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Calcium leak through ryanodine receptor is involved in neuronal death induced by mutant huntingtin

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative disorder caused by an abnormal expansion of polyglutamine (polyQ) tract in huntingtin (htt) protein. Although altered calcium (Ca^{2+}) homeostasis is suggested in HD, its molecular mechanisms have remained poorly understood despite their important role in the pathogenesis. In this study, we examined involvement of ryanodine receptor (RyR), an endoplasmic reticulum-resident Ca^{2+} channel, in mutant htt-induced neuronal death. Inhibitors of RyR attenuated cell death induced by mutant htt, while co-expression of RyR enhanced htt toxicity. Intracellular Ca^{2+} imaging revealed that mutant htt caused excessive basal Ca^{2+} release (Ca^{2+} leak) through RyR leading to depletion of internal Ca^{2+} store. Ca^{2+} leak was also observed in striatal and cortical neurons from R6/2 HD model mice. Moreover, expression of FK506-binding protein 12 (FKBP12), a RyR stabilizer, suppressed both Ca^{2+} leak and cell death. These results provide novel evidence suggesting altered RyR function is involved in neuronal cell death, and its stabilization might be beneficial for treatment of HD.

1. Introduction

Huntington's disease (HD) is an autosomal dominant, fatal neurodegenerative disorder caused by an expansion of polymorphic CAG repeat encoding polyglutamine (polyQ) in huntingtin (htt) [1]. In this disease, progressive neuronal loss in the striatum and cerebral cortex resulted in abnormal involuntary movements and cognitive dysfunction [2]. Proteolytic N-terminal fragments of mutant htt containing the polyQ expansion are found to form inclusion bodies in affected neurons, and HD mice expressing exon1 of htt show neurological symptoms [3], suggesting that the truncated fragment of htt plays a crucial role in HD pathogenesis. It is generally thought that expanded polyQ stretch has been shown to confer toxic properties on the disease proteins through conformational transition to a β-sheet-dominant structure, leading to assembly of the host proteins into insoluble β-sheet-rich aggregates [4]. Toxic function of expanded polyQ has been assigned to various cellular regulatory systems including gene transcription, ubiquitin-proteasomal system, mitochondrial energy metabolism and calcium (Ca^{2+}) homeostasis [1]. However, molecular mechanisms centrally involved in mutant htt-induced neuronal death are not fully understood.

Altered Ca^{2+} homeostasis is one of the striking features of HD [5]. Our previous study demonstrated that perturbed intracellular Ca^{2+} homeostasis is associated with polyQ-induced neuronal death [6]. Endoplasmic reticulum Ca^{2+} stores are an important source of Ca^{2+} in neurons, and Ca^{2+} release through the inositol 1,4,5-triphosphate receptor (IP3R) or the ryanodine receptor (RyR) plays crucial role in neuronal survival and function [7]. Several groups have reported that altered function of IP3R is involved in polyQ toxicity [8–13]. It is noteworthy that a part of these studies showed therapeutic effect of dantrolene (Dan) on neurological phenotypes and cell loss in polyQ-disease model mice [8–11], although the target of Dan is not IP3R, but is RyR. Since Dan is a clinically approved RyR inhibitor, and its beneficial effects are widely demonstrated in various CNS disease models [14–16], it is expected to be clinically developed for HD and other related neurodegenerative diseases with no effective therapy currently available. However, the role of RyR, which is a target of Dan, in polyQ-induced neurodegeneration remains to be explored.

Abbreviations: HD, Huntington's disease; polyQ, polyglutamine; tNhtt, truncated N-terminal huntingtin; RyR, ryanodine receptor; IP3R, inositol 1,4,5-triphosphate receptors; Dan, dantrolene; RR, ruthenium red; Ry, ryanodine; DHBP, 1,1′-diheptyl-4, 4′-bipyridinium dibromide; 2APB, 2-aminoethoxydiphenyl borate; PEI, polyethyleneimine; TG, thapsigargin; $[Ca^{2+}]_i$, cytoplasmic Ca^{2+}; SERCA, sarco/endoplasmic reticulum Ca^{2+} pumps; FKBP12, FK506-binding protein..

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Here we demonstrated the involvement of RyRs in the mechanisms underlying mutant htt-induced neuronal death. We found that mutant htt causes Ca^{2+} leak from RyR in transiently transfected HD cellular model and neurons from R6/2 HD model mice. Moreover, expression of RyR stabilizing protein FK506-binding protein 12 (FKBP12) attenuated both Ca^{2+} leak and cell death. Our results provide novel evidence that altered RyR function may contribute to the neurodegeneration in HD.

2. Materials and methods

2.1. Materials

The sources of materials used in this work were as follows: 1,1′-diheptyl-4, 4′-bipyridinium dibromide (DHBP) from TOCRIS Cookson (Bristol, UK), 2-aminoethoxydiphenyl borate (2APB) from Calbiochem Corp. (La Jolla, CA), and thapsigargin from Research Biochemicals Inc. (Natiek, MA). All other materials were obtained either from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or from Sigma–Aldrich Corp (St. Louis, MO).

2.2. Plasmids

The truncated N-terminal huntingtin (tNhtt) tagged with green fluorescent protein (EGFP) expression constructs pEGFP-tNhtt17Q and pEGFP-tNhtt150Q were provided by N. Nukina [17]. Expression construct pBFP-tNhtt encoding blue fluorescent protein (BFP) was prepared by replacing the EGFP region of pEGFP-tNhtt with the BFP region from pQBI50fC1 vector. The expression plasmids of full-length RyR1 and FLAG-FKBP12 were from G. Meissner [18] and K. Miyazono [19], respectively.

2.3. Cell culture and transfection

Cortical or striatal neurons were prepared from neonatal Sprague–Dawley rats. All the experimental procedures conformed to the guidance set by the committee at the Research Center of Laboratory Animals, Hokkaido University. The tissues were digested with 0.05% trypsin followed by addition of feeding medium (Eagle's minimum essential medium [MEM] supplemented with 5% fetal calf serum [FCS], 5% heat-inactivated horse serum [HS], 10 mM HEPES pH 7.4, 20 mM glucose, 25 U/ml penicillin and 25 μg/ml streptomycin). Mechanically dissociated cells were plated on polyethyleneimine (PEI)-coated plastic plates or glass-cover slips and incubated at 36 °C in a humidified atmosphere of 5% CO_2/95% air. On day 2, Cytosine-β-D-arabinofuranoside (final 2.5 μM) was added to reduce the non-neuronal proliferation, and half of the medium was replaced with fresh serum-free medium at day 4. Cortical or striatal neurons were transfected at day 5–7 as described previously [20]. For co-transfection experiments an equimolar ratio mixture of the plasmids was used. Neuronal expression of tNhtts was confirmed by staining with anti-MAP2 (microtubule-associated protein 2, Sigma) antibody as described previously [6]. Human embryonic kidney 293t cells (293t cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% FCS, penicillin and streptomycin.

2.4. Evaluation of cell death

Cortical or striatal neurons were incubated with 5 g/ml ethidium bromide (EtBr) in HEPES-buffered saline (HBS: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 1 g/L glucose, pH 7.2) for 5 min. Neurotoxicity is shown as a percentage of the number of neurons stained with EtBr out of that of EGFP-positive neurons. Cell viability of 293t cells was evaluated by dye extrusion with trypan blue as well. Experiments were repeated at least three times and representative results are presented.

2.5. Fluo-3 Ca^{2+} imaging

The cells were loaded with 3 μM fluo-3/AM (Dojindo Laboratories, Kumamoto, Japan) in HBS and 0.04% pluronic F147 (Molecular Probes Inc, Eugene, OR) for 30 min at 36 °C for 293t cells or 27–28 °C for mouse neurons. Ca^{2+} leak through RyR was assessed as described previously with slight modifications [21]. 2APB (final 100 μM) was added to the fluo-3 loading buffer. The glass-cover slips were mounted on a modified Sykes-Moore chamber (Bellco Biotechnology Inc., Vineland, NJ), placed on an inverted microscope (Nikon Diaphoto 300, Nikon). The data was analyzed using the software associated with ARGUS 50 (Hamamatsu Photonics Co., Hamamatsu, Japan). For striatal or cortical neurons from adult mice, HEPES-buffered Hank's balanced salt solution (H-HBSS) was used as a substitute for HBS.

2.6. Preparation of adult dissociated neurons

Striatal or cortical neurons were prepared from 13 to 14 weeks heterozygous transgenic R6/2 male mice (Jackson Laboratories, Bar Harbor, ME) or age-matched controls. Striatum and frontal cerebral cortex were removed from 350 μm coronal brain slices prepared by using Mcllwain Tissue Chopper (Mickle Laboratory Engineering Co, Inc, Surrey, UK), and dissociated mechanically in Krebs–Ringer Bicarbonate buffer (KRBB) containing 0.1% bovine serum albumin. The cells were collected and incubated in a feeding medium (50% MEM, 25% H-HBSS, 25% heat-inactivated HS and 25 mM glucose) for 2 days, and then subjected to experiments.

2.7. Statistical analysis

Differences between two groups were analyzed by a two-tailed Student's *t* test. For comparison between more than two groups, ANOVA followed by *post hoc* Turkey's test was used.

3. Results

3.1. Inhibitors of ryanodine receptor protect neurons against toxicity of mutant huntingtin

In this study, we used cortical neurons transiently transfected with wild-type (17Q) or mutant (150Q) truncated N-terminal huntingtin (tNhtt) tagged with enhanced green fluorescent protein (EGFP). EGFP-tNhtt150Q formed intracellular inclusion body in the neurons (Fig. 1A), arrowheads, which is a typical hallmark of HD pathology, and caused neuronal cell death in a polyQ-length-dependent manner (Fig. 1B). By using this model, we explored the role of the inositol 1,4,5-triphosphate receptor (IP3R) and the ryanodine receptor (RyR) in mutant htt-induced neuronal death. We examined the effects of inhibitors of RyR or IP3R and found that dantrolene (Dan), ryanodine (Ry), 1,1′-diheptyl-4,4′-bipyridinium dibromide (DHBP) and ruthenium red (RR), all inhibitors of RyRs, significantly suppress mutant htt-induced neuronal death (Fig. 1C). In contrast, 2-aminoethoxydiphenyl borate (2APB), an inhibitor of IP3Rs, failed to protect these neurons. The protective effect of Dan was abolished by 4-chloro-m-cresol (CMC), a potent activator of RyRs, confirming that the effect of Dan is indeed mediated by inhibition of RyR (Fig. 1D). It was also found that Dan effectively attenuated mutant htt-induced cell death of striatal neurons (Fig. 1E). These results indicate that inhibition of Ca^{2+} release from RyRs, but not IP3Rs, attenuates neuronal death induced by mutant htt.

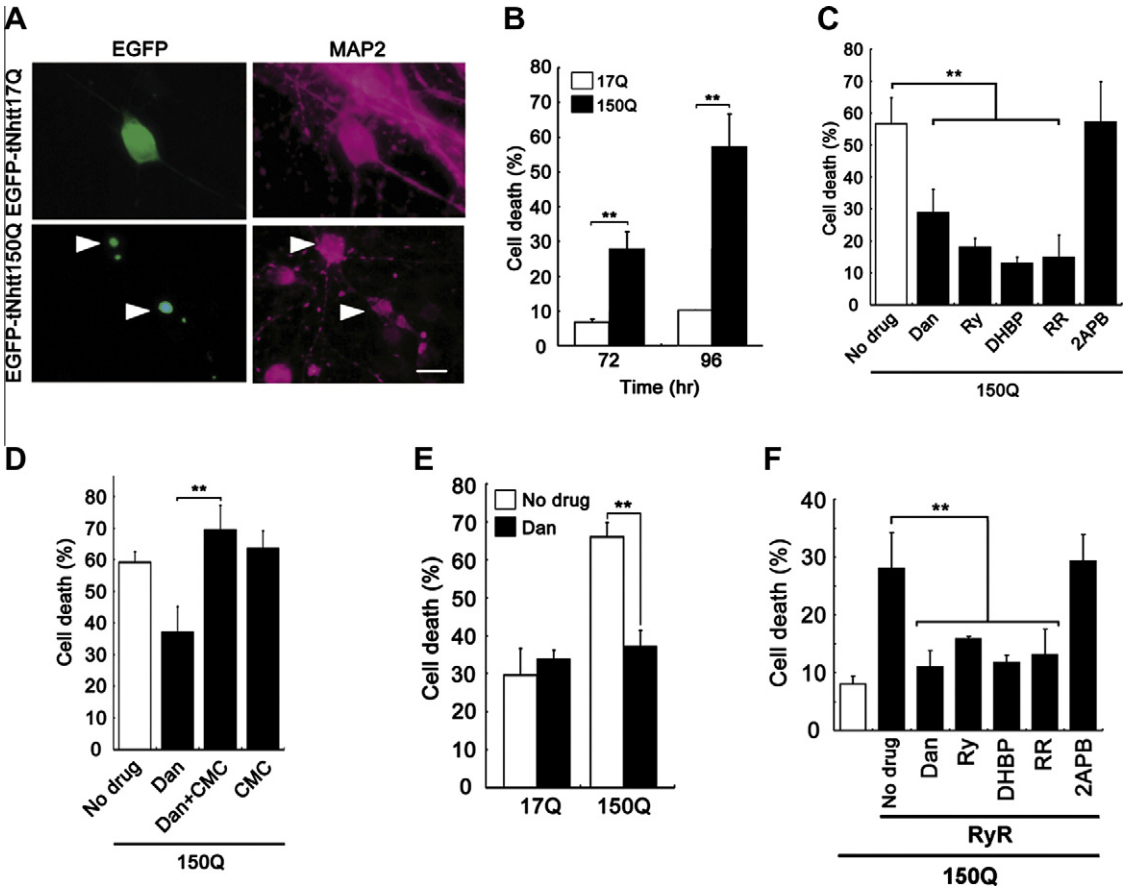

Fig. 1. Blockade of ryanodine receptor (RyR) attenuates neuronal cell death induced by mutant huntingtin (htt). (A) Immunofluorescence analysis. Cortical neurons transfected with EGFP-tagged truncated N-terminal huntingtin 17Q (EGFP-tNhtt17Q) and 150Q (EGFP-tNhtt150Q) were stained with the neuronal marker MAP2. Arrowheads indicate mutant htt inclusions. Bar, 10 μm. (B) Time- and length-dependency of mutant htt-induced neuronal death. (C) Suppression of mutant htt-induced neuronal death by inhibition of RyRs. Cortical neurons transfected with EGFP-tNhtt150Q (150Q) were treated with RyR inhibitors such as dantrolene (Dan, 30 μM), ryanodine (Ry, 10 μM), 1,1′-diheptyl-4,4′-bipyridinium dibromide (DHBP, 50 nM) or ruthenium red (RR, 10 nM). Inositol 1,4,5-triphosphate receptors (IP3Rs) inhibitor 2-aminoethoxydiphenyl borate (2APB, 10 μM) did not show any effects. (D) Counteracting effect of RyR activator 4-chloro-m-cresol (CMC) on the protective effect of Dan. Cortical neurons were treated with Dan (30 μM), CMC (10 μM), or both. (E) Protective effect of Dan in striatal neurons. Concentration of Dan was 30 μM. (F) Potentiation of mutant htt toxicity by expression of RyR1. HEK 293t cells bearing no endogenous RyR, were co-transfected with EGFP-tNhtt150Q (150Q) and RyR1. Concentration of the drugs was same as C. The mean ± SD ($n = 3$). $^{**}p < 0.01$.

To obtain direct evidence that RyRs actually mediate the toxicity of mutant htt, we next investigated the effect of RyR expression on mutant htt toxicity. Co-expression of EGFP-tNhtt150Q and RyR1, which has target site for Dan inhibition [22], and is reported to increase in the caudate nucleus of HD patients [23], significantly increased cell death of HEK 293t (293t) cells bearing no endogenous RyRs. We further confirmed that this increase was suppressed by RyRs inhibitors, but not with IP3Rs inhibitor (Fig. 1F). Taken together, we concluded that abnormal Ca^{2+} release through RyR1 is induced by mutant htt and involved in mutant htt-induced neuronal cell death.

3.2. Mutant htt causes abnormal Ca^{2+} leak through RyRs

We moved on to measure the level of cytoplasmic calcium ($[Ca^{2+}]_i$) upon activation of RyRs to examine if there is any dysfunction regarding Ca^{2+} release from RyR expressing mutant htt. The cells co-expressing blue fluorescent protein (BFP)-tNhtts and RyR1 responded to caffeine, which activates RyRs to release Ca^{2+} from ER to cytoplasm, by rapid increases in $[Ca^{2+}]_i$ 24 h after transfection, and the responses were similar between wild-type (Q17) and mutant (Q150) htt (Fig. 2A and B). However, the cells expressing mutant htt became to poorly respond to this stimulation at 48 h, and these cells failed to respond by 72 h.

These results raise the possibility that the internal Ca^{2+} store might be depleted under these conditions by enhanced spontaneous Ca^{2+} release (Ca^{2+} leak). Ca^{2+} levels of cytoplasm and ER is balanced by uptake through SERCAs (sarco/endoplasmic reticulum Ca^{2+} pumps) and release through RyRs and IP3Rs (Fig. 3A). Therefore, Ca^{2+} leak through RyR could be evaluated by measuring $[Ca^{2+}]_i$ increase under treatment with thapsigargin (TG), an inhibitor of SERCAs, following pretreatment with 2APB, an inhibitor of IP3Rs [21]. Upon these treatments, there was a slight increase in $[Ca^{2+}]_i$ in the cells expressing wild-type htt and RyR1 (17Q), which indicates spontaneous Ca^{2+} release through RyR1. Compared with this, there was a large increase in $[Ca^{2+}]_i$ in the cells expressing mutant htt and RyR1 (150Q, Fig. 3B and C). This $[Ca^{2+}]_i$ increase was suppressed by treatment with RyR inhibitor DHBP (150Q+DHBP), suggesting that excessive Ca^{2+} leak through RyR is induced by mutant htt.

Next we further sought to obtain evidence that abnormal Ca^{2+} leak from RyRs occurs in a well-established animal model as well. Striatal or cortical neurons were prepared from 13- to 14-weeks-old R6/2 HD model mice expressing exon1 of htt with 144 repeats of polyQ tract, and subjected to Ca^{2+} imaging. The neurons from R6/2 (R6/2) mice, but not those from age-matched wild-type mice (WT), showed a gradual increase in $[Ca^{2+}]_i$ levels following application of TG (Fig. 3D) and there was a significant difference in relative

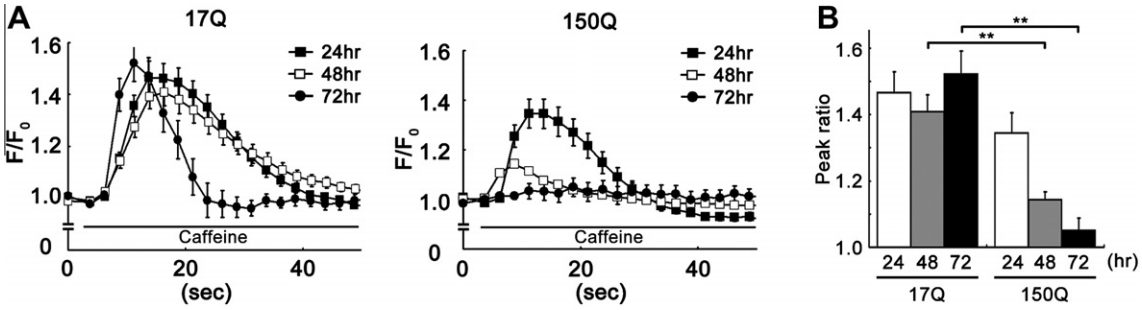

Fig. 2. Caffeine-sensitive Ca^{2+} store declines in the cells expressing mutant htt. (A) Cytoplasmic Ca^{2+} increase upon treatment with an activator of the RyR. HEK 293t cells were co-transfected with RyR1 and blue fluorescent protein (BFP)-tagged tNhtt17Q (17Q) or 150Q (150Q) for 24, 48 or 72 h, and then loaded with calcium indicator fluo-3. Fluo-3 fluorescence of BFP-positive cells was monitored following treatment with caffeine (25 mM). (B) Peak values of caffeine responses in A. A maximal value for F/F_0 was calculated for caffeine responses of each cell. The mean ± SE (n = 16–26 cells). $^{**}p < 0.01$.

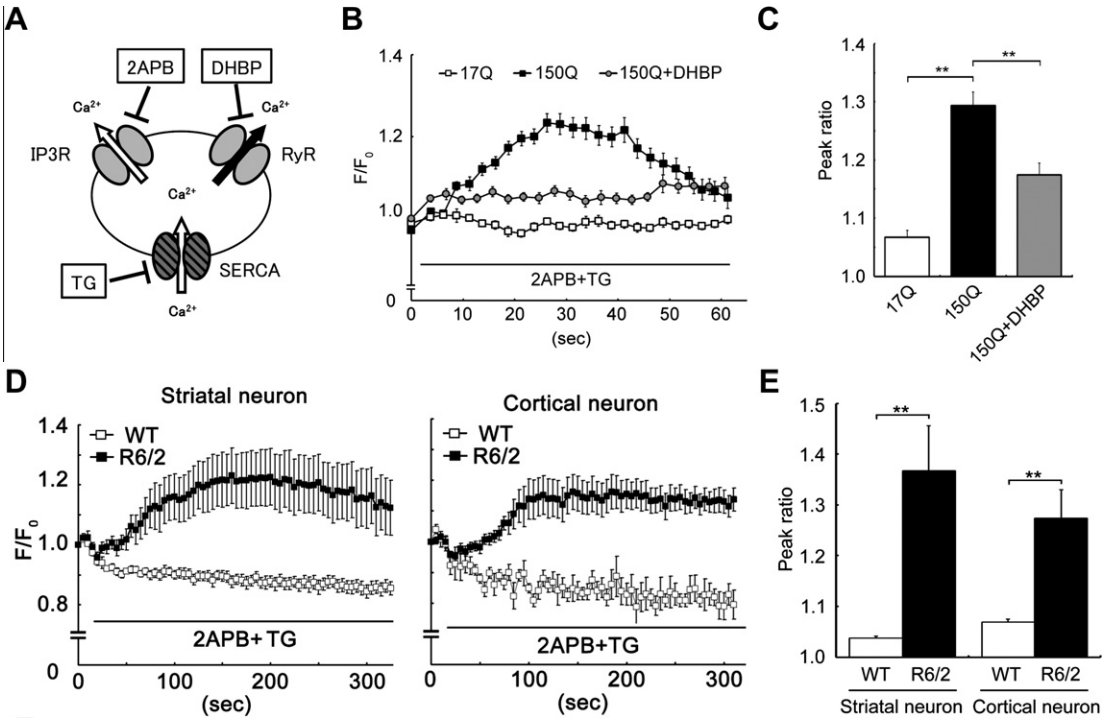

Fig. 3. Mutant htt induces abnormal Ca^{2+} leak through RyRs. (A) Schematic drawing of Ca^{2+} flow between cytoplasm and ER. Ca^{2+} leak through RyR was assessed by measurement of cytoplasmic Ca^{2+} increase upon treatment with thapsigargin (TG), an inhibitor of SERCAs (ER Ca^{2+} pumps), and 2APB, an inhibitor of IP3Rs. (B) Abnormal Ca^{2+} leak through RyR in the cells expressing mutant htt. HEK 293t cells were transfected with RyR1 and BFP-tNhtt17Q (17Q) or 150Q (150Q). At 24 h of transfection, the cells were loaded with fluo-3 in the presence of 100 μM 2APB and then cytoplasmic Ca^{2+} levels were measured following treatment with 1 μM TG. Enhanced Ca^{2+} increase of mutant htt-expressing cells was suppressed by RyR inhibitor DHBP (50 nM). The mean ± SE (n = 13–29). (C) Maximal value of F/F_0 in B. (D) Abnormal Ca^{2+} leak in striatal or cortical neurons from R6/2 HD model mice. The neurons prepared from 13- to 14-week-old wild-type (WT, n = 15) or R6/2 mice (R6/2, n = 14) were incubated with 3 μM fluo-3 and 100 μM 2APB. Then, cytoplasmic Ca^{2+} levels were measured following treatment with 0.5 μM TG. (E) Quantification of the results in D. The mean ± SE. $^{**}p < 0.01$.

peak ratios of these responses between R6/2 and wild-type mice (Fig. 3E). Taken together, these results show that mutant htt induces enhanced Ca^{2+} leak through RyRs in the HD pathogenesis.

3.3. Expression of RyR stabilizer FKBP12 suppresses mutant htt-induced abnormal Ca^{2+} leak and cell death

We further examined whether stabilization of RyR1 could suppress abnormal Ca^{2+} leak and mutant htt-induced cell death. It is well-known that FK506-binding protein 12 (FKBP12), a member of immunophilin family, physically interacts and stabilizes RyR1 by decreasing channel open probability, playing the role for endogenous RyR stabilizer [24]. Therefore, we co-expressed FKBP12 with mutant htt, and found that mutant htt-induced Ca^{2+} leak through RyR1 was effectively suppressed by FKBP12 in 293t cells (Fig. 4A and B). Consistent with these results, FKBP12 decreased cell death of 293t cells, primary cultured cortical and striatal neurons (Fig. 4C and D), suggesting that stabilization of RyR channel function protects neurons against mutant htt toxicity.

4. Discussion

Here we have shown that inhibitors of RyRs attenuate cell death induced by mutant htt. Mutant htt caused Ca^{2+} leak from RyR1 followed by depletion of Ca^{2+} store in the ER, and expression of RyR stabilizer FKBP12 suppressed both Ca^{2+} leak and cell death. From these results we concluded that abnormal Ca^{2+} leak from RyRs may contribute to neuronal death in HD.

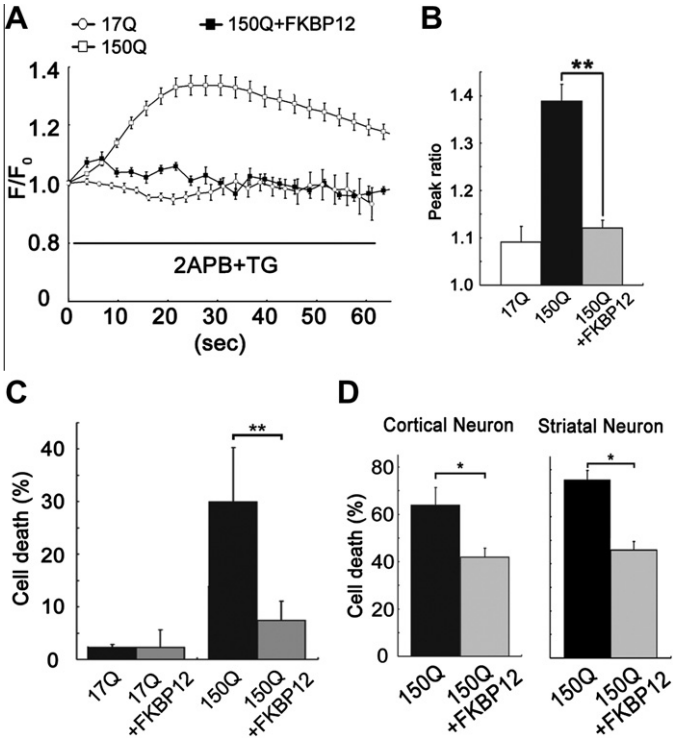

Fig. 4. Expression of RyR stabilizer FK506-binding protein 12 (FKBP12) suppresses mutant htt-induced abnormal Ca^{2+} leak and cell death. (A) Suppression of abnormal Ca^{2+} leak from RyR by FKBP12. HEK293t cells were co-transfected with RyR1 and BFP-tNhtt17Q (17Q), RyR1 and BFP-tNhtt150Q (150Q), or RyR1, BFP-tNhtt150Q and FKBP12 (150Q+FKBP12). Ca^{2+} leak was assessed at 48 h of transfection. The mean ± SE (n = 19–21). (B) Quantification of the results shown in A. (C) Suppression of mutant htt-induced cell death by FKBP12 in HEK 293t cells. Cell viability was evaluated 96 h after transfection. The mean ± SD (n = 3). (D) Attenuation of mutant htt-induced neuronal death by expression of FKBP12. Primary cultured cortical or striatal neurons were transfected for 96 h and cell viability was evaluated. The mean ± SD (n = 3). $^{*}p < 0.05$,$^{**}p < 0.01$.

To our knowledge, this study is the first report suggesting that Ca^{2+} leak through RyR contributes to pathogenesis of HD. Ca^{2+} leak from RyR1 has mainly been reported in muscular diseases such as malignant hyperthermia and muscular dystrophies. In these conditions, PKA (cAMP-dependent protein kinase)-dependent phosphorylation, oxidation or nitrosylation of RyR is shown to cause dissociation of FKBP proteins from the channels resulting in Ca^{2+} leak [25]. Although we have not assessed the status of RyR, there are several reports implicating that similar modification of RyR might be induced by mutant htt. For instance, increased activity of PKA [26] and nitric oxide synthase was found in R6/1 HD model mice [27] and increased reactive oxygen species and nitric oxide production was shown in a htt-expressing cellular model [28]. It has also been reported that FKBP12 mRNA level was decreased, whereas the expression of RyR1 increased, in caudate nucleus of grade 1 HD patient [23].

Then, how does Ca^{2+} leak induce neuronal cell death? Continuous Ca^{2+} leak might cause elevated cytosolic Ca^{2+} levels, which is observed in a YAC mouse model and R6/2 mouse [29,30]. Elevated cytosolic Ca^{2+} might lead mitochondrial depolarization through Ca^{2+} overload that cause energy disruption. Ca^{2+} overload also induces the release of cytochrome c that leads subsequent apoptotic pathways. It is reported that mitochondria of mutant htt-expressing cells show increased Ca^{2+} sensitivity of the permeability transition pore [12]. Not only mitochondrial damage, but also Ca^{2+} leak might amplify the toxicity of mutant htt in other ways. For example, elevated cytosolic Ca^{2+} may cause Calpain activation leading to generate toxic fragments [31]. Moreover, if the reduction in ER Ca^{2+} levels occurs, ER stress may be induced and eventually lead to cell death [32].

In addition to Ca^{2+} leak, we also found that caffeine-sensitive Ca^{2+} store might be depleted in mutant htt-expressing cells. Because Ca^{2+} leak was observed prior to declined caffeine responses in our cellular model (Figs. 3 and 4), continuous Ca^{2+} leak might result in Ca^{2+} depletion of caffeine-sensitive stores. Since RyR is considered to play a pivotal role in neuronal functions including excitation, neurotransmitter release, synaptic plasticity [33], stabilization of RyR might be effective not only in neuronal death but also in neuronal dysfunction of HD.

Currently there is no treatment for attenuating or reversing HD. Our results strongly indicate that dantrolene or other similar RyR inhibitors may be beneficial for HD patients. Notably, dantrolene is one of the clinically approved drugs and its neuroprotective effect has been shown in other neurological disease models including spinocerebellar ataxia type 2 and type 3, Alzheimer's disease and ischemia [9,10,14,15]. This study revealed novel mechanism of action whereby dantrolene attenuate neurodegeneration through suppression of Ca^{2+} leak from RyR and further support the notion that dantrolene should be potential therapeutic agent for the treatment of HD.

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