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miR-29a differentially regulates cell survival in astrocytes from cornu ammonis 1 and dentate gyrus by targeting VDAC1

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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.

1. 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 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 and 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 and Barres, 2013; Barreto et al., 2011). Critically, astrocyte homeostasis is tightly coupled to neuronal cell fate following ischemic injury (Nedergaard and 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

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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.

2. Methods and materials

2.1. 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% CO_2 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.

2.2. 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 h) 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 μM, Sigma Chemicals) and propidium iodide (PI, 5 μM, 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 200× 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 and 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 × 100.

2.3. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated with TRIzol® (ThermoFisher Scientific) from cultures 24 h 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 (ΔCt) and comparisons calculated as the inverse log of the ΔΔCT to provide relative fold change (Livak and 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 ΔΔCT. All PCR experiments were repeated 3 times, each using separate sets of samples.

2.4. Immunoblotting

Immunoblotting of 20 μg 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 (Stary 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.

2.5. 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%