Mutant huntingtin reduces vesicular zinc level by inhibiting the binding of Sp1 to ZnT3 promoter

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Abstract

Background

Synaptic dysfunction caused by mutant huntingtin greatly contributes to Huntington's disease (HD) pathogenesis. HD patients show cognitive impairment as well as uncontrolled movements. Vesicular zinc is closely linked to modulating synaptic transmission and maintaining cognitive ability. However, whether does mutant huntingtin affect zinc homeostasis in the brain or not? This will be of great significance for further revealing the pathogenesis of HD.

Methods

N171-HD82Q transgenic mice and cultured BHK cells expressing N-terminal mutant huntingtin fragment containing 160 glutamines (160Q BHK cells) were used to investigate the effect of mutant huntingtin on zinc homeostasis and its molecular mechanisms.

Results

Herein, we have demonstrated that the density of synaptic vesicular zinc decreases in the cortex, striatum and hippocampus of N171-82Q mice. Given that vesicular zinc concentration depends on the abundance of zinc transporter 3 (ZnT3) on the membrane of synaptic vesicles, ZnT3 expression is detected in the brain of N171-82Q mice and 160Q BHK cells. Mutant huntingtin leads to a dramatical decrease in ZnT3 mRNA and protein levels in the three brain regions of these mice aged from 14 to 20 weeks. Significantly, Sp1 activates ZnT3 transcription via its binding to the GC boxes in ZnT3 promoter. Nevertheless, mutant huntingtin inhibits the binding of Sp1 to the promoter of ZnT3 gene and down-regulates ZnT3 expression. Furthermore, the overexpression of Sp1 ameliorates inhibition of ZnT3 gene transcription by mutant huntingtin.

Conclusions

Collectively, this first study to reveal a significant loss of synaptic vesicular zinc and ZnT3 expression caused by mutant huntingtin in the early stage of HD. Our findings have revealed the molecular mechanism underlying this change. Mutant huntingtin inhibits the binding of Sp1 to ZnT3 gene promoter to reduce ZnT3 expression. The imbalance of vesicular zinc homeostasis may be closely associated with synaptic dysfunction and cognitive deficits in HD. This work sheds novel mechanistic insights into the pathogenesis of HD and promises a potential therapeutic strategy for HD.

Background

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by the pathological expansion of CAG repeats (> 36) in the first exon of the HD gene encoding huntingtin (Htt) [1–8]. Neuropathologically, the neuronal loss occurs primarily in the striatum and the cortex in the early stages of HD. However, other brain regions, such as the hippocampus, hypothalamus, brainstem, and spinal cord, are also affected in advanced stages [2–6, 9]. HD symptoms occur usually in mid-life, comprising movement, cognitive and psychiatric impairments inexorably progressing to death within two decades [1, 7]. Psychiatric deteriorations and cognitive deficits precede the onset of motor disorder [7], and even the onset of both psychiatric and motor symptoms often occur years prior to detectable neuronal loss [7, 8].

Synaptic dysfunction greatly contributes to HD pathogenesis [10, 11]. Neurotransmitter release significantly alters in the transgenic HD mouse models [12, 13]. Neuronal and synaptic dysfunction precedes cell death by many years in the HD patients [14, 15] and animal models [16]. Furthermore, some pharmacological interventions focus on targeting early synaptic disturbances, which has been proven to restore synaptic function [17–19] and delay progression to neurodegeneration in HD transgenic mice [20, 21]. Therefore, disturbed synaptic function accounts for the early symptoms of HD and triggers neuronal death in later stages of the disease [11, 22, 23]. It is vital to investigate the mechanism of synaptic damage in HD disease.

The divalent cation zinc in the brain contributes to efficient synaptic transmission. Approximately, 85% of total brain zinc is tightly bound to metalloproteins. 10 ~ 15% of total brain zinc is highly localized in synaptic vesicles of excitatory glutamatergic neurons [24, 25]. This pool of zinc, the ionic zinc, is either free or chelatable. It can be detected with simple histochemical method such as neo-Timm's sulfide-silver method [26, 27], and is thus often called histochemically reactive zinc. Neurons containing histochemically detectable zinc are present in many regions of the brain, including the neocortex, striatum, hippocampus, amygdale and olfactory bulb [28]. Vesicular zinc is closely linked to modulating synaptic transmission. It serves as a signal factor to play an important role in modifying glutamatergic neurons [29–31]. Zinc releases from glutamatergic neuron terminals, which may protect neurons from excitotoxicity of glutamate to attenuate the excess amount of presynaptic glutamate release [32]. Zinc deficiency affects neurogenesis and trigger neuronal apoptosis. Therefore, this can result in learning and memory deficits [32].

More importantly, the homeostasis of zinc in the brain is tightly regulated. The zinc transporters (ZnTs) mainly function to efflux zinc out of cytoplasm or into intracellular organelles [33]. Among them, zinc transporter 3 (ZnT3), the primary vesicular zinc transporter, is located on the membrane of synaptic vesicles to transport zinc ions into presynaptic vesicles from the cytosol [34]. The concentration of vesicular zinc depends on the abundance of ZnT3 [34, 35]. Targeted deletion of ZnT3 gene eliminates zinc from synaptic vesicle, which leads to age-dependent deficits in learning and memory ability [36] and neurodegeneration [36–38]. Consequently, ZnT3-dependent zinc homeostasis in synaptic vesicles takes an important role in maintaining synaptic function.

An imbalance of vesicular zinc homeostasis is associated with the pathogenesis of multiple neurodegenerative diseases, including Parkinson's disease (PD) [39, 40], Alzheimer's disease (AD) [39, 41, 42, 43, 44] and amyotrophic lateral sclerosis (ALS) [45]. These diseases have in common with HD features and mechanisms that the misfolded proteins cause neuronal death at the late onset of the disorder. Altered homeostasis of essential elements such as iron, chromium and selenium has been observed in the HD patients [46, 47] and mice model [48]. Specially, increased level of zinc is detected in the blood of HD patients, indicating that mutant Htt (mHtt) might impair zinc homeostasis [46]. Nevertheless, whether does mHtt affect zinc homeostasis in the brain or not? This will be of great significance for further revealing the pathogenesis of HD. Here, we have demonstrated that mHtt reduces ZnT3 expression by inhibiting the binding of Sp1 to ZnT3 gene promoter to down-regulate vesicular zinc level in the brain of N171-82Q HD transgenic mice. Disruption of vesicular zinc homeostasis will ultimately contribute to synaptic dysfunction and neurodegeneration in HD.

Materials And Methods

Huntington's disease transgenic mice

B6C3-Tg (HD82Gln) 81Dbo/J (N171-HD82Q) HD transgenic mice (Jackson Laboratories) express a cDNA encoding a 171 amino acid N-terminal fragment of huntingtin containing 82 CAG (Q) repeats [48]. At the age of 4 weeks, mice were genotyped by polymerase chain reaction (PCR) of tail DNA genotyped according to the Jackson Laboratories protocol to determine hemizygosity for the HD transgene. Wild-type littermates were used as controls.

Plasmids For the generation of three ZnT3 promoter reporter constructs, pGL3-ZnT3(−283~+10) vector containing both GC-1 and GC-2 elements, pGL3-ZnT3(−193~+10) vector containing GC-2 element, and pGL3-ZnT3 (−171~+10) vector without any GC-rich element, the promoter fragments of wild type mice ZnT3 gene (GenBank accession number 22784) were amplified using the PCR technique and subcloned into pGL-3 basic vector harboring a luciferase reporter gene (Promega). The pEGFP-exon-1 20Q-Htt and pEGFP-exon-1 160Q-Htt plasmids [49], pEBGN-Sp1 plasmid, nuclear factor κB (NF-κB) p50 and NF-κB (p65) vectors, Wilms' tumor (WT1) gene vectors (WT1+KTS, WT1-KTS) and pMIC-Sp1 were kindly provided by Xiao-Jiang Li (USA), Gerald Thiel (Germany), Neil D. Perkins (UK), Holger Scholz (Germany), and Jacqueline Marvel (France), respectively.

Cell culture and transfection

Baby hamster kidney cells (BHK cells), a specific cell line originally derived from kidney tissue of baby hamster, were cultured in Dulbecco's modified Eagle's medium of high glucose and supplemented with 10 % fetal bovine serum (GIBCO, Australia), 100 µg/ml penicillin and 100 units/ml streptomycin in a humidified incubator at 37 °C under 5 % CO₂ and 95 % air. Having been 90 – 95 % confluent BHK cells, 4×10⁵ cells / well were planted in the six-well plate. After 24 h, they were transiently transfected with

plasmids using Lipofectamine TM 2000 (Invitrogen). The cells were replaced with fresh culture medium six hours after transfection.

Flame atomic absorption spectrometry (FAAS) The 20-week-old N171-82Q mice and age-matched WT mice (n = 3) were sacrificed after anesthesia with pentobarbital sodium intraperitoneal injection (40 – 45 mg / kg body weight). After the brain tissues were dissected and weighed, samples were washed and digested in ultrapure nitric acid. All the samples were evaporated to dryness, and were resuspended in 2 % nitric acid. After the resuspending of the samples, the total zinc was detectable by SpectrAA-240FS (VaRIAN).

Autometallography (AMG)

The 20-week-old N171-82Q mice and age-matched WT mice (n = 3) were anesthetized and then perfused intracardially with 0.3 % sodium sulfide solution in a 0.1 M sodium phosphate buffer (PB, pH 7.4) 150 ml, followed by transcardial perfusion with 250 ml 4 % paraformaldehyde in 0.1 M PB, and finally with the same sodium sulfide solution again for 150 ml [50]. After perfusion, the brains were removed and post-fixed with 4 % paraformaldehyde in 0.1 M PB overnight. The brains were placed in 30 % sucrose overnight.

For light microscopy (LM), the samples were cut into 10 µm coronal sections in a cryostat and placed on Farmer fluid cleaned glass slides and AMG developed [51]. The sections were immersed in a citrate buffered silver lactate/hydroquinone developer containing a protective colloid (gum arabic), and incubated in a 26 °C water bath shaking for 1 h, and then were stopped with a 5 % thiosulphate solution 10 min. The sections were rinsed with warm water 38 °C for 10 min in order to remove the gelatine membrane [52]. Images were taken on a Nikon microscope (Digital Camera DXM 1200).

For electron microscopy (EM), mice were transcardial perfusion with 250 ml 4 % paraformaldehyde added by 0.25 % glutaraldehyde after being perfused with sodium sulfide solution. The samples were sectioned at 40 µm on a cryostat and were incubated in an AMG developer. Selected samples for ultrastructurally analyzed were fixed with 1% osmium acid in PB for 30 min, dehydrated in a series of alcohols and embedded in epon. Then, the samples were cut into 60 nm ultrathin sections that were stained with uranyl acetate (15 min) and lead citrate (30 min). Finally, the sections were examined with a transmission electron microscope (FEI Tecnai G² Type 12)

Immunohistochemistry

The 20-week-old N171-82Q mice and age-matched WT mice (n = 3) were deeply anesthetized and perfused transcardially with saline, followed by 4 % paraformaldehyde in 0.1 M PB 150 ml. The brains were removed and further post-fixed with the same fixatives for 8 h at 4 °C. The samples were cryoprotected in 30 % sucrose at 4 °C 12 h and sectioned at 30 µm using a freezing microtome. For immunohistochemical localization of ZnT3, free-floating sections were treated with 0.3 % TritonX-100 and 3 % hydrogen peroxide (H₂O₂) in PB for 30 min to reduce endogenous peroxidase activity and were

pre-incubated for 30 min with 3 % bovine serum albumin (BSA) to reduce nonspecific staining. The sections were incubated overnight at 4 °C with polyclonal rabbit anti-ZnT3 antibodies (Proteintech) diluted 1:100 in BSA contained 2 % goat serum and 0.3 % TritonX-100. After twice washing with 0.01 M PBS, the sections were incubated with biotinylated anti-rabbit IgG (Vector Labs, Burlingame, CA) diluted 1:200 and a avidin-biotin complex (Vector ABC Elite; Vector Labs) 1:100 for 2 h at room temperature. Then, the sections were incubated 0.02 % diaminobenzidine (Sigma-Aldrich) and 0.005 % hydrogen peroxide in 0.05 M Tris-HCl buffer for 10 to 15 min. Images were taken on a Nikon microscope (Digital Camera DXM 1200).

Western blot analysis

Brain tissues from 14-, 18-, and 20-week-old N171-82Q mice and age-matched WT mice (n = 4 at each age) and BHK cells at different time after transfection (n = 4 at each time interval) were homogenized in an ice-cold lysis buffer included 50 mM Tris (pH 8.0), 150 mM NaCl, 1 % Triton X-100, protease inhibitor cocktail (Sigma-Aldrich) and 100 mg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). Then the resulting homogenate was centrifuged at 12,000 *g* for 30 min at 4 °C. The extracted proteins were separated on 10 % SDS-polyacrylamide gels and were transferred onto NC membranes (Amersham Biosciences UK Limited) in an electrotransfer device (90 V, 90 min). Then the membranes were blocked in 5 % nonfat milk in 0.01 M PBS for 1 h and incubated in primary antibody overnight at 4 °C. The dilutions of primary antibodies were 1:1000 for ZnT3, 1:1000 for Sp1 (Abcam), 1:10000 for γ -tubulin and 1:5000 for GAPDH. Followed the membranes were washed with 5 % nonfat milk and immersed in the horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature with constant agitation. The immunoreactive bands were visualized by exposure to an enhanced chemiluminescence (ECL) kit (Pierce Thermo Scientific).

RT-PCR

Total RNA from BHK cells, wild type and HD transgenic mice was extracted with Trizol reagent (Invitrogen). ZnT3 and Sp1 mRNA expression were amplified by reverse-transcription polymerase chain reaction, and β -actin mRNA was taken as an internal control. The following PCR conditions were used: 94 °C for 3 min; 30 cycles of denaturing at 94 °C for 30 s, annealing for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 3 min. Photos of the amplified genes were taken after agarose gel (1 %) electrophoresis. The primers and amplification used in the PCRs were listed in the Table 1.

siRNA transfection

The sense Sp1 siRNA (5'-GGAUGGUUCUGGUCAAAUA-3') and control non-silencing siRNA were produced by RIBOBIO company (China). The BHK cells were grown in Opti-MEM till 60 % confluent. The cells were transfected with siRNA (50 nM) using Lipofectamine TM 2000 (Invitrogen). The cells were harvested at 48 h after transfection. RT-PCR and Western blot assay were respectively performed to detect protein and mRNA levels of Sp1.

Dual-luciferase reporter gene assay

BHK cells were seeded in 12-well tissue culture plates at 2×10^5 per well in 1 ml of Opti-MEM (Invitrogen) at 24 h before transfection. For the detection of targeting relationship of Sp1 and ZnT3, pEBGN-Sp1 was co-transfected with reporter plasmids pGL3-ZnT3 (-283 ~ +10), pGL3-ZnT3 (-193 ~ +10), or pGL3-ZnT3 (-171 ~ +10), respectively, into BHK cells. pEBGH empty vector was served as a control. For examining the effect of mHtt on ZnT3 transcription activity, different concentration of mHtt vector (0.5 μ g, 1 μ g, 2 μ g) was co-transfected with reporter plasmid pGL3-ZnT3 (-171 ~ +10) into BHK cells. 20Q Htt vector was served as a control. Cells were harvested at 48 h after transfection and lysed for 30 min in 500 μ l of passive lysis buffer according to the manufacturer's instructions of dual-luciferase reporter assay system (Promega). The 20 μ l lysate were added with 100 μ l luciferase assay reagent II. Relative luminescence units (RLUs) produced by firefly luciferase activity were measured by using LB9507 luminometer. Then 100 μ l Stop&Glo reagent was added to the mixtures and activated the Renilla luciferase. Luciferase activity of pRL-TK Renilla luciferase plasmid was used to determine the background for each luciferase reaction. Luciferase activity = RLU (firefly luciferase) / RLU (Renilla luciferase). All experiments were performed three times, and the mean relative luciferase activity was obtained.

Chromatin immunoprecipitation assay (ChIP) and reverse transcription-quantitative PCR (RT-qPCR)

BHK cells were seeded in 10 cm dishes and were fixed with 37 % formaldehyde for 10 min in 37 °C to cross bonding the target protein and the corresponding genomic DNA according to manufacturer's instructions of ChIP Assay kit (Beyotime institute biotechnology P2078). Then 1.1 ml Glycine Solution (10 $\times$) was added to the dishes for 5 min at room temperature. Cells were washed twice with 0.01 M PBS (includes 100 mM PMSF) and harvested with 1 ml the same solution. After centrifugation, cells were resuspended with 0.2 ml SDS Lysis Buffer (includes 100 mM PMSF) on ice for 10 min. Released DNA was sonicated three times for 10 s. 1 % of the sample volume was saved as input for the later normalization. The chromatin solution was incubated with Sp1 antibody (ChIP Grade, Abcam) or a nonspecific control antibody (normal rabbit IgG, Beyotime Institute Biotechnology). After incubating with the antibody for overnight at 4 °C, 60 μ l Protein A+G Agarose / Salmon Sperm DNA was added and shaken for 3 h. Then, the beads were washed with wash buffer and antibody-coupled protein was washed twice by incubation in 250 μ l elution buffer (1 % SDS, 0.1 M NaHCO₃) for 10 min. After centrifugation, 5 M NaCl (20 μ l) was added to the supernatant and incubated at 65 °C for 4 h. After decrosslinking, DNA was purified with PCR/DNA clean up kit (Beyotime Institute Biotechnology D0033). After purification, RT-qPCR was performed to analyze immunoprecipitated DNA using the ZnT3 promoter primers (Table 1). 4 μ l of the purified DNA was subjected to 40 cycles of PCR amplification in a volume of 20 μ l (denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 3 min) by using SYBR Green Master Mix (Bio-Rad). The amount of amplified DNA was expressed as percent of the input. All ChIP assays were performed three times.

Statistical analysis

Statistical analyses were carried out using SPSS 17.0 software for one-way ANOVA followed by Student's *t* test. All values were represented as mean $\pm$ S.D. Differences were considered significant if $p < 0.05$.

Results

N171-82Q mice display significant loss of total zinc and vesicular zinc in the brain tissue

The flame atomic absorption spectrometry (FAAS) and autometallography (AMG) were respectively applied to explore the effect of mHtt on total and vesicular zinc level in the brain tissue of N171-82Q mice. Comparison of the 20-week-old N171-82Q mice with age-matched wild type (WT) mice showed that total zinc level was extremely low in the cortex, striatum and hippocampus examined in N171-82Q mice, compared to controls (Fig. 1).

AMG results showed that histochemically reactive zinc was significantly decreased in all three brain regions examined in the 20-week-old N171-82Q mice, compared to controls (Fig. 2). In the wild type (WT) mice, intense AMG staining was seen in the CA1, CA2 and CA3 region of hippocampus (Fig. 2A, B), cortex (Fig. 2E) and striatum (Fig. 2E, F). However, the N171-82Q mice presented faint zinc staining in the corresponding brain regions (Fig. 2C, D, G, H).

At the ultrastructural level, the AMG reactive zinc granules were present within synaptic vesicles in striatum of the WT mice (Fig. 3A), whereas the number of positive vesicles was rarely, if at all, seen in striatum of the N171-82Q mice (Fig. 3B). These collective findings indicates that zinc is unbalanced in the HD brain and suggests a role of zinc in HD pathogenesis.

ZnT3 expression is decreased in N171-82Q mice and BHK cells expressing mutant huntingtin

ZnT3 is required for zinc transport into synaptic vesicles and that vesicular zinc concentration is regulated by the amount of ZnT3 present on synaptic vesicle membranes [34–36]. Thus, we hypothesized that lower level of vesicular zinc may result from reduced ZnT3 in the N171-82Q mice. To test this assumption, we analysed ZnT3 expression in N171-82Q mice and age-matched WT mice. Light microscopic immunohistochemistry revealed that ZnT3 immunoreactivities were intensely present in the hippocampus, striatum and cortex (Fig. 4A, B) of the 20-week-old WT mice. Especially in the dentate gyrus (DG) and CA3 area of hippocampus, where are rich in axonal terminal boutons, intense ZnT3-immunoreactive granules were seen. In contrast, age-matched N171-82Q mice displayed weak ZnT3-immunoreactivities in the same brain regions (Fig. 4C, D).

To further identify whether ZnT3 expression was decreased in the N171-82Q mice, we investigated ZnT3 protein and mRNA level in different brain regions of the N171-82Q mice and age-matched WT mice. ZnT3 protein and mRNA levels in the cortex, hippocampus and striatum were markedly reduced in 14-, 18-, and 20-week-old N171-82Q mice, in comparison with age-matched WT controls (Fig. 5A, B). These results coincide with our assumption that mHtt disturbs ZnT3 expression in the N171-82Q mice.

Following this viewpoint, we further determined ZnT3 levels in cultured BHK cells expressing N-terminal mHtt containing 160 polyglutamine (160Q cells) or 20 polyglutamine (20Q cells). Being consistent with above findings in the N171-82Q mice, identified significant deficit in ZnT3 expression level was detected in 160Q cells (Fig. 6). Associated with the above results, we can reasonably conclude that mHtt causes the loss of vesicular zinc by affecting ZnT3 expression.

Sp1 positively regulates ZnT3 expression through activating ZnT3 gene promotor

Gene transcriptional dysregulation is greatly involved in the mechanisms of neurodegeneration in HD [53, 54]. Whether does down-regulation of ZnT3 expression result from transcriptional inhibition or not in HD? We analyzed ZnT3 gene promoter via bioinformatics. The data hints that the promoter of mouse ZnT3 gene contains two GC-rich boxes, which might be Sp1 binding sites (Fig. 7A). Sp1, a key transcription factor isolated from human being, has been shown to recognize related GC motifs within promoter regions [55]. One GC-rich box (GC-1) in ZnT3 gene promoter is from -269 bp to -261 bp, the other (GC-2) is from -184 bp to -174 bp (Fig. 7A). These putative Sp1 sites are conserved among human, rat, and mouse. To verify this hypothesis that Sp1 might be a transcription factor of ZnT3 gene, we first examined whether Sp1 affects the ZnT3 promoter activity. For dual-luciferase reporter gene assay, we constructed three ZnT3 promoter driven Luciferase constructs, pGL3-ZnT3 (-283 ~ +10) containing both GC-1 and GC-2 sequence, pGL3-ZnT3 (-193 ~ +10) containing GC-2 sequence and pGL3-ZnT3 (-171 ~ +10) without putative Sp1 binding sites (Fig. 7B). These constructs were respectively co-transfected with Sp1 expressing vector pEBGN-Sp1 into BHK cells. The reporter activities of pGL3-ZnT3 (-283 ~ +10) and pGL3-ZnT3 (-193 ~ +10) were significantly up-regulated in Sp1 transfected cells (Fig. 7B). However, there were no differences in the reporter activities of pGL3-ZnT3 (-171 ~ +10) between Sp1 and empty vector transfected cells (Fig. 7B), suggesting that Sp1 affects the promoter activity of ZnT3 gene through GC boxes.

To further demonstrate Sp1 transactivates ZnT3 gene by binding GC boxes in the promoter, we pursued chromatin immunoprecipitation (ChIP) assay. Sp1 antibody precipitated more DNAs containing these ZnT3 promoter sequences with GC-1 or GC-2 than a mock immunoprecipitation with control IgG (Fig. 7C). This result indicates that Sp1 positively regulates ZnT3 transcription activity by binding directly to GC boxes.

Following this viewpoint, the role of Sp1 in the regulation of ZnT3 expression was examined in BHK cells. Sp1 overexpression promoted significantly the expression of endogenous ZnT3 mRNA and protein in WT BHK cells (Fig. 8). Whereas, the other transcription factors including NF- κ B and WT1 did not affect ZnT3 expression in BHK cells (Fig. S1), indicating that Sp1 acts as an important transcription factor of ZnT3 gene.

In contrast, ZnT3 protein and mRNA level were significantly reduced in BHK cells transfected with Sp1 siRNA, but not with the non-targeting control siRNA (Fig. 9). We confirmed the reduction of Sp1 expression in the BHK cells transfected with Sp1 siRNA (Fig. S2). Consequently, these results imply that Sp1 plays a key role in transcriptional regulation of the ZnT3 gene.

Sp1 overexpression ameliorates inhibition of ZnT3 gene transcription by mutant huntingtin

We further investigated the effect of Sp1 on ZnT3 gene transcription in 160Q BHK cells. Sp1 overexpression increased significantly ZnT3 mRNA levels in 160Q BHK cells, compared to 20Q BHK cells (Fig. 10A, B).

Furthermore, we examined the effect of mHtt on ZnT3 transcription activity. Different concentrations of mHtt vector (0.5 µg, 1 µg, 2 µg) were respectively co-transfected with reporter plasmid pGL3-ZnT3 (– 283 ~ + 10) into BHK cells. Dual-luciferase reporter gene assay showed that 160Q Htt reduced the ZnT3 promoter activity, compared to 20Q Htt (Fig. 10C). However, overexpression of Sp1 ameliorated ZnT3 promoter activity in 160Q cells (Fig. 10D). These data indicate that Sp1 reverses the suppression of mHtt to ZnT3 transcription activity in BHK cells.

Mutant huntingtin inhibits the binding of Sp1 to ZnT3 promoter

Mutant huntingtin has been demonstrated to inhibit the binding of Sp1 to target genes in HD [56–59], which might also be the mechanism of the effect of mHtt on ZnT3 expression. In this report, we examined the binding of Sp1 to ZnT3 promoter in 160Q and 20Q BHK cells. ChIP assay displayed that there was a significant decrease in Sp1-immunoprecipitated DNA consisting of ZnT3 promoter in 160Q cells, compared to 20Q cells [Fig. 11]. In consequence, mHtt inhibits ZnT3 expression by disturbing the binding of Sp1 to the ZnT3 gene.

Discussion

Impairment of synaptic function contributes to the HD pathogenesis. Vesicular zinc is of significance for synaptic function. Herein, we identified a significant loss of total zinc, especially vesicular zinc in the brain of the HD transgenic N171-82Q mice. Furthermore, a reduction of ZnT3 expression was observed in these mice and 160Q BHK cells. ZnT3, an important protein located on the membrane of synaptic vesicles, affects synaptic function *via* various mechanisms in neurons [60]. Most important of all, ZnT3 is responsible for the movement of zinc from the cytoplasm to the synaptic vesicles. Vesicular zinc level is dependent on ZnT3 protein abundance. Zinc is eliminated from synaptic vesicles in the brain of ZnT3 knockout mice [61, 62]. In the present study, we found that vesicular zinc detected by AMG staining is dramatically decreased in the striatum, hippocampus and cortex of the N171-82Q mice compared to age-matched controls, suggesting that the reduced ZnT3 expression in HD greatly disturbs zinc homeostasis in synaptic vesicles.

It is well-known that brain zinc homeostasis is strictly controlled to guarantee physiological function under healthy conditions. Vesicular zinc serves as a signal factor in a subclass of glutamatergic neurons, which is linked to glutamate signaling and cognitive activity [63, 64]. Specially, vesicular zinc can inhibit glutamate release. In mutant mice with lacking vesicular zinc, glutamate release inhibition induced by zinc is absent [65]. Many evidences support a key role of glutamate-mediated excitotoxic cell death in HD pathogenesis [66–68]. Thus, it appears likely that loss of vesicular zinc in the N171-82Q mice aggravates

glutamate-mediated excitotoxic neuron death. On the other side, vesicular zinc signaling meets the requirement for cognitive and emotional behavior [36]. Movement impairment and cognitive deficits occur prior to neuron degeneration in HD patients [8, 69]. Our results reveal that vesicular zinc level is lower in the hippocampus of the N171-82Q mice compared to WT mice. Consequently, the decrease of vesicular zinc might be responsible for the cognitive decline in HD.

Beside affecting vesicular zinc, the reduced ZnT3 expression may also impair synaptic structures and proteins. In ZnT3 knockout mice, there is a decrease in total dendritic spines per neuron. Similarly, synaptic plasticity-related proteins, such as presynaptic synaptosome-associated protein 25 (SNAP25) and postsynaptic PSD95, are markedly decreased in the absence of ZnT3 [36]. According to the previously-reported literatures, both SNAP25 and PSD95 are also defective in HD [69, 70]. Of note is that the N171-82Q mice showed significant reductions in ZnT3 protein and mRNA levels at a relatively early stage of this disease (about 14 weeks). Subtle alterations in synaptic function can lead to the early symptoms of HD [71]. In consequence, we conclude that reduction of ZnT3 expression in the N171-82Q mice results in synaptic dysfunction.

However, how does mHtt cause the reduction of ZnT3 expression in HD? Subsequently, the mechanism about this issue was further illuminated in this study. One of distinctive characteristics of mHtt is to form aggregates or inclusions, which directly recruit synaptic proteins [72, 73]. Here, we firstly examined whether mHtt aggregates could recruit ZnT3 protein. Co-immunoprecipitation results showed that ZnT3 protein was not detected in aggregates, suggesting that down-regulation of ZnT3 does not come from the recruit of mHtt. Additionally, mHtt affects gene expression *via* altering the activity of transcription factors or abnormally interacting with ones [74–76]. Two major transcriptional pathways, namely CRE and Sp1-mediated transcription, have been extensively studied in HD [53]. Increasing findings demonstrate that several genes containing Sp1-binding motifs in their promoters are down-regulated in HD [55, 77, 78]. In the present study, one important finding was that Sp1 transactivated ZnT3 gene by binding the GC boxes in the promoter. We also detected whether mHtt could affect Sp1 expression to reduce ZnT3 mRNA level. Interestingly, the N171-82Q mice displayed an increasing Sp1 expression compared to WT mice, which was consistent with previous reports [79]. The result indicates that inhibition of ZnT3 expression does not result from decreased Sp1 protein.

More importantly, Sp1 is found to interact with Htt [57, 80–82]. mHtt binds to C-terminal region of Sp1, which inhibits the interaction of Sp1 with targeted gene promoters [81, 82]. Here, we confirmed that mHtt inhibits the binding of Sp1 to the GC boxes in the ZnT3 gene promoter so as to reduce ZnT3 expression. Similar mechanism has been verified in previous studies. For example, Sp1 acts through its binding to the GC boxes in the nerve growth factor receptor (NGFR) promoter, followed by activating the expression of NGFR. mHtt inhibits the binding of Sp1 to the NGFR promoter to decrease its transcription [81]. As is mentioned above, the N171-82Q mice showed the defective of vesicular zinc at a relatively early stage of this disease. Given that Sp1 disruption also occurs early in human HD pathogenesis, even in postmortem tissues of pre-symptomatic grade [77, 81]. We reasonably infer that mHtt blocks the binding of Sp1 to ZnT3 promoter to decrease the expression of ZnT3, thereby disrupting vesicular zinc homeostasis. In

order to further confirm this mechanism, we investigated the influence of overexpression of Sp1 on ZnT3 mRNA level in 160Q BHK cells. As expected, overexpression of Sp1 enhanced the transcriptional activity of ZnT3 gene to up-regulate its mRNA level in our experiments. Thus, overexpression of Sp1 greatly attenuates inhibition of the binding of Sp1 to ZnT3 gene promoter by mHtt. Obviously, increasing endogenous Sp1 protein in the N171-82Q mice might be a reactive result as reduction of binding between Sp1 and ZnT3 gene promoter. According to the above data, we present the mechanism of ZnT3 down-regulation and its effect on vesicular zinc in HD in Fig. 12.

Conclusions

In conclusion, this work has identified significant reductions in vesicular zinc and its molecular mechanism in HD. ZnT3 expression is decreased in the brain at the early stage of the N171-82Q mice, indicating that altered neuronal zinc homeostasis is an early event in HD pathogenesis. Sp1 serves as one important transcription factor of ZnT3 gene to activate its transcription. However, mHtt down-regulates ZnT3 expression by inhibiting the binding of Sp1 to the GC boxes in ZnT3 promoter. This further leads to a decrease in the transportation of zinc into synaptic vesicles from cytoplasm. The decline of vesicular zinc results in synapse dysfunction and cognitive impairment. Our findings provide novel insights to elucidate the pathogenesis of HD and pave new therapeutic avenues for the treatment of HD.

Abbreviations

HD: Huntington's disease; ZnT3: zinc transporter 3; Htt: huntingtin; mHtt: mutant huntingtin; BHK cells: Baby hamster kidney cells; FAAS: Flame atomic absorption spectrometry; AMG: Autometallography; ChIP: Chromatin immunoprecipitation assay.

Declarations

Subheading

A loss of vesicular zinc in Huntington's disease

Supplementary information

Supplemental information includes two figures.

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Authors' Contributions

He Li and Cuifang Ye conceived the design of the study. Li Niu performed all experiments. Shiming Yang and Weixi Wang contributed to the cloning of constructs and electron microscopy. Cuifang Ye supervised all experiments, performed the data analysis and wrote the paper with help from He Li. All authors read and approved the final manuscript.

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Availability of data and material

The data and materials are available from corresponding author on reasonable request.

Ethics approval

All procedures for animal use were approved by the institutional Animal Care and Committee of Tongji Medical College, Huazhong University of Science and Technology.

Consent for publication

All authors have approved of the contents of this manuscript and provided consent for publication.

Competing interests

The authors declare that there is no conflict of interest.

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Tables

Table 1 Primers, amplicon size and annealing temperature of ZnT3, Sp1 and β -actin for RT-PCR and ChIP-RT-qPCR

Name	Primer Sequences		Amplicon size	Annealing temperature
	Forward	Reverse		
β -actin	GTCGTACCACAGGCATTGTGATGG	GCAATGCCTGGGTACATGGTGG	492bp	58°C/63°C
β -actin	TTTCCAGCCTTCCTTCTTGGGTATG	ATAGAGGTCTTTACGGATGTCAACG	100bp	58°C/63°C
ZnT3	GGAGGTGGTTGGTGGGTATTTAGC	CCATTAGCAGATTGGCACAGACAGC	317bp	58°C
Sp1	AGAACCCACAAGCCCAGACAATC	CTCCTTCTCCACCTGCTGTCTCA	330bp	63°C
ZnT3 promoter -338 ~ -232 GC-1	GTCGTACCACAGGCATTGTGATGG	GCAATGCCTGGGTACATGGTGG	106bp	60°C
ZnT3 promoter -253 ~ -112 GC-2	TTTCCAGCCTTCCTTCTTGGGTATG	ATAGAGGTCTTTACGGATGTCAACG	141bp	60°C

Supplementary Information Legends

(Supplementary information) Figure 1 Effect of transctiption factors overexpression on ZnT3 expression in WT BHK cells. Overexpression of Sp1 increases ZnT3 protein levels. However, overexpression of other transctiption factors, WT1 and NF- κ B, does not affect ZnT3 expression.

(Supplementary information) Figure 2 Sp1 expression level in WT BHK cells transfected with Sp1 siRNA. Sp1 siRNA effectively knocks down Sp1 expression in WT BHK cells. A and B are Western blots analysis of ZnT3 expression and RT-PCR analysis of ZnT3 mRNA level in BHK cells transfected with different concentrations of Sp1 siRNA vectors, respectively. Con-Si: control siRNA; Sp1-Si: Sp1 siRNA

Figures

Figure 1

Total zinc level in N171-82Q (TG) mice brain. Total zinc decreases in cortex, striatum and hippocampus of the 20-week-old TG mice compared to age-matched wild type (WT) mice. $n = 3$. *, $p < 0.05$ compared to WT mice.

Figure 2

Free zinc level in TG mice brain. AMG stain is abundant in hippocampus and striatum of the 20-week-old WT mice, but poor in age-matched TG mice brain. A, C, E and G are hippocampus of WT mice, hippocampus of TG mice, striatum of WT mice, and striatum of TG mice, respectively. B, D, F and H are the magnified images indicated by boxed areas in A, C, E and G, respectively. h: hilus; luc: s lucidum; or: s oriens; rad: s radiatum; Cor, cortex; Str, striatum. Scale bars: 500 μm in A, C, E and G; 125 μm in B, D, F and H.

Figure 3

Electron microscopy images of vesicular zinc in TG mice striatum. Zinc density in synaptic vesicles dramatically decreases in striatum of the 20-week-old TG mice compared to age-matched WT mice. A and B are WT mice and TG mice, respectively. Arrowheads show postsynaptic membrane. T1: axonal terminal containing AMG positive granules; T2: axonal terminal without AMG positive granules; d: dendrite. Scale bars: 0.2 μm .

Figure 4

Immunohistochemistry determination of ZnT3 expression in TG mice brain. The 20-week-old TG mice display weak ZnT3-immunoreactivities in the brain compared to age-matched WT mice. A, B, C and D are hippocampus of WT mice, striatum of WT mice, hippocampus of TG mice, and striatum of TG mice, respectively. DG: dentate gyrus; ml: molecular layer. Scale bar: 500 μm .

Figure 5

ZnT3 expression level in TG mice brain. ZnT3 protein expression and mRNA level decrease in cortex, striatum and hippocampus of TG mice aged from 14 to 20 weeks compared to age-matched WT mice. A1 and A2 are Western blots analysis of ZnT3 expression and the quantitative representation of ZnT3 band intensity normalized to GAPDH, respectively. B1 and B2 are RT-PCR analysis of ZnT3 mRNA level and the relative amounts of ZnT3 mRNA normalized to β-actin, respectively. Hip: hippocampus. n=4. *, p < 0.05 compared to WT mice.

Figure 6

ZnT3 expression level in 160Q BHK cells. ZnT3 protein expression and mRNA level markedly decrease in 160Q BHK cells at different times after transfection compared to 20Q BHK cells. A1 and A2 are Western blots analysis of ZnT3 expression and the quantitative representation of ZnT3 band intensity normalized to GAPDH, respectively. B1 and B2 are RT-PCR analysis of ZnT3 mRNA level and the relative amounts of ZnT3 mRNA normalized to β-actin, respectively. n=4. *, p < 0.05 compared to 20Q BHK cells.

Figure 7

Analysis of ZnT3 gene transcriptional activity. Sp1 activates ZnT3 transcription by binding to GC boxes in ZnT3 gene promoter. A is the scheme chart of the mouse ZnT3 gene promoter sequence. Potential Sp1 binding sites (GC-1 and GC-2) are underlined. B shows Dual-luciferase (Luc) report gene assay of the targeting relationship of Sp1 and ZnT3. $n=3$ *, $p < 0.05$ compared to BHK cells transfected with empty pEBGN vector. C1-C3 show ChIP-RT-qPCR analysis of Sp1 binding to ZnT3 promoter. The input lane represents 0.01% of total chromatin used in ChIP. The relative amounts of ZnT3 promoter were normalized to input DNA. GC-1: ZnT3 promoter -338 ~ -232; GC-2: ZnT3 promoter -253 ~ -112. $n=3$ *, $p < 0.05$ compared to control IgG.

Figure 8

ZnT3 expression level in BHK cells overexpressing Sp1. ZnT3 protein expression and mRNA level greatly increase in BHK cells overexpressing Sp1. A1 and A2 are RT-PCR analysis of ZnT3 mRNA level and the relative amounts of ZnT3 mRNA normalized to β-actin, respectively. B1 and B2 are Western blots analysis of ZnT3 expression and the quantitative representation of ZnT3 band intensity normalized to GAPDH, respectively. Con: control. n=4. *, p < 0.05 compared to BHK cells transfected with empty vector.

Figure 9

ZnT3 expression level in BHK cells transfected with Sp1 siRNA. ZnT3 protein expression and mRNA level decrease in BHK cells transfected with Sp1 siRNA. A1 and A2 are Western blots analysis of ZnT3 expression and the quantitative representation of ZnT3 band intensity normalized to GAPDH, respectively. B1 and B2 are RT-PCR analysis of ZnT3 mRNA level and the relative amounts of ZnT3 mRNA normalized to β-actin, respectively. n=4. *, p < 0.05 compared to BHK cells transfected with control siRNA vector.

Figure 10

ZnT3 expression level in 160Q BHK cells overexpressing Sp1. Forced overexpressed Sp1 reverses the suppression of ZnT3 mRNA by mHtt in BHK cells. A and B are RT-PCR analysis of ZnT3 mRNA level and the relative amounts of ZnT3 mRNA normalized to β-actin, respectively. n=3. *, p < 0.05 compared to 20Q cells. #, p < 0.05 compared to 160Q cells. C and D are dual-luciferase report gene assay of ZnT3 promoter transcriptional activity in 160Q BHK cells and Sp1-overexpressed 160Q BHK cells transfected with reporter plasmid pGL3-ZnT3 (-283 ~ + 10). The promoter activity of ZnT3 is down-regulated in BHK cells transfected with different concentrations of 160Q Htt vector. Sp1 overexpression up-regulates ZnT3 promoter transcriptional activity in 160Q BHK cells. n=3, *, p < 0.05 compared to 20Q cells.

Figure 11

ChIP assays of Sp1 binding to ZnT3 promoter in 160Q BHK cells. mHtt inhibits the binding of Sp1 to ZnT3 promoter containing GC-1 box or GC-2 box. A and B display ChIP-qRT-PCR analysis of Sp1 binding to ZnT3 promoter containing GC-1 box or GC-2 box, respectively. The relative amounts of ZnT3 were normalized to input DNA. n=3. *, $p < 0.05$ compared to BHK cells transfected with 20Q Htt.

Figure 12

The mechanism of ZnT3 down-regulation and its effect on vesicular zinc in HD. B shows inhibition of the binding of transcription factor Sp1 to ZnT3 gene promoter by mHtt in HD. Down-regulated ZnT3 expression reduces the transport of zinc into synaptic vesicles in the axonal terminal. Zinc dyshomeostasis contributes to synaptic dysfunction. Instead, A shows the relationships between Sp1, ZnT3 and vesicular zinc in neuron. Sp1 binds to ZnT3 gene promoter and up-regulates its expression. Vesicular zinc is dependent on ZnT3 on the membrane of synaptic vesicles. SV, synaptic vesicle.

Supplementary Files

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