CHAPTER 11

INOSITOL 1,4,5-TRIPSHOSPHATE RECEPTOR, CALCIUM SIGNALLING AND HUNTINGTON'S DISEASE

I. BEZPROZVANNY*

Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, USA

Abstract: Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder that has no cure. HD primarily affects medium spiny striatal neurons (MSN). HD is caused by polyglutamine (polyQ) expansion (exp) in the amino-terminal region of a protein huntingtin (Htt). The connection between polyQ expansion in Htt^{exp} and MSN neurodegeneration remains elusive. My laboratory discovered that mutant Htt^{exp} protein specifically binds to the carboxy-terminal region of the type 1 inositol 1,4,5-trisphosphate receptor ($InsP_3R1$), an intracellular Ca^{2+} release channel. Moreover, we found that Htt^{exp} association with $InsP_3R1$ causes sensitization of $InsP_3R1$ to activation by $InsP_3$ in planar lipid bilayers and in primary MSN. Mutant Htt^{exp} has also been shown to activate Ca^{2+}-permeable NR2B-containing NMDA receptors. All these results suggested that deranged neuronal Ca^{2+} signaling may play an important role in pathogenesis of HD. In support of this idea, we demonstrated a connection between abnormal Ca^{2+} signaling and apoptosis of MSN cultured from YAC128 HD mouse model. These results indicate that $InsP_3R$ and other Ca^{2+} signaling proteins should be considered as potential therapeutic targets for treatment of HD

Keywords: calcium signaling, huntingtin, neurodegeneration, polyglutamine expansion, inositol 1,4,5-trisphosphate, NMDA receptor, apoptosis, mitochondria, memantine

1. HUNTINGTON'S DISEASE (HD) AND HUNTINGTIN (HTT)

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder with the age of onset between 35 and 50 years and inexorable progression to death 15–20 years after onset. The symptoms include motor abnormalities including

*Dr. Ilya Bezprozvanny, Dept. of Physiology, ND12.502, UT Southwestern Medical Center at Dallas, Dallas, TX 75390-9040, E-mail: Ilya.Bezprozvanny@UTSouthwestern.edu

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chorea and psychiatric disturbance with gradual dementia (Vonsattel and DiFiglia, 1998). Neuropathological analysis reveals selective and progressive neuronal loss in the striatum (caudate nucleus, putamen and globus pallidus) (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). GABAergic medium spiny striatal neurons (MSN) are the most sensitive to neuronal degeneration in HD (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). Positional cloning efforts demonstrated that at the molecular level the cause of HD is polyglutamine (polyQ) expansion in the amino-terminal of a 350 kDa evolutionary conserved cytosolic protein called huntingtin (Htt) (1993). Clinical signs of HD develop if the length of polyQ track in Htt exceeds a pathological threshold of 35Q. The CAG repeat length is inversely correlated with age of onset (Langbehn et al., 2004). Htt is widely expressed in the brain and in non-neuronal tissues and not particularly enriched in the striatum (Li et al., 1993; Strong et al., 1993; Sharp et al., 1995). Htt plays an essential function in development, as deletion of the Htt gene in mice is embryonic lethal (Duyao et al., 1995; Nasir et al., 1995). Analysis of Htt primary sequence suggests that Htt is likely to function as a signaling scaffold (MacDonald, 2003), but the precise function of Htt in cells is not known. In order to elucidate the pathogenesis of HD, a number of transgenic HD mouse models have been generated (Menalled and Chesselet, 2002; Rubinsztein, 2002).

The key question in HD research is how does polyQ-expanded huntingtin (Htt^{exp}) kill MSN? The answer to this question is a prerequisite to development of effective HD therapies. The HD mutation at least in part creates a "gain of function". A number of toxic functions have been assigned to Htt^{exp}, including effects on gene transcription, induction of apoptosis, disruption of key neuronal functions such as proteasomal function, ubiquitination, axonal transport, endocytosis and synaptic transmission. The evidence in favor of these hypotheses are reviewed elsewhere (Tobin and Signer, 2000; Menalled and Chesselet, 2002; Ross, 2002; Rubinsztein, 2002; Harjes and Wanker, 2003; Sugars and Rubinsztein, 2003; Li and Li, 2004). All of these models are consistent with a toxic function of Htt^{exp} in neurons, but none of these models explain the selective vulnerability of MSN in HD. In this review I discuss recently emerging results that support the concept that HD may be a disease of deranged calcium (Ca^{2+}) signaling.

2. HTT^{EXP} SENSITIZES $INSP_3R1$ TO $INSP_3$

The inositol (1,4,5)-triphosphate receptor ($InsP_3R$) is an intracellular calcium (Ca^{2+}) release channel that plays an important role in neuronal Ca^{2+} signaling (Berridge, 1998). Three isoforms of $InsP_3R$ have been identified (Furuichi et al., 1994). The type 1 receptor ($InsP_3R1$) is the predominant neuronal isoform. Mice lacking $InsP_3R1$ display severe ataxic behavior (Matsumoto et al., 1996), and mice with a spontaneous mutation in the $InsP_3R1$ gene experience convulsions and ataxia (Street et al., 1997), suggesting a major role of $InsP_3R1$ in neuronal function. In the search for novel $InsP_3R1$-binding partners we performed a yeast two-hybrid screen with $InsP_3R1$ carboxy-terminal bait and isolated Htt-associated protein 1A

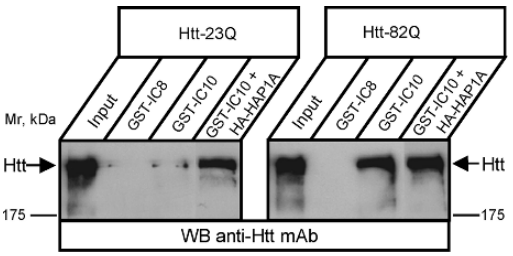

Figure 1. Mutant Htt^{exp} specifically binds to InsP$_3$R1 carboxy-terminal fragment.
IC8 (D2590-F2627) and IC10 (F2627-A2749) fragments of rat InsP$_3$R1 carboxy-terminal tail were expressed as GST fusion proteins in bacteria and utilized in pull-down experiments. Wild type Htt-23Q and mutant Htt-82Q were expressed in HEK293 cells. HA-HAP1A protein was expressed in COS7 cells and included in pull-down reaction as indicated. The precipitate Htt protein was detected by Western blotting with anti-Htt monoclonal antibodies. Adapted from (Tang et al., 2003)

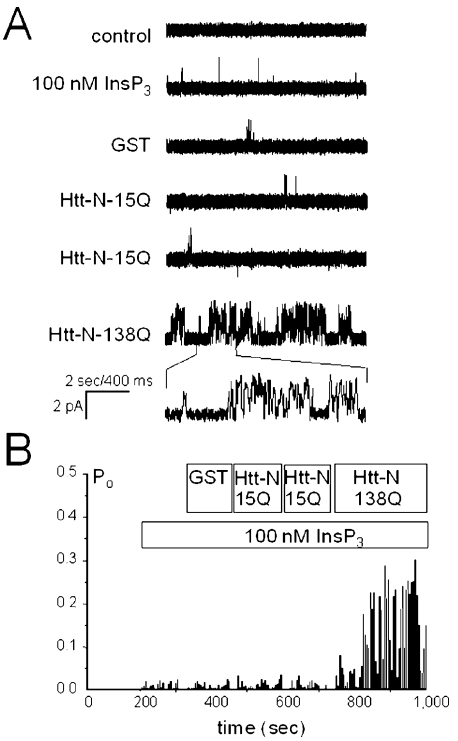

Figure 2. Htt^{exp} amino-terminal fragment sensitizes InsP$_3$R1 to activation by InsP$_3$ in planar lipid bilayers.
(A) Effects of GST, GST-Htt-N-15Q and GST-Htt-N-138Q on activity of recombinant InsP$_3$R1 in planar lipid bilayers at 100 nM InsP$_3$. Each current trace corresponds to 10 sec (2 sec for expanded traces) of current recording from the same experiment. (B) The average InsP$_3$R1 open probability (P_o) in the presence of 100 nM InsP$_3$ is calculated for a 5 sec window of time and plotted for the duration of an experiment. The time of InsP$_3$, GST, GST-Htt-N-15Q, and GST-Htt-N-138Q additions are shown. Adapted from (Tang et al., 2003)

(HAP1A) (Tang et al., 2003). In biochemical experiments, we demonstrated the formation of $InsP_3R1$-HAP1A-Htt ternary complex *in vitro* and *in vivo* (Tang et al., 2003).

What is an effect of Htt^{exp} mutation on ability of Huntingtin to associate with $InsP_3R1$? On $InsP_3R1$ function? In a series of pull-down experiments we discovered that mutant Htt^{exp}, but not wild type Htt, binds directly to the $InsP_3R1$ carboxy-termini (Figure 1). Furthemore, in planar lipid bilayer reconstitution experiments we demonstrated sensitization of $InsP_3R1$ to $InsP_3$ in the presence of Htt-138Q amino-terminal fragment (Figure 2a, 2b) or full-length Htt-82Q

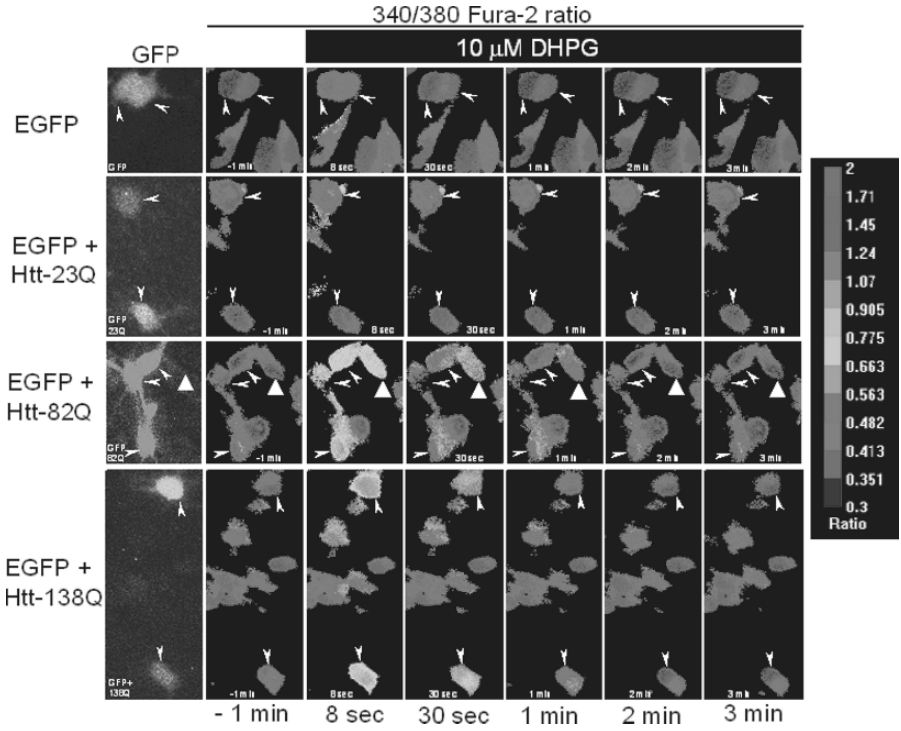

Figure 3. Htt^{exp} facilitates $InsP_3R1$-mediated Ca^{2+} release in cultured MSN.
The images show Fura-2 340/380 ratios in transfected rat MSN. Pseudocolor calibration scale for 340/380 ratios is shown on the right. The recordings were performed in Ca^{2+}-free ACSF containing 100 μM EGTA. GFP images (1st column) were captured before Ca^{2+} imaging to identify transfected cells (arrowheads). $InsP_3R1$-mediated Ca^{2+} release was initiated by addition of 10 μM DHPG, a specific mGluR1/5 agonist. Ratio recordings are shown for DHPG-induced Ca^{2+} transients in MSN neurons transfected with EGFP (first row), EGFP + Htt-23Q (second row), EGFP + Htt-82Q (third row), and EGFP + Htt-138Q (fourth row). The 340/380 ratio images are shown for MSN neurons 1 min before (2nd column), and 8 sec, 30 sec, 1 min, 2 min, and 3 min after application of 10 μM DHPG as indicated. Adapted from (Tang et al., 2003) (See Colour Plate 18)

(Tang et al., 2003). These effects were specific for Htt^{exp}, as the Htt-15Q amino-terminal fragment (Figure 2a, 2b) or full-length Htt-23Q (Tang et al., 2003) had no effect on InsP$_3$R1 sensitivity to InsP$_3$.

In experiments with primary cultures of rat MSN we demonstrated facilitation of InsP$_3$R1-mediated Ca^{2+} release in the presence of Htt-82Q and Htt-138Q proteins, but not in the presence of Htt-23Q protein (Figure 3). The ability of Htt^{exp} to sensitize InsP$_3$R1 to activation by InsP$_3$ correlated with ability of Htt^{exp}, but not Htt, to associate directly with InsP$_3$R1 carboxyl-terminal region (Tang et al., 2003). Thus, we reasoned that potentiating effect of Htt^{exp} on InsP$_3$R1-mediated Ca^{2+} release is due to direct association of Htt^{exp} with InsP$_3$R1 carboxyl-terminus.

From these results we proposed that upregulation of InsP$_3$R1 by Htt^{exp} may be a contributing factor to Ca^{2+} overload and degeneration of MSN in HD (Tang et al., 2003). MSN are highly enriched for mGluR5, a member of the group I mGluRs (Testa et al., 1995; Kerner et al., 1997; Tallaksen-Greene et al., 1998; Mao and Wang, 2001, 2002). Stimulation of group I mGluR in MSN leads to the generation of InsP$_3$ and release of Ca^{2+} (Figure 3). The alterations in ER enzymes that have been observed in HD postmortem brains (Cross et al., 1985) are consistent with malfunction of ER Ca^{2+} handling in HD MSN neurons.

3. HTT^{EXP} ACTIVATES NR2B-CONTAINING NMDA RECEPTORS

MSN abundantly express NR2B subtype of NMDA receptors (Monyer et al., 1994; Landwehrmeyer et al., 1995; Portera-Cailliau et al., 1996). In contrast to NMDA receptors containing NR2A subtype, NR2B-containing NMDA receptors have significant permeability for Ca^{2+} and activation of these receptors may have a dramatic effect on intracellular Ca^{2+} signals in MSN. Importantly, studies from Lynn Raymond's and Michael Hayden's laboratories suggested that expression of mutant Htt^{exp} protein facilitates activity of NR2B subtype of NMDAR receptors in a heterologous HEK293 cells expression system (Chen et al., 1999). Interestingly, the potentiating effect of Htt^{exp} was specific for the NR1/NR2B NMDAR subtype and not for the NR1/NR2A NMDAR subtype (Figures 4a, 4b). Using the same HEK293 cells expression system it was also demonstrated that cells co-transfected with NMDAR and Htt-138Q plasmids were more sensitive to NMDA-induced apoptosis than the cells co-transfected with NMDAR and Htt-15Q or GFP (control) plasmids (Zeron et al., 2001). Similar to effects on NMDAR currents (Figures 4a, 4b), potentiating effects of Htt-138Q on excitotoxic cell death were more pronounced in the presence of the NR1/NR2B NMDAR subunit combination than in the presence of the NR1/NR2A subunit combination (Zeron et al., 2001).

Further support for the potentiating effects of Htt^{exp} on NMDAR activity was obtained by Lynn Raymond's and Michael Hayden's laboratories in the analysis of YAC72 HD mouse model (Hodgson et al., 1999). NMDA-evoked currents (Figure 4c, 4d) (Zeron et al., 2002) and NMDA-mediated Ca^{2+} transients (Zeron et al., 2004) were significantly increased in striatal neurons from YAC72 mouse when compared to wild type controls. Consistent with the HEK293 cells expression

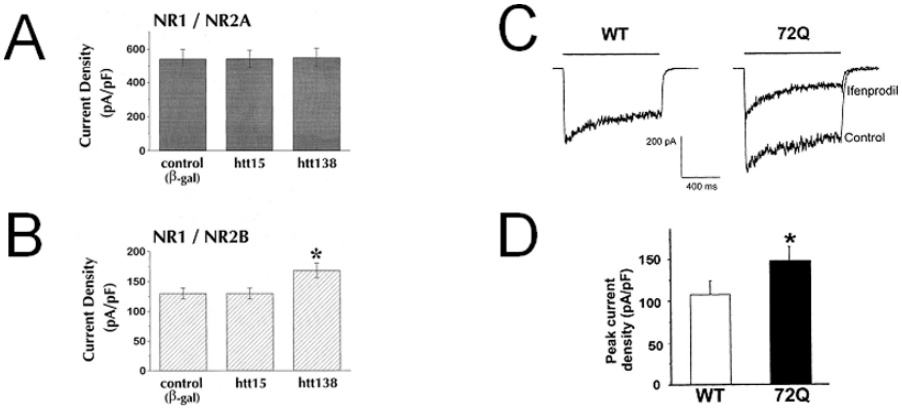

Figure 4. NR2B NMDAR are upregulated by Htt^{exp}.
(A). A combination of NR1 and NR2A NMDAR subunits was co-transfected into HEK293 cells together with β-gal plasmid (control), Htt-15Q, or Htt-138Q plasmids. The NMDA-induced currents in transfected cells were measured by whole-cell recordings and normalized to cell capacity. No significant differences in the size of NMDA-evoked currents were observed between 3 groups of cells. (B). A combination of NR1 and NRB NMDAR subunits was co-transfected into HEK293 cells together with β-gal plasmid (control), Htt-15Q, or Htt-138Q plasmids. The NMDA-induced currents in transfected cells were measured by whole-cell and normalized to cell capacity. The size of NMDA-evoked current is significantly higher in cells co-transfected with Htt-138Q (asterisk) than in other 2 groups of cells. (C) The size of NMDA-evoked current in primary MSN from non-transgenic (WT) and YAC72Q (72Q) mouse. Inhibition of NMDA-evoked current by ifenrpodil is shown for YAC72 mouse MSN.(D). An average NMDA-evoked peak current density in MSN from non-transgenic (WT) and YAC72 (72Q) mouse. Panels A and B are adapted from (Chen et al., 1999). Panels C and D are adapted from (Zeron et al., 2002)

data (Figures 4a, 4b), the NMDAR currents in striatal neurons potentiated in the presence of YAC72 transgene were selectively blocked by NR1/NR2B-specific antagonist ifenprodil (Figure 4c).

How does Htt^{exp} activate NMDAR? Experiments in a heterologous expression system demonstrated that Htt binds to a modular adaptor protein PSD95 and that Htt^{exp} binds to PSD95 less strongly than Htt (Sun et al., 2001). The PDZ domains of PSD95 bind to the carboxy-terminal region of the NMDAR NR2 subunit. The association of PSD95 with the NR2 subunit leads to recruitment of Src tyrosine kinase, tyrosine phosphorylation of NMDAR and an increase in NMDAR currents (Ali and Salter, 2001). It was proposed that weakened association of Htt^{exp} with PSD95 increases the pool of PSD95 available for interactions with NR2 subunits, leading to hyperphosphorylation of NMDAR by Src kinase. Consistent with this hypothesis, tyrosine hyperphosphorylation of NR2B subunits was observed in a heterologous expression system in the presence of a Htt-48Q construct (Song et al., 2003). Moreover, inhibition of NR2B phosphorylation by the Src tyrosine kinase inhibitor SU6656 attenuated Htt-48Q-facilitated apoptotic cells death in rat hippocampal neuronal cell line HN33 (Song et al., 2003). Future experiments will be needed to determine if PSD95 and Src mediated pathway is responsible for

NMDAR potentiation by Htt^{exp} *in vivo*. An alternative hypothesis may involve direct or cytoskeleton-mediated effects of Htt^{exp} on NMDAR gating or changes in NMDAR surface expression and/or localization in the presence of Htt^{exp}.

4. CA^{2+} SIGNALING AND APOPTOSIS OF HD MSN

Several lines of evidence indicate that glutamate-mediated excitotoxicity plays a role in neurodegeneration of HD MSN. Striatal injection of kainic acid induced death of MSN and yielded one of the first animal models of HD (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976). Importantly, effects of kainate required presence of corticostriatal neurons (McGeer et al., 1978), suggesting that glutamate release is required for kainate-induced MSN cell death. More direct evidence for an involvement of NMDAR was obtained when HD-like lesions were observed following striatal injection of the NMDAR agonist quinolinic acid (Beal et al., 1986; Hantraye et al., 1990; Beal et al., 1991). Consistent with the excitotoxicity hypothesis, striatal neurons from YAC72 mouse were sensitized to neuronal death induced by quinolinic acid and NMDA (Zeron et al., 2002). Moreover, excitotoxic cell death of YAC72 MSN was blocked by ifenprodil (Zeron et al., 2002), supporting a direct involvement of NR1/NR2B NMDAR subtypes in HD.

Based on the results described above (sections 2 and 3) we previously suggested that overactivation of $InsP_3R1$-mediated Ca^{2+} release and NR2B-mediated Ca^{2+} influx in HD MSN may lead to Ca^{2+} overload and apoptosis of these neurons (Bezprozvanny and Hayden, 2004). To test this "Ca^{2+} hypothesis of HD" my laboratory recently used TUNEL assay to compare glutamate-induced apoptosis of MSN cultured from wild type mice and mice expressing mutant human Htt-128Q gene (YAC128 mouse (Slow et al., 2003)). The mice expressing normal human Htt-18Q gene (YAC18 (Hodgson et al., 1999)) was used as a control in these experiments. At 14 DIV all 3 groups of MSN were challenged by an 8 h application of glutamate (from 0 to 250 μM) to mimic physiological stimulation. Following exposure to glutamate, MSN were fixed, permeabilized and scored for apoptotic cell death using TUNEL staining. We determined that in basal conditions (no glutamate added) approximately 10% of MSN in all 3 experimental groups were apoptotic (TUNEL-positive) (Figures 5a, 5b). Addition of 25 μM or 50 μM glutamate increased the number of apoptotic cells to 15–20% in all 3 experimental groups (Figures 5a, 5b). Addition of 100 μM or 250 μM glutamate increased apoptotic death to 60–70% for YAC128 MSN (Figures 5a, 5b), but only to 25–30% for wild type and YAC18 MSN (Figures 5a, 5b). Thus we reasoned that exposure to glutamate concentrations in 100 – 250 μM range leads to selective apoptosis of YAC128 MSN (Tang et al., 2005).

The "*in vitro* HD" model described above (Figure 5) enabled us to test a connection between abnormal Ca^{2+} signaling and apoptosis of HD MSN. We found that inhibition of mGluR1/5 receptors (by a mixture of MPEP and CPCCOEt) reduced the glutamate-induced apoptosis of YAC128 MSN to WT MSN levels (Figure 6). NMDAR-inhibitor (+)MK801 or NR2B-specific antagonist ifenprodil

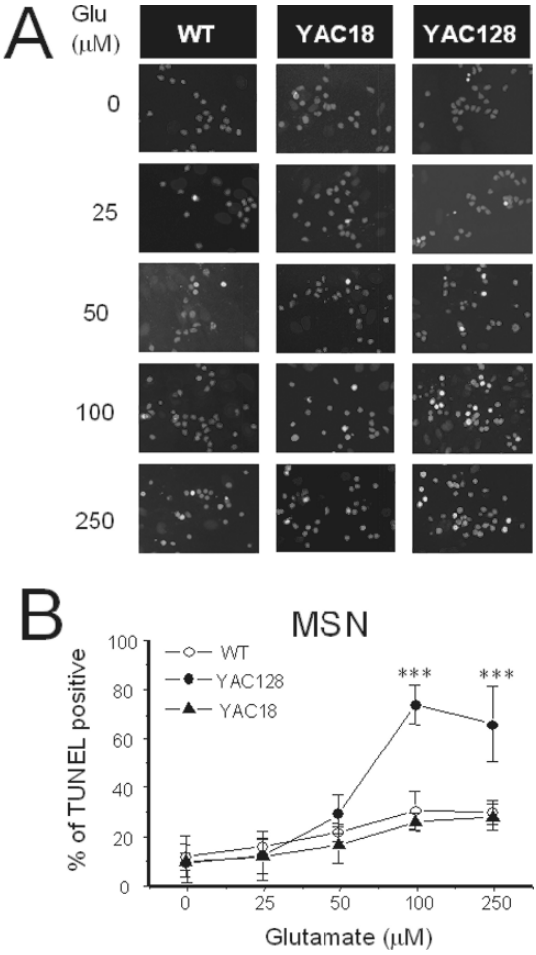

Figure 5. In vitro HD assay.
(A) 14 DIV MSN from wild type (WT), YAC18 and YAC128 mice were exposed to a range of glutamate concentrations for 8 h, fixed, permeabilized and analyzed by TUNEL staining (green) and propidium iodide counterstaining (PI). (B) The fraction of TUNEL-positive MSN nuclei was determined as shown on panel A and plotted against glutamate concentration for wild type (WT) (open circles), YAC128 (filled circles), and YAC18 (filled triangles) mice. At each glutamate concentration the data are shown as mean ± SD (n = 4–6 microscopic fields, 200–300 MSN per field). At 100 μM and 250 μM glutamate the fraction of TUNEL-positive MSN is significantly ($p < 0.05$) higher for YAC128 than for WT or YAC18. Similar results were obtained with 10 independent MSN preparations. Adapted from (Tang et al. 2005) (See Colour Plate 19)

had similar neuroprotective effects (Figure 6). Consistent with direct involvement of $InsP_3R1$, preincubation of the MSN cultures with a membrane-permeable $InsP_3R$ blocker 2-APB (Maruyama et al., 1997) protected YAC128 MSN from glutamate-induced apoptosis (Figure 6). All these results supported an idea that

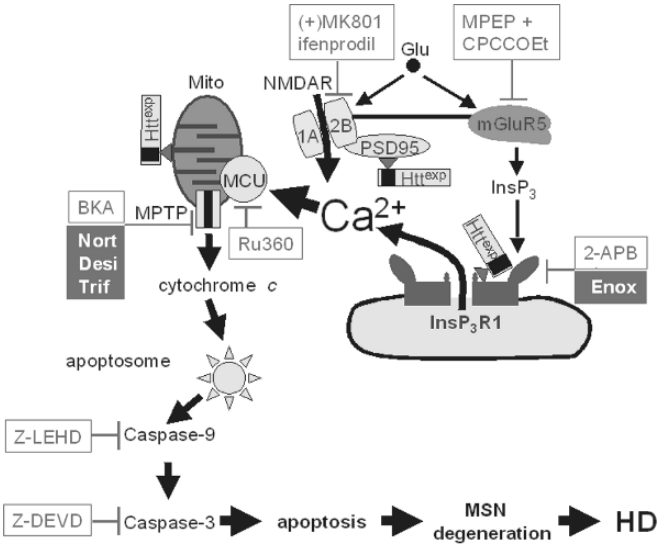

Figure 6. Ca^{2+} hypothesis of HD.
Glutamate released from corticostriatal projection neurons stimulates NR1A/NR2B NMDAR and mGluR5 receptors abundantly expressed in striatal MSN (Landwehrmeyer et al., 1995; Testa et al., 1995). Htt^{exp} affects Ca^{2+} signaling in HD MSN by sensitizing InsP$_3$R1 to activation by InsP$_3$ (Tang et al., 2003), stimulating NR2B/NR1 NMDAR activity (Chen et al., 1999; Sun et al., 2001; Zeron et al., 2002), and destabilizing mitochondrial Ca^{2+} handling (Panov et al., 2002; Choo et al., 2004). As a result, stimulation of glutamate receptors leads to supranormal Ca^{2+} responses in HD MSN and mitochondrial Ca^{2+} overload. Once mitochondrial Ca^{2+} storage capacity is exceeded, mitochondrial permeability transition pore (MPTP) opens, leading to release of cytochrome c into the cytosol and activation of caspases 9 and 3. Activation of caspase-3 leads to progression of apoptosis, MSN degeneration and HD. The model is supported by ability of blockers (shown in red) to reduce glutamate-induced apoptosis of YAC128 MSN to wild type levels in our experiments. The blockers which were effective in our experiments are: NMDAR blocker (+)MK801 and NR2B-specific blocker ifenprodil; mGluR1/5-specific blockers MPEP and CPCCOEt; membrane-permeable InsP$_3$R1 blockers 2-APB and Enoxaparin; MCU blocker Ru360, MPTP blockers BKA, Nortriptyline, Desipramine and Trifluoperazine, membrane-permeable caspase-9 blocker Z-LEHD-FMK and caspase-3 blocker Z-DEVD-FMK. Adapted from (Tang et al., 2005) (See Colour Plate 20)

glutamate-induced Ca^{2+} overload plays a key role in induction of apoptotic cell death of HD MSN.

How do supranormal Ca^{2+} signals induce apoptosis of HD MSN? The best known link between Ca^{2+} overload and apoptosis involves mitochondrial Ca^{2+} overload and activation of intrinsic apoptotic pathway (Choi, 1995; Hajnoczky et al., 2003; Orrenius et al., 2003; Rizzuto et al., 2003). Consistent with this idea, we found that glutamate-induced apoptosis of YAC128 MSN in our experiments can be prevented by Ruthenium 360 (Ru360), an inhibitor of mitochondrial Ca^{2+} uniporter/channel (MCU) (Figure 6).

The observation of dysfunctional mitochondria in HD mouse models and in HD patients (Panov et al., 2002; Choo et al., 2004) provides further support

to mitochondrial involvement in HD pathogenesis. We further found that the glutamate-induced apoptosis of YAC128 MSN was prevented by mitochondrial permeability transition pore (MPTP) inhibitor bongkrekic acid (BKA) and by memrbane permeable inhibitors of caspases-9 and 3 (Figure 6). These data support a model that links Htt^{exp} mutation, abnormal Ca^{2+} signaling and apoptosis of HD MSN (Figure 6) (Tang et al., 2005). Many similar conclusions have been reached in the studies of NMDA-induced apoptosis of YAC72 and YAC128 MSN performed recently by Lynn Raymond's laboratory (Zeron et al., 2004; Shehadeh et al., 2005, 2006).

5. CA^{2+}-RELATED TARGETS AND TREATMENT OF HD

Despite efforts by many laboratories and cloning of Huntingtin in 1993, there is still no cure for HD. The proposed model (Figure 6) suggests that Ca^{2+} signaling blockers, such as NR2B-specific inhibitors of NMDAR and blockers of mGluR5 and $InsP_3R1$, may be beneficial for the treatment of HD. Inhibitors of Htt^{exp} association with $InsP_3R1$ may potentially be used as a more specific HD therapeutic. These concepts are currently being tested in my laboratory. When compared to inhibitors of apoptosis an advantage of using Ca^{2+} signaling blockers and inhibitors of $InsP_3R1$-Htt^{exp} association is that they may stop pathological process at its earliest point, before severe neuronal dysfunction triggers apoptotic cell death. In our recent study we demonstrated that clinically relevant NMDA receptor inhibitor memantine protected YAC128 MSN from glutamate-induced apoptosis in "in vitro HD" model (Wu et al., 2006). Interestingly, a 2-year-long human clinical study suggests that memantine also has an ability to retard the progression of HD based on observed UHDRS scores (Beister et al., 2004). Further evaluation of memantine and other clinically-relevant Ca^{2+} inhibitors will be required to establish if Ca^{2+} pathway constitute a useful target for treatment of HD.

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