Protective effect of sodium butyrate on the cell culture model of Huntington disease*

Zhang Baorong[1]**, Tian Jun[1], Yin Xinzhen[1], Luo Wei[1] and Xia Kun[2]

(1. Department of Neurology, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China; 2. National Laboratory of Medical Genetics of China, Central South University, Changsha 410078, China)

Accepted on December 26, 2006

Abstract This study aimed to develop a cell culture model of Huntington disease and observe the effect of sodium butyrate on this cell culture model. Exon 1 of both a wild type and a mutant IT15 gene from the genomic DNA of a healthy adult and a patient with Huntington disease was amplified and cloned into the eukaryotic expression vector pEGFP-C1. Human neuroblastoma SH-SY5Y cells were transiently transfected with these recombinant plasmids in the absence and presence of sodium butyrate (0.1, 0.2, 0.5, 1.0 mmol/L). The MTT assay was used to measure cell viability. The results indicated that the N-terminal fragment of mutant huntingtin formed perinuclear and intranuclear aggregates and caused a decrease of SH-SY5Y cell viability. Sodium butyrate inhibited the decrease of SH-SY5Y cell viability caused by the N-terminal fragment of mutant huntingtin. This suggests that sodium butyrate has a protective effect on this cell culture model of Huntington disease.

Keywords: Huntington disease, huntingtin, sodium butyrate.

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder that is characterized by motor dysfunction, cognitive decline and psychiatric disturbance[1]. The striatum is the primary area of neuronal degeneration in HD[2]. This disease is caused by an unstable expansion in the number of CAG trinucleotide repeats in exon 1 of the IT15 gene on chromosome 4, which encodes an expansion of a polyglutamine (poly Q) region near the N-terminus of huntingtin (Htt)[3]. In the normal population, the number of CAG repeats varies from 6 to 35, but a length of 37 and over usually causes HD. How an expansion of polyglutamine repeats in the N-terminal region of mutant huntingtin causes selective degeneration of neurons in the brain remains uncertain, although several mechanisms have been proposed[4]. There is currently no effective treatment to prevent the progress of HD. However the histone deacetylase (HDAC) inhibitor, sodium butyrate, has been found to play a therapeutic role in a number of HD models[5].

We had genetically confirmed 5 HD patients from a Chinese family, in which an 18-year-old patient had an expanded CAG allele of 68 repeats in the IT15 gene. To model the pathogenesis of Huntington disease, in this study, we transfected human neuroblastoma SH-SY5Y cells with plasmid constructs containing exon 1 of the IT15 gene of the HD patient, and investigated the effect of sodium butyrate on this cell culture model of HD.

1 Materials and methods

1.1 Plasmid construction

Exon 1 of IT15 gene with 17 and 68 CAG repeats was generated by PCR using genomic DNA isolated from lymphoblastoid cell lines constructed from a healthy adult and an 18-year-old HD patient using the sense primer 5′ gcgcagatctatggcgaccctggaaaagc 3′ and the antisense primer 5′ gcgcgaattcggcggctgaggaagctgagga 3′. PCR products were subcloned into the eukaryotic expression vector pEGFP-C1 to form recombinant plasmids: GFP-Htt-19Q (17 CAG repeats) and GFP-mHtt-70Q (68 CAG repeats). The constructs were identified by restriction enzyme digestion and sequencing.

1.2 Cell culture and transfection

Human neuroblastoma SH-SY5Y cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum. For transfection, cells were grown to 80%—90% confluence on coverslips for 24 h. The

* Supported by Natural Science Foundation of Zhejiang Province (Grant No. J20060713)

** To whom correspondence should be addressed. E-mail: brzhang@zju.edu.cn

cells were transfected with pEGFP-C1 or GFP-Htt-19Q or GFP-mHtt-70Q mediated by lipofectamine^{tm} 2000 according to the manufacturer's instructions. At 48 h post transfection, the percentages of cells with aggregates were determined by counting the cells with visible aggregates and the total number of cells exhibiting green fluorescence. All experiments were carried out in triplicate. In each experiment, 20 view fields were selected randomly and transfected cells were counted. One-way ANOVA was used for statistical analysis by using SPSS 11.0.

1.3 Histone deacetylase inhibitors

The sodium butyrate was dissolved in water. Different concentrations of sodium butyrate (0.1, 0.2, 0.5, 1.0 mmol/L) were added to the culture medium every 24 h beginning the day after transfection.

1.4 Cell viability assay

The MTT assay was used to measure cell viability. Transfected cells in 96-well plates were treated with 5 mg/mL 3-(4, 5-dimethylthiazolel-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) at 48 h post-transfection and incubated for another 4 h. The supernatant was then removed and the formazan crystals were suspended in 200 μL DMSO. Absorbence was measured on an enzyme-linked immunosorbent assay reader at 570 nm. Non-transfected cells were taken as control with 100% viability. The relative cell viability (%) compared to control cells was calculated by $[\text{absorbency}]_{sample}/[\text{absorbency}]_{control} \times 100\%$. A one-way ANOVA was used for statistical analysis by using SPSS 11.0.

1.5 Western blot analysis

At 48 h post-transfection the cells in the 6-well plate were washed with PBS and suspended in 80 μL sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 60 mmol/L Tris-HCL, pH 6.8, 25% glycerol and 5% 2-mercaptoethanol). The suspension was boiled for 10 min. The cell extracts were resolved by electrophoresis on 12% polyacrylamide gels and transferred onto the PVDF membrane. The membrane was blocked for 1 h at room temperature with a blocking solution containing 5% non-fat milk. The membrane was incubated with 1∶1000 dilution of rabbit anti-GFP polyclonal antibody for 1 h at room temperature and then treated with 1∶5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. Bands were detected using enhanced chemiluminescence.

2 Results

2.1 The eukaryotic expression vector of exon 1 of the IT15 gene

Two fragments of approximately 170 bp and 320 bp were generated by PCR amplification of the genomic DNAs from a healthy adult and an HD patien respectively (Fig. 1). The recombinant plasmids, GFP-Htt-19Q and GFP-mHtt-70Q, were confirmed by restriction endonuclease analysis. Two fragments of approximately 170 bp and 320 bp were digested by *Eco*RI and *Bgl*II from GFP-Htt-19Q and GFP-mHtt-70Q respectively (Fig. 2). Results showed that exon 1 of a wild type and a mutant IT15 gene were both successfully subcloned to the pEGFP-C1 vector. Meanwhile, the constructs were confirmed by sequencing.

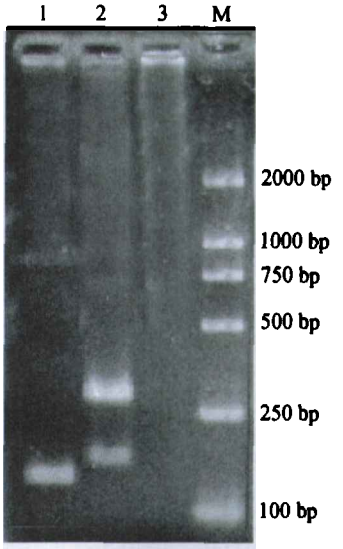

Fig. 1. PCR products of exon 1 of the IT15 gene. 1, healthy adult; 2, HD patient; 3, negative control; M, DL2000 marker.

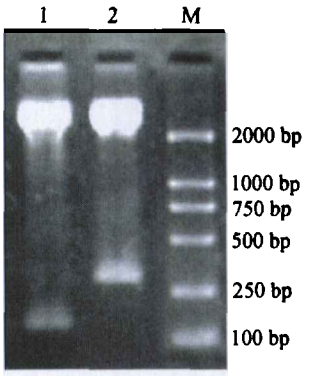

Fig. 2. The recombinant plasmids digested by *Eco*RI and *Bgl*II. 1, GFP-Htt-19Q; 2, GFP-mHtt-70Q; M, DL2000 marker.

2.2 Expression of the N-terminal fragments of huntingtin in SH-SY5Y cells

Fluorescence microscopic observation showed that the cells transfected with pEGFP-C1 were diffusely labeled with green fluorescence in the cytoplasm and in the nucleus. The cells transfected with GFP-Htt-19Q were diffusely labeled with green fluorescence in the cytoplasm throughout the experiment. The cells transfected with GFP-mHtt-70Q were diffusely labeled with green fluorescence in the cytoplasm initially, but 32 h after transfection aggregates appeared in the perinuclear region and in the nucleus. The green fluorescences of aggregates were very bright (Fig. 3). Sodium butyrate did not affect the formation of mutant huntingtin aggregates (Fig. 4). Immunoblotting with anti-GFP antibodies demonstrated a 29 kD band for pEGFP-C1, a 34 kD band for GFP-Htt-19Q and a 40 kD band for GFP-mHtt-70Q (Fig. 5), which identified the expression of the N-terminal fragments of normal and mutant huntingtin in SH-SY5Y cells.

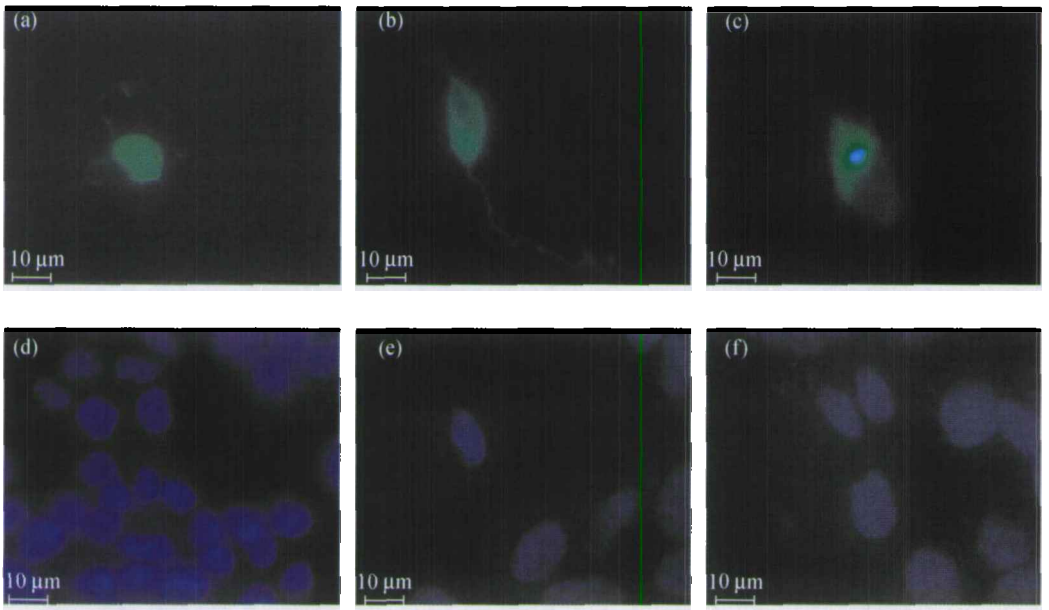

Fig. 3. Expression of the N-terminal fragments of huntingtin in SH-SY5Y cells at 48 h post transfection. SH-SY5Y cells were transfected with pEGFP-C1 (a), (d), GFP-Htt-19Q (b), (e), and GFP-mHtt-70Q (c), (f). Nuclei were labeled with DAPI and appeared blue.

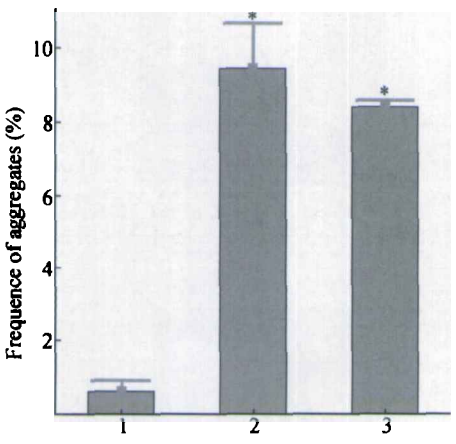

Fig. 4. The effect of sodium butyrate on the formation of mutant huntingtin aggregates. 1, The cells transfected with GFP-Htt-19Q; 2, the cells transfected with GFP-mHtt-70Q; 3, the cells transfected with GFP-mHtt-70Q at 0.5 mmol/L sodium butyrate. * $P<0.001$ vs the cells transfected with GFP-Htt-19Q, $n=3$. There was no significant difference between the cells transfected with GFP-mHtt-70Q and the cells transfected with GFP-mHtt-70Q at 0.5 mmol/L sodium butyrate.

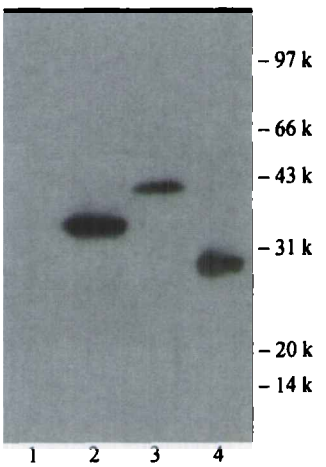

Fig. 5. Western blot analysis of the N-terminal fragments of huntingtin expression in SH-SY5Y cells. 1, Negative control; 2, the cells transfected with GFP-Htt-19Q; 3, the cells transfected with GFP-mHtt-70Q; 4, the cells transfected with pEGFP-C1.

2.3 Protective effect of sodium butyrate against neurotoxicity induced by the N-terminal fragment of mutant huntingtin

In cell viability, no significant difference was

found compared to the control when the cells were transfected with pEGFP-C1 or GFP-Htt-19Q. Also no significant change was detected in the cells transfected with pEGFP-C1 or with GFP-Htt-19Q at any concentration of sodium butyrate (0.1, 0.2, 0.5, 1.0 mmol/L). While in cell viability, the cells transfected with GFP-mHtt-70Q demonstrated a statistically significant decrease compared with that in the control (Fig. 6) and also a statistically significant increase at all sodium butyrate concentrations tested (0.1, 0.2, 0.5, 1.0 mmol/L) (Fig. 7). This results suggested that the N-terminal fragment of mutant huntingtin was toxic to SH-SY5Y cells and sodium butyrate could relieve the toxicity of the N-terminal fragment of mutant huntingtin. Sodium butyrate had the protective effect on this cell culture model of HD.

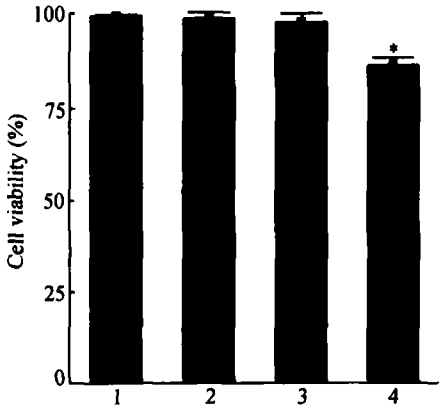

Fig. 6. A decrease in SH-SY5Y cell viability caused by the N-terminal fragment of mutant huntingtin. 1, Control; 2, the cells transfected with pEGFP-C1; 3, the cells transfected with GFP-Htt-19Q; 4, the cells transfected with GFP-mHtt-70Q (* $P < 0.001$, vs control, $n = 5$).

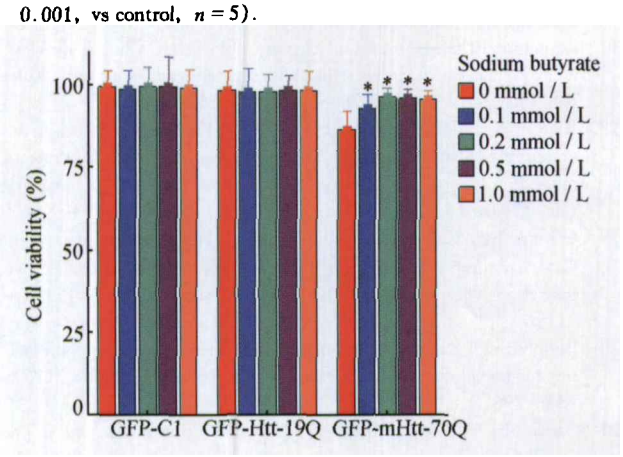

Fig. 7. The effect of sodium butyrate on SH-SY5Y cell viability. * $P < 0.05$, vs the cells transfected with GFP-mHtt-70Q at 0 mmol/L sodium butyrate, $n = 5$.

3 Discussion

A critical step in the pathogenesis of HD is the progressive truncation of mutant huntingtin to form the N-terminal fragments containing the polyglutamine repeats[6]. These N-terminal fragments of mutant huntingtin involved in nuclear location and the formation of aggregates are believed to be the toxic species[7–9]. We modeled HD pathogenesis with expression of the N-terminal fragment of mutant huntingitn in SH-SY5Y cells in this study, and observed that the N-terminal fragment of mutant huntingtin formed perinuclear and intranuclear aggregates in SH-SY5Y cells and caused a decrease in SH-SY5Y cell viability. We had developed a cell culture model that was able to model major pathogenic features of HD. This model would be useful for future experiments to study mechanism of neurotoxicity induced by the N-terminal fragment of mutant huntingtin and the formation of mutant huntingtin aggregates. This model would also be useful to search potential therapeutical drugs for HD.

A causal pathway from the mutant huntingtin to neuronal dysfunction and death has not been established and there is currently no effective treatment for this lethal disease. Transcriptional dysfunction may play an important role in the pathogenesis of HD[10]. Histones can be in one of the two antagonist forms, acetylated or deacetylated, regulated by the corresponding enzymes, histone acetylases (HATs) and histone deacetylases (HDACs). Histone acetylation correlates with chromatin remodeling and transcriptional activation. Deacetylation of histone induces transcriptional repression through chromatin condensation[11]. Mutant huntingtin had been found to bind the acetylase domains of two distinct proteins, CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF), to repress the HAT activity[12]. Meanwhile, mutant huntingtin did not affect the HDAC activity in affected neurons[13]. The balance between HAT activity and HDAC activity in affected neurons was disrupted by mutant huntingtin. Histones had been found to be hypoacetylated in transgenic mouse models of HD[14,15] and in PC12 cell model of HD[16]. Suppression of the HDAC activity to restore the balance between HAT activity and HDAC activity in affected neurons had been proposed as a therapeutic candidate for this lethal disease. At present, HDAC inhibitors are primarily used as anticancer drugs clinically[17]. HDAC inhibitors, such as sodium butyrate had been the best clinically studied compounds and was known to readily reach the

brain[18]. Sodium butyrate had been found to slow photoreceptor neuron degeneration in a transgenic *Drosophila* model of HD and extend survival in a dose-dependent manner, improve body weight and motor performance, and delay the neuropathological sequelae in the R6/2 transgenic mouse model of HD[12,14]. Recent studies indicated that expression of full-length mutant huntingtin in immortalized striatal cells was associated with deficits in mitochondrial-dependent Ca^{2+} handling that can be ameliorated by treatment with sodium butyrate[19]. Therefore, we investigated the effects of sodium butyrate on our cell culture model of HD. In our study, sodium butyrate inhibited the decrease in SH-SY5Y cell viability caused by the N-terminal fragment of mutant huntingtin. Our findings supported the hypothesis that sodium butyrate would be a neuroprotective agent for HD[20]. The possible explanation for direct neuroprotection of sodium butyrate is that sodium butyrate restored the balance between HAT activity and HDAC activity in SH-SY5Y cells to increase the level of the acetylated histones and reactivate a number of gene expressions which were repressed by the N-terminal fragment of mutant huntingtin. The results also strengthened the hypothesis that transcriptional dysfunction plays an important role in the pathogenesis of HD.

Intraneuronal aggregates, both intra- and extranuclear, are prominent features of HD. However, whether these aggregates are causal or protective remains hotly debated[21,22]. In our experiments, sodium butyrate inhibited the decrease in SH-SY5Y cell viability caused by the N-terminal fragment of mutant huntigntin, but failed to attenuate the formation of mutant huntingtin aggregates. This suggested that neuron death might not correlate with the formation of mutant huntingtin aggregates. Insoluble aggregates may not be inherently toxic. The explanation of the formation of mutant huntingtin aggregates might be a protective mechanism in neurons to decrease the levels of toxic diffuse forms of mutant huntingtin[23].

Acknowledgments We are grateful to the patients and family for their cooperation.

References

1 Nance MA. Huntington disease: clinical, genetic and social aspects. J Geriatr Psychiatry Neurol, 1998, 11: 61—70
2 Petersen A, Mani K and Brundin P. Recent advances on the pathogenesis of Huntington' disease. Exp Neurol, 1999, 157: 1—6
3 The Huntington's Disease Collaborative Research Group. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell, 1993, 72: 971—983
4 Perez-Navarro E, Canals JM and Gines S. Cellular and molecular mechanisms involved in the selective vulnerability of striatal projection neurons in Huntington's disease. Histol Histopathol, 2006, 21(11): 1217—1232
5 Sadri-Vakili G and Cha JH. Mechanisms of disease: Histone modifications in Huntington's disease. Nat Clin Pract Neurol, 2006, 2 (6): 330—338
6 Fryer DJ and Zoghbi YH. Huntingtin's critical cleavage. Nat Neurosci, 2006, 9 (9): 1088—1089
7 Wellington CL, Ellerby LM, Gutekunst CA, et al. Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. J Neurosci, 2002, 22(18): 7862—7872
8 Gafni J, Hermel E, Young JE, et al. Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/caspase fragments in the nucleus. J Biol Chem, 2004, 279(19): 20211—20220
9 Lunkes A, Lindenberg KS, Ben-Haiem L, et al. Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. Mol Cell, 2002, 10 (2): 224—225
10 Sugars KL and Rubinsztein DC. Transcriptional abnormalities in Huntington disease. Trends Genet, 2003, 19: 233—238
11 Monneret C. Histone deacetylase inhibitors. J Med Chem, 2005, 40: 1—13
12 Steffan JS and Bodai L. Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. Nature, 2001, 413(6857): 739—743
13 Hoshino M, Tagawa K, Okuda T, et al. Histone deacetylase activity is retained in primary neurons expressing mutant huntingtin protein. Eur J Neurochem, 2003, 87: 257—267
14 Ferrante JR, Kubilus JR, Lee J, et al. Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. J Neurosci, 2003, 23(28): 9418—9427
15 Gardian G, Browne SE, Choi DK, et al. Neuroprotective effects of phenylbutyrate in the N171-82Q transgenic mouse model of Huntington's disease. J Biol Chem, 2005, 280: 556—563
16 Igarashi S, Morita H, Bennett KM, et al. Inducible PC12 cell model of Huntington's disease shows toxicity and decreased histone acetylation. Neuroreport, 2003, 14: 565—568
17 Drummond DC, Noble CO, Kirpotin DB, et al. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol, 2005, 45: 495—528
18 Egorin MJ, Yuan ZM, Sentz DL, et al. Plasma pharmacokinetics of butyrate after intravenous administration of sodium butyrate or oral administration of tributyrin or sodium butyrate to mice and rats. Cancer Chemother Pharmacol, 1999, 43: 445—453
19 Oliveira JM, Chen S, Almeida S, et al. Mitochondrial-dependent Ca^{2+} handling in Huntington's disease striatal cells: effect of histone deacetylase inhibitors. J Neurosci, 2006, 26(43): 11174—11186
20 Butler R and Bates GP. Histone deacetylase inhibitors as therapeutics for polyglutamine disorders. Nat Rev Neurosci, 2006, 7(10): 784—796
21 Saudou F, Finkbeiner S, Devys D, et al. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell, 1998, 95: 55—66
22 Waelter S, Boeddrich A, Lurz R, et al. Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. Mol Biol Cell, 2001, 12: 1393—1407
23 Arrasate M, Mitra S, Erik S, et al. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature, 2004, 431: 805—810