. *Brain Res*. 1997; 748:151–156. [PubMed: 9067456]
- Papadopoulos MC, Koumenis IL, Yuan TY, Giffard RG. Increasing vulnerability of astrocytes to oxidative injury with age despite constant antioxidant defenses. *Neuroscience*. 1998; 82:915–925. [PubMed: 9483545]

- Roshan R, Ghosh T, Scaria V, Pillai B. MicroRNAs: novel therapeutic targets in neurodegenerative diseases. *Drug Discov Today*. 2009; 14:1123–1129. [PubMed: 19822222]
- Roshan R, Shridhar S, Sarangdhar MA, Banik A, Chawla M, Garg M, Singh VP, Pillai B. Brain-specific knockdown of miR-29 results in neuronal cell death and ataxia in mice. *RNA*. 2014; 20:1287–1297. [PubMed: 24958907]
- Shoshan-Barmatz V, Ben-Hail D. VDAC, a multi-functional mitochondrial protein as a pharmacological target. *Mitochondrion*. 2012; 12:24–34. [PubMed: 21530686]
- Sims NR, Muiyerman H. Mitochondria, oxidative metabolism and cell death in stroke. *Biochim Biophys Acta*. 2010; 1802:80–91. [PubMed: 19751827]
- Smirnova L, Grafe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. Regulation of miRNA expression during neural cell specification. *Eur J Neurosci*. 2005; 21:1469–1477. [PubMed: 15845075]
- Sary CM, Sun X, Giffard RG. Astrocytes Protect against Isoflurane Neurotoxicity by Buffering pro-brain-derived Neurotrophic Factor. *Anesthesiology*. 2015a; 123:810–819. [PubMed: 26270940]
- Sary CM, Xu L, Sun X, Ouyang YB, White RE, Leong J, Li J, Xiong X, Giffard RG. MicroRNA-200c contributes to injury from transient focal cerebral ischemia by targeting Reelin. *Stroke*. 2015b; 46:551–556. [PubMed: 25604249]
- Szabadkai G, Bianchi K, Varnai P, De Stefani D, Wieckowski MR, Cavagna D, Nagy AI, Balla T, Rizzuto R. Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca^{2+} channels. *J Cell Biol*. 2006; 175:901–911. [PubMed: 17178908]
- Xu L, Emery JF, Ouyang YB, Voloboueva LA, Giffard RG. Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia. *Glia*. 2010; 58:1042–1049. [PubMed: 20235222]
- Xu L, Giffard RG. HSP70 protects murine astrocytes from glucose deprivation injury. *Neurosci Lett*. 1997; 224:9–12. [PubMed: 9132695]
- Xu L, Sapolsky RM, Giffard RG. Differential sensitivity of murine astrocytes and neurons from different brain regions to injury. *Exp Neurol*. 2001; 169:416–424. [PubMed: 11358455]

Highlights

- Astrocytes from hippocampal CA1 exhibit a greater decrease in miR-29a and more cell death from glucose deprivation versus astrocytes from DG.
- Elevations in VDAC1, a miR-29a target, coincide with increased cell death, greater in CA1 astrocytes.
- CA1/DG differences in susceptibility to injury are neutralized by elevating miR-29a, or by knockdown of VDAC1.
- Blocking the miR-29a/VDAC1 interaction augments expression of VDAC1, reducing miR-29a protection.
- These findings suggest that VDAC1 plays a downstream role in the miR-29a response to glucose deprivation injury in astrocytes.

Figure 1. Effect of miR-29a on cell injury in astrocytes cultured from hippocampal cornu ammonis 1 (CA1) and dentate gyrus (DG). **(A)** miR-29a expression in CA1 astrocytes and DG astrocytes in response to 48 hrs of glucose deprivation (GD). **(B)** Micrographs of CA1 astrocytes and DG astrocytes pre-treated with either miR-29a mimic or transfection control and stained with propidium iodide (red, dead cells) and Hoechst (blue, all cell nuclei) after 48 hrs of GD. **(C)** Quantification of cell death by PI/Hoechst staining and cell counting following 48 hrs of GD in CA1 astrocytes and DG astrocytes with or without miR-29a mimic pre-treatment. Bar = 25 μ m. Graphs represent n = 4–8 cultures/condition, all experiments repeated three times. * = versus wash controls. # = versus injury controls. Ψ = versus CA1, condition-matched, all p < 0.05.

Figure 2.

Voltage-dependent anion channel 1 (VDAC1) expression in astrocytes from CA1 and DG following injury. (A) Micrographs of astrocytes cultured from CA1 (top) and DG (bottom) subregions following 8, 24 or 48 hrs of glucose deprivation (GD). Cells were triple stained with propidium iodide (red, dead cells) DAPI (blue, all cell nuclei) and anti-VDAC1 (green). (B) Areas that exhibited greater numbers of PI-positive cells tended to co-localize with areas of higher VDAC1 expression (arrow). Quantification of cell death (C) and VDAC1 (D) expression in CA1 and DG astrocytes following 8, 24 or 48 hrs of GD injury. (E) VDAC1 (red) co-localizes with the mitochondrial indicator ATP5a (green) in astrocytes following 48 hrs of GD injury. Bar = 15 μ m. Graph represents pooled data from 3 independent experiments, n = 4 cultures/treatment. * = versus wash controls. Ψ = versus CA1, condition-matched. P < 0.05 for all symbols. CA1 = cornu ammonis 1; DG = dentate gyrus; DAPI = 4', 6'-diamidino-2-phenylindole.

Figure 3.

Effect of VDAC1 knockdown in astrocytes from CA1, DG, and cortex. (A)

Immunofluorescence of astrocytes cultured from CA1 and DG hippocampal subregions pre-treated with 0, 30, or 50 pmol VDAC1 small interfering RNA (siRNA), and subjected to 48 hrs GD. Cells were stained for VDAC1 expression (green), cell death (red, PI) and normalized for total cell count by counter-staining with DAPI (blue). (B) Quantification of post-injury VDAC1 expression in CA1 and DG astrocytes with VDAC1 siRNA pre-treatment. (C) Quantification of cell death in CA1 and DG astrocytes pre-treated with VDAC1 siRNA. (D) VDAC1 protein expression following 48 hrs of GD in CA1 and DG astrocytes, with and without pre-treatment with VDAC1 siRNA. (E) Quantification of cell death from GD in CA1 and DG astrocytes with or without VDAC1 siRNA pre-treatment. Bar = 25 μ m. Graphs represent n = 4–6 cultures/condition, all experiments repeated in triplicate. * = versus wash controls. # = versus post-injury controls. Ψ = versus CA1, condition-matched. $P < 0.05$ for all symbols. CA1 = cornu ammonis 1; DG = dentate gyrus. DAPI = 4',6'-diamidino-2-phenylindole; GD = glucose deprivation; VDAC1 = voltage-dependent anion channel 1.

Figure 4.

(A) Voltage-dependent anion channel 1 (VDAC1) staining before and following 48 hrs of GD in astrocytes from CA1 and DG with either miR-29a mimic or transfection control pre-treatment. Cells are counterstained with DAPI for total cell count. (B) Immunoblot of VDAC1 protein expression following 48 hrs of GD in CA1 and DG astrocytes, with or without miR-29a mimic pre-treatment. (C) Quantification of VDAC1 protein expression following 48 hrs of GD in CA1 and DG astrocytes, with or without miR-29a mimic pre-treatment. Bar = 15 μ m. Graph represents pooled data from 4 independent experiments, n = 4 cultures/treatment. * = versus wash controls, # = versus injury controls. Ψ = versus CA1, condition-matched. $P < 0.05$ for all symbols. CA1 = cornu ammonis 1; DG = dentate gyrus; DAPI = 4',6'-diamidino-2-phenylindole. GD = glucose deprivation.

Figure 5.

Inhibition of miR-29a/VDAC1 binding in CA1 and DG astrocytes. (A) Hippocampal astrocytes transfected with miR-29a/VDAC1 target site blocker (TSB) are identified by double nuclear labeling with 6-FAM^{TM} fluorescent reporter (green) and DAPI, which stains all nuclei blue. (B) Immunofluorescent staining for VDAC1 (red) indicates localization of expression in cells with a high degree of green 6-FAM^{TM} reporter fluorescence (arrows). (C) Quantification of VDAC1 staining in hippocampal astrocytes from CA1 and DG subregions transfected with control scramble sequence, or TSB. Cells transfected with TSB were identified by the presence of reporter fluorescence. (D) Cell death in hippocampal astrocytes from CA1 (left) and DG (right) transfected with miR-29a or control scramble sequence, with and without TSB co-transfection. Cell death was assessed 24 hrs following transfection (pre-injury) and following 48 hrs of glucose deprivation (GD) injury. Bars = 10 μ m. Graphs represent n = 4–6 cultures/condition, all experiments repeated in triplicate. * = versus wash controls. # = versus post-injury controls. P < 0.05 for all symbols. CA1 = cornu ammonis 1; DG = dentate gyrus. DAPI = 4',6'-diamidino-2-phenylindole; VDAC1 = voltage-dependent anion channel 1.