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RelA/p65 Blocks Histone Deacetylase-3 Neurotoxicity and Protects Neurons Against Neuronal Death Induced by PolyQ-Expanded Huntingtin and Ataxin-1 in a p65 Phospho S276 Dependent Manner

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Advisor: Dr. Jonathan E. Ploski

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Neurodegenerative diseases have a large negative impact to human society. Symptoms of neurodegenerative diseases includes memory loss, impaired recognition, motor disfunction due to dysregulated neuronal loss in different brain regions. However, the neurobiological basis of these brain diseases is not fully understood and there are no cures or effective treatments. Polyglutamine (Poly-Q) disorders is a class of neurodegenerative diseases that are caused by polyglutamine expansion within the protein coding regions of specific genes. Huntington's disease (HD), Spinal Cerebellar Ataxia Type 1 (SCA1) and Spinal Cerebellar Ataxia Type 3 (SCA3) are three common diseases among Poly-Q disorders. To better understand the neurobiology of these disorders and further to develop a potential treatment, I focused my dissertation research on p65 which was reported by our laboratory to prevent low-potassium (LK) condition induced program cell death in cerebellar granule neurons (CGN). I discovered that p65 was protective against toxicities in disease models of these disorders. p65 was also showed to prevent neuronal death under oxidative stress in cortical neurons. The protective effect of p65 depended on the Serine at aa276. Mutating p65 S276 to a non-phosphorylatable Alanine (S276A) abrogated the protection of p65 in models

of Huntington's disease and conditions of LK; however, mutating to phosphomimetic Aspartate (S276D) completely protected neurons.

TABLE OF CONTENTS

LIST OF FIGURES

This is dedicated to my parents Yunli Zhang and Yushang Li

CHAPTER 1

INTRODUCTION

Neurodegenerative diseases are a major health, social and economic problem. A defining feature of these disorders is the abnormal neuronal loss in specific brain regions. However, the mechanisms underlying these devastating diseases are not known, and there are no effective treatments or cures for them. It is widely believed that the abundant neuronal loss in neurodegenerative disease is due to apoptosis, but deregulated autophagy and necroptosis might also contribute. One class of neurodegenerative disorders are the CAG repeat disorders. These are inherited disorders in which an expansion in a CAG repeat leads to the production of a mutant protein with an expanded polyglutamine stretch. For my dissertation research, I focused on a protective molecule that protects in those diseases. By studying the mechanism that the molecule protects, I was hoping to discover which process in the cell those disorders may affect and furthermore, may benefit the development of potential treatments. p65, a subunit of NF-κB complex, was reported by our laboratory to prevent LK induced programmed cell death in CGNs[1]. To have a better idea of p65 mediated protection, I transfected p65 in models of Huntington's disease, SCA1, SCA3 and homocysteic acid (HCA) induced cell death model. HCA induces oxidative stress in neurons[2]. I discovered p65 protected against all those neuronal toxic conditions. Then I studied potential mechanisms p65 offers neuro- protection via three avenues. 1) protein-protein interaction; 2) post-translational modifications and 3) activation of NF-κB. I

discovered that Lys218/221 acetylation and Ser276 phosphorylation are necessary for p65 to protect neurons.

A large number of molecules have been found to contribute to cell loss in neurodegenerative diseases and do so to varying extents. Among these are the NF-κB transcription factor and the histone deacetylases, a class of enzymes that deacetylate histones and many other non-histone proteins. Provided below are descriptions of the role of NF-κB and HDACs focusing on their effects on the regulation of neurodegeneration.

1.1.Huntington's disease

Huntington's disease is a rare neurodegenerative disorder caused by CAG expansion in the Huntingtin (Htt) gene. In unaffected individuals, the repeat number of polyQ is no more than 35 whereas HD patients usually have over 39 polyQ repeats. There is a strong inverse correlation between the number of CAG repeats and the patient's age at onset[3]. The polyQ expansion gives the mut-Huntingtin (mut-Htt) protein a toxic gain of function. Htt is a very large protein with 3144 amino acids (~350 kDa) and is widely expressed in the brain and outside of it which plays an essential role in development. The full-length mut-Htt protein is initially cytosolic. Upon cleavage by caspase 6 at amino acid 586, the N-terminus fragment of mut-Htt has a preference to translocate into the nucleus forming aggregates and causing neuronal death[4],[5]. Since the 586 amino acid fragment is much smaller and easier to study, we and many other labs use this fragment of mut-Htt to study the pathology of HD. Aggregation is one of the main histopathological hallmarks of HD. Aggregates of mut-Htt has been found in transgenic mouse model and human brain of HD[6].

Although initially thought to be toxic, it is now believed that large aggregates of mut-Htt serve to sequester soluble mut-Htt which might be the toxic form of the protein. It has been shown that nuclear targeting of mut-Htt not only increases its toxicity in neuronal cell lines[7] but also produces HD-like phenotypes in transgenic mice[8], [9]. Studies found that the first 17-amino-acid domain (N17) acts as a nuclear export sequence(NES)[10]. Conservation of four amino acids within N17 domain is critical to its cytoplasmic localization[10]. And adding an extra NES on the mut-Htt suppresses neuronal cell death[11]. There are two phosphorylation sites within the N17 domain and both of them reduce mut-Htt induced toxicity[12]. It has been reported that IκB kinase (IKK) phosphorylates Htt at Ser13 and 16 within the N17 domain. Mimicking phosphorylation at Ser13 and 16 of mut-Htt reduce its toxicity[13]. Although the normal functions of Htt is not clear, it is believed that Htt is involved in transcription, vesicle trafficking and cell survival. There is a proline-rich domain located right after the polyQ domain of Htt, suggesting that Htt protein may act as a transcription factor[14]. Htt also interacts with a large number of proteins. It is believed that the polyQ expansion impacts these interactions, which then would affect the important functions of normal Htt. For example, while wt-Htt interacts efficiently with the transcriptional co-regulator, CBP, to regulate transcription, mut-Htt interacts less efficiently. Similarly, a reduction in interaction of mut-Htt with HIP1 is thought to impair axonal transport. Htt was reported to interact with NF-κB subunit p65, p50 and p53, but not with p105[15]. Edoardo et.al found that Htt's role of transporting NF-κB from the synapse to the nucleus is impaired in Huntington's disease. Also, it has been reported that Htt could regulate transcription of many neuronal genes including the well-known neurotrophin brain-derived neurotrophic factor (BDNF) [16], [17]. Transcriptional dysregulation of many genes has been found in HD[18], [19]. Zuccato et al fully described reduction of BDNF transcription in HD cells and in animal models[16]. Htt

can also function as a scaffolding protein transporting transcription factors along microtubules. In the case of HD, scientists believe that the normal function of Htt is impaired by the polyQ expansion, leading to dysregulation of homeostasis in the cell.

1.2.Spinocerebellar ataxia type 1

Spinocerebellar ataxia type 1 (SCA1) is another polyglutamine expansion disorder that specifically affects cerebellar neurons. SCA1 is caused by polyglutamine expansion within the coding region of the gene Ataxin1 (Atx1). Although Atx1 is ubiquitously expressed in the brain, purkinje cells in the cerebellum are primarily damaged in the disease. Symptoms of spinocerebellar ataxia type 1 include motor deficits, cognitive impairment and depression[20]. Atx1 is shown to be important in neural progenitor cell proliferation[21], [22]. Similar to what occurs in HD, SCA1 pathology includes abnormalities in transcription and impaired RNA metabolism[23]. Nuclear inclusion is a common feature in neurons of SCA1. Furthermore, it has been demonstrated that the mut-Atx1 has a nuclear distribution that differs from that of wt-Atx1. Transfection of Atx1 in cell lines showed that wt-Atx1 formed small aggregates while mut-Atx1 formed fewer but bigger aggregates in the nuclei of most of the cells[24]. The functional self-association domain of Atx1 is essential to its aggregation. Deletion of this region results in a protein that does not form inclusion bodies and has reduced SUMOylation[25]. As with mut-Htt, the aggregation of Atx1 into inclusions is thought to be a protective mechanism against the toxic oligomeric form of the protein. Posttranslational modifications like phosphorylation and SUMOylation has been reported in Atx1[25]. Large SUMO-positive aggregates have been found in SCA1 transgenic mice. Atx1 SUMOylation was mapped to at least five lysine residues: Lys16, Lys194, Lys 610, Lys697 and

Lys746. Increased polyglutamine expansion decreases SUMOylation level of Atx1. Phosphorylation of Atx1 at Ser776 also affects its SUMOylation[25]. Studies suggested that SUMO modification may affect Atx1 nuclear trafficking efficiency[26]. There are at least 5 phosphorylation sites of Atx1 reported in mice[27]. Among them, Ser239 and Ser776 were suggested to play an important role in SCA1 pathology. Ser239 phosphorylation exacerbates the toxicity of polyglutamine expanded Atx1 in a *Drosophila* model. Phosphorylation of Ser776 was suggested to stabilize Atx1[28]. Animals with a phospho-mimicking Asp776 exhibited a more severe disease phenotype than normal SCA1 mice due to a stabilized mut-Atx1[29]. One aspect of SCA1 pathogenesis is that mut-Atx1 has an exaggerated ability to repress gene transcription with HDAC3. Both wt- and mut-Atx1 were reported to interact with HDAC3 and HDAC3 was redistributed to nuclear inclusions when HDAC3 was co-expressed with either forms of Atx1[30]. A proteome-scale map study indicated that Atx1 interacts with c-Rel[31]; however, the function of this interaction or interactions of Atx1 with other NF-κB subunits still needs to be explored.

1.3.HDAC3

Transcription dysregulation has been found in many disorders. Histone deacetylases (HDACs) play an important role in the process of gene transcription regulation. Several studies demonstrated that pharmacological inhibition of histone deacetylases have protective effect in different animal models of neurodegenerative disease[32], [33]. Our lab previously found that HDAC3 was neurotoxic[34]. The neurotoxic effect of HDAC3 requires it to be phosphorylated by GSK3β and its ability to interact with HDAC1. Higher HDAC1 and HDAC3 interactions are associated with conditions of neurodegeneration both *in vitro* and *in vivo.* It was also reported that

Htt interacts with HDAC3, and this interaction is associated with a reduction in apoptosis which is consistent with Htt's ability to provide neuroprotection by sequestering HDAC3. It was also reported that mut-Htt possesses a much weaker interaction with HDAC3 than wt-Htt[35]. These results indicate that HDAC3 plays a role in HD induced neuronal death. A previous publication from our laboratory hypothesized and provided evidence that under normal condition, Htt sequesters HDAC3 in the cytosol[36]. However, in the HD condition, impaired interaction between mut-Htt and HDAC3 leads to neurotoxicity caused by transcriptional alterations involving genes regulated by the HDAC1-HDAC3 complex[35]. However, it currently remains unknown what genes are regulated by the HDAC1-HDAC3 complex.

1.4.NF-κB

Another protein implicated in neuronal death is NF-κB. Five different NF-κB subunits have been identified including p65/RelA, p50(cleaved from p105), RelB, c-Rel and p52(cleaved from p100). Inactivated forms of NF-κB dimers are sequestered in the cytoplasm by the inhibitor IκB which masks the DNA binding domain and the nuclear localization domain of NF-κB. Activation of NF-κB in the nervous system can be caused by cytokines (TNF-alpha), chemokines and stress. NF-κB can also be activated by neurotrophins like NGF[37] and neurotransmitters like glutamate[38]. Upon stimulation, IκB is phosphorylated by IκB kinase(IKK) complex for ubiquitination and subsequent proteasomal degradation[39]. Released NF-κB complex translocates to the nucleus and binds to κB sites on promoters, activating transcription of target genes. The most common NF-κB subunits found in the nervous system are p65 and p50[40]. However, c-Rel containing complexes are also detected in the central nervous system[41], [42].

Constitutive NF-κB activity has been detected in central nervous system[43] indicating NF-κB plays an essential role in neurons. Scientists generally believe that NF-κB has dual functions in neurons: it is a transcription regulator and also functions as a signal transducer to transmit signals from the synapse to the nucleus[44]. Both pro- and anti-apoptotic effects of NF-κB has been previously described[40], [45], [46]. Bhakar et al. found that NF-κB activation protected neurons from damage and induced anti-apoptotic genes including Bcl-xL, IAP1 and IAP2[47]. Sarnico et al. suggested that the pro- and anti-apoptotic effects of NF-κB were likely to be due to activation of dimers of different subunits by demonstrating that activation of c-Rel containing dimers increased neuron resistance to ischemia[41]. NF-κB could also induce production of reactive oxygen species and excitotoxins in glia cells[48]–[50]. NF-κB pathway dysregulation has been found in human HD myeloid cells[51]. Levels of many cytokines such as IL-1β and TNF-α is lower in HD human tissue and R6/2 HD mice[52]. Although many people are attempting to explain the role of NF-κB in neurodegenerative disease, there remains much about NF-κB that needs to be investigated. Our laboratory discovered that survival of cerebellar granule neuron(CGN) was blocked by inhibitors of NF-κB and overexpression of IκB[53]. These results, were consistent with findings from other laboratories[43], [47], indicating that NF-κB activation is essential for neuronal survival. However, surprisingly, neither the level of endogenous NF-κB subunits nor that of IκB are altered under apoptotic inducing stimuli [53]. And some laboratories have described pro-apoptotic effects of NF-κB in neurons[54], [55].

Since p65/p50 is the most common dimer in neurons, p65 has drawn a lot of attention from scientists. Our lab has published that overexpression of p65 could protect neurons from apoptosis induced by K^+ deprivation in CGN[53]. However, no change was discernible in total cellular,

nuclear or cytoplasmic p65-immunoreactivity following K^+ deprivation. Interestingly, EMSA results showed that DNA-binding activity of p65 was altered in CGNs primed to die indicating p65 may mediate apoptosis through transcription regulation. Posttranslational modification of p65 contributes an essential role on NF-κB activation and function. There are several acetylation and phosphorylation sites on p65. Most acetylation of p65 facilitate its removal from κB-DNA[56] and p300 plays a central role on p65 acetylation. It was shown that p65 is acetylated on Lys122,123 by p300 and PCAF and deacetylated by HDAC3 and the acetylation of both sites are important for p65's ability to be released from κB-DNA.[56]. Rothgiesser et al also demonstrated that p300 acetylates p65 at Lys310,314,315 and acetylation of Lys314 was important to p65 chromatin-association[57]. Conversely, acetylation of Lys221 significantly increased affinity of p65 binding to DNA[58]. It has also been reported that acetylation of p65 could be regulated by its phosphorylation[59]. Phosphorylation of p65 on Ser276 or Ser534 increased assembly of phospho-p65 with p300, which enhanced acetylation on Lys310[59]. Many scientists support the idea that after translocation into the nucleus, p65 will be phosphorylated to enable at least in part its ability to induce transcription activation. Phosphorylation at Ser468 and Ser276 of p65 has been reported to be important for its transactivation ability[60], [61]. Phosphorylation at Ser529 by casein kinase II (CKII) has the same effect on NF-κB transactivation[62], [63]. It has been reported that both IKKs directly phosphorylate the transactivation domain of p65 at Ser534[62]. And Ser534 phosphorylation is necessary for its nuclear translocation and transactivation of anti-apoptotic genes[64]–[66]. Another important site of phosphorylation in p65 is Ser311. Inactivating mutations of Ser311 in mouse fibroblasts severely impaired p65 transcription, abrogated interaction of p65 with its coactivator and blocked its anti-apoptotic function[67]. Interaction of p65 with HDAC3 has been reported by many labs[68]–[70].

CHAPTER 2

MATERIALS AND METHODS

2.1.Materials

Unless specified, all cell culture reagents were purchased from Life Technologies (Carlsbad, CA) and all chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). Poly-L-Lysine for coating neuronal culture plates was purchased from Trevigen. Antibodies used in these studies are listed as follows: GFP (SC-9996a, SC-8334; Santa Cruz Biotechnology), Flag (F1804; Sigma-Aldrich), HA (OASG03275; Aviva Systems Biology), V5(13202S; Cell signaling Technology), Myc (2276, 9B11; Cell signaling Technology), IgG (SC-69786; Santa Cruz Biotechnology), IgG(2729S; Cell signaling Technology) NFκB p65 (SC-372, SC-8008; Santa Cruz Biotechnology, 6956S, 8242S; Cell signaling Technology), HDAC3 (OAAB09604; Aviva Systems Biology). For western blotting, primary antibody was diluted 1:1000 in 5% bovine serum albumin (BP1600; fisher scientific). Peroxidase-conjugated secondary antibodies were goat-anti-rabbit IgG and goat-anti-mouse IgG (Pierce). For immunocytochemistry, primary antibodies were diluted from 1:100 to 1:200. Secondary antibodies were Alexa Fluor 488(115-545-146), Dylight 594 (115-585-146) from Jackson ImmunoResearch Laboratories. Agarose beads for co-immunoprecipitation were purchased from Santa Cruz Biotechnology (SC-2003). The IKK inhibitors BMS-345541(B9935) and IKK-16 (SML1138) were purchased form Sigma Aldrich. Inhibitors of Akt/PI3K pathway wortmannin (9951S) and LY294002 (9901S) were purchased from Cell signaling Technology.

2.2.Plasmids

pEBB-CMV-HA p65, pEBB-CMV-HA p65-K5R/Q were gifts from Dr Michael Kracht, Mudolf-Buchheim-lnstitute of Pharmakologie. RelA RHD cFlag pcDNA3 was a gift from Stephen Smale (RRID:Addgene 20015). GFP-RelA (Addgene plasmid # 23255), T7-RelA(K310R) (Addgene plasmid # 23250), T7-RelA(KR) (Addgene plasmid # 23251), T7-RelA(S276A) (Addgene plasmid # 24153) were gifts from Warner Greene. pRP[Exp]-EF1A-V5-mRela (Vector # : VB180525-1214ybg), pRP[Exp]-EF1A-V5-mRela S276D (Vector # : VB180531-1054esr), pRP[Exp]-EF1A-V5-mRela S276A (Vector # : VB180531-1053pwu), pRP[Exp]-EF1A-V5-mRela K310R (Vector # : VB180531-1057skh), pRP[Exp]-EF1A-V5-mRela K310Q (Vector # : VB180601-1165qgu) were purchased from VectorBuilder. pcDNA3-myc-Ataxin3Q28 and pcDNA3-myc-Ataxin3Q84 were gifts from Henry Paulson (Addgene plasmid # 22125 and #22124 respectively). HDAC3 Flag was a gift from Eric Verdin (Addgene plasmid # 13819). Atx1-wt 30Q and Atx1-mut 82Q were gifts from Chih-Cheng Tsai. pCMV2- TLE1-flag was a gift from Dr. Michael G Rosenfeld, Howard Hughes medical Inst.

2.3.Primary cortical neuronal culture treatment and transfection

Cortical neurons were cultured from E17-18 Wistar rat embryos and plated in Neurobasal medium (A3582901) with B27 supplement (17504044). Neurons were transfected 5 days after plating using the calcium phosphate method. The neurons were subjected to apoptotic stimuli by adding 1 mM homocysteic acid (HCA). Inhibitors were added immediately after transfection. The proportion of cells undergoing apoptosis was quantified by DAPI staining 24 hours later. Viability

of successfully transfected cells was normalized to number of cells successfully transfected with GFP encoding transgene as described previously[71]. Transfections for each experiment were performed in duplicates and the experiments were repeated at least three times.

2.4.Primary cerebellar granule neuron (CGN) culture treatment and transfection

CGNs were cultured from P7-9 Wistar rat pups and plated in Eagle's basal medium containing 10% FBS, 25 mM KCl, 2 mM glutamine and 0.2 % gentamicin as described previously[72]. Antimitotic agent cytosine arabinofuranoside was added 18-22 hours after plating at a concentration of 10 µ. Neurons were transfected 5 days after plating using the calcium phosphate method. Twenty-four hours after transfection, cultures were switched to serum-free BME containing glutamine and gentamycin with 29 mM KCl (HK medium) or 4 mM KCl (LK medium). Inhibitors were added with HK/LK medium. The proportion of cells undergoing apoptosis was quantified under fluorescent microscope 24 hours later. DAPI staining was used distinguish dead cells from live cells. Nuclei with condensed morphology and bright DAPI staining was consider as a dead cell; nuclei with a spread and dark DAPI staining was counted as a live cell. Viability of successfully transfected cells was normalized to number of cells successfully transfected with GFP as described previously[71]. Transfections for each experiment were performed in duplicates and the experiments were repeated at least three times.

2.5.Cell line culture and transfection

Cell lines HEK293T (CRL-11268, RRID: CVCL_1926), N2A (CCL-131, RRID: CVCL_0470) and HT22 (SCC129, CVCL_0321) were obtained from ATCC. HEK293T and N2A

were cultured in DMEM supplemented with 10 % FBS and HT22 was cultured in High Glucose DMEM supplemented with 10 % FBS. Cell lines were transfected in Opti-MEM medium using EndoFectin Max (EF013, GeneCopoeia) according to manufacturer's instructions. Transfection efficiency was about 70-80 %.

2.6.Western blot analysis and immunoprecipitation

Cell lysates were prepared using 1 x cell lysis buffer (Cell Lysis Buffer 10 X #9803, Cell signaling Technology), 50 µ of protein was used per lane and transferred from SDS-PAGE gel to PVDF membrane. After transfer, PVDF membrane was blocked with 5 % milk in TBST at room temperature for 1 hour followed by 3 times TBST washes. Primary antibody incubation was in 4 degree overnight followed by 3 times TBST washes. Secondary antibody was used at 1:10,000 dilution in 5 % milk at room temperature for 1 hour followed by 3 times TBST washes. Immuno-reactive signal was detected by Clarity Western ECL Substrates (1705060, Bio-Rad) according to manufacturer's instructions. For immunoprecipitation, protein A/G agarose beads were pre-incubated with IgG or antibodies then with cell lysates at 4 degree overnight. After washing beads 3 times with 1 x cell lysate buffer, 3 x Laemmli sample buffer was added and boiled at 98 degree for 5 min. Supernatants was subjected to western blot analysis.

CHAPTER 3

RESULTS

3.1. Increasing p65 levels protects against neurotoxicity induced by polyQ-expanded huntingtin, ataxin-1 and ataxin-3.

Our laboratory previously found that NF-κB subunit p65 protects CGN against LK induced apoptosis. To further study this protective molecule, I first examined if p65 could confer protection in other cell death and disease models. Our laboratory has previously described that expression of mut-Huntingtin (mut-Htt) in primary cortical neurons causes ~40 % cell death in 24 hrs[71]. I first overexpressed p65 in this Huntington's disease model and found the toxicity is completely blocked by co-expression of p65 (Fig. 1A). Since oxidative stress is one of the causes of neuronal death in HD and many other neurodegenerative disorders, I further examined the protective ability of p65 in an oxidative stress model in neurons. I overexpressed p65 in cortical neurons treated with homocysteic acid (HCA) which induces oxidative stress. I discovered that HCA induced neuronal death was also abrogated by p65 (Fig. 1F). To further determine if p65 protects exclusively in this Huntington's disease model or if it is also neuroprotective in other neurodegenerative disease models, I also examined if p65 is neuroprotective in two other polyQ disease models. I found that both mutant ataxin-1(Fig. 1C) and mutant ataxin-3(Fig. 1E) toxicity were blocked by co-expression of p65 in CGNs. Interestingly, ataxin-1 does not co-localize with p65(Fig. 1D).

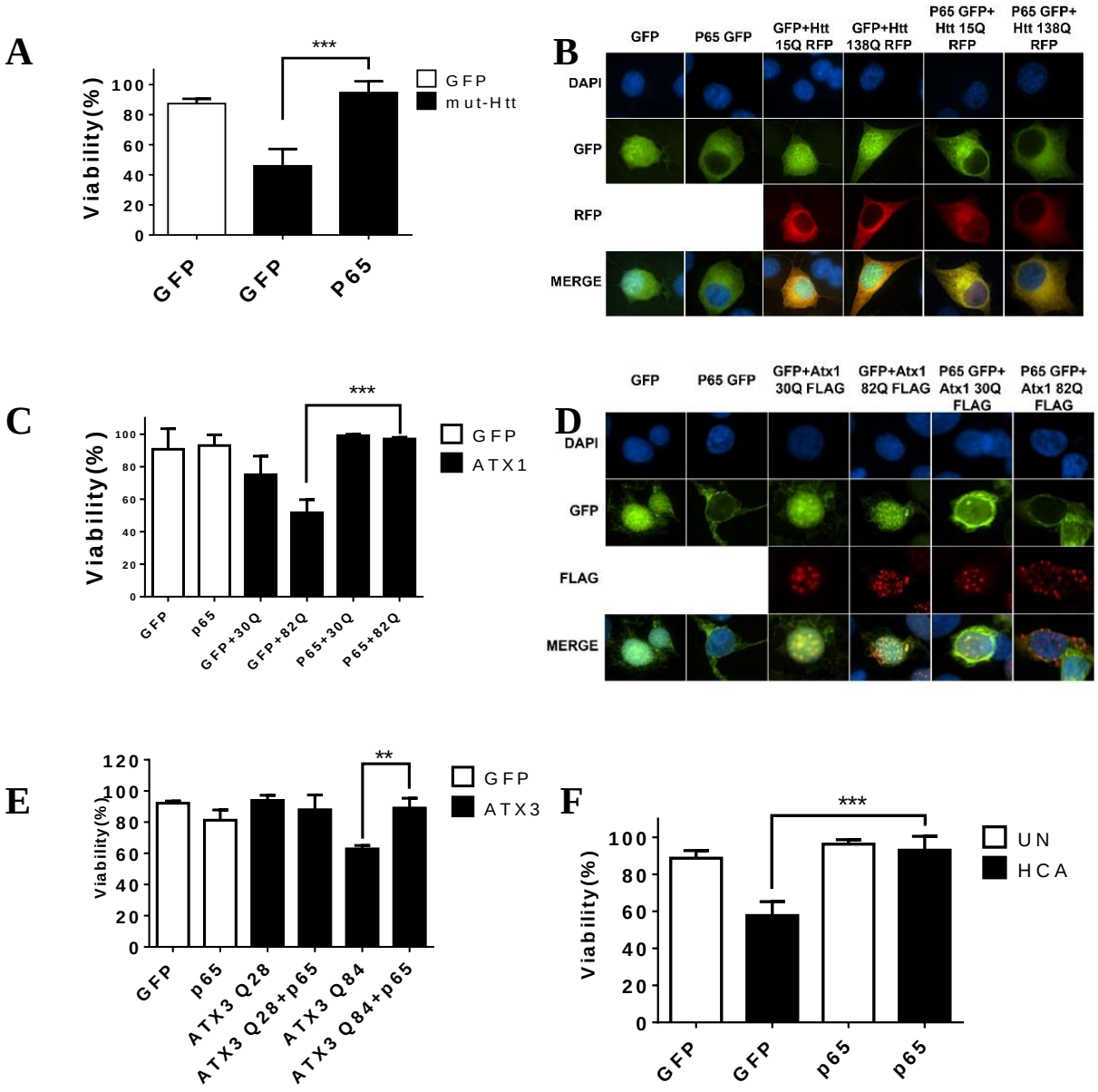


Figure 1. p65 protects neurons from apoptosis.

A. Cortical neurons were co-transfected with mut-Htt and GFP or p65-HA. Viability of transfected neurons were quantified 24h later by immunocytochemistry and DAPI staining. ***B.*** Neuro2A cell line was transfected with p65-GFP and Htt-RFP or mut-Htt-RFP for 48h. Immunocytochemistry was used to investigate co-localization of proteins. ***C.*** CGNs were co-transfected with ATX1 and p65-HA or GFP. Viability of transfected neurons were quantified 24h later by

immunocytochemistry and DAPI staining. ***D.*** HT22 cell line was transfected with p65-GFP and ATX1-14Q-Flag or ATX1-82Q-Flag for 48h. Immunocytochemistry was used to investigate co-localization of proteins. ***E.*** CGNs were co-transfected with ATX3 and p65-HA or GFP. Viability of transfected neurons were quantified 24h later by immunocytochemistry and DAPI staining. ***F.*** Cortical neurons transfected with GFP or p65-HA were treated with media with or without HCA 24h after transfection. Viability of transfected neurons were quantified 24h later by immunocytochemistry and DAPI staining.

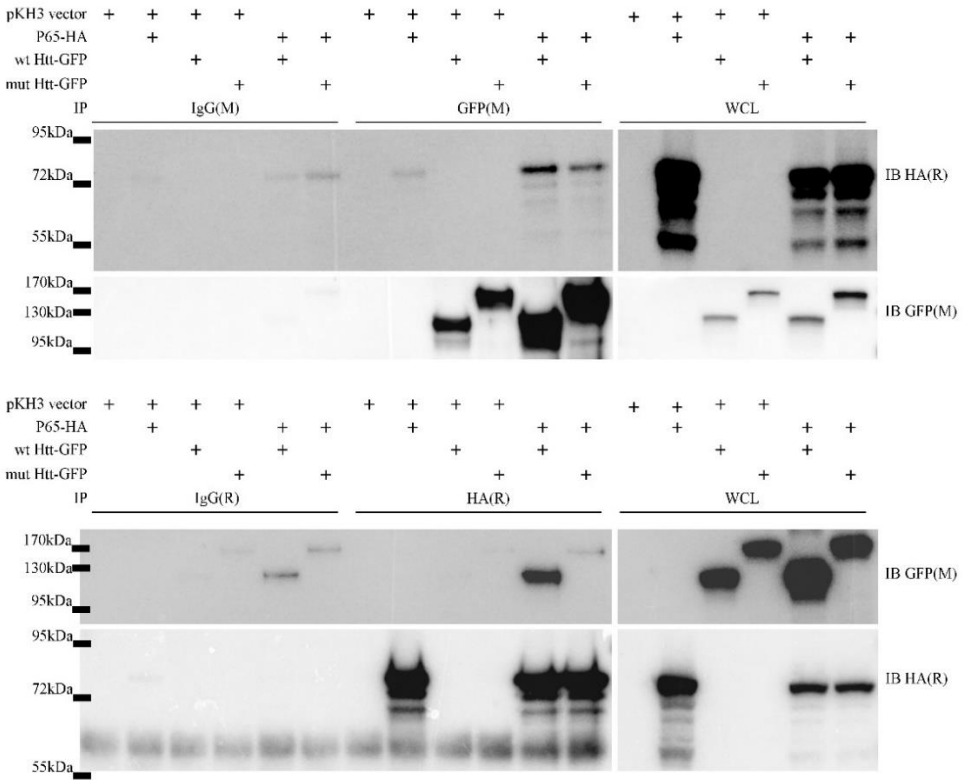
3.2. p65 interacts with the proline-rich domain of Htt.

It has been reported that Htt may normally function to transport p65 from the synapse to the nucleus. This function is impaired in Huntington's disease[73]. So, protein-protein interaction between p65 and Htt may be important during p65 mediated protection against Huntington's disease. I started with overexpressing p65 with Htt in HEK 293T cells followed by immunocytochemistry. I found p65 colocalized with both wt- and mut-Htt (Fig. 1B). This result suggests an interaction may occur. Then I confirmed this possibility with co-immunoprecipitation experiments. I found p65 interacted with both wt- and mut-Htt. Next I attempted to locate the region in Htt/mut-Htt that p65 interacts with. The Htt/mut-Htt contains an N-terminal 17 amino acids (N-17), which have been previously shown to regulate mut-Htt toxicity, followed by the polyQ and two proline rich regions (PRR). To map the region within Htt/mut-Htt that p65 associates with, deletion constructs were co-expressed with p65 (Fig. 2C). Htt construct lacking 1-75 amino acid lost most of its ability to interact with p65 and Htt lacking 47-125 amino acids partially interacts with p65. This suggests the first 1-75 amino acids of Htt are important for interacting with p65. Interestingly, the N-17 region and polyQ tract of wt-Htt both are located in the first 47 amino acids of Htt. The two PRR regions are located at 39-49, 63-78 amino acid region. It is possible that p65 interacts with the two PRRs in Htt. Previous studies found that antibodies and intrabodies binding the PRR of Htt increases its turnover and reduce its toxicity[74], [75].

Although both wild-type and mut-Htt were affected, the rate of turnover was higher for mut-Htt. If the PRRs are truly the region p65 associates with p65, overexpression of p65 may be able to block the PRR of mut-Htt to reduce its toxicity.

Although Atx1 does not have a PRR, I also examined whether p65 interacted with ATX1. In contrast to mut-Htt, p65 didn't colocalize with either Atx1 or mut-Atx1(Fig. 1D) in HEK 293T cells. Co-immunoprecipitation experiments failed to detect interaction with either the normal or polyQ-expanded form of ATX1 (Fig. 2B). This suggests that, at least in the case of mut-ATX1 neurotoxicity, the protective effect of p65 does not require interaction.

A



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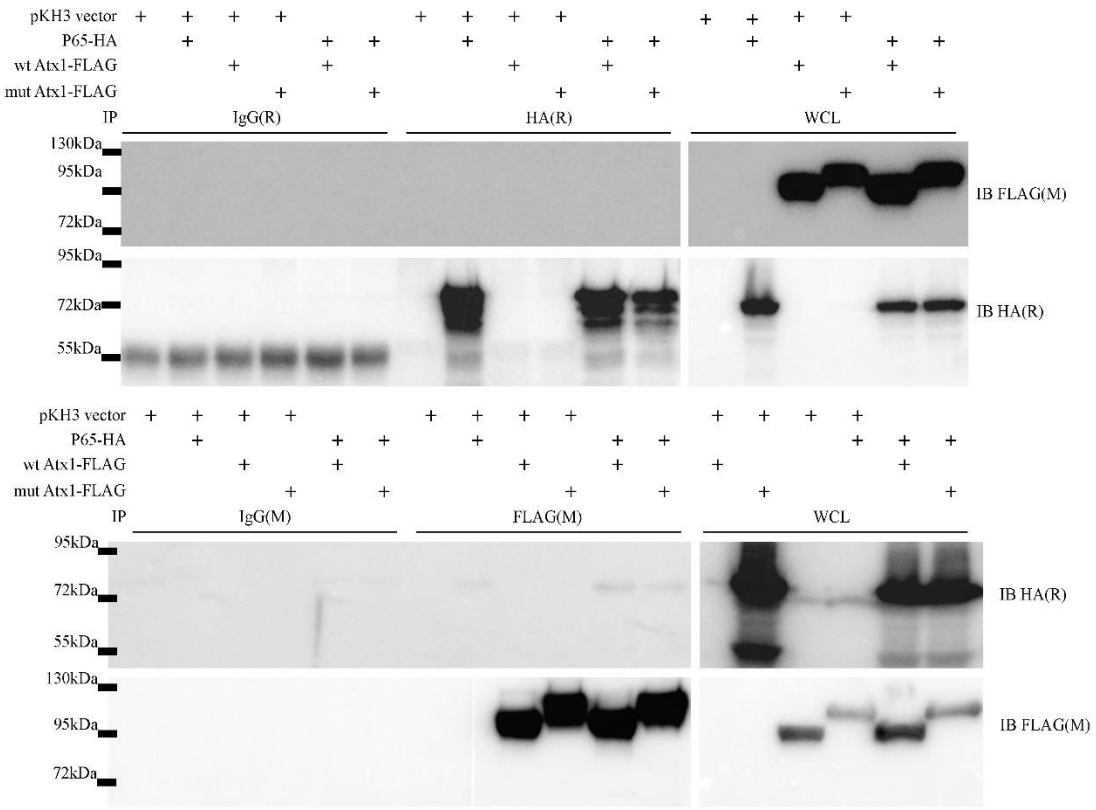
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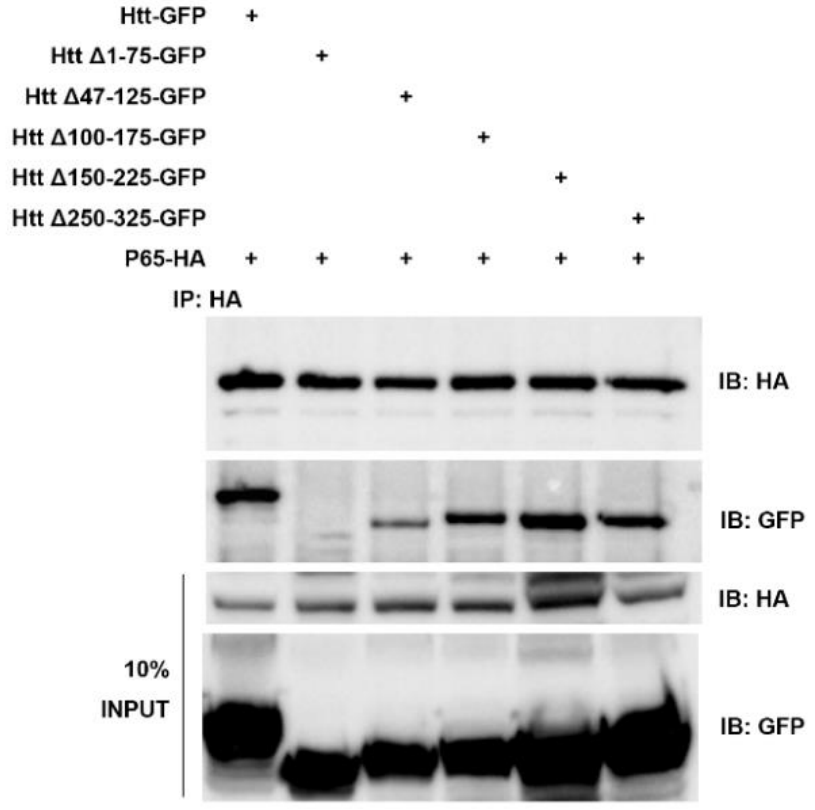
C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Htt Δ100-175-GFP
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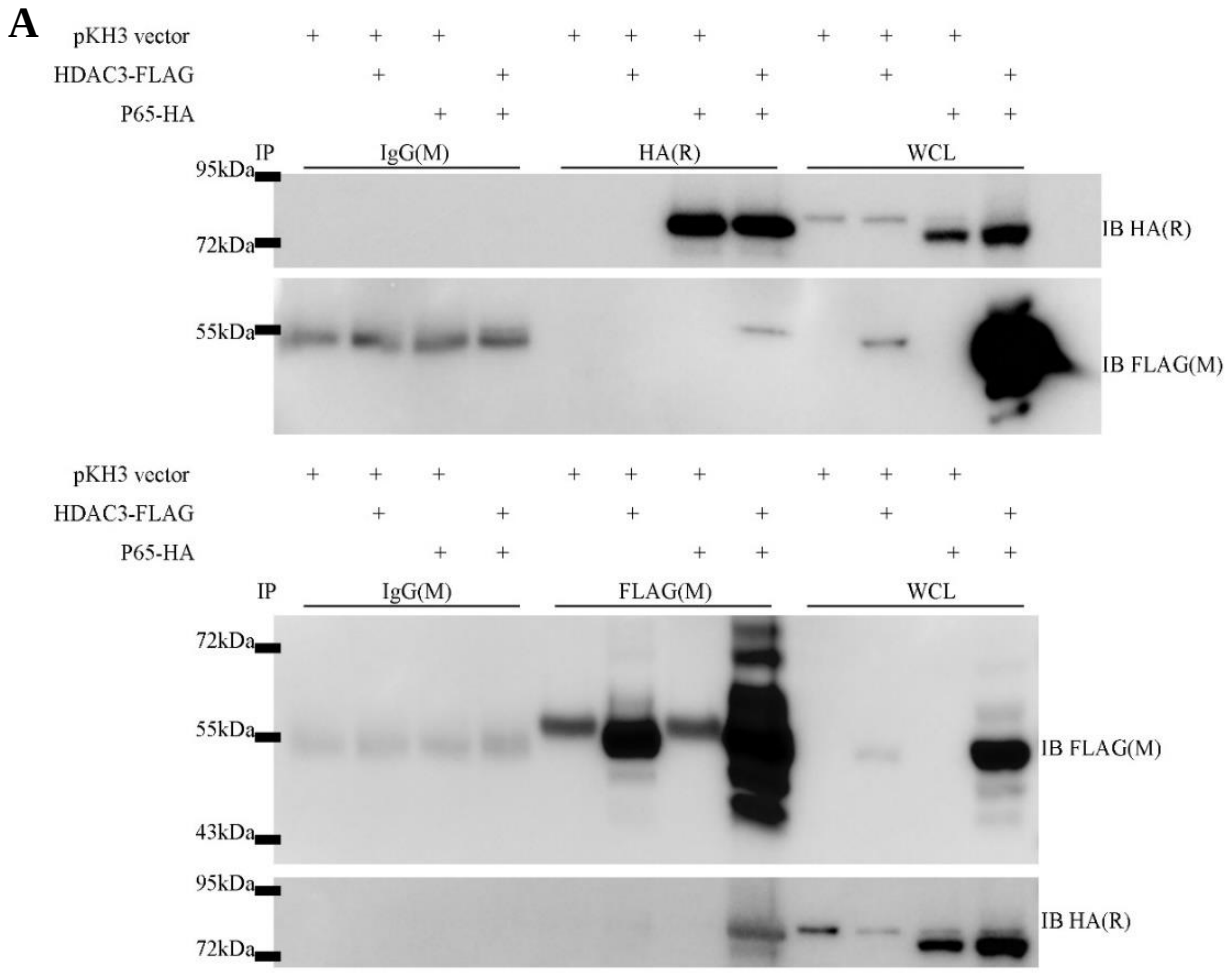
Htt Δ250-325-GFP
P65-HA
IP: HA
IB: HA
IB: GFP
IB: HA
10%
INPUT
IB: GFP

Figure 2. p65 interacted with Htt but not with ataxin1.

A. P65-HA and Htt-GFP or mut-Htt-GFP were co-expressed in HEK293T cell line for 48h. Htt-GFP were immunoprecipitated with GFP antibody. The immunoprecipitate and whole cell lysate were analyzed by Western Blotting using HA and GFP antibody. ***B.*** P65-HA and wt-Atx1-Flag or mut-Atx1-Flag were co-expressed in HEK293T cell line for 48h. Atx1 was immunoprecipitated with Flag antibody. The immunoprecipitate and whole cell lysate were analyzed by Western Blotting using HA, and FLAG antibody. ***C.*** wt-Htt-GFP or deletions of Htt were co-expressed with p65-HA in HEK293T cell line for 48h. P65 was immunoprecipitated with HA antibody. The immunoprecipitates and whole cell lysate were analyzed by Western Blot using GFP and HA antibody.

3.3.p65 interacts with HDAC3 and inhibits its neurotoxic activity.

Accumulating evidence suggests that increased activity of histone deacetylases-3 (HDACs) promotes neurodegeneration. Our laboratory previously found that toxicity by mut-Htt is mediated by HDAC3[36]. Furthermore, HDAC3 binds to and deacetylates p65 at lysines K310, K314 and K315[69]. It is possible therefore that HDAC3 is involved in inhibiting p65-mediated neuroprotection through its deacetylation. As a first step towards testing this possibility I used co-immunoprecipitation analysis to confirm the interaction between p65 and HDAC3. When the two proteins were co-expressed in HEK293 cells, robust interaction was observed (Fig. 3A). However, interaction between endogenous p65 and HDAC3 was not detected in brain tissue (Fig. 3B). I then examined if overexpression of p65 could block the neurotoxic effect of co-expressed HDAC3. As shown in Fig. 3C, I found p65 can also protect HDAC3 induced neuronal death.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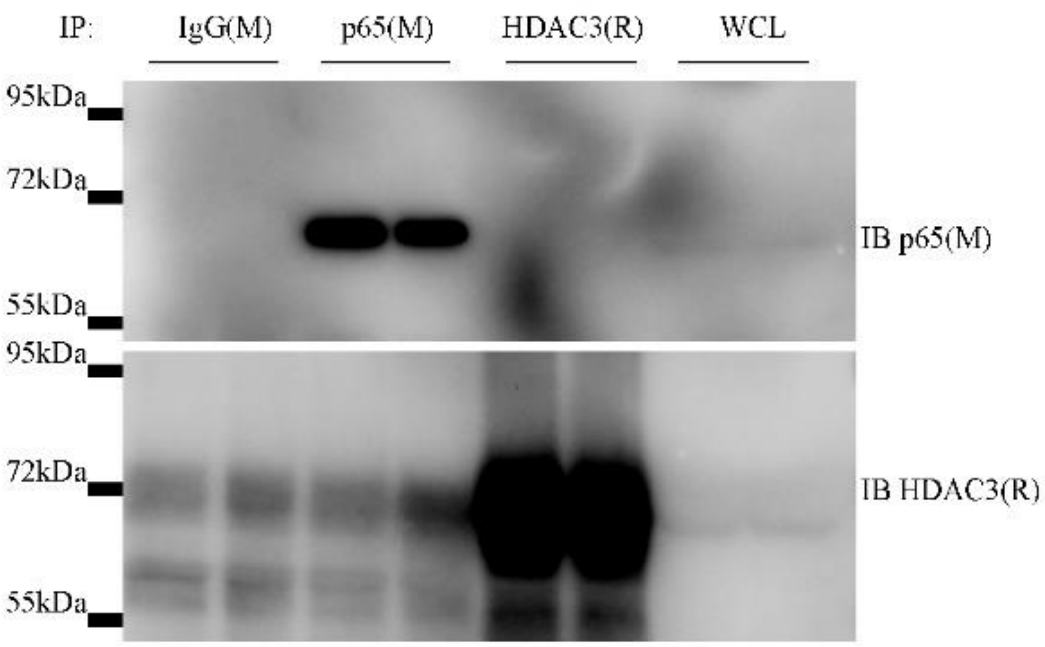
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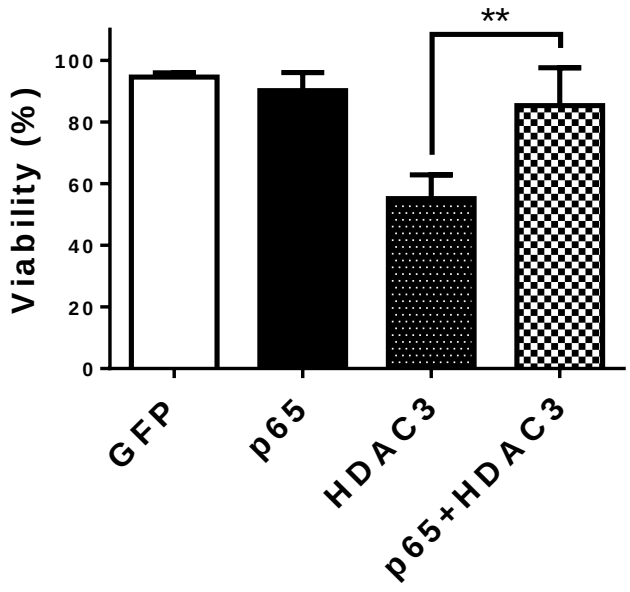
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p65
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p65+HDAC3

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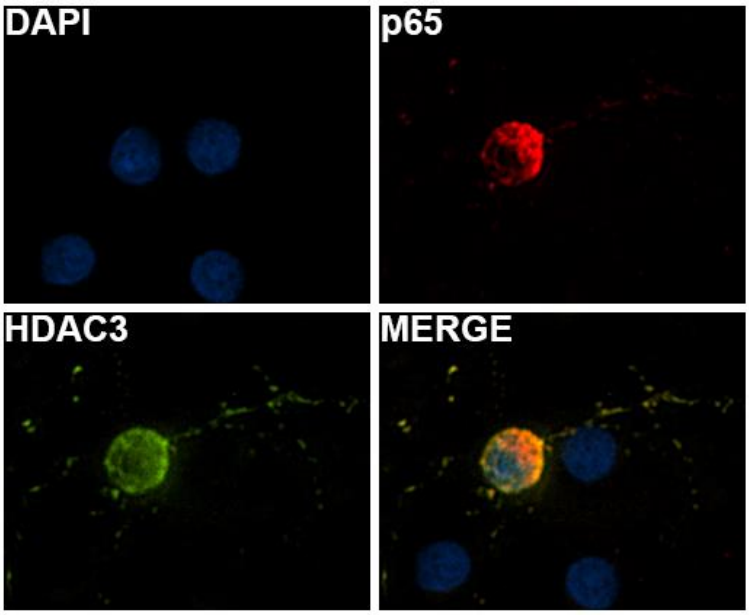


Figure 3. p65 interacted with HDAC3 and inhibited HDAC3 toxicity in CGNs.

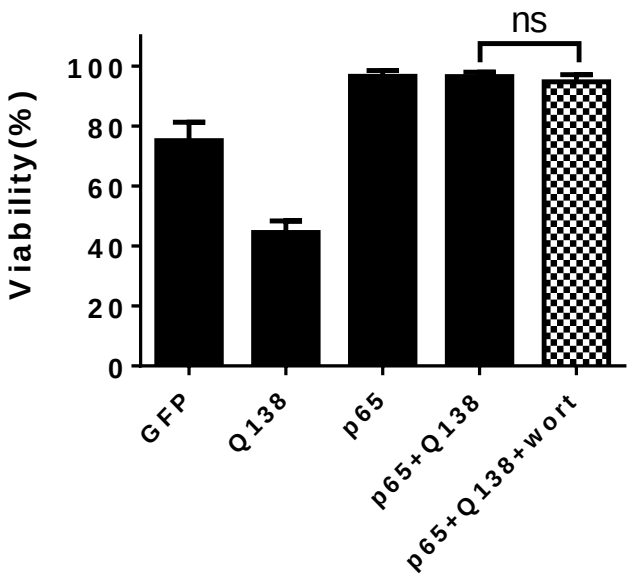
A. HEK293T cell line was overexpressed with HDAC3-FLAG and p65-HA for 48 hours. HDAC3 was immunoprecipitated with FLAG antibody. IgG was used as negative control. Immunoprecipitates were analyzed with HA and FLAG antibody. ***B.*** Protein lysates of rat cortex were collected and immunoprecipitated with HDAC3 and two different p65 antibodies. IgG was used as negative control. Immunoprecipitates were analyzed with p65 and HDAC3 antibody. ***C.*** CGNs were transfected with GFP or co-transfected with GFP or p65-V5 and HDAC3. Neurons were treated with HK media 8 hours after transfection. Cell viability was analyzed after 16 hours. **D.** CGNs were transfected with co-transfected with p65-V5 and HDAC3. Neurons were treated with HK media 8 hours after transfection. Localizations of p65 and HDAC3 were analyzed by immunocytochemistry and DAPI staining.

3.4.p65 mediated protection in neurons is not dependent on the PI3K/Akt pathway.

Activation of PI3K/Akt pathway enhances neuronal survival and slows down progression of neurodegenerative disorders[76]. Phosphorylation of mut-Htt by Akt is essential to its toxicity[77].PI3K/Akt pathway is reported to be affected by progression of Huntington's disease. In the late stage of Huntington's disease, Akt is inactive due to cleavage of Caspase 3[78]. To examine whether p65 mediated protection is related to PI3K/Akt pathway, two PI3K/Akt inhibitor,

wortmannin and LY294002 were tested. However, treatment of both inhibitors failed to stop p65-mediated neuroprotection (Fig. 4).

A



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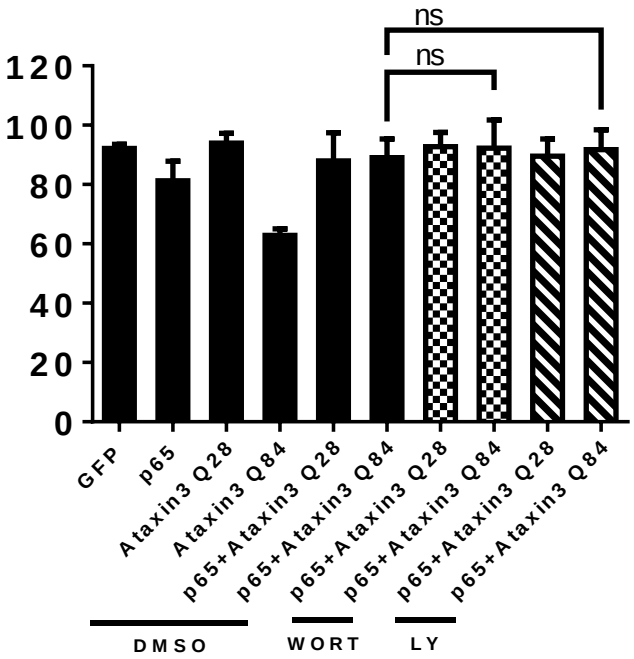


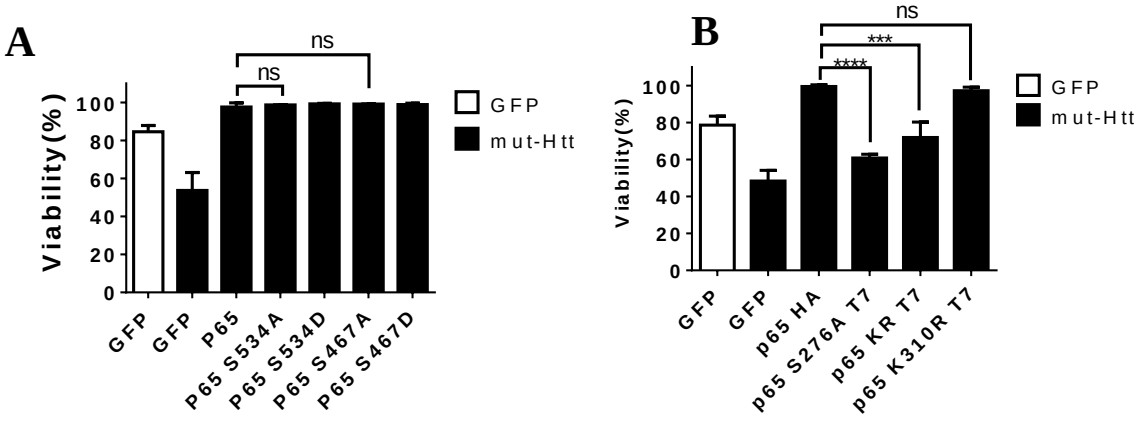
Figure 4. p65 mediated protection in neurons is not dependent on Akt/PI3K pathway.

A. Cortical neurons were transfected with GFP, p65-v5, mut-Htt or co-transfected with p65-HA and mut-Htt. Cells were treated with 200nM wortmannin or DMSO after transfection. Cell viability was analyzed after 24 hours. ***B.*** CGNs were transfected with GFP, p65-v5, wt-ATX3, mut-ATX3 or co-transfected with p65 -HA with wt/mut-ATX3. Cells were treated with HK media containing DMSO, 200nM wortmannin or 10μM LY294002 after transfection. Cells viability was analyzed after 24 hours.

3.5. Effect of posttranslational modifications on the neuroprotective effect of p65.

Since p65 may not promote neuroprotection via protein-protein interactions with mutant Htt or Atx-1/3 or the AKT pathway, I then moved my attention to examining the possibility that specific post-translational modifications of p65 may be critical for its neuroprotective ability. Post-translational modifications can have dramatic consequences on the activity and function of a protein. The activity of p65 can be regulated by phosphorylation at a number of sites[79]. Two phosphorylation sites were reported to stimulate p65 activity - S467 and S536, which are located within the transcriptional activation domain of p65 [79], [80]. To test whether phosphorylation at these sites was required for p65-mediated neuroprotection, I constructed mutant forms of p65 in which each of these serine residues were replaced by non-phosphorylatable alanine residues or phosphomimetic aspartate residues. Surprisingly, I found these mutations had no effect on neuroprotection by p65 (Fig. 5A). It was reported that phosphorylation of p65 S276 can stimulate its activation[81]. So I expressed p65 S276A, another non-phosphorylatable mutation of p65. In contrast to S467 and S536, p65 S276A lost the ability to protect cortical neurons from mut-Htt toxicity (Fig. 5B). This indicates phosphorylation of S276 on p65 is important to mediate p65s ability to be neuroprotective.

Another type of modification that affects p65 activity is acetylation. Among the acetylation sites that have been identified in p65 are K122, K123, K218, K221, K310, K314, and K315. I expressed a construct, p65-K5R in which five lysine residues (K122, K123, K310, K314, and K315) were mutated to a non-phosphorylatable arginine residue. I found that p65-K5R, was just as neuroprotective as wild-type p65 (Fig. 5C) and had same localization with wild-type p65 in neurons. Similarly, a "constitutively acetylated" mutant form of p65, p65-K5Q in which the same five lysine residues were substituted to an acetylation-mimic residue, glutamic acid, was also protective (Fig. 5C). To further explore the role of p65 acetylation in its protection, a construct, p65-KR which mutated K218, 221, 310, was tested for protection against mut-Htt in cortical neurons. Interestingly, protection by p65-KR was severely reduced suggesting that acetylation at one or more of the three mutated sites was necessary for p65-mediated neuroprotection (Fig. 5B). I found that a construct in which only one of these sites is mutated, p65-K310R, was completely protective (Fig. 5B). Together, these results suggest that acetylation at K218 and/or 221 plays a key role in the ability of p65 to be neuroprotective.



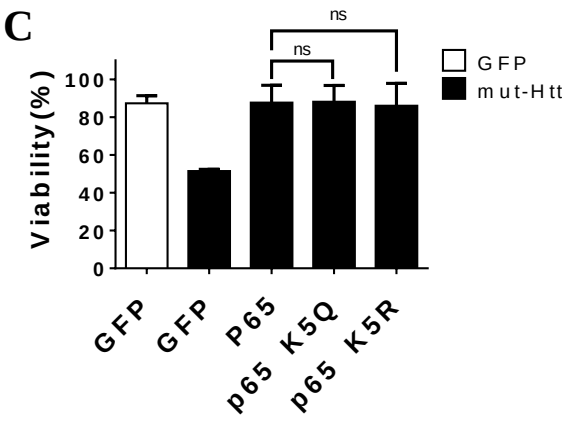


Figure 5. Protectivity of p65 in neurons requires phosphorylation of S276.

A, B. Cortical neurons were co-transfected with mut-Htt and GFP, p65-HA or HA tagged p65 S534A/D, S467A/D, or T7 tagged p65 S276A. Viability of transfected neurons were quantified by immunocytochemistry after 24 hours. ***C.*** Cortical neurons were transfected with GFP or co-transfected with mut-Htt and GFP or HA tagged p65 mutations of K122, K123, K310, K314, and K315(K5Q/R). Cell viability was analyzed after 24 hours. ***D.*** Cortical neurons were transfected with GFP or co-transfected with mut-Htt and GFP or T7 tagged p65 mutations of K218, 221, 310(KQ/R) and K310R-T7. Cell viability was analyzed after 24 hours.

3.6.Inhibition of IKK has no effect on p65 mediated neuroprotection.

Since p65 is the main subunit of the NF-κB complex, transcriptional activation of NF-κB may be involved in p65 mediated protection. The canonical pathway for NF-κB activation involves IKK-mediated phosphorylation of IκB, a protein that sequesters NF-κB dimers in the cytoplasm, which leads to the proteasomal degradation of IκB thus permitting dimeric NF-κB to translocate to the nucleus, bind DNA and regulate transcription. In contrast to most other tissues, brain tissue IKK displays some activity under normal conditions, which is likely responsible for the constitutive NF-κB activity in the brain[59]. It is noteworthy that activity of p65 can also be regulated by other non-canonical mechanisms[82]. To examine the role of IKK in neuroprotection

by p65, I utilized BMS-345541, a widely used, effective and selective chemical inhibitor of IKKα and IKKβ, two essential components of the kinase complex. (Fig. 6). Treatment with BMS-345541 had no effect on p65-mediated neuroprotection against mut-Htt. A lack of effect on p65-mediated neuroprotection was also obtained using IKK16, a structurally unrelated chemical inhibitor of IKK (Fig. 6).

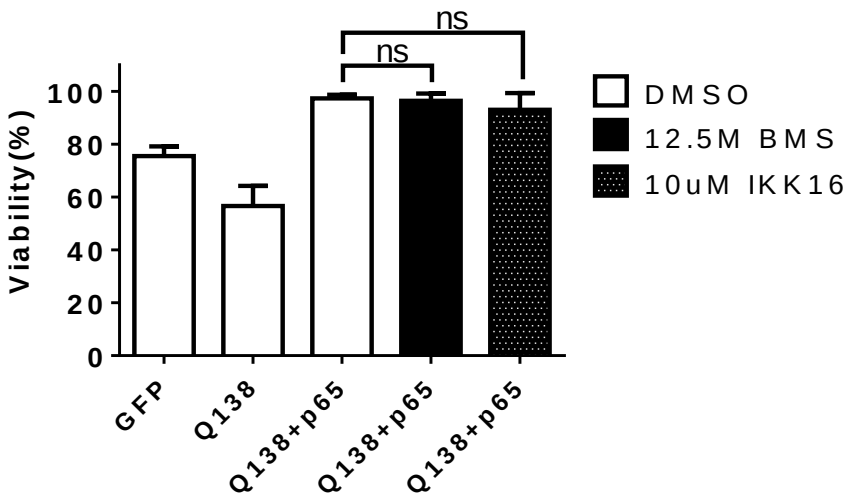


Figure 6. IKK inhibitor didn't block protection mediated by p65.

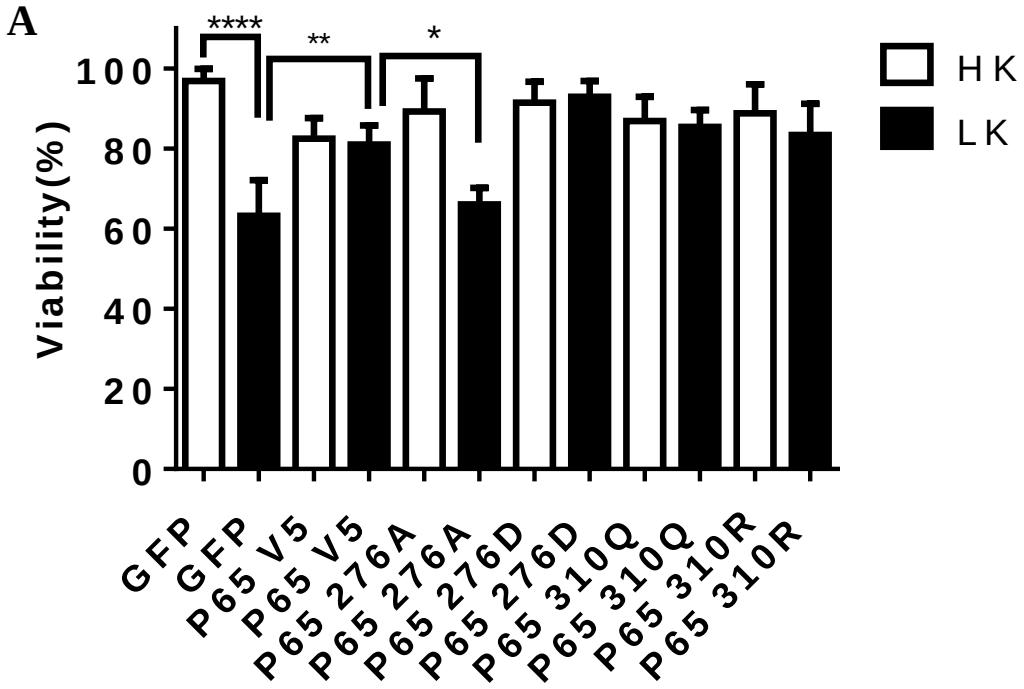
Cortical neurons were transfected with GFP, mut-Htt or co-transfected with p65-HA and mut-Htt. Cells were treated with DMSO, 12.5μM BMS345541 or 10μM IKK-16 after transfection. Cell viability was analyzed after 24 hours.

3.7. V5 tagged p65 plasmids exhibited similar results to other tagged versions of p65.

One issue with the p65 expression plasmids I had utilized was that they were purchased from different sources. Although expressed at comparable levels, these plasmids did not all have the same tag or the same vector backbone. To eliminate potential effects of backbone and tag

between the plasmids in their action of neuroprotection, I generated five of the p65 constructs in the same backbone and all having a V5 tag. As with the previously used HA, Flag and GFP tagged constructs, the newly generated wild type p65 was protective in LK-treated CGNs, although to a slightly lower extent. Likewise, the newly generated V5-tagged p65 S276A failed to protect LK-treated neurons, while the S276D did (Fig. 7).

I extended my studies to the mut-Htt toxicity model. Consistent to the Flag and GFP-tagged plasmids, the V5-tagged p65 perfectly protects CGNs against mut-Htt neurotoxicity. I re-examined the effects of various p65 mutations on mut-Htt neurotoxicity. I found that all of p65 constructs other than p65 S276A were able to protect mut-Htt toxicity in cortical neurons which is consistent with the previous results.



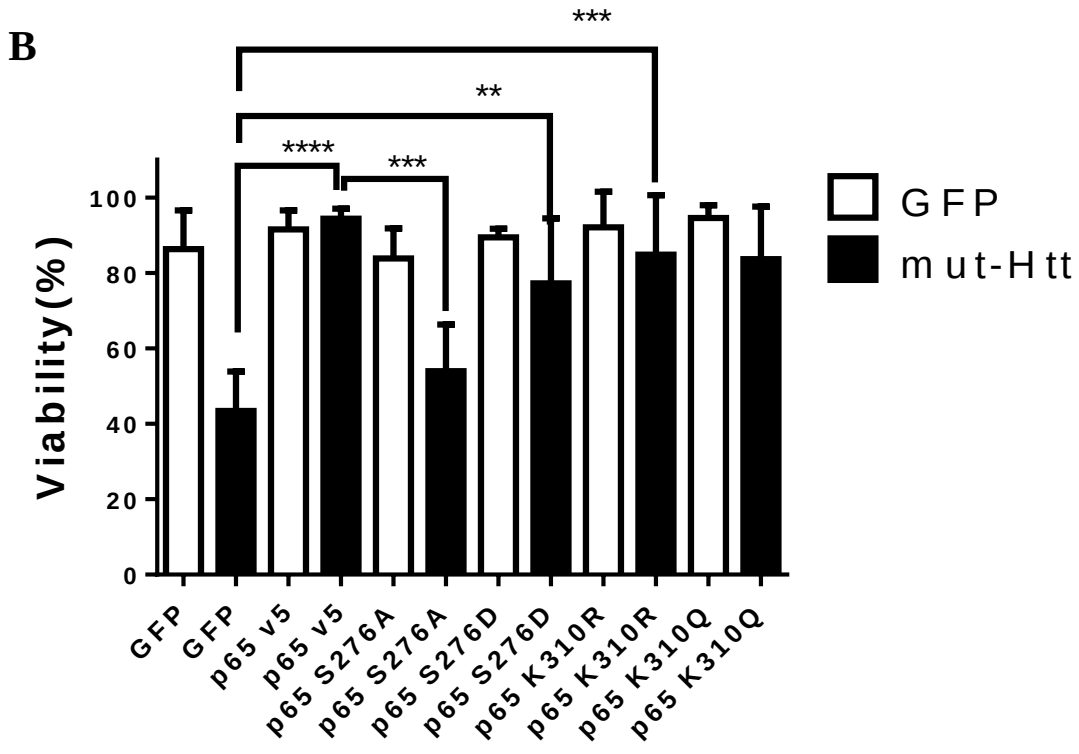


Figure 7 The V5 tagged p65 plasmids exhibited similar results in cortical neurons but not in CGNs.

A. CGNs were transfected with GFP or V5 tagged p65 mutations. Neurons were treated with HK/LK media after 24 hours. Cell viability was analyzed after 24 hours. ***B.*** Cortical neurons were transfected with GFP, mut-Htt, V5 tagged p65 mutations or co-transfected with mut-Htt and V5 tagged p65 mutations. Cell viability was analyzed after 24 hours.

CHAPTER 4

DISCUSSION

For my dissertation research, I discovered that p65 protected neurons in disease models of mut-Htt, SCA1, SCA3 and HDAC3 or HCA induced apoptosis. p65 interacts with Htt but not with Atx1. Using deletions of Htt, I located the domain that is important for binding of p65 to the first 75 amino acids of Htt. I also found inhibitors of PI3K/Akt pathway couldn't block p65 mediated protection which indicates p65 may not protect through Akt. I then used p65 mutation constructs that mutated several phosphorylation or acetylation sites to determine if post-translational modifications are involved in p65 mediated protection. Interestingly, I found abrogating acetylation of Lys218/221 or phosphorylation of Ser276 blocked protection by p65. However, I found inhibition of NF-κB activation using IKK inhibitors didn't abrogate p65 mediated protection. Identifying protective molecules is very important for neurodegenerative disease research because most mechanisms of these disorders are still not known, and there is no treatment for these diseases. Searching for protective molecules and understanding the underlying mechanism facilitates determining the biology of these diseases. Furthermore, studying protective molecules may provide clues for developing potential treatments.

Since p65 interacts with Htt/mut-Htt but not Atx1, protein-protein interactions may be an exclusive mechanism that enables p65 to be protective in models of Huntington's disease. It was

reported that p65 is constitutively activated in neurons[83], and Marcora and colleagues revealed that Htt may mediate the transport of p65 from the synapse to the nucleus[84]. Taken together, interactions between p65 and Htt appears to be essential in neurons. My data from my co-IP experiments indicate that there might be more of an interaction with p65 and wt-Htt than mut-Htt (Fig. 2B). However, these experiments were ambiguous in part due to differing amounts of proteins precipitating due to p65s ability to increase protein expression from some of the plasmids used in these experiments. So therefore, the interactions between wt- and mutant -Htt and p65 are in no way definitive. In this circumstance, mut-Htt may still sequester p65 in inclusions other than transporting to nucleus. Furthermore, I found it possible that p65 interacts with the two PRRs in Htt. PRR is important for Htt to interact with other proteins and blocking this region reduces mut-Htt toxicity[75]. Overexpression of p65 may protect neurons by blocking PRRs in mut-Htt.

HDAC3 was reported to mediate Htt induced neuronal death[36]. Normally Htt sequesters HDAC3 and inhibits toxic activities. When mut-Htt is overexpressed, HDAC3 is released then induces neuronal death. I showed that HDAC3 interacted with p65 and other labs also previously found the same interaction[69]. When overexpressed, p65 may sequester HDAC3 from its toxic activities. As shown in Fig. 3A, WCL of HDAC3 always had much more expression when co-expressed with p65 even though the same amount of DNA was transfected into the cells. The same increase was observed when using different tags for p65. Notably endogenous HDAC3 level was not altered by overexpression of p65. Although the expression level changed, it appeared that HDAC3 may interact with p65 when overexpressed in HEK 293T. However, I was not able to observe the interaction endogenously in the rat cortex. It is possible that the expression level of p65 and HDAC3 are very low in the cortex, making it technically difficult to detect this interaction.

I found that when p65 was mutated on K218, 221, 310 to arginines, it was not able to protect neurons. Mutation of p65 K310R however, didn't affect the protection. These indicated that K218, or K221 or both are involved in p65 mediated protection. Acetylation of K218 and K221 weakens p65 interaction with IκBα while deacetylation at these sites by HDAC3 increases binding to IκBα[85]. If p65 K218,221 deacetylation is essential for p65 mediated protection, disassociating from IκBα should be necessary. Normally after p65 is released from IκBα, p65 will translocate to nucleus to activate transcription. However, IKK inhibitors were not able to abrogate p65 mediated protection. It is possible that while p65 was overexpressed, there were not enough IκB molecule to block all of them. Even if IKK was inhibited, there still was enough p65 that can protect neurons. The other mutation I found that abrogated p65 mediated protection was S276A. It has been reported that S276 phosphorylation by PKAc leads to a structural change of p65 which facilitates interaction with p300/CBP[86], [87]. p300/CBP acetylates p65 at K218,221[88]. So, it is possible that p65 protects neurons when it is phosphorylated at S276 via PKA, which then facilitates the binding of p300/CBP to p65 which then leads to acetylation at K218,221 of p65 allowing it to be release from IκB. p65 may start transcriptional activation or possess other functions after the release from IκB that mediate its ability to be neuroprotective.

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