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Dysregulation of system x_c^- expression induced by mutant huntingtin in a striatal neuronal cell line and in R6/2 mice

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ABSTRACT

Oxidative stress has been implicated in the pathogenesis of Huntington's disease (HD), however, the origin of the oxidative stress is unknown. System x_c^- plays a role in the import of cystine to synthesize the antioxidant glutathione. We found in the *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$ striatal cell lines, derived from neuronal precursor cells isolated from knock-in mice containing 7 or 111 CAG repeats in the huntingtin gene, that there is a decrease in system x_c^- function. System x_c^- is composed of two proteins, the substrate specific transporter, xCT, and an anchoring protein, CD98. The decrease in function in system x_c^- that we observed is caused by a decrease in xCT mRNA and protein expression in the *STHdh*$^{Q111/Q111}$ cells. In addition, we found a decrease in protein and mRNA expression in the transgenic R6/2 HD mouse model at 6 weeks of age. *STHdh*$^{Q111/Q111}$ cells have lower basal levels of GSH and higher basal levels of ROS. Acute inhibition of system x_c^- causes greater increase in oxidative stress in the *STHdh*$^{Q111/Q111}$ cells than in the *STHdh*$^{Q7/Q7}$ cells. These results suggest that a defect in the regulation of xCT may be involved in the pathogenesis of HD by compromising xCT expression and increasing susceptibility to oxidative stress.

1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by an expansion of the CAG region in exon 1 of the *huntingtin* gene (*htt*) (The Huntington's Disease Collaborative Research Group, 1993) affecting approximately 10 in 100,000 people (Rawlins, 2010). The pathogenesis of HD is unknown, however, excitotoxicity (DiFiglia, 1990; Fan and Raymond, 2007; Raymond et al., 2011), oxidative stress (Li et al., 2010), and transcriptional dysregulation (Cha, 2000, 2007; Cui et al., 2006) all appear to play a role.

Disruption of glutamate homeostasis has been implicated in HD (Ferrante et al., 2002; Miller et al., 2008; Petr et al., 2013) and other triplet repeat diseases (Custer et al., 2006). Glutamate transport, thought to be mediated primarily by a family of 5 genes (*eaat1-5*) is important for clearing extracellular glutamate, with important consequences for excitatory signaling and neuronal survival (Rosenberg and Aizenman, 1989; Rosenberg et al., 1992). Recent work suggests that a defect in trafficking to the plasma membrane (Li et al., 2010) and expression (Petr et al., 2013) of the neuronal cysteine and glutamate transporter EAAC1 (EAAT3) is impaired in HD, compromising the ability of neurons to synthesize glutathione. Decreased GSH levels may be an important source of oxidative stress in HD (Browne, 2008; Chen, 2011; Ribeiro et al., 2012). Another glutamate transport system that is important for providing cysteine for synthesis of glutathione is system x_c^-, comprised of the transporter xCT and the glycoprotein CD98 (Sato et al., 1999). Under physiological conditions system x_c^- imports one cystine and exports one glutamate in a Na^+-independent manner (Bannai, 1986). Interestingly, system x_c^- also appears to play a critical role in glutamate homeostasis (Bridges et al., 2012; Kalivas, 2009; Lewerenz et al., 2012). xCT is expressed throughout the brain in both neurons and glia cells, and xCT has the highest expression along border areas of the brain where cerebrospinal fluid is present (Burdo et al., 2006).

We sought to characterize abnormalities in glutamate homeostasis in HD taking advantage of the *STHdh*$^{Q111/Q111}$ neuronal cell line as a model system for the disease (Trettel et al., 2000). Initially

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we confined ourselves to high affinity sodium dependent glutamate transport and transporters (Petr et al., 2013). In the course of that study, we observed a sodium independent component of glutamate transport in these cells. Here, we identify the sodium independent component of glutamate transport in the *STHdh* cells as system x_c^-, and find that both the function and expression of system x_c^- are compromised by the expression of mutant huntingtin, both in the cell lines and in an *in vivo* model. This defect in xCT expression and function has a direct impact on GSH levels and oxidative stress in cells expressing mutant huntingtin.

2. Materials and methods

2.1. Mice

The colony was maintained by the breeding of an ovarian transplanted R6/2 female (BCBA-Tg(HDexon1)62Gpb/1j) with CBA/C57VL/6 males (Jackson Laboratories, Bar Harbor, ME, USA). All mice were maintained at the Children's Hospital Boston Animal Care Facility under standard conditions (12 h light cycle from 7:00 AM to 7:00 PM) with *ad libitum* access to food and water. All experiments were performed in accordance with NIH guidelines and were approved by the Children's Hospital Boston Institutional Animal Care and Use Committee.

2.2. STHdh cells

The *STHdh* cells were generously provided by Dr. Marcy MacDonald (Massachusetts General Hospital, Boston, MA) and have been previously described (Trettel et al., 2000). The cells were grown in 10 cm dishes at 33 °C with 5% CO_2 in DMEM media supplemented with 10% (v/v) heat-inactivated FBS, 4.5 g/L L-glucose, 110 mg/mL sodium pyruvate, 4 mM L-glutamine, 0.5 mg/mL Geneticin, and 100 units/mL penicillin/streptomycin (all Invitrogen, Carlsbad, CA, USA). Cells were used at passage numbers nine to thirteen for all experiments from cells that were frozen at passage seven.

2.3. Glutamate uptake assay

The cells were plated at 2×10^5 cells/well on poly-D-lysine coated 24-well plates. The following day, the cells were washed twice with warm uptake buffer (2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, 10 mM HEPES, 5 mM Tris, 10 mM D-glucose, 140 mM choline chloride) and then 0.5 mL/well of the uptake solution was applied for 10 min [the uptake solution is the uptake buffer with 0.5 μM L-glutamic acid, 0.022 μM L-[^{3}H]-glutamate (PerkinElmer; Waltham, MA, USA), and/or inhibitors]. In some experiments, the concentrations of L-glutamic acid were varied from 0.5 to 300 μM. The following inhibitors were added to the uptake solution in other experiments: 1 mM L-homocysteic acid (HCA), 250 μM sulfasalazine (SSZ), 10 μM (S)-4-carboxyphenylglycine (CPG) (Tocris Bioscience, Ellisville, MO, USA), 100 μM L-cystine, and 200 μM L-cystine (L-CySS). In other experiments, we assayed the effect of upregulation of xCT using 30 μM salubrinal (Enzo Life Sciences, Farmingdale, NY, USA) or 100 μM diethyl maleate (DEM) for 24 h. The uptake was stopped by washing three times with ice cold 1% BSA in uptake buffer. The cells were then lysed in 0.1 mM NaOH. The protein concentration was determined using D_C Protein Assay (Biorad, Hercules, CA, USA) and the radioactivity was measured by liquid scintillation (TRI-CARB 2200CA, ΔPACKARD, Long Island Scientific, Inc.). All chemicals came from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted.

2.4. Western Blots

The *STHdh* cells were washed twice with ice-cold PBS then lysed in 20 mM Tris–HCl pH 7.5, 2.5 mM EDTA, 1% Triton-X 100, 1% deoxycholate, 0.1% SDS, 50 mM NaF, 2 mM sodium orthovanadate, Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail II, and Phosphatase Inhibitor Cocktail III (Sigma–Aldrich, St. Louis, MO, USA). Brain cell lysates were created from a coronal slice in which the striatum was separated from the overlying cortex. The tissue was then lysed in 1% SDS, 50 mM phosphate buffer pH 7.4 with protease and phosphatase inhibitors. The tissue was homogenized using a dounce hand-held homogenizer. Samples were heated at 95 °C for 10 min with Laemmli Sample Buffer containing 400 mM 2-mercaptoethanol. 20 μg of lysate was loaded per well in a 4–20% SDS–PAGE gel (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). The blots were probed with antibodies to xCT (Dun et al., 2006) (1:500), β-actin AC-74 (Sigma–Aldrich, St. Louis, MO, USA, A5316) (1:100,000), and β-III-tubulin (Millipore, Billerica, MA, USA, MAB1637) (1:5,000) in 5% milk in tris buffered saline with Triton-X (TBST) (25 mM Trizma base, 140 mM NaCl, 2.5 mM KCl, pH 7.4, 0.1% Triton-X 100). A blocking peptide for xCT antibody (MVRKPVVATISKGGY) was used at 50 μM to determine the xCT specific bands and was synthesized by Genscript (Piscataway, NJ, USA).

2.5. Quantitative PCR

RNA was isolated using TRIzol reagent following the manufacturer's recommended procedure (Invitrogen, Carlsbad, CA, USA). The isolated RNA was then treated with RQ1 RNase-Free DNase according to the manufacturer's protocol to remove any traces of DNA (Promega, Madison, WI, USA). Single stranded cDNA was created from total mRNA by reverse transcription using iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The sequences of the specific primers were as follows: 5′-GTC TAA TGG GGT TGC CCT TGG-3′ (sense) on exon 5 and 5′-CGC ACT GAT GGT GGT AAA ATA GG-3′ (antisense) on exon 7 for mouse xCT (NM_011990.2). For a control mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) (NM_013552) was used (Gomez et al., 2006). The primers were 5′-GAT CAG TCA ACG GGG GAC ATA-3′ (sense) on exon 4, 5′-GGG GCT GTA CTG CTT AAC CAG G-3′ on exon 6. The qPCR was carried out using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. The PCR cycling parameters were: denaturation at 95 °C for 3 min, amplification and quantification program repeated 40 times (95 °C for 5 s, 56 °C for 10 s, 72 °C for 30 s), melting curve program (65–95 °C with a heating rate of 0.5 °C per second) with a continuous single fluorescent measurement, carried out in the C100 Thermal Cycler and analyzed using CFX96 Manager Software (Bio-Rad, Hercules, CA, USA).

2.6. Glutathione measurements

Cells were seeded at 2×10^5 cells/well on 24-well poly-D-lysine coated plates. On the following day, cells were treated with 1 mM L-homocysteic acid, 100 μM sulfasalazine, 100 μM *tert*-butylhydroquinone (tBHQ) or vehicle for 2, 8, or 24 h. Total GSH was measured using a kinetic assay (Back et al., 1998; Tietze, 1969). Cell growth media was aspirated and then washed with Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA). 200 μL of 0.3 M perchloric acid (PCA) was added followed by gentle shaking on ice for 15 min to lyse the cells. The PCA solution was then transferred to a 1.5 mL microcentrifuge tube containing 50 μL of 3 M potassium bicarbonate, used to neutralize the PCA, and incubated on ice for 30 min. The pellet portion of the neutralized solution was resuspended in RIPA buffer and used to determine total protein