NeuroToxicology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

The effect of manganese exposure in Atp13a2-deficient mice

Sheila M. Fleming[a,*], Nicholas A. Santiago[a], Elizabeth J. Mullin[b], Shanta Pamphile[c], Swagata Karkare[c], Andrew Lemkuhl[c], Osunde R. Ekhator[c], Stephen C. Linn[d], John G. Holden[c], Diana S. Aga[b], Jerome A. Roth[g], Benjamin Liou[e], Ying Sun[e], Gary E. Shull[f], Patrick J. Schultheis[d]

[a] Department of Pharmaceutical Sciences, Northeast Ohio Medical University, Rootstown, OH, United States
[b] Department of Chemistry, SUNY Buffalo, Buffalo, NY, United States
[c] Department of Neurology, University of Cincinnati, Cincinnati, OH, United States
[d] Department of Biological Sciences, Northern Kentucky University, Highland Heights, KY, United States
[e] Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, United States
[f] Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, Cincinnati, OH, United States
[g] Department of Pharmacology and Toxicology, SUNY Buffalo, Buffalo, NY, United States

ARTICLE INFO

Article history:
Received 27 January 2017
Received in revised form 25 May 2017
Accepted 3 June 2017
Available online xxx

Keywords:
Alpha-synuclein
Parkinson's disease
Manganese
Sensorimotor function
Mice
Lipofuscin

ABSTRACT

Loss of function mutations in the P_5-ATPase ATP13A2 are associated with Kufor-Rakeb Syndrome and Neuronal Ceroid Lipofuscinosis. While the function of ATP13A2 is unclear, *in vitro* studies suggest it is a lysosomal protein that interacts with the metals manganese (Mn) and zinc and the presynaptic protein alpha-synuclein. Loss of ATP13A2 function in mice causes sensorimotor deficits, enhanced autofluorescent storage material, and accumulation of alpha-synuclein. The present study sought to determine the effect of Mn administration on these same outcomes in ATP13A2-deficient mice. Wildtype and ATP13A2-deficient mice received saline or Mn at 5–9 or 12–19 months for 45 days. Sensorimotor function was assessed starting at day 30. Autofluorescence was quantified in multiple brain regions and alpha-synuclein protein levels were determined in the ventral midbrain. Brain Mn, iron, zinc, and copper concentrations were measured in 5–9 month old mice. The results show Mn enhanced sensorimotor function, increased autofluorescence in the substantia nigra, and increased insoluble alpha-synuclein in the ventral midbrain in older ATP13A2-deficient mice. In addition, the Mn regimen used increased Mn concentration in the brain and levels were higher in Mn-treated mutants than controls. These results indicate loss of ATP13A2 function leads to increased sensitivity to Mn *in vivo*.

1. Introduction

ATP13A2 is a P_5-ATPase of the P-type ion transport ATPase superfamily (Schultheis et al., 2004). Its substrate specificity and physiological function are unknown, however studies suggest it is involved in lysosomal degradation of proteins, manganese (Mn) homeostasis, and most recently zinc (Zn) transport (Gitler et al., 2009; Chesi et al., 2012; Matsui et al., 2013; Ramonet et al., 2012; Tan et al., 2011; Park et al., 2014; Kong et al., 2014; Tsunemi and Krainc, 2014). Loss of function mutations in ATP13A2 cause Neuronal Ceroid Lipofuscinosis and Kufor-Rakeb Syndrome, the latter an autosomal recessive form of Parkinsonism (PARK9; Ramirez et al., 2006; Farias et al., 2011; Wöhlke et al., 2011; Bras et al., 2012). Studies in yeast and cell culture show an interaction between ATP13A2 and Mn with loss of function mutations resulting in enhanced Mn toxicity and overexpression of ATP13A2 protecting against Mn toxicity (Gitler et al., 2009; Chesi et al., 2012; Schmidt et al., 2009; Covy et al., 2012; Daniel et al., 2015). Similarly, ATP13A2 protects mammalian cell lines and rat primary neuronal cultures from Mn-induced cell death and is upregulated in response to Mn treatment (Gitler et al., 2009; Tan et al., 2011; Covy et., 2012). These data indicate ATP13A2 may be involved in regulating intracellular Mn homeostasis (Tan et al., 2011). Indeed, polymorphisms of ATP13A2 are shown to modify the effects of Mn on motor function in an elderly population (Rentschler et al., 2012).

In addition to its relationship with Mn, *in vitro* studies show ATP13A2 also interacts with alpha-synuclein (aSyn), a presynaptic

* Corresponding author at: Department of Pharmaceutical Sciences, Northeast Ohio Medical University, 4209 State Route 44, RGE, Rootstown, OH 44272, United States.
E-mail address: sfleming1@neomed.edu (S.M. Fleming).

http://dx.doi.org/10.1016/j.neuro.2017.06.005
0161-813X/

protein that abnormally accumulates in lewy bodies in the neurodegenerative disorders Parkinson's disease, multiple system atrophy, and lewy body dementia (Spillantini et al., 1997Polymeropoulos et al., 1997). ATP13A2 expression can suppress aSyn toxicity in yeast and rescue aSyn-induced dopaminergic degeneration in primary neuronal culture (Gitler et al., 2009). However, a recent study did not find neuroprotection when ATP13A2 and aSyn were co-expressed using viral vector technology in the substantia nigra in rats (Daniel et al., 2015). Fibroblasts from PARK9 patients and dopaminergic cell lines made deficient in ATP13A2 exhibit a wide variety of lysosomal dysfunction including reduced lysosomal degradation capacity, accumulation of aSyn and neurotoxicity, impaired lysosomal acidification, decreased proteolytic processing of lysosomal enzymes, accumulation and enlargement of lysosomes, and decreased clearance of autophagosomes (Usenovic et al., 2012; Dehay et al., 2012). Recently, we showed that mice with loss of function of ATP13A2 develop age-dependent sensorimotor deficits, enhanced lipofuscin accumulation, and increased insoluble aSyn in the brain (Schultheis et al., 2013). While another study in a different ATP13A2 knockout mouse found age-dependent motor impairments, gliosis, accumulated ubiquitin protein aggregates, and endolysosomal abnormalities but no aberrant aSyn up to 18 months of age indicating the relationship between ATP13A2 and aSyn remains uncertain *in vivo* (Kett et al., 2015). This uncertainty is further supported by a lack of post mortem studies for Kufor-Rakeb Syndrome.

There are now several studies that also link Mn with aSyn oligomerization and accumulation in neurons *in vitro* and in the brain *in vivo* (Xu et al., 2013; 2014a,b; Xu et al., 2015; Verina et al., 2013). In humans and rodents chronic Mn exposure can result in manganism, a toxic condition that presents with "psychomotor excitement" followed by hypoactivity that resembles aspects of sporadic PD (Shukla and Singhal, 1984). The goal of the present study was to determine if loss of function of ATP13A2 leads to an enhanced sensitivity to Mn exposure *in vivo*.

2. Materials and methods

2.1. Animals

Animal care was conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals, and procedures were pre-approved by the Institutional Animal Care and Use Committee at Northern Kentucky University. Mice lacking ATP13A2 (13a2; Schultheis et al., 2013) were backcrossed to C57BL/6 for 8 generations. Heterozygous male and female mice were bred together to produce wildtype (WT) and homozygous 13a2 mice included in all experiments. Animals were maintained on the C57BL/6-background and littermates were never bred together. The genotype of all WT and 13a2 mice was confirmed by polymerase chain reaction (PCR) amplification analysis of DNA from tail tips. Male and female mice from 27 litters were included in the study. All mice were group housed and provided free access to water and standard rodent chow throughout the experiment except during behavioral testing procedures. Behavioral procedures were conducted during the light cycle.

2.2. Manganese treatment

Manganese chloride tetrahydrate ($MnCl_2\,4H_2O$, adjusted for the molar stoichiometry in the tetrahydrate, Sigma M8054) dissolved in saline (5 mg/kg/day,ip, Sigma) or saline alone were administered daily for 45 days to male and female WT and 13a2 mice. The 5 mg/kg dose and intraperitoneal administration route have been shown to cause modest effects in mice following 30 days of daily administration and reflects more of an environmentally relevant dose rather than a higher occupationally relevant regimen (Stanwood et al., 2009; Moreno et al., 2009; Kim et al., 2017). Behavioral testing was conducted over a two-week period following 30 days of manganese and saline treatment. Mice remained on their respective treatments during behavioral testing. One cohort of mice was tested at 5–9 months of age (WT-Saline = 7, WT-$MnCl_2$ = 8, 13a2-Saline = 8, 13a2-$MnCl_2$ = 8) and a separate cohort was tested at 12–19 months of age (WT-Saline = 8, WT-$MnCl_2$ = 8, 13a2-Saline = 7, 13a2-$MnCl_2$ = 8). All animals were tested at the same time and the ages chosen were based on the initial characterization of these mice to include an older group that is at the cusp of manifesting behavioral impairments and a younger group that is not expected to develop sensorimotor impairments until much older (Schultheis et al., 2013).

2.3. Sensorimotor tests

All mice were tested for spontaneous activity, motor performance and coordination and gait in that order. *Spontaneous Activity.* Spontaneous movements of the mice were measured in a small, transparent cylinder 15.5 cm high and 12.7 cm in diameter (Fleming et al., 2004, 2013; Schultheis et al., 2013). The cylinder was placed on a piece of glass with a mirror positioned at an angle beneath the cylinder to allow a clear view of movements along the ground and walls of the cylinder. Videotapes were viewed and rated in slow motion by an experimenter blind to mouse genotype and treatment. The number of rears, forelimb and hindlimb steps, and time spent grooming over a three-minute period were measured for each mouse.

Motor performance and coordination was measured with the challenging beam traversal test (Fleming et al., 2004, 2013; Schultheis et al., 2013). Briefly, the beam consists of four sections (25 cm each, 1 m total length), each section having a different width. The beam starts at a width of 3.5 cm and gradually narrows by 1 cm increments to a final width of 0.5 cm. Animals were trained to traverse the length of the beam starting at the widest section and ending at the narrowest section. Animals received two days of training prior to testing; on the day of the test a mesh grid (one cm squares) of corresponding width was placed over the beam surface leaving approximately a one cm space between the grid and the beam surface. Animals were then videotaped while traversing the grid-surfaced beam and videotapes were rated on slow motion by an experimenter blind to genotype and treatment. Errors, steps, and time to traverse the beam were measured across five trials and the means of those trials were included in the analysis.

To measure gait, animals were trained to walk through a narrow alley leading into their home-cage. Once trained, paper was placed along the alley floor and each animal's hindlimbs were brushed with non-toxic paint. Animals were then placed at the beginning of the alley. As they walked into their home-cage they left their paw prints on the paper underneath (Schallert et al., 1978; Fleming et al., 2004, 2013; Schultheis et al., 2013). Stride length was determined by measuring the distance between hindlimb prints. Only strides made while continuously walking (no stopping) were included in the analysis. Stride lengths at the beginning and end of the alley were not counted since animals tend to make irregular steps at the beginning and typically stop and make smaller steps just before entering the cage.

Following sensorimotor testing, half of the mice were anesthetized with pentobarbital (100 mg/kg, ip) and intracardially perfused with 0.1 M phosphate buffered saline (PBS) followed by ice cold 4% paraformaldehyde. Brains were removed, post-fixed for 6 h in 4% paraformaldehyde, cryoprotected with 0.1 M phosphate buffer (pH 7.4) containing 20% sucrose for 72 h, and rapidly frozen in isopentane pre-cooled to −70 °C with dry ice. The other half of

Please cite this article in press as: S.M. Fleming, et al., The effect of manganese exposure in Atp13a2-deficient mice, Neurotoxicology (2017), http://dx.doi.org/10.1016/j.neuro.2017.06.005