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Dopamine exacerbates mutant Huntingtin toxicity via oxidative-mediated inhibition of autophagy in SH-SY5Y neuroblastoma cells: Beneficial effects of anti-oxidant therapeutics

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ABSTRACT

Neuronal cell death in Huntington's Disease (HD) is associated with the abnormal expansions of a polyglutamine (polyQ) tract in the huntingtin protein (Htt) at the N-terminus that causes the misfolding and aggregation of the mutated protein (mHtt). Autophagy-lysosomal degradation of Htt aggregates may protect the neurons in HD. HD patients eventually manifest parkinsonian-like symptoms, which underlie defects in the dopaminergic system. We hypothesized that dopamine (DA) exacerbates the toxicity in affected neurons by over-inducing an oxidative stress that negatively impinges on the autophagy clearance of mHtt and thus precipitating neuronal cell death. Here we show that the hyper-expression of mutant (>113/150) polyQ Htt is *per se* toxic to dopaminergic human neuroblastoma SH-SY5Y cells, and that DA exacerbates this toxicity leading to apoptosis and secondary necrosis. DA toxicity is mediated by ROS production (mainly anion superoxide) that elicits a block in the formation of autophagosomes. We found that the pre-incubation with N-Acetyl-L-Cysteine (a quinone reductase inducer) or Deferoxamine (an iron chelator) prevents the generation of ROS, restores the autophagy degradation of mHtt and preserves the cell viability in SH-SY5Y cells expressing the polyQ Htt and exposed to DA.

The present findings suggest that DA-induced impairment of autophagy underlies the parkinsonism in HD patients. Our data provide a mechanistic explanation of the DA toxicity in dopaminergic neurons expressing the mHtt and support the use of anti-oxidative stress therapeutics to restore protective autophagy in order to slow down the neurodegeneration in HD patients.

1. Introduction

Huntington's Disease (HD) is a devastating autosomal dominant neurodegenerative condition characterized by neuronal loss in striatum (particularly of medium-sized spiny neurons (MSNs)), deep layers of the cortex and, when disease progresses, in hypothalamus and hippocampus and other brain regions (Vonsattel et al., 1985). Pathogenesis and progression of HD are strictly correlated with the presence in the affected neurons of cytoplasmatic aggregates and nuclear inclusions of the mutated form of the protein Huntingtin (Htt) and of its N-terminal fragments (DiFiglia et al., 1997; Soto, 2003; Melone et al., 2005). Mutant Htt (mHtt) is characterized by abnormal expansions of a polyglutamine (polyQ) tract to more than 37 Qs (Rubinsztein, 2002). While normal Htt has anti-apoptotic function, mHtt is neurotoxic. The N-terminal fragments of around 150 residues containing the polyQ stretch arising from the proteolytic processing by proteasomes, calpain and aspartyl proteases are even more toxic than full-length Htt (DiFiglia et al., 1997; Ratovitski et al., 2007, 2009; Rossetti et al., 2008). HD patients typically suffer from progressive motor and cognitive impairments, loss of self and spatial awareness, depression, dementia and anxiety over the course of 10–20 years before death. Alteration in dopamine (DA) neurotransmission is clearly involved in motor

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and cognitive symptoms of HD patients (Cepeda et al., 2014). Striatal and cortical loss of DA receptors in early stage of HD patients has been correlated with early cognitive decline, such as attention, executive function, learning and memory (Bäckman and Farde, 2001). The level of DA is elevated in the early stage of the disease (characterized by the chorea), while it decreases in the late stage when Parkinson-like symptoms (akinesia) become apparent (Garrett and Soares-da-Silva, 1992; Kish et al., 1987; Chen et al., 2013). Indeed, the level of DA in HD patients with parkinsonism resembles that of Parkinson Disease patients (Chen et al., 2013). DA is normally present in the striatum at elevated concentration, and is not harmful to normal neurons. However, DA is *per se* an excitotoxic neurotransmitter that triggers oxidative stress and may cause neuronal cell death (Jakel and Maragos, 2000). *In vitro*, DA-mediated oxidative stress was shown to induce apoptosis of striatal MSNs derived from transgenic R6/2 mice, an animal model of HD (Petersén et al., 2001a). It is possible that DA and glutamate synergize for the production of reactive oxygen species (ROS), so enhancing the toxicity of mHtt in MSNs (Cepeda et al., 2014).

The autophagy-lysosomal proteolytic system plays a protective role in HD by removing Htt aggregates (Sarkar and Rubinsztein, 2008). Dysfunctional regulation of this proteolytic system is consistently found in neurodegenerative disorders (Vidoni et al., 2016). Drugs able to increase the level of autophagy promote the clearance of Htt aggregates and relief the clinical symptom in '*in vivo*' model of HD (Rubinsztein, 2006; Sarkar et al., 2007, 2008; Roscic et al., 2011). Autophagy has been reported to be up-regulated *in post mortem* striatum regions of HD patients (Cherra et al., 2010). Abnormal expression of autophagy-related (ATG) proteins in the neurons of a knock-in HD mouse model indicates that alteration of the autophagic flux is an early stress response to mHtt (Heng et al., 2010). The Htt protein itself can affect autophagy by directly interacting with SQSTM1/p62 (which tags the autophagy substrates) (Martinez-Vicente et al., 2010; Bjørkøy et al., 2005) and with ULK1 (which activates the BECLIN 1-PI3KC3 complex that triggers the autophagosome formation) (Rui et al., 2015), or by sequestering in the protein aggregate mTOR, a kinase that negatively regulates ULK1 (Ravikumar et al., 2004). Interestingly, autophagosomes accumulate in primary striatal neurons from HD mice expressing truncated mutant Htt following dopamine-induced oxidative stress (Petersén et al., 2001b).

What is the functional role of autophagy in dopaminergic neurons expressing the mHtt and what is the cytotoxic mechanism of DA that could underlie the onset of parkinsonism in HD patients remain however obscure. As an attempt to fill in this gap of knowledge, we investigated the molecular link between DA-induced oxidative stress and mHtt toxicity in relation to the activation of the autophagy pathway in an '*in vitro*' model of parkinsonian HD. We found that DA-induced ROS production causes the death of dopaminergic human neuroblastoma SH-SY5Y cells expressing transgenic mHtt following the inhibition of autophagosome formation. Preventing ROS generation by N-Acetyl-L-Cysteine (an inducer of quinone reductase) or Deferoxamine (an iron chelator) restores the autophagy flux and the clearance of mHtt, and saves the mHtt-expressing neuronal-like cells from DA toxicity. Thus, *anti*-ROS drugs able to restore autophagy could slow down the progression of HD and prevent the onset of parkinsonian-like symptoms in HD patients.

2. Materials and methods

2.1. Cell culture and treatment

Human dopaminergic neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured under standard culture condition (37 °C; 95 v/v% air: 5 CO_2 v/v%) in 50% Minimum Essential Medium (MEM, cod. M2279, Sigma-Aldrich Corp. St. Luis, MO, USA), 50% Ham's F12 Nutrient Mixture (HAM, cod. N4888, Sigma-Aldrich Corp.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, cod. ECS0180L, EuroClone S. p.A. Milan, Italy), 1% w/v of a penicillin-streptomycin solution (cod. P0781, Sigma-Aldrich Corp.) and 2 mM di L-glutamine (cod. G7513, Sigma-Aldrich Corp.). The cells were seeded (starting density 70.000/cm^2) on sterile Petri dishes or glass coverslip, as indicated, and allowed to adhere for 24–36 h prior to start any treatment. Excitotoxic treatment was performed with 100 μM Dopamine (DA, cod. H8502, Sigma-Aldrich Corp. dissolved in sterilized H_20). Apoptosis was inhibited with 20 μM of the pancaspase inhibitor z-VAD (OMe)-fmk (z-VAD-fmk, cod. 260-020-M005, Alexis Laboratories, San Diego, CA). The cells were pre-incubated 2 h with 2 mM N-Acetyl-L-Cysteine (NAC, cod. A9165, Sigma-Aldrich Corp.) or 1 mM Deferoxamine mesylate salt (DFO, D9533, Sigma-Aldrich Corp.) to prevent ROS generation. Chloroquine (ClQ, 30 μM, cod. C6628, Sigma-Aldrich Corp.) was used to inhibit autophagosome degradation. Pepstatin A (PstA, 100 μM, cod. P 5318, Sigma-Aldrich Corp.) was used as inhibitor of Cathepsin D.

2.2. HD expression constructs and plasmid transfection

The plasmids encoding for Htt (either wild-type or mutant) and for the polyQ tract (see Supplementary Fig. S1A) were generously provided by Prof. F. Persichetti (Università del Piemonte Orientale, Italy). (HD)N1-171Q21 and (HD)N1-171Q150 mammalian expression constructs in pcDNA3/Zeo (+) encode the amino-terminal 171 amino acids of human Htt protein (Accession No. L12392, bp 314–823), with 21 and 150 glutamines, respectively. (HD)N1-171Q21GFP and (HD)N1-171Q150GFP were created by inserting a fragment of pGreen Lantern-1, encoding a GFP-tag, in frame with the carboxy-terminus of the Htt sequence (Persichetti et al., 1999). Full length HD constructs Q21-FL (pC_3F_7HD Q21) and Q113-FL (FL 113Q HD CMV) encode the full length of human huntingtin protein, with 21 and 113 glutamines, respectively. The construct Q21-FL was created by inserting the FLAG-tag in frame with the amino-terminus of the full length human huntingtin protein.

The cells were transfected with the plasmids using the Lipofectamine 3000 Reagent (cod. L3000-015, Life Technologies Ltd, Paisley, UK) as indicated by the purchaser. Briefly, SH-SY5Y were plated in P35 Petri dish at 70.000/cm^2 and let adhere 24 h before transfection. The DNA–Lipofectamine complexes were prepared in 500 μl of Opti-MEM I Reduced Serum Medium (cod. 11058021, Life Technologies Ltd) with 6 μg of plasmid, 5 μl of P3000 reagent and 7.5 μl of Lipofectamine. After 6 h of incubation, the transfection medium was replaced with a serum-containing culture medium (10% FBS), and the cells were cultivated for further 21 h to allow for maximal expression of the transgenic protein prior to any treatment.

2.3. Assessment of cell proliferation, cell viability and cell toxicity

Cell culture growth was assessed by cell counting of adherent viable (trypan blue-excluding) cells, and the doubling time (Dt) was calculated using the software Doubling Time Online Calculator (http://www.doubling-time.com/compute.php).

To test cell viability, the cells adherent on coverslips were labeled with CellTracker TM (CellTracker TMBlue-CMAC 7-amino-4-chloromethylcoumarin; cod. C2110, Life Technologies Ltd), and the blue fluorescence, an indicator of the mitochondrial respiratory activity, was immediately imaged under the fluorescence microscope Leica DMI6000 (Ekkapongpisit et al., 2012; Cagnin et al., 2012).