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Calpastatin ablation aggravates the molecular phenotype in cell and animal models of Huntington disease

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ABSTRACT

Deciphering the molecular pathology of Huntington disease is of particular importance, not only for a better understanding of this neurodegenerative disease, but also to identify potential therapeutic targets. The polyglutamine-expanded disease protein huntingtin was shown to undergo proteolysis, which results in the accumulation of toxic and aggregation-prone fragments. Amongst several classes of proteolytic enzymes responsible for huntingtin processing, the group of calcium-activated calpains has been found to be a significant mediator of the disease protein toxicity. To confirm the impact of calpain-mediated huntingtin cleavage in Huntington disease, we analysed the effect of depleting or overexpressing the endogenous calpain inhibitor calpastatin in HEK293T cells transfected with wild-type or polyglutamine-expanded huntingtin. Moreover, we crossbred huntingtin knock-in mice with calpastatin knockout animals to assess its effect not only on huntingtin cleavage and aggregation but also additional molecular markers. We demonstrated that a reduced or ablated expression of calpastatin triggers calpain overactivation and a consequently increased mutant huntingtin cleavage in cells and *in vivo*. These alterations were accompanied by an elevated formation of predominantly cytoplasmic huntingtin aggregates. On the other hand, overexpression of calpastatin in cells attenuated huntingtin fragmentation and aggregation. In addition, we observed an enhanced cleavage of DARPP-32, p35 and synapsin-1 in neuronal tissue upon calpain overactivation. Our results corroborate the important role of calpains in the molecular pathogenesis of Huntington disease and endorse targeting these proteolytic enzymes as a therapeutic approach.

1. Introduction

Proteolytic fragmentation of disease proteins has been discussed as an important step in the molecular pathogenesis not only of Huntington disease (HD) but also further neurodegenerative disorders (Ono et al., 2016; Weber et al., 2014). The so called *toxic fragment hypothesis* states that this post-translational modification represents an important source for breakdown products of disease proteins which, in comparison to the full-length protein, exhibit an increased toxicity and aggregation propensity (Wellington and Hayden, 1997). In HD, several groups of enzymes have been associated with the cleavage of mutant huntingtin such as caspases, cathepsins, matrix-metalloproteinases and calpains (Ehrnhoefer et al., 2011). Calpains, a class of Ca^{2+}-dependent cysteine proteases, do not only cleave the disease protein, but also exhibit an overactivation in the molecular context of HD (Clemens et al., 2015; Cowan et al., 2008; Gafni and Ellerby, 2002; Gafni et al., 2004; Kim et al., 2001; Paoletti et al., 2008; Weber et al., 2016). Furthermore, this overactivation seems be connected with synaptic dysfunctions via calpain-dependent loss of surface NMDARs (Cowan et al., 2008; Dau et al., 2014; Gladding et al., 2012). In addition, the calpain-

Abbreviations: CAPN, calpain; CAST, calpastatin protein; *Cast*, mouse calpastatin gene; HD, Huntington disease; HDKI, Hdh^{Q111} knock-in mice; Htt, huntingtin protein; polyQ, polyglutamine.

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mediated conversion of the cyclin-dependent kinase 5 (Cdk5) activator p35 to p25 was shown to result in an aberrant Cdk5 activity, which might contribute to the selective striatal cell death in HD (Paoletti et al., 2008). These observations emphasize that calpains represent an important player in the molecular pathogenesis of HD. Targeting these enzymes might constitute a promising therapeutic strategy to treat this dreadful disease and preclinical studies in HD animal models have proven the beneficial effect of directly or indirectly lowering calpain activity (Clemens et al., 2015; Menzies et al., 2014; Weber et al., 2016).

One of the most investigated regulatory mechanisms for calpain activation is the inhibitory function of the endogenous proteinaceous calpain inhibitor calpastatin (CAST) (Hanna et al., 2008; Wendt et al., 2004). Two *in vivo* studies conducted in our institute investigated the effect of a genetic CAST depletion on the pathogenesis of mutant α-synuclein-linked Parkinson disease and Machado-Joseph disease (Diepenbroek et al., 2014; Hübener et al., 2013). Both investigations showed that a CAST knockout led to an increase in calpain activation, an aggravation of disease-associated molecular hallmarks and a worsening of the disease phenotype. As calpains play an important role in HD, we aimed for analysing the molecular effect of a CAST ablation in cell and mouse models of HD.

In this study, we show that knockdown of CAST in mammalian cell culture leads to an increased calpain-mediated cleavage of mutant huntingtin, consequently amplifying the formation of huntingtin aggregates. On the other hand, CAST overexpression in cells reduced both huntingtin cleavage and aggregate formation. Furthermore, crossbreeding Hdh^{Q111} knock-in (HDKI) with $Cast^{-/-}$ mice (Takano et al., 2005; Wheeler et al., 1999) worsened HD-related molecular characteristics *in vivo* including calpain activation, and both fragmentation and aggregation of mutant huntingtin. Thus, our findings corroborate the significant involvement of calpains in the molecular pathogenesis of HD.

2. Methods

2.1. Ethical statement on animal research

All mouse experiments were conducted after approval by the local ethics committee at the Regierungspräsidium Tübingen in accordance with the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU).

2.2. Expression constructs and esiRNAs

For huntingtin overexpression, pCI-neo vectors carrying cDNA for full-length human huntingtin with 15 or 128 glutamines were employed. For knocking down expression of CAST, endoribonuclease-prepared siRNA (esiRNA) directed against human CAST (MISSION® esiRNA EHU096291) or control siRNA against *Renilla* luciferase (MISSION® esiRNA EHURLUC) were utilized (both Sigma Aldrich). Overexpression of CAST was achieved using a pRK5 vector carrying the cDNA for full-length human CAST (hCAST). To obtain a control for the latter plasmid, the CMV promotor of the pRK5-CAST vector was excised using the restriction enzymes SpeI (cutting 5′ of the promotor sequence) and XbaI (cutting 3' of the promotor sequence).

2.3. Cell culture and transfection

Cell culture experiments were performed with HEK293T cells (ATCC: CRL-11268). HEK293T cells were cultured in Dulbecco's modified eagle's medium (DMEM) GlutaMAX™ supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids (MEM NEAA) and 1% Antibiotic-Antimycotic (all Gibco®, Thermo Fisher Scientific) at 37 °C in 5% CO_2. Transfections were conducted for 48 h to 72 h using the Attractene reagent (Qiagen) according to the manufacturer's protocol.

2.4. Cell-based calpain activation and inhibition assays

Cell-based activation of calpains has been described elsewhere (Weber et al., 2017). Culture medium of transfected HEK293T cells was aspirated and replaced by Opti-MEM® I Reduced Serum Media (Gibco®, Thermo Fisher Scientific). For negative control, cells were pre-treated with 10 μM of the calpain inhibitor III (CI III; carbobenzoxy-valinyl-phenylalaninal) (Merck Millipore) for 1 h. Calpain activation was triggered by incubating cells with 0.125 μM of the Ca^{2+} ionophore ionomycin (Sigma-Aldrich) and 5 mM $CaCl_2$ for indicated time points at 37 °C in 5% CO_2. For calpain inhibition assays, cells were solely treated with CI III for 2 h prior to harvesting.

2.5. Fluorescence microscopy

For fluorescence microscopy, HEK293T cells were seeded in poly-L-lysine-coated 24-well cell culture plates (Thermo Fisher Scientific), transfected with respective constructs and cultured for 72 h. Subsequently, cells were pre-fixed with 0.4% PFA in 1× Dulbecco's phosphate-buffered saline (DPBS; Gibco®, Thermo Fisher Scientific) for 15 min at 37 °C and fixed with 4% PFA in DPBS for another 15 min at room temperature. After washing three times with DPBS for 5 min, cells were permeabilized and blocked using 10% BSA in 1 × DPBS with 0.5% Triton X-100 for 1 h at room temperature. Afterwards, cells were incubated with primary antibody mouse anti-huntingtin (1:1000; clone 1HU-4C8, MAB2166, Merck Millipore) in antibody diluent (1% BSA in 1 × DPBS with 0.5% Triton X-100) over night at 4 °C. The primary antibody was aspirated and cells washed four times with 1× DPBS for 5 min at room temperature. Then, secondary fluorescence-labelled antibody donkey anti-mouse Alexa Fluor® 488 (1:500; ab150109, Abcam) in antibody diluent was added. After 1 h incubation at room temperature, cells were washed four times with 1 × DPBS for 5 min. To counterstain nuclei, cells were incubated with 5 μM CyTRAK Orange™ (BioStatus) in 1× DPBS for 30 min at room temperature. Stained cells were stored protected from light and covered with 1× DPBS at 4 °C until imaged. Fluorescent pictures were taken on an inverted Eclipse TS100 microscope (Nikon) equipped with an AxioCam MRm (Zeiss) at a 100× magnification using the imaging software AxioVision LE (AxioVs40 4.8.2.0., Zeiss). Five representative areas were photographed per well. Cells and aggregates were quantified using the manual cell counter plugin of the ImageJ software (Schneider et al., 2012).

2.6. Animals

Heterozygous *Cast* knockout ($Cast^{+/-}$) and heterozygous Hdh^{Q111} knock-in (HDKI) mice were kept on the same C57BL/6N background by breeding heterozygous mice of each strain with C57BL/6N mice purchased from Charles River (Sulzfeld, Germany) for more than 10 generations, as this background strain has also been used in previous studies (e.g. Hübener et al., 2013; Diepenbroek et al., 2014). Mice were genotyped as previously described (Takano et al., 2005; Wheeler et al., 1999). For this study, first homozygous *Cast* knockout ($Cast^{-/-}$) mice were obtained by breeding $Cast^{+/-}$ mice with each other. Then, homozygous $Cast^{-/-}$ and heterozygous HDKI mice were crossbred to obtain animals heterozygous for both the Hdh^{Q111} allele and *Cast* knockout. These mice were further