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Ischemia-induced autophagy contributes to neurodegeneration in cerebellar Purkinje cells in the developing rat brain and in primary cortical neurons in vitro

Alicia K. Au [a], Yaming Chen [a], LinaDu [a], Craig M. Smith [b], Mioara D. Manole [c], Sirine A. Baltagi [d], Charleen T. Chu [e], Rajesh K. Aneja [a,c], Hülya Bayır [a,c,f], Patrick M. Kochanek [a,c,g], Robert S.B. Clark [a,c,h,*]

[a] Department of Critical Care Medicine, The Safar Center for Resuscitation Research, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[b] Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL, USA
[c] Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA
[d] Department of Pediatrics, Washington University School of Medicine, St. Louis, MO, USA
[e] Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[f] Department of Environmental and Occupational Health, Center for Free Radical and Antioxidant Health, University of Pittsburgh, Pittsburgh, PA, USA
[g] Department of Anesthesiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[h] Clinical and Translational Science Institute, University of Pittsburgh, Pittsburgh, PA, USA

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ABSTRACT

Increased autophagy/mitophagy is thought to contribute to cerebellar dysfunction in *Purkinje cell degeneration* mice. Intriguingly, cerebellar Purkinje cells are highly vulnerable to hypoxia–ischemia (HI), related at least in part to their high metabolic activity. Whether or not excessive or supraphysiologic autophagy plays a role in Purkinje cell susceptibility to HI is unknown. Accordingly, we evaluated the role of autophagy in the cerebellum after global ischemia produced by asphyxial cardiac arrest in postnatal day (PND) 16–18 rats, using siRNA-targeted inhibition of Atg7, necessary for microtubule-associated protein light chain 3-II (LC3-II) and Atg12–Atg5 complex formation. Two days before a 9 min asphyxial cardiac arrest or sham surgery, Atg7 or control siRNA was injected intracisternally to target the cerebellum. Treatment with Atg7 siRNA: 1) reduced Atg7 protein expression in the cerebellum by 56%; 2) prevented the typical ischemia-induced formation of LC3-II in the cerebellum 24 h after asphyxial cardiac arrest; 3) improved performance on the beam-balance apparatus on days 1–5; and 4) increased calbindin-labeled Purkinje cell survival assessed on day 14. Improved Purkinje cell survival was more consistent in female vs. male rats, and improved beam-balance performance was only seen in female rats. Similar responses to Atg7 siRNA i.e. reduced autophagy and neurodegeneration vs. control siRNA were seen when exposing sex-segregated green fluorescent protein-LC3 tagged mouse primary cortical neurons to oxygen glucose deprivation in vitro. Thus, inhibition of autophagy after global ischemia in PND 16–18 rats leads to increased survival of Purkinje cells and improved motor performance in a sex-dependent manner.

1. Introduction

Accelerated cerebellar Purkinje cell degeneration is seen in mice with mutations in one of now several identified *pcd* alleles, with the *Purkinje cell degeneration* mouse the first to be characterized [1]. These *pcd* mice demonstrate cerebellar ataxia and gait disturbances beginning around postnatal day (PND) 21 and lose >99% of Purkinje cells by PND 42 [1]. This profound Purkinje cell vulnerability has been reported to be at least in part due to excessive autophagy [2]. Autophagy is an intracellular degradation pathway that is involved in the homeostatic turnover of aging proteins and organelles, including mitochondria. Autophagic degradation of mitochondria—termed "mitophagy", can be triggered by externalization of cardiolipin [3] or severe membrane depolarization [4]. Autophagy proceeds via a complex interplay of autophagy-related genes (Atgs), with Atg7 representing a central player in its induction [5], although an Atg5/7-independent pathway has been reported [6]. Atg7 is an ubiquitin E1-like enzyme that controls the critical step of converting Atg8/microtubule-associated protein light chain 3-I (LC3-I) to LC3-II via covalent attachment of phosphatidylethanolamine [7,8] and for the formation of Atg12–Atg5 complexes [9]. Increased autophagy in the injured brain has been reported after multiple insults, including traumatic brain injury and hypoxia–ischemia (HI) [10–15]. However, the role of autophagy after HI has been controversial, as attempts to elucidate its role after HI have been limited by the lack of specific pharmacological inhibitors, the requirement for basal autophagy in normal neurodevelopment complicating studies in

* Corresponding author at: Safar Center for Resuscitation Research, 3434 Fifth Avenue, Pittsburgh, PA 15260, USA.
E-mail address: clarkrs@upmc.edu (R.S.B. Clark).

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transgenic mice, and limited distribution of small interfering RNA (siRNA) in the brain when injected in vivo [16–19].

In addition to being prone to neurodegeneration via dysregulation of autophagy [2], cerebellar Purkinje cells are exquisitely vulnerable to HI [20,21], both perhaps related to the fact that Purkinje cells have one of the highest metabolic rates of any class of neurons. Purkinje cell vulnerability to HI and proclivity toward autophagy-induced neurodegeneration, in combination with the cerebellum's proximity to the intracisternal space, provided us with the opportunity to directly evaluate the role of autophagy after global brain HI in vivo, using a siRNA strategy. In the present study, we were able to effectively reduce both Atg7 protein abundance and HI-induced autophagy in the cerebellum using injection of Atg7 siRNA into the intracisternal space. We then evaluated the role of autophagy after HI by determining the effect of Atg7 knockdown in the cerebellum on Purkinje cell survival and vestibulomotor performance in our model of pediatric asphyxial cardiac arrest [22,23]. Lastly, we verified the capacity for Atg7 knockdown to inhibit ischemia-induced autophagic neurodegeneration using sex-segregated green fluorescent protein (GFP)–LC3 tagged (GFP–LC3$^{+/-}$) mouse primary cortical neurons exposed to oxygen-glucose deprivation (OGD) in vitro.

2. Materials and methods

2.1. Asphyxial cardiac arrest

Studies were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. A total of 90 rats were used for the in vivo studies reported here, with 72 rats used for siRNA treatment studies.

An established model of pediatric asphyxial cardiac arrest was utilized for this study using PND 16–18 rat pups [22,23]. This developmental age was chosen because in the rat it is similar in terms of cerebral metabolism and blood flow, dendritic pruning, and synaptogenesis, to children 1–4 years of age [24]. PND 16–18 Sprague–Dawley rats (35–40 g) were anesthetized with 3% isoflurane/50% N_2O/balance oxygen in a Plexiglas chamber until unconscious. Rats were intubated with an 18-gauge angiocatheter and mechanically ventilated with 1% isoflurane/50% N_2O/balance oxygen for surgery. Tidal volumes and ventilatory rates were adjusted to maintain $PaCO_2$ 35 to 45 mm Hg. Femoral arterial and venous catheters (PE10) were inserted via inguinal cut down. Mean arterial blood pressure, heart rate, and temperature were monitored continuously. Vecuronium (1 mg/kg, intravenously) was administered 10 min before asphyxia. Isofluorane/N_2O was discontinued 2 min before asphyxia to allow washout of anesthetic. 1 min before asphyxia, the FiO_2 was reduced to 0.21 to avoid hyperoxygenation. The endotracheal tube was disconnected from the ventilator for 9 min to induce asphyxial cardiac arrest. Resuscitation consisted of reconnecting the tracheal tube to the ventilator at a FiO_2 of 1.0, intravenous administration of epinephrine 0.005 mg/kg and sodium bicarbonate 1 mEq/kg, and rapid manual chest compression until return of spontaneous circulation. Vascular catheters were removed, rats were extubated, observed for 1 h, then returned to their mothers. Shams underwent all procedures except asphyxia and resuscitation.

To characterize neurodegeneration in the cerebellum in this model a separate group of male rats were sacrificed at 24 or 72 h after asphyxial cardiac arrest with naïve rats serving as control, and processed for histological assessment of neurodegeneration (n = 3/group) or electron microscopy (n = 3/group). Hemotoxylin and eosin staining and Fluorojade labeling of degenerating neurons were performed in paraffin sections, and ultrastructural analysis was performed in 65 nm ultrathin sections of cerebellum by transmission electron microscopy as previously described [22,25].

2.2. Intracisternal administration of siRNA

Intracisternal (i.c.) injection was performed using a modification of the technique described by Consiglio et al. [26] for application in rat pups. Naïve postnatal day (PND) 14–16 Sprague–Dawley rats were anesthetized with 3% isoflurane/50% N_2O/balance oxygen in a Plexiglas chamber until unconscious. The head was flexed and using sterile technique a 27-gauge butterfly needle was inserted vertically and centrally approximately 2-mm posterior to the intra-aural line. The i.c. space was identified by the appearance of CSF in the tubing, and verified by aspiration of ~25 μl of CSF (to accommodate injection). A total of 800 pmol of Atg7 siRNA (5′-GCAUCAUCUUUGAAGUGAA-3′; Sigma, PDSIRNA) or non-targeting control siRNA (Fischer, D-001206-13-20) were combined with 25 μl of jetSI (Polyplus Transfection, 55-126), a commercial cationic polymer transfection reagent, formulated with dioleoylphosphatidyl ethanolamine (DOPE) (Sigma, P1223) for i.c. injection. Commercially obtained control siRNA consisted of missense sequences which have not been shown to have off-target effects. A syringe containing 25 μl of siRNA containing solution was injected slowly over 1 min to prevent leakage from the puncture site, then the needle was withdrawn. Inhaled anesthetics were discontinued and animals were allowed to recover with their mothers prior to randomization to asphyxial cardiac arrest or sham surgery.

The dose and timing of i.c. siRNA injection was based on pilot experiments in separate naïve rats. A volume of 25 μl of solution—artificial CSF containing Evans blue dye, was the maximum tolerated by rats at this age. Evans blue dye was distributed prominently within and around the cerebellum 24 h after injection. In our hands, the maximum amount of siRNA that can be added to 25 μl is 800 pmol. Subsequent experiments in naïve rats (n = 3/treatment group) using doses of 250–800 pmol of Atg7 siRNA injected 24–48 h prior to sacrifice, demonstrated that Atg7 knockdown was optimized with 800 pmol siRNA 48 h after i.c. injection (56%). After i.c. injection Atg7 knockdown was seen in the cerebellum, but not the cortex or hippocampus using western blot (data not shown). Accordingly, for the remainder of experiments 800 pmol siRNA was injected i.c. 48 h before asphyxia or sham surgery and we focused on effects in the cerebellum.

2.3. Western blot analysis

For determination of effective siRNA knockdown, western blot analysis was performed as previously described [25,27] on cerebellar samples from male naïve rats 48 h after siRNA injection, and in male rats 24 h after asphyxial cardiac arrest (72 h after Atg7 or control siRNA injection). Briefly, rats were anesthetized and transcardially perfused with ice-cold saline. Brains were removed and the cerebellum was isolated. Samples were homogenized with lysis buffer (20 mM HEPES-KOH, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM PMSF, 2 mg/ml aprotinin). Lysates were serially centrifuged to separate cellular proteins, with the P2 fraction containing autophagosomes, mitochondria, and small organelles. Samples were stored at −80 °C in 10% glycerol. Protein concentration was determined using a Bradford-based protein assay. Proteins were loaded into 15% acrylamide gels, separated electrophoretically, then transferred to a polyvinyl difluoride membrane. The membranes were incubated in 1:1000 dilution of monoclonal antibody against Atg7 (Sigma, A2856) or LC3 (MBL International, M115-3) at room temperature for 1 h, washed in phosphate-buffered saline (PBS) containing 0.1% Tween 20, then incubated in the appropriate secondary antibody for 1 h. The membranes were then incubated in chemiluminescence reagents, and exposed to X-ray film. After imaging membranes were washed and re-incubated using an antibody against cytochrome oxidase IV (COX IV) to serve as a loading control. Relative optical densities (ROD) of Atg7, LC3-II, and COX IV were calculated for each sample.

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