Neurobiology of Disease 76 (2015) 1–12

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Frataxin knockdown in human astrocytes triggers cell death and the release of factors that cause neuronal toxicity

Frida Loría [a], Javier Díaz-Nido [a,b,c,*]

[a] Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Madrid, Spain
[b] Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain
[c] Center for Biomedical Research on Rare Diseases (CIBERER), Madrid, Spain

ARTICLE INFO

Article history:
Received 10 July 2014
Revised 15 November 2014
Accepted 21 December 2014
Available online 29 December 2014

Keywords:
Friedreich's ataxia
Astrocytes
Cell death
Apoptosis
Neurotoxicity
Neurodegeneration

ABSTRACT

Friedreich's ataxia (FA) is a recessive, predominantly neurodegenerative disorder caused in most cases by mutations in the first intron of the frataxin (FXN) gene. This mutation drives the expansion of a homozygous GAA repeat that results in decreased levels of FXN transcription and frataxin protein. Frataxin (Fxn) is a ubiquitous mitochondrial protein involved in iron–sulfur cluster biogenesis, and a decrease in the levels of this protein is responsible for the symptoms observed in the disease. Although the pathological manifestations of FA are mainly observed in neurons of both the central and peripheral nervous system, it is not clear if changes in non-neuronal cells may also contribute to the pathogenesis of FA, as recently suggested for other neurodegenerative disorders. Therefore, the aims of this study were to generate and characterize a cell model of Fxn deficiency in human astrocytes (HAs) and to evaluate the possible involvement of non-cell autonomous processes in FA. To knockdown frataxin *in vitro*, we transduced HAs with a specific shRNA lentivirus (shRNA37), which produced a decrease in both frataxin mRNA and protein expression, along with mitochondrial superoxide production, and signs of p53-mediated cell cycle arrest and apoptotic cell death. To test for non-cell autonomous interactions we cultured wild-type mouse neurons in the presence of frataxin-deficient astrocyte conditioned medium, which provoked a delay in the maturation of these neurons, a decrease in neurite length and enhanced cell death. Our findings confirm a detrimental effect of frataxin silencing, not only for astrocytes, but also for neuron–glia interactions, underlining the need to take into account the role of non-cell autonomous processes in FA.

Introduction

Friedreich's ataxia (FA) is a hereditary disorder caused in most cases by the expansion of GAA repeats in the first intron of the frataxin (FXN) gene, which dampens the expression of frataxin (Fxn), a protein involved in the biogenesis of iron–sulfur clusters and the regulation of oxidative stress (Campuzano et al., 1996; Campuzano et al., 1997; Pastore and Puccio, 2013). FA is characterized by progressive neurodegeneration that mainly affects the dorsal root ganglia, the corticospinal and spinocerebellar tracts of the spinal cord, and the deep cerebellar nuclei (Delatycki and Corben, 2012; Koeppen and Mazurkiewicz, 2013). Until recently, neurodegenerative diseases have been regarded as exclusively neuronal disorders and the contribution of non-neuronal cells to FA has been largely ignored. However, there is now considerable evidence that the selective death of certain types of neurons is not only due to intrinsic changes within the vulnerable neurons but also, to additional changes within neighboring non-neuronal glial cells (Verkhratsky et al., 2012). Indeed, non-cell autonomous mechanisms mediated by glial dysfunction appear to contribute to the vulnerability of neurons in many neurodegenerative diseases (Liu et al., 2005; Ilieva et al., 2009; Lu et al., 2009; Rodriguez and Verkhratsky, 2011; Tao et al., 2011; Di Malta et al., 2012; Garden and La Spada, 2012; Meyer et al., 2014). In the case of FA, specific silencing of Fxn in glial cells triggers a phenotype in *Drosophila* similar to that observed after ubiquitous Fxn reduction, characterized by altered lipid metabolism in glial cells, degeneration of neurons, severely impaired locomotor activity and reduced lifespan (Navarro et al., 2010). Thus, it seems plausible that non-cell autonomous processes might contribute to neurodegeneration in FA.

Astrocytes are the most abundant glial cell type in the central nervous system (CNS), and fulfill a wide variety of complex and essential functions responsible for maintaining brain homeostasis (Barres, 2008; Belanger et al., 2011), such as: controlling fluid movement between the intracellular and extracellular space; protecting neurons against oxidative stress; regulating energy metabolism and blood flow; and modulating neuronal activity through the expression of cytokines, growth factors and transporters (Sofroniew and Vinters, 2010; Allaman et al., 2011). These cells are crucial for the correct functioning

Abbreviations: FA, Friedreich's ataxia; Fxn, frataxin; HAs, human astrocytes; shRNA, short hairpin RNA; ACM, astrocyte conditioned medium.

* Corresponding author at: Centro de Biología Molecular Severo Ochoa (CBMSO), Universidad Autónoma de Madrid, Campus de Cantoblanco, C/Nicolás Cabrera 1, 28049 Madrid, Spain. Fax: +34 911964420.
E-mail address: javier.diaznido@uam.es (J. Díaz-Nido).

Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2014.12.017
0969-9961/

of neurons, microglia, oligodendrocytes and endothelial cells. Nevertheless, while the functional effects of Fxn depletion in neurons have been studied to some extent (Palomo et al., 2011; Mincheva-Tasheva et al., 2014), less is known about the consequences of Fxn loss in astrocytes. Using an *in vitro* approach, we have characterized the effects of Fxn depletion in cultured human astrocytes (HAs). Accordingly, we describe the detrimental effects of Fxn deficiency in astrocytes, and we provide evidence that this astrocyte dysfunction has a negative impact on neuronal maturation and survival. Therefore, our results provide evidence of the crucial contribution of non-cell autonomous glial interactions in the neurodegeneration observed in FA.

Materials and methods

Lentiviral production and titration

To establish an *in vitro* model of Fxn knockdown in human astrocytes, lentiviral vectors expressing short hairpin RNA (shRNA) sequences were purchased from Sigma-Aldrich (Madrid, Spain) and they contained either the sequences against the human FXN gene (shRNA37: Mission® shRNA, Gene Bank accession number NM_000144), or a non-specific scrambled control (SCR: Mission® Non-Target shRNA, SHC002). Lentiviral packaging, stock production and titration was performed as described previously (Follenzi and Naldini, 2002).

Cell cultures

Human astrocyte cultures

Primary human astrocytes (HAs: generously donated by Dr. Vega García-Escudero and Dr. Filip Lim, Centro de Biología Molecular Severo Ochoa, Madrid, Spain) were obtained from ScienCell Research Laboratories (Cat no 1800; Carlsbad, CA, USA). The cells were cultured in astrocyte medium (Sciencell Cat no 1801) in 2 μg/cm^2 poly-L-lysine-coated (PLL: Sigma-Aldrich) 100 mm culture dishes and handled according to the manufacturer's recommendations. Before any experimentation started, the HAs were characterized by immunostaining for two well-known astrocyte markers: rabbit anti-GFAP (1:500; Promega Biotech, Madrid, Spain) and mouse anti-S100β (1:1000; Sigma-Aldrich). Confluent cells were detached in 0.25% Trypsin/0.5 mM ethylenediaminetetraacetic acid (EDTA), and they were seeded in 60 mm dishes or in multi-well plates (with or without glass coverslips) at a density between 5000 and 7500 cells/cm^2. The cells were left in medium for at least 2 days *in vitro* (DIV) and then transduced with the lentiviral vectors or left as controls.

Astrocyte conditioned medium (ACM)

Conditioned medium from HAs was prepared as described previously, with some modifications (Lafon-Cazal et al., 2003). Equal number of cells were plated in astrocyte medium and transduced with SCR or shRNA37 lentiviral vectors. After 48 h, the medium was replaced with neuronal medium and after a further 48 h, ACM was collected at 96 h post transduction. Upon collection, the ACM was centrifuged twice to eliminate cell debris, filtered and frozen at −20 °C.

Primary neuronal cultures

Primary cortical neurons were prepared from C57BL/6 mice (embryonic days 16–18), as described previously (Tapia et al., 2010). Briefly, pregnant mice were sacrificed by cervical dislocation, the fetuses were removed and decapitated, and the meninges were stripped off the exposed brain. The cortices were removed and digested in trypsin (0.25%: Life Technologies, Madrid, Spain) containing DNaseI (1 mg/ml: Roche Diagnostics, Madrid, Spain) for 15 min at 37 °C, and then dissociated by trituration with a fire-polished Pasteur pipette. The dissociated cortical cells were plated in multiwell dishes previously coated with PLL (1 mg/mL) or on PLL-coated coverslips, and maintained in Neurobasal® medium (Life Technologies) supplemented with 10% horse serum at densities ranging from 40,000 to 60,000 cells/cm^2. To analyze the possible effects of conditioned medium from Fxn-depleted astrocytes on neuronal growth and maturation, the medium was changed after 3 h to either neuronal medium (Neurobasal medium supplemented with B-27® [Life Technologies], Glutamax™ [Life Technologies] and penicillin/streptomycin) or to ACM. The cells were then incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability

Cell viability was quantified by measuring calcein acetoxymethyl ester/propidium iodide (calcein/PI) uptake (Mattson et al., 1995). In living cells, the non-fluorescent calcein is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases, while non-viable cells can be distinguished by a bright red fluorescence emitted when PI intercalates into double-stranded nucleic acid in cells with a disrupted plasma membranes. Briefly, cells were incubated at 37 °C for 20 min with 1 μM of Calcein AM (Molecular Probes, Life Technologies) and 4 μM of PI (Sigma-Aldrich), and they were then visualized on an Axiovert 200 fluorescence inverted microscope (Zeiss). Three randomly selected fields were captured and analyzed per experimental condition, and the experiments were performed in duplicate on three independent cultures. Cell viability was calculated as the percentage of green cells relative to the total number of cells (both red and green).

Protein extraction and western blots

Following treatment, the cells were rinsed once with phosphate buffer saline (PBS) and homogenized in lysis buffer: 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 100 mM sodium chloride (NaCl), 100 mM sodium fluoride (NaF), 1% Triton X-100, 1 mM sodium orthovanadate (Na_3VO_4), 5 mM EDTA and the COMPLETE™ protease inhibitor cocktail (Roche Diagnostics). The resulting soluble fractions were centrifuged at 16,000 × *g* for 10 min at 4 °C, and the supernatants were taken as the whole cell lysate, mixed with electrophoresis buffer and boiled for 5 min. The protein concentration was measured using the Bio-Rad DC protein assay, according to the manufacturer's instructions (Bio-Rad Laboratories, Madrid, Spain). For western blots, equal amounts of protein (10–30 μg/well) were separated on 6–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (SDS–PAGE) and immobilized on nitrocellulose membranes (GE Healthcare, Barcelona, Spain), following standard procedures. The membranes were probed overnight at 4 °C with primary antibodies diluted in a blocking solution (PBS containing 0.1% Tween-20 and 5% non-fat dried milk). The membranes were then washed and exposed for 1 h at room temperature to the appropriate secondary peroxidase-conjugated antibody (1:5000), visualizing the specific protein bands by chemiluminescence. Following detection, the membranes were stripped of antibodies using a commercial kit (Restore Western Blot Stripping Buffer; Fischer Scientific, Madrid, Spain) and then re-probed for other proteins. The primary antibodies used in this study were: rabbit anti-Frataxin (1:1000; clone H155; Santa Cruz Biotechnology, Heidelberg, Germany), goat anti-cyclin-dependent kinase inhibitor 1A; p21 (1:1000; Santa Cruz Biotechnology), mouse anti-p53 (1:1000; DO-7; BD Biosciences, Madrid, Spain), rabbit anti-Caspase-3 (1:500; #9662; Cell Signaling, Boston, MA, USA). The western blots were quantified by densitometry using NIH ImageJ (Bethesda, MD, USA) open source software and the absolute values (arbitrary units) from each experimental group were normalized to those obtained for mouse anti-β-actin (1:5000; Sigma-Aldrich) used as a control for loading. The results are presented in arbitrary units expressed as the change relative to their respective controls that were run simultaneously.