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Down regulation of huntingtin affects expression levels of interaction partners and morphological changes in neuronal and HeLa cells

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Introduction

Huntington's Disease (HD) is a neurodegenerative disorder caused by an abnormally expanded polyglutamine tail in the amino-terminal region of huntingtin (htt). Pathogenic mechanisms involve a gained toxicity of mutant htt and a potentially reduced neuroprotective function of the wild-type allele. Among the molecular abnormalities reported, HD cells are characterized by the presence of aggregates, transcriptional deregulation, altered mitochondrial membrane potential and disturbed calcium (Ca^{2+}) signalling. The biological function of htt has not been completely elucidated. It is reported that short interfering RNA (siRNA) mediated inhibition of endogenous htt results in an aberrant configuration of the endoplasmic reticulum (ER) network in vitro in different cell lines [1]. We aimed to investigate htt down-regulation mediated effects on the ER and actin in human neuronal cell lines, combined with gene expression profiling of 14 different htt interaction partners. In order to compare differences between human neuronal SH-SY5Y cells and human epithelial HeLa cells on the gene expression level, we performed gene expression profiling by quantitative PCR, and cell morphology was visualized by fluorescence-microscopy.

Materials and Methods

Cell culture
Human epithelial HeLa cells and human neuronal SH-SY5Y cells were obtained from ATCC. For cell culture HeLa cells were maintained in DMEM medium (Sigma-Aldrich) supplemented with 10% FCS and SH-SY5Y cells were grown in DMEM : Ham's F12 1:1 (Sigma-Aldrich) supplemented with 15 % FCS. The cells were grown at 37°C and 5% CO_2.

Immunofluorescence staining
For immunofluorescence staining, cells were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes. Then cells were quenched with 50mM Tris-Cl, 100 mM NaCl pH 8,0 and permeabilized with PBS with 0,2% Triton X-100. After 1 hour blocking with 3% BSA, the cells were incubated with the first antibodies (anti-htt 181-810 (Chemicon) 1:500 and anti-calnexin (Sigma-Aldrich) 1:100 in 1%BSA) for at least 1 hour. After 5x washing with PBS the second antibodies were added (anti-mouse-Alexa 488 (Sigma-Aldrich) 1:400 and anti-rabbit-TRITC (Sigma-Aldrich) 1:400). After an incubation of 1 hour the cells were washed 5x with PBS and covered with Mowiol. 24 hours later the slides were checked and photographed with a Zeiss Aviovert 200 M microscope.

siRNA transfection
Cells were transfected in six well plates, using 5 µL Lipofectamine ™RNAiMAX (Invitrogen) and 20 nM huntingtin siRNA (Invitrogen) per well, according to the manufacturers protocol.

Real-time RNA quantification
The RNA was isolated by QIAGEN RNeasy® Mini Kit according to the manufacturers protocol. The reverse transcription was run with the TaqMan® Reverse Transcription Reagent (Applied Biosystems), and the quantification of RNA was assessed by real-time PCR with the LightCycler® 2.0 Detection System (Roche). QuantiTect Primers as well as QuantiTect SYBR Green PCR Kit (QIAGEN), were used for the experiments. For normalization a G6PDH mRNA internal control was run for each sample.

Results

Basal expression profiling

The basal expression profiling of the htt interaction partner DRD1, DRD2, DARPP32, ADORA2A, HTR2A, SH3GL3, GIT1, HAP1, HIP1, HIP14, PSD95, CREB, SP1, p53 as well as HSP40, HSP70, HSP90 was determined in SH-SY5Y and HeLa cells. These genes were all expressed in SH-SY5Y, most of the genes showing low expression levels. In HeLa cells HAP1 and PSD95 were not detected and the receptors DRD1, DRD2 ADORA2A and HTR2A were expressed only at very low level.

Expression profiling

Htt down-regulation efficiency and gene expression profiles of genes affected by htt down-regulation in SH-SY5Y cells and HeLa cells are shown in figure 1 and figure 2.

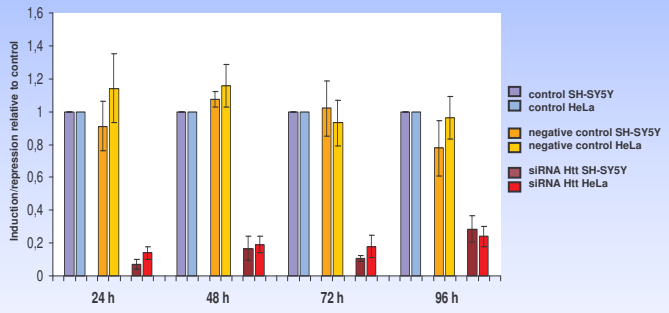

Fig. 1: Detection of htt endogenous expression levels following siRNA mediated htt down-regulation compared to the control in SH-SY5Y cells and HeLa cells. After htt siRNA transfection, the cells were incubated for 24 h, 48 h, 72 h and 96 h in growth medium. Expression was determined by real-time qRT-PCR with the LightCycler® 2.0 (Roche) capillary system. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) and was subjected to LightCycler® qRT-PCR analysis as described in Materials and Methods. Data were normalised to G6PDH expression as internal control. As a negative control, the cells were transfected with Stealth Negative Control siRNA (Invitrogen). Shown are mean values with standard deviation of two independent experiments.

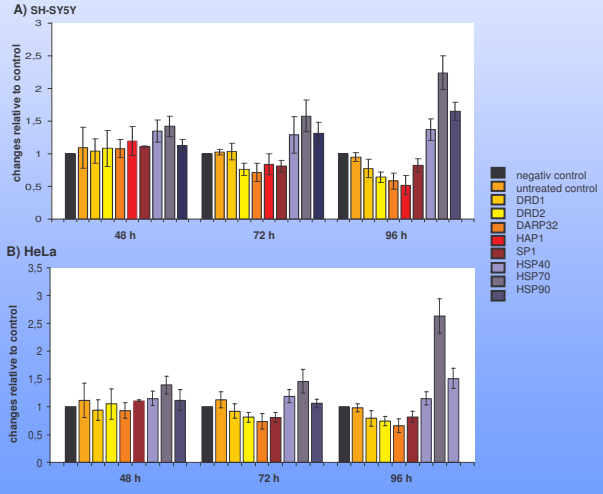

Fig. 2: Influence of siRNA mediated htt down-regulation on endogenous expression levels of htt interaction partner and heat shock proteins compared to the negative control in A) SH-SY5Y cells and B) HeLa cells. After htt siRNA transfection, the cells were incubated for 48 h, 72 h and 96 h in growth medium. Expression was determined by real-time qRT-PCR with the LightCycler® 2.0 (Roche) capillary system. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) and was subjected to LightCycler® qRT-PCR analysis as described in Materials and Methods. Data were normalised to G6PDH expression as internal control. As a negative control, the cells were transfected with Stealth Negative Control siRNA (Invitrogen). Shown are mean values with standard deviation of two independent experiments.

Morphological observations

The morphology of the cells was microscopically observed and immunofluorescence staining of htt, the specific ER marker calnexin and actin was detected by fluorescence-microscopy.

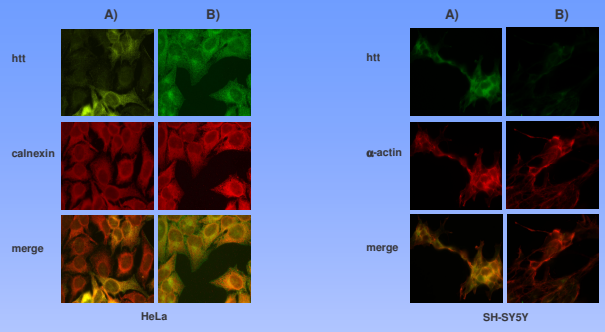

Fig. 3: Detection of immunofluorescence staining of htt, the specific ER marker calnexin as well as α-actin by Zeiss Axiovert 200 M fluorescence-microscopy in HeLa and SH-SY5Y cells 96 h after transfection. A) Cells down-regulated with 20 nM htt siRNA (Invitrogen). B) Cells transfected with Stealth Negative Control siRNA (Invitrogen).

Conclusion

- Not all htt interaction partners were expressed in HeLa cells.
- siRNA mediated down-regulation of htt affected gene expression of several genes like DARPP32, DRD2, SP1 and HAP1. Similar expression patterns were detected in both cell lines.
- The turn-over of htt was estimated at about 72 h by immunofluorescence staining of the protein and Western Blot. This corresponds also to the effects seen on gene expression level.
- No differences in calnexin location and quantity as well as actin filament structure was observed by immunofluorescence staining and Western Blot.
- HSP70 induction following htt down-regulation indicates stress activation.
- Actually longer timepoints and aditionnally differentiated SH-SY5Y are analysed.

Acknowlegements

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References

[1] Kazuya Omi et al.: *siRNA-mediated inhibition of endogenous Huntington disease gene expression induces an aberrant configuration of the ER network in vitro* . Biochemical and Biophysical Research Communications 338 (2005) 1229–1235