MUTANT HUNTINGTIN ENHANCED SENSITIVITY TO N-METHYL-D-ASPARTATE RECEPTOR-MEDIATED EXCITOTOXICITY

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B. Sc., The University of British Columbia, 1997

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT

FOR THE DEGREE OF

DOCTOR   OF PHILOSOPHY

In

THE FACULTY OF GRADUATE STUDIES

Graduate Program in Neuroscience

We accept this thesis as conforming to the required standards

The University of British Columbia

2002

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Date __April 25/02__

ABSTRACT

Previous work suggests N-methyl-D-aspartate receptor (NMDAR) activation may be involved in degeneration of medium spiny striatal neurons in Huntington's disease (HD). Here, we determined whether expression of huntingtin (htt) containing the polyglutamine expansion augments NMDAR-mediated excitotoxicity. First, human embryonic kidney (HEK) 293 cells co-expressing mutant huntingtin (htt-138Q) and either NR1A/NR2A- or NR1A/NR2B-type NMDARs exposed to 1 mM NMDA showed a significant increase in excitotoxic cell death compared to controls [cells co-expressing htt-15Q or green fluorescent protein (GFP)], but the difference was larger for NR1A/NR2B. Moreover, agonist-dependent cell death showed apoptotic features for cells co-expressing htt-138Q and NR1A/NR2B, but not for cells expressing htt-138Q and NR1A/NR2A. Furthermore, NR1A/NR2B-mediated apoptosis was not seen with co-expression of an N-terminal fragment of mutant htt. Since NR1A/NR2B is the predominant NMDAR subtype in neostriatal medium-sized spiny neurons (MSNs), we hypothesized that enhancement of NMDA-induced apoptotic death in NR1A/NR2B-expressing cells by full-length mutant htt may contribute to selective neurodegeneration in HD. To test this hypothesis we compared NMDAR-induced cell death in striatal neurons from a yeast artificial chromosome (YAC) transgenic mouse model of HD expressing full-length mutant huntingtin with striatal neurons from the same strain of wild-type mice. Excitotoxic death of MSNs from the transgenic mice was increased after NMDA but not AMPA. NMDAR-mediated cell death was completely blocked by the NR2B subtype-selective antagonist ifenprodil, and was also associated with increased caspase-3 activity relative to wildtype (WT) MSNs. Importantly, there was no enhancement of NMDAR-mediated cell death in cerebellar granule neurons expressing mutant huntingtin, demonstrating cell type specificity and also consistent with NMDAR subtype specificity. Although caspase-3 can cleave huntingtin to form smaller fragments we did not observe an increase in the proteolysis of huntingtin after exposure to NMDA in WT or mutant htt

expressing striatal cultures. Enhanced mitochondrial membrane depolarization and intracellular calcium levels were observed upon NMDAR application in MSNs expressing mutant htt. NMDAR excitotoxicity was partially inhibited by application of cyclosporin A in MSNs, and inhibition was greater in MSNs from transgenic mice than WT mice. Together, our data support a role for NR2B-subtype NMDAR activation as an upstream trigger for mitochondrial dysfunction and caspase-3 activation. These results may help explain selective neuronal degeneration in HD.

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LIST OF ABBREVIATIONS

A	Agonist
aa	Amino acid
ABC	Avidin-biotin-HRP complex
AFC	7-amino-4-trifluoromethyl coumarin
AIF	Apoptosis-inducing factor
AM	Acetoxymethyl ester
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AMPAR	AMPA receptor
ANT	Adenine nucleotide translocase
Apaf-1	Apoptosis protease-activating factor 1
APV	(+)-2-amino-5-phosphonopentanoic acid
AT	Adenine and thymine nucleotide base pairs
ATP	Adenosine triphosphate
β-gal	β-galactosidase
BCA	Bicinchoninic acid
BES	N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
BSA	Bovine serum albumin
BSS	Balanced salt solution
C	Control
$^\circ$C	Degrees centigrade
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine 5'-monophosphate

CCD	Charge-coupled devices
CCP	Carbonyl cyanide m-chlorophenylhydrazone
cDNA	Complementary deoxyribonucleic acid
CGNs	Cerebellar granule neurons
Chapsyn 110	Channel-associated proteins of the synapses-110
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CNS	Central nervous system
Cyto	Cytoplasm
DAB	3,3'-diaminobenzidine
DARPP-32	Dopamine- and cyclic adenosine 3', 5'-monophosphate-regulated Phosphoprotein, 32 kDa
dATP	Deoxyadenosine triphosphate
DED	Death effector domain
DIV	Days *in vitro*
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EAAs	Excitatory amino acids
EC_{50}	Effective concentration to achieve 50 percent maximal response
ECL	Enhanced chemiluminescence system
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis[β-aminoethyl ether]-N,N,N'N'-tetraacetic acid
EtOH	Ethanol
FITC	Fluorescein
FBS	Fetal bovine serum

GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GluR	Glutamate receptor
h (or hr)	Hour
HAP-1	Huntingtin-associated protein-1
HBSS	Hank's balanced salt solution
HD	Huntington Disease
HEK293 cells	Human embryonic kidney 293 cells
HEPES	4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid
HIP-1	Huntingtin-interacting protein-1
Hippi	HIP-1 protein interactor
hippo	Hippocampus
HRP	Horse radish peroxidase
HRPT	Hypoxanthine phosphoribosyltransferase
HSP	Heat shock protein
htt	Huntingtin
I	Inhibitor
ICAD	Inhibitor of caspase-activated DNAase
IFN	Ifenprodil
iGluR	Ionotropic glutamate receptor
K^+	Potassium
KCl	Potassium chloride
K_d	Dissociation constant

KA	Kainic acid
KAR	KA receptor
Da	Daltons
M	Molar
$MgCl_2$	Magnesium chloride
Mem	Membrane
MEM	Minimum essential medium
mGluR	Metabotropic glutamate receptor
MK-801	Dizocilpine or (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
mL	Milliliter
μL	Microliter
mM	Millimolar
μM	Micromolar
MMP	Mitochondrial membrane potential ($\Delta\psi_m$)
mRNA	Messenger ribonucleic acid
ms	Milliseconds
MSNs	GABAergic medium-sized spiny striatal neurons
mV	Millivolts
Na^+	Sodium
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	Sodium phosphate, dibasic
NaH_2PO_4	Sodium phosphate, monobasic
$NaHCO_3$	Sodium bicarbonate

NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NR1	NMDA receptor subunit-1
NR2	NMDA receptor subunit-2
ONPG	o-nitrophenyl-β-D-galactopyranosidase
P	Postnatal day
PARP	Poly(ADP-ribose) polymerase
PI	Propidium Iodide
PKC	Protein kinase C
PBS	Phosphate-buffered saline
PDZ domain	**PSD/D**iscs-large/**ZO**-1 domain
PMSF	Phenylmethylsulfonyl fluoride
PolyQ	Polyglutamine expansion
PSD-95	Postsynaptic density-95
PTP	Permeability transition pore
PVDF	Polyvinylidene difluoride
Q	PolyQ repeats
Rho-123	Rhodamine-123
ROIs	Regions of interest
SAP-102	Synapse-associated protein-102
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	Seconds
SEM	Standard error of the mean

SH3	Src homology 3
stauro	Staurosporine
TBS	Tris-buffered saline
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP-fluorescein nick end labelling
VDAC	Voltage-dependent anion channels
wk	Week
WT	Wild-type (FVB/N mice)
YAC	Yeast artificial chromosome

ACKNOWLEDGEMENTS

I would first like to thank Dr. Lynn Raymond, who is the finest illustration of a clinician-scientist and all-around human being I have known. Her knowledge, commitment, supportiveness, and open-mindedness have played a major role in my success in her laboratory. I hope to live by her extraordinary example and flourish as a future clinician-scientist. I would like to thank all the past and present members of our laboratory who have certainly demonstrated how well a group can cooperate and work together. Included in this group are; Dr. Nansheng Chen, Tao Luo, Dr. Chris Price, Dr. Claudia Krebs, Bo Li, Jackie "Sunshine" Shehadeh, all of whom have provided invaluable insights and selfless assistance. Drs. M.R. Hayden, Blair Leavitt, Roshni Singarari and, most of all, Cheryl L. Wellington, have been extraordinary and inspirational collaborators and friends. Without their assistance this work would not be possible. Drs. K.G. Baimbridge and G. Rintoul have been wonderful collaborators and provided immense assistance. Drs. Timothy H. Murphy and Steve R. Vincent have shared immeasurable knowledge, guidance and quality control for my project and it has been pleasurable working with them. I would also like to thank the members of my supervisory committee, Drs. Wolfram Tetzlaff and Jane Roskams, for their helpful insights and guidance. Especially to Dr. Wolfram Tetzlaff, for encouraging my interest to pursue a career in research. Dr. Fred Lam, has been a remarkable inspiration and an extraordinarily generous and caring friend. I am very lucky to have you in my life. Dr. Claudia Krebs, for being a great collaborator and a true friend. You have always had my best interests at heart. My friends Toby and Crystal Proctor, as well as being my biggest fans, have shown me the true meaning of life and love. My parents, brother and the rest of my family who have shown me immense love, understanding and support, especially through rough times. Joe Choi, you have always been a great friend and supporter. You have given me some exceptional opportunities in life. Thanks to all the members of my defense committee, Drs. Ian J. Reynolds, John Church, Jane Roskams, Jon Stoessl, and David

CHAPTER 1

Introduction

1.1 Huntington Disease

Huntington Disease (HD) is characterized by motor impairment, cognitive decline and emotional and neuropsychiatric disorders (Harper, 1991). HD is one of eight currently known progressive neurodegenerative diseases identified as a CAG triplet repeat disorder, each of which have unique regions of neuronal degeneration (reviewed by Sieradzan and Mann, 2001). HD prevalence is estimated to be between 5 and 10 per 100, 000 persons (Vonsattel and DiFiglia, 1998). In HD, an unstable CAG expansion in exon 1 of the IT15 gene of chromosome 4p1.6 translates into an expansion (>35) of the polyglutamine (polyQ) tract in the N-terminal region of the protein, huntingtin (htt), found to cause the disease (HDCRG, 1993). This molecular defect leads to dramatic loss of medium-sized spiny GABAergic projection neurons (MSNs) of the caudate and putamen nuclei, as well as less profound loss in the rest of the basal ganglia and selected neuronal populations in other brain regions such as cortical layers III, V and VI and certain subcortical structures (Vonsattel and DiFiglia, 1998).

The full length protein with >35 polyQ repeats is found to cause the disease pathology and repeat lengths of 36-180 have been associated with the phenotype (Kremer et al., 1994; Rubinsztein et al., 1996; Sathasivam et al., 1997). A correlation has been demonstrated between the length of the CAG repeat and the onset of HD; a larger CAG repeat is associated with an earlier disease onset (Andrew et al., 1993; Brinkman et al., 1997; Sieradzan et al., 1997). Adult onset HD with CAG repeats <60 usually presents in middle life, normally ~39 years of age, and progresses for ~15-20 years until death (Harper 1991). Juvenile onset HD is associated with CAG repeats of >60 and presents differently, with little or no chorea, akinetic-rigid syndrome, seizures or dementia, usually with accelerated clinical progression (Brandt et al., 1996). In adult

onset HD, disease duration and type of presentation seem to be independent of the CAG number (Sieradzan et al., 1997). In normal and unaffected subjects, the CAG segment of the HD gene is unstable and undergoes changes in meioses leading to alteration in CAG length--both increases and decreases--and the largest increases are seen in paternal transmission (reviewed by Vonsattel and DiFiglia, 1998). In this chapter, I intend to review the current findings on physiology and molecular biology of HD pathology relevant to this study.

1.1.1 Selective Neurodegeneration in Huntington Disease

Degeneration of the neostriatum in HD has been extensively described (Vonsattel et al., 1985). Using a five-point grading system, correlating closely with the extent of clinical disability, they assessed postmortem HD brains macroscopically and microscopically, grading the ascending order of severity of degeneration as grades 0-4. They observed the highest severity of degeneration in the caudate and putamen, especially the dorsal-medial-caudal regions (Vonsattel et al., 1985). In grade 1 brains >50% of neurons in the caudate were lost and astrogliosis was notable (Vonsattel et al., 1985). In grade 3 brain there was notable neuronal loss in the putamen and globus pallidus as well (Vonsattel et al., 1985). In grade 4, >95% of neurons were lost from all regions of the striatum, including the caudate, putamen, globus pallidus and nucleus accumbens, and a 28% increase in number of astrocytes was observed (Vonsattel et al., 1985).

HD preferentially affects the principal input and projection neurons, the medium-sized GABAergic striatal neurons (MSNs), which comprise 95% of neurons in the neostriatum (Surmeier et al., 1988) and are the focus of our studies. The enkephalin and D2 dopamine receptor-expressing 'indirect' pathway neurons projecting to the external globus pallidus are affected earlier than the substance P and D1 dopamine receptor 'direct' pathway neurons projecting to the internal globus pallidus (reviewed by Sieradzan and Mann, 2001). In contrast,

large aspiny cholinergic interneurons, medium aspiny NADPH-diaphorase/somatostatin/neuropeptide Y/nitric oxide synthase and parvalbumin interneurons are spared (reviewed by Sieradzan and Mann, 2001) in the striatum. There is only a 30% reduction in neocortical regions including the associative frontal, temporal and parietal, and primary somatosensory cortices (reviewed by Sieradzan and Mann, 2001). The large pyramidal neurons of cortical layers III, V and VI that project directly into the striatum are selectively lost, but this is only seen in grade 4 brains (reviewed by Sieradzan and Mann, 2001). In grades 3 and 4, atrophy has also been found in the thalamus, subthalamic nucleus, substantia nigra, white matter, amygdala, and to a lesser extent, the hippocampus; in this region 35% of the CA1 pyramidal neurons are lost (Sieradzan and Mann, 2001; Spargo et al., 1993; Vonsattel and DiFiglia, 1998).

1.1.2 Huntingtin is Widely Expressed with No Known Function

It is interesting that htt, and its known interacting proteins, are widely expressed throughout the central nervous system (Ross, 1995; Nance, 1997) and expression levels are not particularly increased in MSNs (DiFiglia et al., 1995; Fusco et al., 1999; Li et al., 1993). It is unknown why MSNs are particularly targeted for degeneration in HD. The normal function of the non-pathogenic or normal htt protein is also unknown, although there is evidence to suggest it is involved in vesicular trafficking (DiFiglia et al., 1995; Sharp et al., 1995; Wood et al., 1996). It is also required during early embryonic development, since HD gene knock out mice die at E7.5-8.5 (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). In addition, recent data show that normal huntingtin appears to exert an anti-apoptotic effect *in vitro* after serum deprivation or metabolic stress (Rigamonti et al., 2000) or *in vivo* in normal HD gene mouse models after excitotoxic challenge [Y. Zhang and R.M Friedlander (personal communication)] or in mutant htt mice with increased human wild-type htt levels (Leavitt et al., 2001).

One well-accepted hypothesis to explain selective neuronal degeneration is that the polyQ expansion triggers a toxic gain-of-function of htt through altered interactions with cellular proteins that are selectively expressed in particular neuronal populations (Ross, 1995). This has been proposed because HD shows autosomal dominant inheritance. Also, a case has been reported in which one copy of the HD gene was inactivated by a translocation but the individual did not have HD symptoms regardless of having only 50 % of normal HD gene expression (Ambrose et al., 1994; Persichetti et al., 1996). Furthermore, the null mutation of the HD gene in mice is embryonically lethal (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995), whereas in humans homozygous mutation of the HD gene results in HD and not embryonic lethality (Gusella and MacDonald, 1996; Myers et al., 1989; Wexler et al., 1987). Finally, humans homozygous or heterozygous for the HD mutation show no significant differences in disease onset or progression (Gusella and MacDonald, 1996; Myers et al., 1989; Wexler et al., 1987). In summary, evidence suggests that in addition to a toxic gain-of-function of mutant htt, a parallel depletion of normal htt, i.e. through proteolysis [Y. Zhang and R.M. Friedlander (personal communication)], may result in a detrimental loss-of-function, adding to the degenerative effects in the striatum.

1.1.3 Huntingtin Forms Intracellular Aggregates

Another interesting characteristic of HD, and most other CAG triplet repeat disorders, is the presence of protein inclusions and aggregates found in the cytoplasm, perinuclear region and nucleus (reviewed by Sieradzan and Mann, 2001). Nuclear inclusions have become a characteristic phenotype of HD, as well as other polyQ repeat disorders. N-terminal fragments of htt that include the polyQ tract may be formed by caspase-mediated cleavage of the full-length htt protein and lead to protein aggregation; these fragments have been reported in brains of HD patients, cell lines, and transgenic mice, and are proposed to play a role in pathogenesis of HD

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(Davies et al., 1997; DiFiglia et al., 1997; Mangiarini et al., 1996; reviewed by Wellington et al., 1997). However, upstream processes which may trigger caspase activation, htt cleavage, and cell death remain uncharacterized, and it is still unknown how MSNs are specifically targeted for degeneration in HD.

1.2 Huntingtin and Its Interacting Proteins

The full length HD gene encodes the ~350 kDa protein, htt, and contains a CAG repeat in exon 1 (HDCRG 1993) encoding an N-terminal polyglutamine (polyQ). Htt is cytoplasmic, found in dendrites, cell bodies and terminals, and is ubiquitously expressed in the central nervous system and not enriched in the striatum (DiFiglia et al., 1995; Ferrante et al., 1997; Li et al., 1993; Sharp et al., 1995). In the striatum, htt immunoreactivity is weak in the striosomes and evident in the matrix, and variable in staining intensity among MSNs (Ferrante et al., 1997). Furthermore, there is no disparity in htt immunoreactivity between all striatal neuron types (Kumar et al., 1997), demonstrating that differential htt expression is not the cause for selective degeneration of MSNs. Mutant and normal htt co-distribute in all regions and in both gray and white matter (Aronin et al., 1995). Htt associates with vesicular and trans-Golgi-network membranes and microtubules and therefore may play a role in vesicle transport. (DiFiglia et al., 1995; Velier et al., 1998). The normal function of htt is unknown but complete gene knock-out results in embryonic lethality and heterozygous knock-out mice (+/-) have varying phenotypes, which are not like the clinical and pathological features of HD (Duyao et al., 1995; Nasir et al., 1995; White et al., 1997; Zeitlin et al., 1995). These studies and genetic studies in humans suggest that the polyQ expansion triggers a toxic gain-of-function, perhaps through altered interactions with cellular proteins, leading to cellular dysfunction and death.

1.2.1 Functional Role of Huntingtin Fragments

Due to the observation of widespread aggregation of truncated htt containing the expanded polyQ, there has been much speculation as to the importance of the polyQ in the context of its expression within the entire full-length htt protein or within N-terminal fragments of htt. Initially it was thought that the role of the rest of the htt protein was to simply modulate a pathological process driven by the polyQ tract. It was demonstrated that the polyQ itself is toxic to living cells, since a large CAG repeat ectopically expressed in the hypoxanthine phosphoribosyltransferase (HRPT) mouse gene causes formation of widespread neuronal inclusions, and a progressive neurological phenotype leading to early death (Ordway et al., 1997). However, these observations are exaggerated when the polyQ expansion is expressed in the context of the HD protein. Initial experiments *in vitro* and *in vivo* in mice (R6/2 mice) analyzed effects of the polyQ, within the N-terminal region of a truncated htt protein (Mangiarini et al., 1996). There were substantially more neuronal inclusions and a progressive neurological phenotype more characteristic of HD in these mice indicating the importance of the polyQ expansion expressed within the context of the N-terminal region of htt (Mangiarini et al., 1996; reviewed by Reddy et al., 1998b). Most importantly, it has been shown that the expression of the polyQ within the context of the entire full-length htt protein provides *in vivo* mouse models with characteristics most closely resembling HD pathology and behaviour (Hodgson et al., 1999; Reddy et al., 1998a). Therefore, use of mouse models of HD have provided clues as to the importance of the presence of the entire full-length htt gene containing the CAG repeat in the pathogenesis of HD.

It still remains controversial whether the entire htt gene containing the CAG repeat is necessary to fully explain HD pathogenesis and selective vulnerability but it seems clear that the N-terminal portion of htt containing the polyQ expansion found in HD brains and in mice models may be responsible for increased cellular toxicity and accelerated pathology of HD (reviewed by Wellington and Hayden, 2000). It has been shown *in vitro* that full-length mutant htt can be

cleaved into shorter fragments, containing the N-terminal with the polyQ expansion. Such fragments are found to enhance cells' vulnerability to apoptosis (Cooper et al., 1998; Hackam et al., 1998; Li et al., 1999; Martindale et al., 1998) *in vitro*. *In vivo* studies confirm that HD brains express N-terminal fragments of htt recognized with an antibody raised against the N-terminal region of htt, but not with an antiserum directed to an internal site of htt downstream of the polyQ (Gourfinkel-An et al., 1997; Sapp et al., 1997). Again, these data show indirect evidence for the importance that N-terminal fragments may play in HD pathogenesis.

Contrary to this evidence, regional expression patterns for N-terminal htt fragments and aggregates show no correlation to the pattern of selective neuronal loss in HD. The incidence of nuclear inclusions and dystrophic neurites is found to be polyQ-length dependent - found in 38-52 % of neurons in juvenile-onset HD cases and in only 3-6 % of neurons in adult-onset HD cases (Vonsattel and DiFiglia, 1998). Also, there is no correlation of nuclear inclusions with affected regions in HD; nuclear inclusions are found in only 1-4 % of striatal neurons in human HD cases and of those, only 4 % of nuclear inclusions in the striatum are found in the MSNs (Fusco et al., 1999; Gutekunst et al., 1998; Kuemmerle et al., 1999; Sieradzan et al., 1999).

The truncation of htt may be mediated in part by caspase-3 and –6, as well as calpains [Goldberg et al., 1996; Kim et al., 2001; Wellington et al., 2000; Gafni and Ellerby, 2002; C.L Wellington and M.R. Hayden (personal communication)]. From this evidence, we can say that protease activation and cleavage of htt may be important to neuronal death in HD but the molecular triggers of protease activation and means of selective targeting of the MSNs remain unknown. It is likely that multiple pathways are involved to explain the exquisite degeneration of the MSNs but there are many clues that point to particular protein candidates that are selectively expressed in this neuronal population that may co-operate to cause the pathology of the disease.

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1.2.2 Functional Role of Huntingtin Aggregates

It is thought though that when truncated htt, a unique 40 kDa fragment (DiFiglia et al., 1997), translocates to the nucleus it is specifically found to be toxic (Gutekunst et al., 1999; Hodgson et al., 1999; Kim et al., 1999; Li et al., 1999; Saudou et al., 1998) and it remains controversial whether aggregation is necessary for cell death. Thus it is uncertain whether truncated htt containing the polyQ is more important to HD pathology in non-aggregated form or aggregated form. It remains unclear whether insoluble aggregates of htt have protective or degenerative properties, possibly affecting regulation of gene transcription, protein interactions, and protein transport within the nucleus and cytoplasm (Vonsattel and DiFiglia, 1998). Short fragments of htt have been shown to self-aggregate in cell lines, transgenic mice and HD brains (Davies et al., 1997; DiFiglia et al., 1997; Hackam et al., 1998; Martindale et al., 1998; Scherzinger et al., 1997). However, there is also evidence that some full-length htt is recruited into nuclear aggregates and that nuclear inclusions are not entirely made up of cleaved protein (DiFiglia et al., 1997; Persichetti et al., 1999; Martindale et al., 1998). There are a few mechanisms proposed to explain the tendency for mutant htt to aggregate. First, htt could undergo transglutamination, forming cross-links via isopeptide bonds (Green, 1993). Also, aggregation of htt could occur through SH3 domain-dependent mechanisms through the htt proline-rich motifs (Sittler et al., 1998). Furthermore, the polyQ regions could act as 'polar zippers', joining together through hydrogen bonds between their amide groups into stable hairpin structures forming pleated sheets of β-strands (Perutz et al., 1994).

The elongation of the polyQ track in htt may alter the configuration of the protein and hence modify its solubility by increasing misfolding or its interaction with other cellular proteins (Vonsattel and DiFiglia, 1998). It has been found that mutant htt may disrupt normal functions within the nucleus as the elongated polyQ track may disrupt the nuclear matrix, or interfere with gene transcription mechanisms, or sequester functionally important proteins in aggregates of

mutant htt (Sieradzan and Mann, 2001). Mutant htt has also been found to interact with the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), possibly leading to a loss of energy metabolism (Burke et al., 1996). Htt is found to be localized together with ubiquitin to neuronal intranuclear inclusions and dystrophic neurites in HD cortex and neostriatum, but not in the HD globus pallidus, cerebellum or control brains (Gourfinkel-An et al., 1997; Sapp et al., 1997). The co-distribution of ubiquitin with mutant htt in the aggregates suggests that ubiquitin-dependent proteolysis of mutant htt is incomplete (Vonsattel and DiFiglia, 1998). Furthermore, aggregates and nuclear inclusions have been shown to contain chaperone proteins (e.g., members of the heat shock protein family, such as HSP40 and HSP70), and components of the proteasome system, but there is no clear consensus regarding their role in the formation and clearance of htt aggregates (Sieradzan and Mann, 2001).

Overall, the importance of the protein context within which the polyQ expansion may be found has largely remained controversial. It remains uncertain whether N-terminal fragments of htt containing the polyQ may play a role in HD. Our studies will help delineate the role of truncated mutant htt in the pathogenesis of HD.

1.2.3 Huntingtin Interacting Proteins

Relevant to our studies is the identification of possible interactions that htt may have with cytoskeletal or membrane-associated proteins. It had been previously found through yeast two hybrid screens that the N-terminal region of htt interacts with ubiquitously expressed cytoskeletal proteins such as huntingtin-associated proteins (HAP-1, 2) and huntingtin-interacting proteins (HIP-1, 2, 3) in a polyQ length-dependent manner (Kalchman et al., 1997; Li et al., 1995). To date, no proteins have been found to interact with the C-terminal region of htt (Gusella and MacDonald, 1998). Also, due to its subcellular distribution, htt is thought to play a role in vesicle trafficking, as well as surface expression or function of membrane proteins (Bhide et al.,

1996; DiFiglia et al., 1995; Gutekunst et al., 1999; Sharp et al., 1995; Wood et al., 1996), via interactions with the cytoskeleton since both HAP- and HIP- molecules interact with vesicle- or membrane-associated proteins (Kalchman et al., 1997; Li et al., 1995; Wanker et al., 1997). Htt's associated proteins are also not particularly enriched in the MSNs (Gutekunst et al., 1998). Although the huntingtin protein is shown to interact with intracellular proteins and may play a role in membrane trafficking, vesicle transport, and/or cytoskeletal functions, the mechanistic intracellular pathway through which the protein acts to specifically target the death of the MSNs has not been elucidated.

HIP-1 is a particularly interesting htt partner for several reasons. HIP-1 mRNA has been found to be ubiquitously expressed, but HIP-1 protein expression was only found in the brain, mostly in the cortex, and less in the cerebellum and striatum (Kalchman et al., 1997; Wanker et al., 1997). Furthermore, subcellular fractionation revealed colocalization of HIP-1 and htt in neuronal compartments (Kalchman et al., 1997). The C-terminus of HIP-1 shares homology with talin, a mammalian membrane cytoskeletal-associated protein involved in cell-cell interactions (Kalchman et al., 1997; Ross and Gilliland, 1999). HIP-1 also contains a novel death effector domain (DED), thought to provide the molecular basis of HIP-1 toxicity (Hackam et al., 2000). Recently, HIP-1 has been shown bind to a protein named HIP-1 protein interactor (Hippi) through similar DED domains, forming pro-apoptotic Hippi-HIP-1 heterodimers, capable of recruiting procaspase-8 and launching apoptosis through a caspase-dependent pathway (Gervais et al., 2002; see 1.6.2 for discussion on caspase-dependent cell death). There is no known function of HIP-1, although it is 45 % homologous to Sla2p, an actin-binding protein family member in yeast (Hackam et al., 2000; Kalchman et al., 1997). Sla2p is a peripheral membrane protein participating in actin organization, endocytosis, and required for the functioning of the cytoskeleton in yeast (Engqvist-Goldstein et al., 1999; Yang et al., 1999). Since the binding of HIP-1 and htt is inversely correlated to the length of the polyQ, there may be an impairment of

the membrane cytoskeletal function in HD (Kalchman et al., 1997). HIP-1 remains an intriguing potential interacting protein partner for htt that remains largely unexplored.

Interestingly, a recent study showed a direct interaction between htt and postsynaptic density protein-95 (PSD-95) (Sun et al., 2001). PSD-95 and its family members have been shown previously to participate in the clustering of ion channels and their attachment to intracellular signaling molecules at synapses (Firestein et al., 1999). Relevant to this thesis, PSD-95 and members of the PSD-95 family have been shown to bind directly to the NR2 subunit of the N-methyl-D-aspartate receptor (Brenman et al., 1996; Muller et al., 1996; see section 1.4.3 below). As well, PSD-95 family members can also bind to the actin cytoskeleton (Allison et al., 1998). PSD-95 has a type II SH3 domain which can bind to the N-terminal proline-rich region adjacent to the polyQ domain of normal htt (Sun et al., 2001). When the polyQ is expanded, mutant htt and PSD-95 fail to interact, thereby freeing more intracellular PSD-95 in the cytoplasm which could increase ion channel clustering and the efficiency of signal transduction (Sun et al., 2001).

1.3 Model Systems of HD

1.3.1 HEK293 Cell Lines

As in any study, one needs somewhere to begin to study their hypothesis, preferably a simple model. HEK293 cells are a homogenous cell population that can be manipulated easily. HEK293 cells are effective to examine toxicity, ion channel properties, and protein overexpression (Anegawa et al., 1995; Boeckman and Aizenman, 1996; Cik et al., 1993; Raymond et al., 1996). Heteromeric NMDAR complexes of NR1A and NR2A or NR2B expressed in HEK293 cells show agonist-evoked current amplitudes and biophysical properties similar to those found in neurons (Chen et al., 1997; reviewed by Dingledine et al., 1999).

Therefore, this expression system has been widely used as a model to determine links between NMDAR ion channel properties and excitotoxicity (Anegawa et al., 1995; Boeckman and Aizenman, 1996; Cik et al., 1993; 1994; Raymond et al., 1996). We had previously reported that expression of full-length mutant htt in HEK293 cells resulted in enhanced current amplitude for NR1A/NR2B but not NR1A/NR2A (Chen et al., 1999b; see Figure 1). We elected to use this model system to help determine the role that NMDARs may play in cell death in HD.

1.3.2 YAC Transgenic Mice

Recently, several transgenic HD mouse models have been created (reviewed by Reddy et al., 1999b). The first of these was a mouse transgenic for exon 1 of the human HD gene containing a CAG repeat length of ~150 controlled by the human HD promoter (line R6/1 and R6/2). These mice show some abnormal behaviors, die early, have decreased brain size (the brains weigh ~20 % less than their littermates), but do not reproduce the pathology of HD, as shown by absence of striatal degeneration (Davies et al., 1997). Despite lacking striatal degeneration, it is interesting that the R6 mice have shown electrophysiological abnormalities similar to those found in mice models expressing full-length human htt (Levine et al., 1999; Cepeda et al., 2001a). The R6 mouse model shows that the truncated form of htt may cause neuronal dysfunction but that expression of full-length mutant htt may be important for mimicking HD pathology. This is confirmed by the development of mice models that contain the full-length human HD gene (Hodgson et al., 1999; Reddy et al., 1998a). Mice generated by Reddy et al., (1998a) that express full-length HD cDNA with expanded CAG repeats at high levels under the control of a cytomegalovirus promoter, show progressive neurobehavioral peculiarities and human HD-like apoptosis and gliosis, demonstrating that the entire htt protein is important in the pathogenesis more resembling that found in HD patient brains.

A model, developed by the M.R. Hayden laboratory using yeast artificial chromosome (YAC) technology in FVB/N mice, expresses full-length human htt with expanded polyQ and including all regulatory elements, controlled by the endogenous human HD gene promoter. This strategy yields normal developmental and cell-specific regulation of htt expression. The HD clones were created to contain CAG sizes similar to those seen in either adult onset (YAC46 with 46 CAG repeats) or juvenile onset (YAC72 having 72 CAG repeats) HD (Hodgson et al., 1999). These mice show distinct HD phenotypes behaviorally and pathologically-i.e. hyperkinetic movements and striatal neuronal degeneration, which precedes nuclear translocation and aggregation of htt (Hodgson et al., 1999). A YAC72 mouse (line 2498) expressing the mutant human htt at higher levels demonstrated the behavioural phenotype as early as 6 weeks, while specific striatal degeneration and intranuclear aggregation was seen at 12 months (Hodgson et al., 1999). A lower expressing YAC72 mouse (line 2511) (the line used in my experiments) showed progressive electrophysiological abnormalities at 6 months, behavioural phenotype (mild hyperkinetic movement disorder characterized by progressive spontaneous hyperactivity during the dark phase of open field testing and some unidirectional circling) at 7 months, followed by selective striatal neurodegeneration beginning at about 12 months of age with intranuclear htt N-terminal fragments, but no detectable aggregates present (Hodgson et al., 1999), suggesting that aggregation of htt fragments may not be important to HD pathogenesis. It is interesting that unlike degeneration observed in the striata of human HD patients seen as a medial-to-lateral gradient (Vonsattel et al., 1985), degeneration in the striatum of 12 month old YAC72 was most severe laterally (Hodgson et al., 1999). This may be reflective of an important difference between the transgenic mouse model and humans and an argument against the use of this mouse HD model. The HD mice have been found to exhibit abnormal electrophysiological recordings, showing a hyperexcitable response to high frequency stimulation at Schaeffer collateral-CA1 synapses of the hippocampal slices from 6 month old YAC72 mice, which could be inhibited by

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application of 2-amino-5-phosphonovalerate (APV), an NMDAR antagonist (Hodgson et al., 1999). Furthermore, elevated basal calcium levels in acutely dissociated hippocampal neurons from 10 month old YAC46 mice were observed (Hodgson et al., 1999). It is interesting to note that no degeneration of the CA1 region of the hippocampus in YAC46 mice up to 20 months of age was observed, in contrast to findings in the CA1 region in human HD (Spargo et al., 1993). In spite of some differences in the neuropathological findings between the YAC mice and human HD, this model more closely mimics the human disease than other available models so we elected to use the YAC mice in our experiments.

Electrophysiological recordings from acutely dissociated striatal neurons from 6-11 wk old mice done previously in our laboratory by N.Chen show that NMDA-evoked peak current density is significantly larger in striatal neurons from YAC72 than wild type FVB mice, and most of the current could be blocked by ifenprodil (see Figure 2 below; Zeron et al., 2002), a specific antagonist for the receptors of the NR1/NR2B subtype (Williams, 1993). Thus, previous findings in cell lines and recent findings in neurons from transgenic HD mice lead us to test whether NMDAR may play a role in the pathogenesis of HD.

1.4 Glutamate Receptors

Glutamate is one of the most ubiquitous neurotransmitters in the adult central nervous system (CNS), mediating most excitatory neurotransmission in the brain. Glutamate has been shown to play a role in synaptogenesis, learning and memory, in addition to a number of pathological conditions such as ischemia, epilepsy, and neurodegenerative diseases (Albin and Greenamyre, 1992; Dingledine et al., 1999). Aspartate, kynurenic acid and quinolinic acid are also thought to play a role as excitatory neurotransmitters. (Dingledine et al., 1999) Glutamate levels during synaptic transmission are estimated to reach levels of 1-3 mM (Bergles et al., 1999). Glutamate is cleared from the synapse by uptake by neuronal and glial glutamate

transporters or through diffusion (Deutch et al., 1999). Upon uptake into neurons and glia, glutamate is enzymatically converted into glutamine that can be transported, taken up and stored in neurons until it is enzymatically converted back to glutamate for subsequent release. During stress, such as injury or disease of the CNS, glutamate can reach excessive amounts in the synaptic cleft from increased release directly from neurons, or shutdown of neuronal and glial glutamate transporters by pro-oxidant conditions (Schubert and Piasecki, 2001). Excessive extracellular glutamate levels can have detrimental effects on cell health and survival by overstimulating its neurotransmitter receptors. As well, high extracellular glutamate can inhibit the function of the glutamate/cystine antiporter, preventing the formation of glutathione and resulting in the increase in intracellular free radicals and cellular toxicity (Schubert and Piasecki, 2001).

Glutamate activates ionotropic glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluRs). Ionotropic GluRs control ion channels and are responsible for the majority of excitatory neurotransmission and a great deal of CNS pathology (Dingledine et al., 1999). Metabotropic glutamate receptors control the activity of intracellular enzymes via G-protein-linked pathways. iGluRs can be further categorized into N-methyl-D-aspartate receptors (NMDARs) and non-N-methyl-D-aspartate receptors, the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs) and the kainate receptors (KARs). The igluRs are distinguishable pharmacologically, electrophysiologically, and pathophysiologically. Ultimately, the presence of both iGluRs and mGluRs in normal neuronal transmission, and their precise assembly and spatial organization in the post-synaptic membrane, can determine the function of a synapse. A change in normal glutamate receptor function can have widespread implications for the performance of the synapse. For the purposes of our study, we will focus mainly on the iGluRs, and specifically, the NMDARs.

1.4.1 Metabotropic Glutamate Receptors

The mGluR family consists of 3 groups of G-protein-coupled receptors, which modulate excitatory synaptic transmission. Group I subtype (mGluR1, mGluR5) of mGluRs activate the phospholipase C pathway, eliciting hydrolysis of phosphoinositide and intracellular calcium mobilization (Moldrich et al., 2001). Group II (mGluR2, mGluR3) are negatively coupled to adenylate cyclase and inhibit the production of cAMP (Moldrich et al., 2001). Group III mGluRs (mGluR4, mGluR6-8) subtypes also show negative coupling to the adenylate cyclase and cAMP second messenger system (Moldrich et al., 2001). MSNs mainly express mGluR5 and mGluR3 receptors (Vonsattel and DiFiglia, 1998). mGluRs have been shown to modulate neurotransmission (Flavin et al., 2000; Cartmell and Schoepp, 2000), inhibit ion channels (Cartmell et al., 1998; Conn and Pin, 1997), and modulate ionotropic GluR-mediated excitotoxicity (Nicoletti et al., 1996; Calabresi et al., 1999; Pellicciari and Costantino, 1999). A positive interaction between NMDARs and group I mGluRs, via PKC interaction, has been shown in MSNs but not striatal interneurons, suggesting that this may partially account for the selective vulnerability of MSNs (Calabresi et al., 1999). The role that mGluRs play in the pathogenesis of HD is unexplored in this study and largely remains to be discovered.

1.4.2 Non-N-Methyl-D-Aspartate Receptors

Thought to mediate the fast excitatory neurotransmission in the brain, the non-NMDAR iGluRs are composed of two subtypes, the KARs and the AMPARs. The majority of these receptors are selectively permeable to monovalent cations, although a minor subset shows moderate calcium permeability as well (reviewed by Dingledine et al., 1999). The glutamate-evoked currents of these receptors deactivate and desensitize rapidly, having decay time constants around 1 ms and 5-15 ms, respectively (reviewed by Dingledine et al., 1999). Molecular cloning has identified four subunits of AMPAR (GluR1-4) and 5 subunits of KARs

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(GluR5-7 KA-1, -2). The bulk of the evidence indicates AMPARs are tetrameric complexes containing combinations of these subunits (Dingledine, et al., 1999). The expression of the GluR2 subunit within functional AMPARs has been demonstrated to suppress calcium permeability of these receptors. The GluR2/GluR3 subunits are expressed at high levels in MSNs but appear to be absent in NADPH-diaphorase-positive interneurons (Bernard et al., 1997; Martin et al., 1993; Tallaksen-Greene and Albin, 1996; Kim et al., 2001). The high expression of the GluR2 subunit suggests that the MSNs express few calcium-permeable AMPARs (Kim et al., 2001). The GluR1 subtype is predominantly expressed in the striosomes and in aspiny interneurons (Nansen et al., 2000; Vonsattel and DiFiglia, 1998). GluR5/6/7 are expressed in MSNs (Nansen et al., 2000) and NADPH-diaphorase-enriched medium aspiny interneurons may also be enriched in KARs (Nansen et al., 2000; Vonsattel and DiFiglia, 1998). The AMPARs play the dominant role in rapid synaptic transmission, whereas KARs may play a modulatory role (Dingledine et al., 1999). It is interesting that administration of AMPA/kainate receptor agonists directly into the brain, specifically in the striatum, results in excitotoxic lesions (Coyle et al., 1983; Coyle and Schwarcz, 1976; McGeer and McGeer, 1976). These animal models show phenotypes with some similarity to HD neuropharmacologically and behaviourally. In contrast, animal models that utilize NMDAR agonists or mitochondrial toxins intrastriatally show better resemblance to HD phenotype in humans as outlined in section 1.5.1 below. The role that non-NMDARs may play in the pathogenesis is largely undiscovered. In our study, we examined the role that AMPARs may play in HD pathogenesis in an *in vitro* model.

1.4.3 N-Methyl-D-Aspartate Receptors

The last class of iontropic glutamate receptors, and the central focus of our studies, is the NMDARs. A variety of data suggests that NMDARs are involved in many aspects of neuronal development, synaptic plasticity, learning and memory, and death after injury (Dingledine et al.,

1999). NMDARs have been implicated in a variety of neurological disorders that include epilepsy, ischemic brain damage, and speculatively neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, and amyotrophic lateral sclerosis (Dingledine, et al., 1999; Cull-Candy et al., 2001). It had been found previously that the high calcium influx resulting from overactivation of NMDARs triggers calcium-dependent degradative enzymes and creation of reactive oxygen species, leading to cellular dysfunction and death (reviewed by Albin and Greenamyre, 1992; Coyle and Puttfarcken, 1993, Sattler and Tymianski, 2001). For the purpose of this study, we were most interested in the role that NMDARs may play in the pathogenesis of HD, as determined by their unique properties and differential distribution of subtypes.

NMDARs are composed of combinations of the NR1 subunit splice variants (NR1A-H) and one or more of the NR2A, NR2B, NR2C and/or NR2D subunits forming a tetrameric complex; the modulatory NR2 subunits largely determine the ion channel and pharmacological properties of the receptor (Dingledine et al., 1999). Recently, NR3A and NR3B subunits have been cloned that have an inhibitory effect when expressed in recombinant NMDARs (Das et al., 1998; Andersson et al., 2001; Goebel and Poosch, 1999; Nishi et al., 2001; Perez-Otanol et al., 2001). NMDAR subunits are predicted to have three transmembrane domains (M1, M2, and M4) and a cytoplasmic-facing re-entrant second membrane loop (Dingledine et al., 1999). The residues in the re-entrant loop control permeation properties of the ion pore (Dingledine et al., 1999). The N-terminus is extracellularly located and the C-terminus is found intracellularly (Dingledine et al., 1999).

Activation of NMDARs is shown to depend on agonist binding (i.e. two molecules of L-glutamate or L-aspartate or quinolinic acid or NMDA) to the NR2 subunit congruent with co-agonist binding of two molecules of glycine on the NR1 subunit (Dingledine et al., 1999). Activation is also governed by the voltage-dependent magnesium block (reviewed by Dingledine

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et al., 1999), whereby the membrane must be depolarized (>-30 mV) amply to alleviate the block. NMDARs are highly permeable to Ca^{2+} as well as Na^+ and K^+ (Ascher and Nowak, 1986). In comparison with AMPARs, NMDARs have slower kinetics (2 orders of magnitude slower) (Dingledine et al., 1999; Cull-Candy et al., 2001) accounting for the slower component of glutamatergic excitatory postsynaptic potentials (EPSPs) in the CNS. NMDARs open maximally about 10 ms after glutamate is released into the synaptic cleft, and continue to open and close for hundreds of milliseconds until agonist is unbound (Cull-Candy et al., 2001).

NMDAR subtypes are determined by the NR2 subunit and exhibit distinct ion channel and pharmacological properties (e.g., open channel probability and zinc block) (Chen et al., 1999a,b; Chen et al., 1997; Paoletti et al., 1997; Williams et al., 1996; Wyllie et al, 1998; Dingledine et al, 1999). For example, with the same application of a glutamate stimulus and the same density of receptors, NR1/NR2A exhibit larger peak current amplitude and deactivate more quickly than NR1/NR2B, but both show equivalent total (integrated) current influx (Dingledine et al., 1999; Chen et al., 1999a). Moreover, NR1/NR2B-mediated currents are enhanced by expression with mutant full-length htt in HEK293 cells, whereas those of NR1/NR2A are not (Chen et al., 1999b; see Figure 1 below).

At least one of the 8 splice variants of the NR1 subunits (NR1A-H) is expressed in all neurons, but the NR2 subunits have more discrete distributions (Charton et al., 1999; Seeburg, 1993) in the brain, which change during development. Prenatally the NR2B and NR2D mRNAs predominate, whereas NR2A and NR2C mRNAs are first detected near birth (Monyer et al., 1994). Postnatally, NR2B mRNA levels disappear and NR2C levels increase in the cerebellar granule cell layer (Monyer et al., 1994). In the adult hippocampus, NR2A and NR2B mRNAs dominate in the CA1 and the dentate gyrus, and NR2B dominates in the CA3 area, while interneurons express NR2C and NR2D (Monyer et al., 1994; Scherzer et al., 1998). In the adult rat cortex, the pyramidal-like neurons of layers II/III, V and VI are intensely immunoreactive for

the NR2B subunit (Scherzer et al., 1998; Charton et al., 1999), whereas NR2A is highly expressed in layers III and VI (Scherzer et al., 1998).

Notably, MSNs primarily express the NR1A splice variant with NR2B subunits (Ghasemzadeh et al., 1996; Kuppenbender et al., 1999; Landwehrmeyer et al., 1995; Rigby et al., 1996), whereas other forebrain regions express combinations of both NR2A and NR2B with a variety of NR1 splice variants (Portera-Cailliau et al., 1996), and the cerebellum and brain stem lack NR2B (Hollmann and Heinemann, 1994; Monyer et al., 1994; Portera-Cailliau, et al., 1996; Thompson et al., 2000; Kovacs et al., 2001). Furthermore, striatal interneurons other than MSNs have a variable, and usually much lower, level of NR2B/2A mRNA and protein and express NR2D-NMDAR mRNA (Chen and Reiner, 1996; Landwehrmeyer et al., 1995). The NR1A splice variant is the full-length clone containing both C-terminal exons, which are involved in receptor clustering, contains protein kinase C phosphorylation sites and binds to calmodulin (Dingledine et al., 1999). Thus, it is possible that the relatively unique enrichment of the NR1A and NR2B subunit in the striatal MSNs plays a role in the selective neuronal vulnerability in HD. This idea is important to the scope of our studies.

1.4.3.1 N-Methyl-D-Aspartate Receptors and Interacting Proteins

Alterations in the cytoskeletal protein design are involved in normal neuronal functions such as neurite outgrowth, cell migration, axonal transport and neurotransmitter release (Shorte, 1997). Cytoskeletal proteins are structurally and functionally important for receptor targeting or clustering, as well as for modulation of receptor activity and activation of signaling pathways (Dingledine et al., 1999). Furthermore, NMDARs have been found to be functionally modulated and/or have altered distribution through interactions with cytoskeletal proteins (Dingledine et al., 1999). The cytoskeleton is composed of microtubules, actin filaments, intermediate filaments

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and many associated proteins, such as α-actinin (Allison et al., 2000). The actin scaffolding is thought to play an important role in anchoring glutamate receptors to the cytoskeletal network (Allison et al., 2000), thereby helping to localize the receptors to their appropriate site of function (Allison et al., 1998). As well, NMDARs are found to be modulated by the actin cytoskeleton in a calcium-dependent manner (Allison et al., 1998; Rosenmund and Westbrook, 1993). Conversely, NMDAR-dependent neuronal membrane depolarization and calcium influx have been shown to change the state of actin polymerization in neurons (Shorte, 1997). If the NMDARs change the actin equilibrium in favor of depolymerization, then the binding of actin filament bundling proteins to NMDARs will be impaired, altering calmodulin binding to NMDAR and modulating receptor function (Ehlers et al., 1996).

The interaction of actin and NMDAR is thought to be indirect through several bridging proteins, including α-actinin (Allison et al., 1998; Dunah et al., 2000; Wyszynski et al., 1998). Alpha-actinin is part of the spectrin superfamily of actin-binding and crosslinking proteins (Chan et al., 1998; Viel, 1999) and α-actinin subtypes 1 and 2 have been shown to directly bind to the C-terminus of NR1 and NR2B subunits *in vitro* (Vallenius et al., 2000; Wyszynski et al., 1998). Since both actin and α-actinin interact, and have been shown to influence NMDAR function and interact with NMDARs, then htt could influence this interaction in a polyQ length-dependent manner if it were found that any htt interacting proteins directly or indirectly interacted with NMDARs or any NMDAR-associated cytoskeletal proteins, such as α-actinin or actin. This possibility will be explored further in the Discussion.

Another NMDAR-interacting cytoskeletal protein candidate for mediating interaction with htt is PSD-95. Sun and associates (2001) have examined its potential role in HD. They showed that normal htt is associated with NMDARs and KARs via PSD-95; the SH3 domain of PSD-95 mediates binding to htt, and an expanded polyQ interferes with htt binding to PSD-95 (Sun et al.,

2001). They hypothesized that as polyQ length increases, htt's direct interaction with PSD-95 decreases allowing more PSD-95 to be free to interact with the C-terminus of NMDARs, leading to enhanced clustering of the NMDARs at the synapse, thereby increasing the total number of receptors receiving transmitter. Other candidate proteins, as yet unexplored, are other members of the PSD-95 family and calmodulin; shown to interact with htt within a large complex in the presence of calcium (Bao et al., 1996).

1.5 The Excitotoxic Hypothesis of Huntington Disease

Overactivation of receptors by excitatory amino acid (EAA) neurotransmitters resulting in neuronal death was called excitotoxicity (Olney et al., 1971; Choi, 1992; Doble, 1999) from early experiments done in the 1950s. Lucas and Newhouse in 1957 discovered that when high doses of glutamate were systemically administered to mice there was retinal neurodegeneration. Since then, many experiments have shown evidence for neuronal death after overstimulation by EAAs, resulting in selective degeneration of brain regions and this could be blocked by specific glutamate receptor antagonists (Watson et al., 1989; Meldrum and Garthwaite, 1990). Disruption of glutamate homeostasis has been linked to many neurological disorders, thereby making the glutamatergic system of great interest as a therapeutic target (Lipton and Rosenberg, 1994).

1.5.1 Striatal Lesion Models of Huntington Disease

A role for glutamate excitotoxicity in HD has long been postulated (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976). Although it is not known why the GABAergic medium-sized spiny neurons (MSNs) of the striatum are preferentially targeted for degeneration in HD, a body of evidence supports a role for excitotoxic cell death mediated by the release of glutamate from cortical afferents and activation of the ionotropic glutamate receptors: the NMDARs,

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AMPARs, and KAR (DiFiglia, 1990; Beal, 1992). A variety of data suggests that NMDARs are involved in pathogenesis of HD. The high calcium influx resulting from overactivation of NMDARs triggers calcium-dependent degradative enzymes and creation of reactive oxygen species, leading to cellular dysfunction and death (reviewed by Albin and Greenamyre, 1992; Coyle and Puttfarcken, 1993). Results of radiolabelled ligand binding assays in human postmortem tissue indicated that striatal glutamate receptors show disproportionate loss in HD, with a 50-60 % decrease in binding in the striata and no loss observed in the cortex (Young et al., 1988; Albin et al., 1990). Moreover, NMDAR binding was significantly decreased in this region even in the presymptomatic stage of the disease, suggesting that neurons which highly express these receptors may be most vulnerable (Young et al., 1988; Albin et al., 1990). It was found that glutamate or quinolinic acid levels were not enhanced in the striatum of HD patients (Reynolds et al., 1988; Beal et al., 1990); therefore, increased levels of glutamate receptor agonists most likely do not cause excitotoxicity in HD, contrary to cerebral ischemia. In rodents, intrastriatal injection of the non-NMDAR agonist kainic acid resulted in death of most neurons with preservation of glia and afferents (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976), while NMDAR agonists, such as quinolinic acid, selectively destroyed MSNs but spared interneurons that are known to be resistant to degeneration in HD (Beal et al., 1986; Ferrante et al., 1985). Furthermore, injection of quinolinic acid in the striata of non-human primates resulted in behavioural, neurochemical, and neuropathological abnormalities that mimicked the changes seen in HD (Hantraye et al., 1990). Finally, systemic injection of mitochondrial toxins such as malonate and 3-nitropropionic acid also caused striatal degeneration typical of HD; this neuronal loss was blocked by the NMDAR antagonist MK-801 (Beal et al., 1993b; Greene et al., 1993). Furthermore, subtoxic concentrations of malonate co-injected with non-toxic concentrations of glutamate and NMDA caused large lesions, suggesting that bioenergetic defects may intensify excitotoxicity (Greene and Greenamyre, 1995). Together, these data

support the hypothesis that excitotoxicity, especially that mediated by NMDARs, may play a critical role in the pathogenesis of HD.

1.5.2 Selectivity of Neurodegeneration May Depend on NMDARs in Huntington Disease

The results of studies outlined above also raise the question of whether mutant htt expression can cause increased activity of NMDARs or their downstream effectors of cell death, and how such interactions might explain selective neuronal vulnerability. Recent data strongly support roles for caspase activation, and/or formation of toxic N-terminal fragments of htt containing the polyQ tract, in the neuronal degeneration underlying HD and related neurodegenerative diseases caused by genes with a triplet repeat expansion (reviewed by Wellington et al., 1997; see sections 1.1.3, 1.2.1 and 1.6.2.1). However, each of these diseases has a distinct pattern of selective neuronal death, so that the upstream events that trigger caspase activation and protein cleavage may be unique for each disease and related to interactions between mutant protein and other proteins that are selectively expressed in vulnerable neuronal populations. We have previously reported enhancement of NMDAR-mediated current amplitude in cell lines expressing full-length mutant htt and the NR1A/NR2B but not NR1A/NR2A subtype of NMDARs (Chen et al., 1999b; see Figure 1 below). This was interesting since MSNs primarily express the NR1A and NR2B subunits (Kuppenbender et al., 1999; Landwehrmeyer et al., 1995), whereas other forebrain regions express combinations of both NR2A and NR2B with a variety of NR1 splice variants, and the cerebellum and brain stem lack NR2B (Hollmann and Heinemann, 1994; Kovacs et al., 2001; Monyer et al., 1994; Thompson et al., 2000). Recent data obtained in a mouse HD model expressing the N-terminal region of htt containing an expanded polyQ utilizing acute striatal slices show enhanced current density and percent change in intracellular calcium in response to NMDA application compared to wildtype striatal neurons (Laforet et al., 2001). Another study done by Cepeda and associates, 2001a, compare two

different mouse models, one utilizing only the N-terminal region of htt containing the polyQ and the other mouse model containing the full-length htt protein and the polyQ (the same model mouse we use). Using either model, they found NMDAR-dependent enhancement in current amplitude and intracellular calcium flux in MSNs expressing the expanded polyQ (Cepeda et al., 2001a). Electrophysiological recordings from acutely dissociated striatal neurons from 6-11 wk old YAC transgenic HD mice done previously in our laboratory by N. Chen (see Figure 2 below; Zeron et al., 2002) show that NMDA-evoked peak current density is significantly larger in striatal neurons from a mouse model containing the full-length htt protein and the polyQ than striatal neurons from wild type FVB mice. Furthermore, most of the current could be blocked by ifenprodil, a specific antagonist to the NR2B subunit (Zeron et al., 2002). Therefore, evidence suggests that NMDARs may play a role in the pathogenesis of HD. We will explore this further in our study.

1.6 Mechanisms of Cell Death

1.6.1 Classic Modes of Cell Death

It is thought that the mode of cell death that occurs in HD is apoptosis, due to the late onset and slow progression of HD, as well as evidence of DNA fragmentation in post-mortem HD brains and in a quinolinic acid striatal-lesion rat model (Dragunow et al., 1995; Portera-Cailliau et al., 1995; Wellington and Hayden, 2000). This still remains controversial (reviewed by Alexi et al., 2000) and we felt it was important to examine the mode of cell death closely in our studies.

Classically, apoptosis and necrosis have been distinguished on the basis of different morphological and biochemical properties. Early plasma membrane preservation in concert with organized chromatin condensation and DNA fragmentation identify apoptosis morphologically, whereas overt cytolysis and tissue inflammation distinguish necrosis (Arends et al., 1990; Kerr et al., 1972; Kerr and Harmon, 1991; Searle et al., 1982; Wyllie et al., 1980).

1.6.1.1 Apoptosis and Necrosis Continuum

Recent studies show that these classic characterizations of mutually exclusive modes of cell death are not so easily delineated as once previously thought (Gwag et al., 1997; Martin et al., 1998; Portera-Cailliau et al., 1997; Sohn et al., 1998; Wyllie et al., 1980). For example, biochemical features of apoptosis and morphological characteristics of necrosis have been detected simultaneously following insults in the adult brain and developing brain (Beilharz et al., 1995; Ferrer et al., 1995; Goto et al., 1990; Hill et al., 1995; Pollard et al., 1994; Portera-Cailliau et al., 1995; Tominaga et al., 1993). In a stimulating review on apoptosis, Roy and Sapolsky, 1999, discuss the relative rarity of the 'classical' apoptotic profile seen experimentally since in many cases both necrotic and apoptotic characteristics are present in the same cell, or that only a subset of apoptotic characteristics will appear in a cell. Therefore there may be a continuum between apoptosis and necrosis, and these may not be mutually exclusive forms of cell death (Roy and Sapolsky, 1999). The latter authors propose that since apoptosis is an active process, requiring time and energy for cytoskeletal proteolysis, changes in cellular morphology, DNA condensation and fragmentation, and the alteration of surface antigens, there must be enough energy resources to carry out the entire apoptotic pathway (Roy and Sapolsky, 1999). According to their model, if there is not enough energy to stimulate the apoptotic pathway, necrosis will occur and if there is not enough energy to complete the entire apoptotic pathway only parts of the pathway will occur. Support for this is seen in the ATP dependency for caspase-9 activation. Therefore, one could predict that there may be two ways to cleave an identical substrate - the apoptotic way will predominate when energy is plentiful, and the necrotic way will govern when energy is lacking (Roy and Sapolsky, 1999). Furthermore, the subcellular localization of the initiation of an apoptotic process may predict the profile of the pathway as well (Roy and Sapolsky, 1999). A deeper understanding of the dynamics of cell death modes may prove

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helpful in delineating the type of cell death occurring in HD and enable insight into possible therapeutic interventions (Alexi et al., 2000).

1.6.1.2 Ionic Homeostasis

Important to our studies, it has been shown that changes in the intracellular homeostasis of several physiological ions, such as Ca^{2+} or K^+, appear to be important modifiers or mediators of apoptosis (reviewed by Yu et al., 2001). Under normal physiological conditions, calcium levels are highly regulated, since calcium ions are important intracellular messengers regulating a number of cellular functions, such as cell growth and differentiation, membrane excitability, exocytosis, and synaptic plasticity (Berridge, 1998; Clapham, 1995). The basal intracellular calcium concentration is about 20, 000 times lower than the extracellular calcium levels in order to obtain a high signal-to-noise-ratio for efficient calcium signalling to occur (Carafoli, 1987; Clapham, 1995). Calcium homeostasis is normally acquired by synchronicity between calcium influx processes (through NMDAR or voltage-gated calcium channels), calcium efflux processes (through calcium/ATPase pump or sodium/calcium antiporter), calcium storage organelles (mitochondria, endoplasmic reticulum and nucleus), and calcium binding proteins (e.g., calbindin) (Sattler and Tymianski, 2000). Increases in intracellular calcium have been found to play a role in both apoptosis and necrosis (reviewed by Yu et al., 2001).

The neuronal degeneration that occurs after intense glutamate stimulation is thought to be dependent on calcium influx through NMDARs (Choi, 1987). It is unclear whether calcium influx through NMDARs is uniquely toxic due to the receptor's subcellular localization (Tymianski et al., 1993) or because of its high permeability to calcium (Hartley et al., 1993). There appears to be some consensus that there is a correlation between intracellular calcium concentration and toxicity. There are many downstream effects proposed to explain how high intracellular calcium levels may compromise cell viability. There are many calcium-dependent

enzymes, such as nitric oxide synthase and calpain, which may mediate excitotoxicity (Dawson et al., 1991; Siman et al., 1989). As well, calcium overload in mitochondrial stores causes bioenergetic failure, production of reactive oxygen species, and mitochondrial membrane depolarization with opening of the permeability transition pore (PTP) - all proposed to be downstream mediators of cell death (reviewed by Nicholls and Budd, 2000). Increased intracellular calcium levels can also activate a variety of enzymes involved in cell death, such as phospholipase A_2, proteases and endonucleases (Leist and Nicotera, 1998; Orrenius et al., 1996) and increase the cellular production of free radicals (Castilho et al., 1999; Hatanaka et al., 1996; Lafon-Cazal et al., 1993; Reynolds and Hastings, 1995). Importantly, it has been shown that mitochondrial uptake of calcium after NMDAR stimulation is necessary to mediate excitotoxic cell death in cultured forebrain neurons (Stout et al., 1998). Use of a calcium ionophore in striatal cultures showed that increases in intracellular calcium are important as a trigger for caspase activation and mitochondrial dysfunction (Petersen et al., 2000). Overall, enhanced intracellular calcium through activation of NMDARs can have detrimental effects on cell survival.

1.6.1.3 Excitotoxic Cell Death

In vitro and *in vivo* studies in the CNS suggest that both apoptotic and necrotic neuronal death can occur during excitotoxicity, depending on the duration and intensity of the initiating insult. The findings indicate that mild excitotoxic insults lead to recoverable mitochondrial membrane depolarization, reversible energy compromise, and apoptosis. In contrast, strong stimuli result in irreversible mitochondrial depolarization and energy loss, and ionic imbalance with subsequent swelling and necrosis (Ankarcrona et al., 1995; Ayata et al., 1997; Bonfoco et al., 1995; Larm et al, 1997). Roy and Sapolsky (1999) outlined a few independent measures that appear to determine whether apoptosis is likely to occur: first, for the same insult, the greater the

amount of energy available, the more likely apoptosis will occur; second, for the same insult, the younger the neuron, the more likely apoptosis will happen; third, for the same duration of exposure to an excitotoxin, the less toxin delivered, the more likely apoptosis will occur; and last, for the same concentration of an excitotoxin, the shorter exposure time, the greater the likelihood that apoptosis will occur. These aspects were important determinants in our examination of cell death in the experiments discussed in this paper.

1.6.2 Protease Role in Cell Death

Caspases (aspartate-directed cysteine proteases) are proteolytic enzymes that have been implicated in cell death (Tenneti et al., 1998; Troy et al., 1997; Troy et al., 1996; Yuan et al., 1993; Du et al., 1997). The caspase family consists of 14 members to date, and corresponds to the mammalian homologues of ced-3 from *Caenorhabditis elegans* (Humke et al., 1998; Yuan et al., 1993). Caspase-3 has been found to be an important mediator of neuronal apoptosis and final executioner of the caspase-dependent apoptotic pathway (Kuida et al 1996; Tenneti and Lipton, 2000). Caspase-3 can be activated by other proteases such as calpains, or caspase-9 or caspase-8 (reviewed by Nicholson, 1999). Peptide inhibitors of caspases lessen ischemic and excitotoxic neuronal damage *in vivo* (Chen et al., 1998; Hara et al., 1997) in addition to traumatic brain injury (Yakovlev et al., 1997). Caspase activation may play an important role in neurodegenerative disease such as Alzheimers and amyotrophic lateral sclerosis (Yuan and Yankner, 2000). Caspase activation has been shown to exert an apoptotic effect by proteolysis of proteins such as poly(ADP-ribose) polymerase (PARP), inhibitor of caspase-activated DNAase (ICAD) and acinus-enhancing apoptotic DNA fragmentation and chromatin condensation (McGinnis et al., 1999; Sahara et al., 1999; Nagata, 2000).

1.6.2.1 Caspase Activation in Huntington Disease

Caspases are activated in cells undergoing apoptosis and have been implicated in polyQ-dependent cell death in cell culture and mouse models where expression of mutant htt increases susceptibility to apoptotic stimuli (Wellington and Hayden, 2000). Striatal neurons in the HD brain have been observed to be labeled by Tdt-mediated dUTP-biotin nick end-labelling (TUNEL) (Dragunow et al., 1995; Portera-Cailliau et al., 1995), suggesting that cell death in human HD may be through apoptosis. Apart from the direct apoptotic effect caspase activation may have in neurons affected in HD, full-length htt protein has been shown to be a caspase substrate, with cleavage producing an N-terminal fragment containing the polyQ tract (reviewed by Sieradzan and Mann, 2001). Caspase-3 and caspase-6, and possibly caspase-9 and –10 (but not caspase-7 or –8), have been shown *in vitro* to be capable of cleaving htt downstream of the polyQ, creating N-terminal fragments the size thought to be neurotoxic in cell culture (Wellington et al., 1998; Wellington et al., 2000). A cross between R6/2 transgenic HD mice, expressing exon 1 of the HD gene, with an expanded polyQ, and mice expressing a dominant negative caspase gene, which inhibits caspase-1 and probably other caspases, are resistant to htt protein-induced neuronal damage, as evidenced by prolonged survival and delayed manifestations of the HD phenotype (Ona et al., 1999). Caspase-1 has two consensus sites within the N-terminal 538 amino acids of htt (Wellington et al 1998; Wellington et al., 2000) but no consensus sites within the N-terminal 17 amino acids of htt and the polyQ that is expressed in the R6/2 transgenic HD mice. These data confirm that caspase cleavage of htt may be important to the progression of HD.

There are four consensus sites for caspase-3 within the N-terminal region of htt; the one at amino acid position 513 appears to be the most important site, with the 552 position used less efficiently and the other two not at all in cultured cells (Wellington et al., 1998; Wellington et al., 2000). When htt is mutated at the caspase-3 consensus sites, it is cleavage-resistant. Caspase-6 cleaves at position 586 (Wellington et al., 2000). The proteolytic cleavage products of htt by

caspase-3 appear to be around 100 kDa (Wellington et al, 1998; Wellington et al 2000), which is much larger than the 40 kDa N-terminal fragment identified in HD brains (DiFiglia et al., 1997). Other proteolytic enzymes may therefore be involved in creating the smaller htt fragment after initial caspase-3 cleavage. Also, an activated form of caspase-8 was isolated from the insoluble fraction of the striatum, but not the cerebellum, of HD brains suggesting this caspase may be recruited into aggregates of mutant htt and may proteolyze proteins other than htt in the cell (Sanchez et al., 1999). *In vitro* models demonstrate caspase activation likely involves apoptosis, but in chronic neurodegenerative disorders such as HD, a low-grade process, or 'cumulative-damage hypothesis', leading to cell dysfunction but not immediate cell death might be involved (Sieradzan and Mann, 2001). Procaspases, such as the proenzyme form of caspase-3, have approximately 60-fold less activity than the activated enzymes (Robitaille et al., 1997) and therefore may mediate htt cleavage chronically, without effecting rapid cell death (Wellington et al 1998, Wellington et al 2000). On the other hand, a 'one hit model' has been proposed instead where affected neurons are always in an abnormal, or 'mutant steady', state and random changes in the expression or function of particular genes or proteins can cause neuronal death (Clark et al., 2000). Thus, perhaps caspase activation above a particular level may determine the vulnerability of a MSN to die in HD. Whether caspase activation leads to the direct stimulation of the apoptotic pathway, or indirectly, by causing the production of toxic N-terminal fragments of htt, the roles of caspases in HD remain highly speculative. In this study we will attempt to address this topic.

1.6.2.2 Protease Activation After NMDAR Stimulation

NMDAR activation after mild insults has been found to induce time-dependent activation of caspase-3-like proteases and apoptosis (Tenneti and Lipton, 2000). Furthermore, the calcium influx evoked by NMDAR activity results in activation of the calcium-dependent protease,

calpain. Calpain has been found to cleave caspase-3 (McGinnis et al., 1999; Wolf et al., 1999) and other caspase family members, including –7, -8 and –9 (Chua et al., 2000; Ruiz-Vela et al., 1999), and Bcl-2 family members (Nakagawa and Yuan, 2000; Wood and Newcomb, 1999), as well as C-terminal regions of NR2 NMDARs subunits, without directly changing receptor function (Guttmann et al., 2001). Calpains have been implicated mainly in excitotoxic neuronal death and necrosis (Bednarski et al., 1995; Seubert et al., 1989; Siman and Noszek, 1988). More recently, a study has found a direct link between the early, excitotoxic, calcium-mediated activation of m-calpain after cerebral hypoxia-ischemia and the synergistic activation of caspase-3, supporting a hypothetical pathway of "pathological apoptosis" (Blomgren et al., 2001). These data support the idea that enhanced levels of calcium into the cell through NMDAR activation trigger calpain activation and thus caspase-3 activity. Recently, it has been shown that calpain activity is enhanced in HD brains (Gafni and Ellerby, 2002). Furthermore, evidence shows that one of the major N-terminal htt fragments found in HD brains is derived from calpain cleavage (Gafni and Ellerby, 2002), a result supported by work done *in vitro* (Kim et al., 2001). Thus, NMDAR stimulation may play a role in calpain and caspase activation leading to indirect or direct activation of the apoptotic pathway.

1.6.3 Mitochondrial Role in Cell Death

1.6.3.1 Mitochondrial Homeostasis

During cytoplasmic calcium overload, the mitochondrion is the main organelle responsible for calcium sequestration. Mitochondria can undergo harmful calcium loading after NMDAR stimulation (Budd and Nicholls, 1996; White and Reynolds, 1997), which results in increases in intracellular free calcium concentration above the buffering capacity of neurons (Dubinsky, 1993), whereby mitochondria may play an important role in apoptotic signaling

(Kluck et al., 1997; Yang et al 1997). Mitochondria have a large capacity for calcium uptake, through the membrane potential-driven calcium uniporter, and may be neuroprotective by removing calcium from the cytoplasm (reviewed by Nicholls and Budd, 2000). In the presence of physiological concentrations of phosphate, an osmotically inactive calcium-phosphate complex forms - without precipitation - in the mitochondrial matrix during critical calcium loading (Nicholls and Budd, 2000). With the calcium-phosphate complex, mitochondria act to buffer calcium whenever the cytosolic calcium concentration rises above the set point. The set point is the value at which mitochondrial uptake and efflux of calcium are balanced - in some cases varying between 0.3 and 1 μM - and calcium is released below this point (Nicholls and Budd, 2000). Thus, a NMDAR-induced rise of intracellular calcium above a certain threshold level could be detrimental to mitochondrial normal calcium buffering capacity, altering mitochondrial homeostasis, leading to the loss of ATP production, an increase in free radical production, organelle swelling, and causing mitochondrial membrane depolarization and permeabilization (Nicholls and Budd, 2000). It has been shown that mitochondrial uptake of calcium after NMDAR stimulation is necessary to mediate excitotoxic cell death in cultured forebrain neurons (Stout et al., 1998). Mitochondrial membrane permeabilization is thought to differentially affect the outer mitochondrial membrane, which becomes protein-permeable; in contrast, the inner mitochondrial membrane, which continues to retain matrix proteins, dissipates the mitochondrial transmembrane potential (reviewed by Ferri and Kroemer, 2001). The exact mechanisms of mitochondrial membrane permeabilization are still being debated as to the order and involvement of the inner and outer membrane and the protein players in mitochondrial membrane permeabilization.

1.6.3.2 Mitochondrial-Dependent Apoptosis

Mitochondria may also act as a link between the action of the initiator caspases and the downstream effector caspases. In nonneuronal cells, mitochondria have been shown to release apoptogenic factors, such as cytochrome c (Kluck et al 1997, Yang et al., 1997) and apoptosis-inducing factor (AIF) (Susin et al., 1996), from the intra-mitochondrial membrane space during mitochondrial membrane permeabilization, which can accelerate caspase activation. AIF is a 57, 000 Da flavoprotein which induces chromatin condensation when added to isolated nuclei by activating a DNAase located in the nucleus (Kroemer et al., 1998). Cytochrome c, a component of the respiratory chain, when released to the cytosol can bind with Apaf-1, dATP and procaspase-9, forming a complex initiating a caspase cascade, involving caspase-3 activation (Li et al, 1997; Green and Reed, 1998).

Some caspases and Bid and Bax (pro-apoptotic Bcl-2 family members) have been shown to induce cytochrome c and AIF release (Li et al., 1998; Bossy-Wetzel and Green, 1999; Marzo et al., 1998) while Bcl2 prevents their release (Kluck et al., 1997; Yang et al., 1997; Susin et al., 1996). There is great speculation as to the mechanism of release from the mitochondria: pore-forming proteins such as Bax could allow the transport of cytochrome c out of the inner membrane space into the cytoplasm (Marzo et al., 1998), or there is an opening of a permeability transition pore (PTP) in the inner mitochondrial membrane leading to the rupture of the outer mitochondrial membrane (Zamzami et al., 1996), or perhaps the PTP and the voltage-dependent anion channel in the outer mitochondrial membrane act together (Shimizu et al., 1999). The PTP is thought to consist of voltage-dependent anion channels (VDAC), which are found in the outer membrane, and the adenine nucleotide translocase (ANT), which is found in the inner membrane (reviewed by Ferri and Kroemer, 2001). Furthermore, procaspases and Smac-α/DIABLO are also thought to be released during mitochondrial membrane permeabilization, which can activate caspases (Ferri and Kroemer, 2001). Two recent reports show enhanced cytochrome c release in

cultured cells expressing mutant htt, indicating cytochrome c release and mitochondrial dysfunction leading to caspase activation may occur in HD (Li et al., 2000; Jana et al., 2001).

1.6.3.3 Mitochondrial Role in Huntington Disease

It is thought that impaired energy metabolism may play a role in the degeneration of MSNs in HD. Regional and selective neostriatal neuronal loss can be induced by systemic injection of 3-nitropropionic acid, which causes irreversible inhibition of the succinate dehydrogenase-complex II of the mitochondrial respiratory chain, or of malonate, which also causes inhibition of succinate dehydrogenase (Beal et al., 1993a; Greene et al., 1993). The neuronal loss can be prevented by addition of MK-801, a use-dependent NMDAR antagonist, demonstrating that mitochondria may be involved downstream of NMDAR activation, in secondary excitotoxicity (Beal et al., 1993a; Greene et al., 1993). Furthermore, subtoxic concentrations of malonate co-injected with non-toxic concentrations of glutamate and NMDA caused large lesions (Greene and Greenamyre, 1995), suggesting that bioenergetic defects may intensify excitotoxicity, and that mild metabolic changes can cause severe damage by rendering neurons more vulnerable to endogenous levels of glutamate. Reports of increased cerebral lactic acid levels in HD patients and decreased succinate dehydrogenase activity in postmortem HD striata support the hypothesis of impaired energy metabolism in HD (Vonsattel and DiFiglia, 1998). Impairment of mitochondrial function results in decreases in high-energy phosphate stores and a deterioration of membrane potential leading to the relief of the voltage-dependent Mg^{2+} block of NMDAR activation by glutamate and therefore increasing the total amount of NMDAR activity (Vonsattel and DiFiglia, 1998). Another study showed that polyQ-induced cell death in primary rat neurons could be blocked by co-expression with Bcl-2 or Bcl-x_L (anti-apoptotic Bcl-2 family members), also supporting a role for mitochondria in HD (Sanchez et al., 1999).

A.V. Panov and J.T. Greenamyre have recently shown (personal communication) that mitochondrial calcium homeostasis is compromised in HD. Lymphoblast mitochondria from HD patients are shown to have a lower membrane potential and depolarize at lower calcium loads than controls (ibid). Similarly, brain mitochondria from YAC transgenic HD mice show mitochondrial defects preceding the onset of known physiological, pathological or behavioral abnormalities (ibid). They suggest that mitochondrial calcium abnormalities occur early in HD pathogenesis and may be a direct effect of mutant htt on the mitochondria (A.V. Panov and J.T. Greenamyre, personal communication). Hopefully, our study will help to define the role that mitochondria may play in HD.

1.6.3.4 NMDAR-Dependent Mitochondrial Dysfunction

It has been shown that NMDAR activation can also influence mitochondrial function. Recently, it has been shown that NMDAR-mediated, but not staurosporine-mediated, apoptosis is preceded by depolarization of the mitochondrial membrane potential and ATP loss in cerebrocortical neurons (Budd et al., 2000). Apoptosis, mitochondrial membrane depolarization, ATP loss and caspase-3 activation could be blocked with bongkrekic acid, which inhibits the adenine nucleotide translocator, a supposed component of the mitochondrial permeability pore (Budd et al., 2000). Cytochrome c was still released, suggesting that the cytochrome c release pathway may not play a role in caspase-3 activation or apoptosis (Budd et al., 2000). This also supports a role for mitochondrial dysfunction after NMDAR overactivation in HD. We hope that our data can begin to explain the roles that NMDARs and mitochondria may play in the pathogenesis of HD.

1.7 Research Hypothesis

Twenty-five years of research suggests that N-methyl-D-aspartate receptors (NMDARs) may play a role in the pathology of Huntington's disease (HD). Overactivation of these receptors may account for some of the neurodegeneration found in HD (Young et al., 1988; Beal et al., 1991; 1992). NMDARs can be functionally modulated by interactions with intracellular proteins such as cytoskeleton-associated proteins (Kim et al., 1996; Kornau et al., 1995; Muller et al., 1996; Rosenmund and Westbrook, 1993; Paolettti et al, 1994; Wyszynski et al., 1997). Furthermore, huntingtin (htt) is found to interact with intracellular proteins thought to play a role in membrane trafficking and vesicle transport and/or cytoskeletal functions (Kalchman et al., 1997; Li et al., 1995; Wanker et al., 1997). Interestingly, there is no particular enrichment of htt or its interacting proteins in the striatum (DiFiglia et al., 1995; Ferrante et al., 1997; Gutekunst et al., 1998; Li et al., 1993; Sharp et al., 1995) but there is increased expression of the modulatory type NMDAR subunit NR2B relative to other NR2 subunits in MSNs (Ghasemzadeh et al., 1996; Landwehrmeyer et al., 1995; Rigby et al., 1996), which is relatively unique among populations of mature neurons (Hollmann and Heinemann, 1994). Previously, our laboratory (Chen et al., 1999b) found that in cell lines co-expressing mutant htt and NR1/NR2B-type NMDARs there was a significant increase in glutamate-evoked current density compared to cells co-expressing wild type htt; in contrast, cells co-expressing NR1/NR2A-type NMDARs with mutant htt showed similar current densities compared to cells with wild type htt (see Figure 1 below). Furthermore, hippocampal neurons from mice transgenic for the full-length mutant HD gene showed increased basal calcium levels (Hodgson et al., 1999), which may be indicative of events such as overactivated calcium-permeable channels (e.g. NMDARs) or of mitochondrial stress. As well, studies have demonstrated enhancement of NMDAR-mediated current density and intracellular calcium levels in MSNs from several mouse models for HD (Cepeda et al., 2001a; Laforet et al., 2001). Finally, electrophysiological recordings from acutely dissociated striatal

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neurons from 6-11 wk old mice done previously by N. Chen showed that NMDA-evoked peak current density was significantly larger in striatal neurons from YAC72 than wild type FVB mice, and most of the current could be blocked by ifenprodil, a selective antagonist for the NR1/NR2B subtype of NMDARs (see Figure 2 below) (Zeron et al., 2002). Therefore, we hypothesize that mutant htt may augment NMDAR function (particularly that of the NR1/NR2B-NMDAR subtype) in MSNs leading to calcium-induced cellular dysfunction and cell death.

Initially, I examined whether the increase in current density evoked by glutamate observed in the HEK293 cell model system, expressing mutant htt and the NR1/NR2B NMDAR subtype, correlated with an enhanced vulnerability to excitotoxic cell death. In the next set of experiments I studied MSNs in primary neuronal culture to determine whether expression of mutant htt in YAC72 mice resulted in increased vulnerability to NMDAR-mediated excitotoxicity. In the final sets of experiments, I investigated the downstream effects of the overactivation of NMDARs including caspase activation and mitochondrial dysfunction.

FIGURE 1. Peak current density is enhanced in HEK293 cells coexpressing NR1A/NR2B with htt-138Q but not NR1/NR2A (Chen et al., 1999b). Peak current response was obtained during fast perfusion of 100 μM glutamate in the presence of 50 μM glycine using whole-cell patch-clamp recordings (Chen et al., 1999b). Peak current amplitude was normalized to cell capacitance in recordings from cells coexpressing NR1A/NR2A or NR1A/NR2B and the indicated proteins (Chen et al., 1999b). Data are mean ± S.E.M. values from 60, 80, or 68 cells (NR1A/NR2A) and 106, 110, or 96 cells (NR1A/NR2B) for β-gal, htt-15Q, and htt-138Q transfections, respectively (Chen et al., 1999b). *p = 0.028 by unpaired t test, significantly different from control and htt-15Q (Chen et al., 1999b). Figure adapted from Chen et al., 1999b.

NR1A/NR2A

Current Density (pA/pF) vs Full-length htt conditions: Control (β-gal), 15Q, 138Q

NR1A/NR2B

Current Density (pA/pF) vs Full-length htt conditions: Control (β-gal), 15Q, 138Q
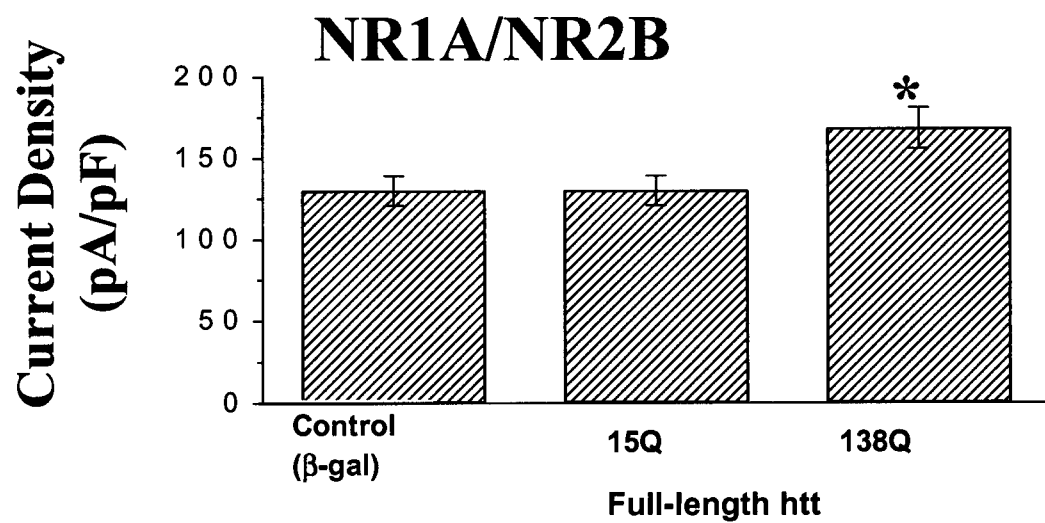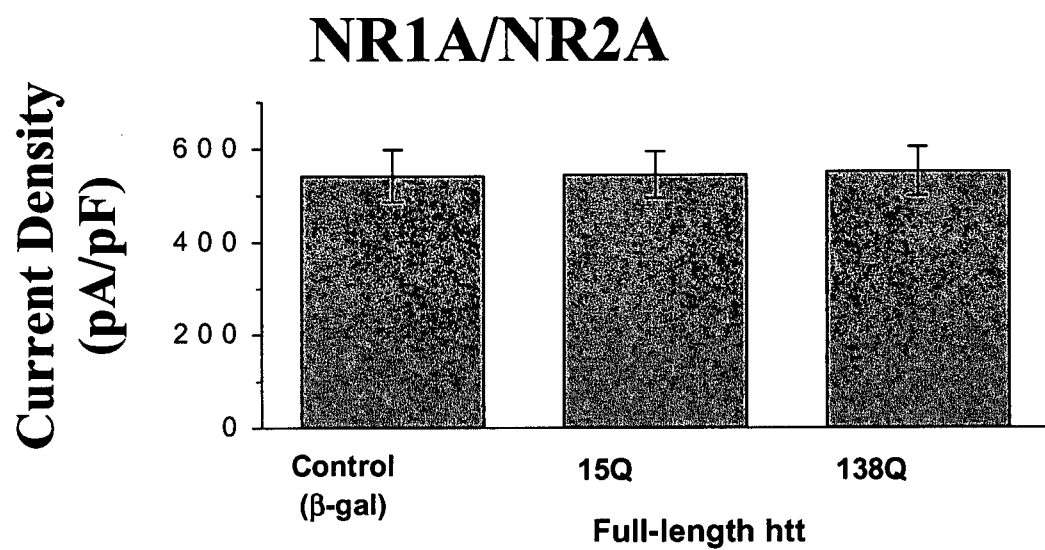
FIGURE 2. Increased peak NMDA-evoked current recorded from acutely dissociated YAC72 striatal neurons compared with WT controls as obtained by N. Chen (published in Zeron et al., 2002). **(A)** Representative traces of NMDA-evoked current recorded from a single WT or 72Q MSN. Bar represents application of 1 mM NMDA, in the presence of 50 µM glycine. In right panel "control" indicates application of NMDA alone, and "ifenprodil" indicates application of NMDA with 10 µM ifenprodil. Note that greater than 50 % of peak current is inhibited by ifenprodil, indicating that majority of current is carried by NR1/NR2B-type NMDARs. **(B)** Peak current density for 1 mM NMDA-evoked currents recorded from MSNs acutely dissociated from the striata of 6-11 week old animals. Bars represent mean ± S.E.M. measured from N=39 and 42 different cells from 10 and 13 different animals for WT and YAC72, respectively. *P=0.015 compared with WT, using unpaired t test. Figure adapted from Zeron et al., 2002

WT   72Q

200 pA

400 ms

Ifenprodil

Control

Peak current density (pA/pF)

*

WT   72Q
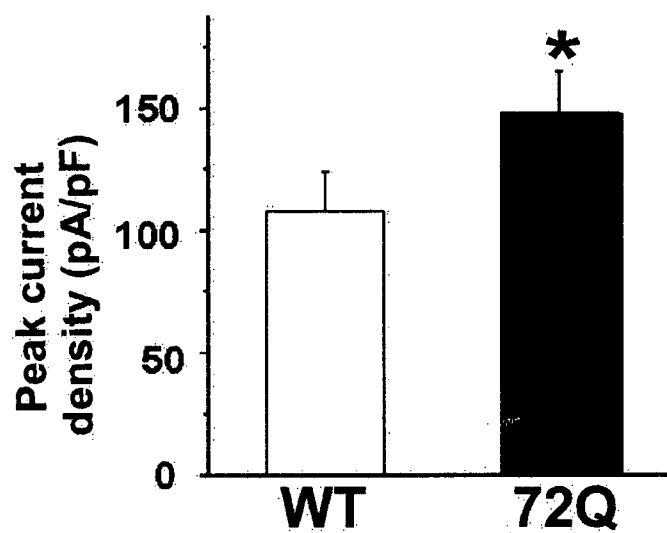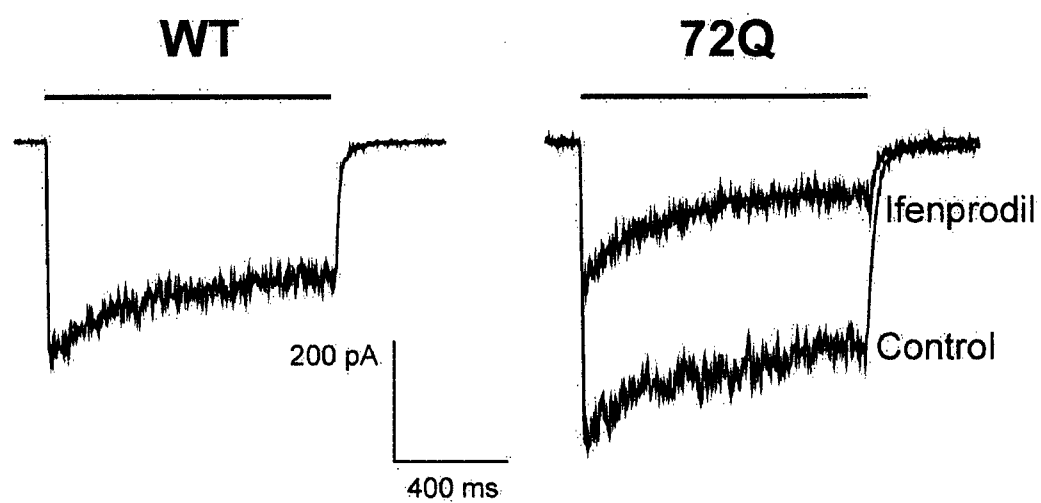
CHAPTER 2

Materials and Methods

HEK 293 Cell Lines:

2.1 Cell Culture and Transfections

HEK293 cells were cultured and transfected as previously described by Chen et al., 1997. Cells were grown in minimum essential medium (MEM) (Gibco# 11700) containing Earle's salts and supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 2 mM α-glutamine and maintained at 37°C in 5 % CO_2 in a humidified atmosphere. After confluency was attained, cells were propagated every 3-4 days and plated at a density of ~2 x 10^6/mL. Cells were rinsed with warm phosphate-buffered saline (PBS) and triturated in 9 mL of fresh media after 1 mL 1 x trypsin addition. Cells were plated in new plates and further diluted as necessary for transfection. Cells were transfected using either the calcium phosphate precipitation or Lipofectamine method.

2.1.1 Calcium Phosphate Precipitation

For the calcium phosphate method of transfection, cells were plated at a density of ~1 x 10^6 cells/mL in 6-well culture dishes or 10 cm culture plates pre-coated with 100 μg/mL poly-D-Lysine (M.W. = ~135,000), and grown for 9-12 h before the transfection. To insure that cells expressing NMDARs and the marker protein β-galactosidase (β-gal) also expressed adequate levels of htt (or control protein GFP) we used a 4:3:3:8 ratio of cDNAs encoding β-gal; NR1A; NR2 (A or B); and green fluorescent protein (GFP), or htt (full-length with 15Q or 138Q- htt-15Q or htt-138Q); using a total of 18 μg of plasmid DNA per 6-well plate. Plasmids were

precipitated in 1/10 total plasmid volume of 3 M sodium acetate and 3 times the total plasmid volume of 100 % EtOH and briefly spun followed by addition of 1 mL 100 % EtOH and centrifugation at 14000 rpm for 15-20 min. at 4°C. The EtOH was decanted and the DNA pellet allowed to air dry. Then cells were preincubated in 3 % CO_2 for 20 min while the DNA pellet was dissolved in 1 mL of solution containing: 11.3 mM Tris-base, 1.44 mM EDTA, 125 mM $CaCl_2$, 25 mM BES, 140 mM NaCl and 0.75 mM Na_2HPO_4 in MILLIQ H_2O. 1 mL of transfection solution was added per 10 cm plate or 150 µL per 35 mm plate. Previously, we showed overexpression of NMDARs and htt by western blot analysis after transfection under similar conditions (Chen et al., 1999a,b). Cells were transfected for ~8 h (no longer than 12 hours) in a 3 % CO_2 atmosphere and then washed twice with PBS and incubated in fresh medium with saturating concentrations of NMDAR antagonists [1 mM (±)-2-amino-5-phosphonopentanoic acid (APV) or 100 µM memantine]. NMDAR antagonists were added post-transfection in order to prevent glutamate and glycine (present in the media) -induced cell death in cells that express functional NMDARs (Anegawa et al., 1995; Raymond et al., 1996).

2.1.2 Lipofectamine Transfection

Cells that were transfected by the Lipofectamine method were plated at a density of ~2 x 10^6 cells/mL on 6-well culture dishes for toxicity assays, or 10 cm plates for protein expression assays, pre-coated with 100 µg/mL poly-D-Lysine and grown for ~24 h before the transfection. We used a 2:3:3:6 ratio of cDNAs encoding β-gal; NR1A; NR2 (A or B); and GFP, or htt (full-length htt-15Q or htt-138Q; or else an N-terminal fragment containing 548 amino acids, htt-N548-15Q or htt-N548-128Q); using a total of 14 µg of plasmid DNA per 6-well plate. The cDNA encoding full-length htt was 10,366 base pairs, whereas that encoding the N-terminal fragment of htt was only 1955 base pairs, so the molar quantity of cDNA was markedly higher

for truncated than for full-length htt. Again, Western blot analysis confirmed overexpression of htt under these conditions (not shown). Using a ratio of 1 µg cDNA: 6 µL Lipofectamine (Gibco): 100 µL OPTI-MEM (Gibco), cells were transfected for 6 h in a 5 % CO_2 atmosphere. After the transfection, cells were washed twice with PBS and incubated with fresh medium and saturating concentrations of NMDAR antagonists [1 mM APV (high affinity antagonist for the NR2A-NMDAR subtype) and 250 µM 5,7-dichlorokynurenic acid (high affinity antagonist at the NR1-NMDAR glycine site)]. In each experiment, the transfection efficiency was determined by staining for β-galactosidase activity as previously described (Raymond et al., 1996). A sister plate of cells were fixed for 5 min in solution containing 37 % formaldehyde and 25 % glutaraldehyde in PBS and rinsed 3 times with PBS. Cells were stained for β-galactosidase activity using a solution containing: 1.22 mM X-galactosidase in 24 uL DMSO added to 2 mM $MgCl_2$ and 8 mg potassium ferricyanide and 10.5 mg potassium ferrocyanide in 5 mL PBS, and incubated in solution overnight at 37°C. Cells were counted positively transfected if they appeared blue in color under brightfield microscopy. Plates of cells showing less than 50 % transfection efficiency were not used for further experiments.

2.2 Excitotoxicity Induction

36-42 h after the start of the transfection the cells were washed twice with warm PBS and then incubated in a physiological salt solution containing: 140 mM NaCl, 1.4 mM $CaCl_2$, 5.4 mM KCl, 1.2 mM NaH_2PO_4, 21 mM glucose, and 26 mM $NaHCO_3$, pH 7.4. A saturating concentration of antagonist (see above) was reapplied to half the wells and 1 mM NMDA (with 50 µM glycine), was applied to the remaining half. The cells were incubated for 6-h in humidified 5 % CO_2, 37 °C atmosphere. Cell death induced by withdrawal of antagonist alone was not explored since previous data showed that cells expressing recombinant receptors were

vulnerable to death induced by low levels of agonist found in the medium or else released from dying cells (Anegawa et al., 1995; Raymond et al., 1996).

2.3 Toxicity Assay: β-galactosidase Assay

After the 6-h incubation with agonist or antagonist, survival of transfected cells was measured by analyzing β-galactosidase activity using a colorimetric assay (Sambrook et al., 1998). Medium containing floating cells and β-galactosidase released into the media (representative of non-viable cells) was collected, and then the remaining adherent cells (representative of viable cells) were scraped into ice-cold 0.1 M sodium phosphate solution: 0.082 M Na_2HPO_4-$2H_2O$ and 0.018 M NaH_2PO_4-$2H_2O$ in H_2O (pH 7.5) and collected into separate tubes. Both sets of samples were sonicated twice for 10 s, then a 0.1 mL aliquot from each tube was added to 0.54 mL of the o-nitrophenyl-β-D-galactopyranoside (ONPG) substrate mixture containing: 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, 13 mM ONPG in 0.1 M sodium phosphate solution pH 7.5, and then incubated for 20 min at 37 °C. The reaction was stopped by adding 1 mL of 1 M $NaHCO_3$ to each tube, and the color change was assessed by measuring absorbance at 420 nm using a Pharmacia Biotech Ultraspec 3000 spectrophotometer. A standard curve was constructed from serial dilutions of purified β-galactosidase to confirm that the absorbance measurements made for the experimental samples were within the linear range of β-gal activity. Cell survival was determined as the absorbance measured from adherent cells relative to the total (adherent cells + incubation media containing released β-gal and floating cells). Viability of adherent vs. floating cells was confirmed by trypan blue staining. There were very few intact floating cells, but for those we observed, ~90 % were non-viable, and only ~15 % of cells adherent to the plate were non-viable. To examine NMDAR-mediated toxicity specifically, we normalized cell survival obtained for the agonist condition to that obtained for

the antagonist condition to calculate relative cell survival. Relative cell death was calculated as 1 − relative cell survival.

2.4 Assessment of Apoptosis: Hoechst Dye Assay

After the 6-h incubation, apoptosis was determined morphologically using Hoechst 33258 dye, pentahydrate (bis-benzimide) (Molecular Probes), a cell permeant dye that binds to the DNA (AT and double-stranded DNA selective) in nuclei of all cells through the bis-benzimide. The cells were washed once with warm PBS and then fixed in 3 % paraformaldehyde solution, pH 7.3, for 1-h. After a series of washes with PBS, the cells were stained with 2.5 µg/mL Hoechst 33258 dye for 10 min at 4 °C, washed using SlowFade Antifade Kit (Molecular Probes), and sealed on a microscope slide. The cells were analyzed in a blinded fashion under fluorescence at 356 nm using a 40X Plan-NEOFLUAR objective on a Zeiss Axiophot microscope. Cells were scored as apoptotic only if they displayed a globular, or blebbed, nucleus; all other cells were scored as being non-apoptotic, including those with chromatin condensation alone.

2.5 Western Blot Analysis

HEK293 cells were cultured in 10 cm plates and transfected as previously described. 24 hours after start of the transfection cells were collected. For analysis of NMDAR expression, cells were rinsed twice in PBS and collected in 1 mL of Harvest Buffer which contained: 1 mM EDTA, 1 mM EGTA, 100 U/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) in PBS, on ice and then sonicated. Samples were spun at 14000 rpm for 20 min at 4 ^{0}C. The pellet containing the membrane-associated fraction was resuspended in 500 µL of Solubilization Buffer containing: Harvest Buffer and 1 % Triton-X-100, and sonicated for 10s. Samples underwent end-over-end rotation (RoTo) for 30 min and centrifuged at 14000 rpm for 20 min at 4 ^{0}C where

supernatant was saved. 25 μL of sample was kept for bicinchoninic acid (BCA) protein analysis (Pierce) in 225 μL Solubilization Buffer using the Enhanced Tube Protocol (5-250 μg/ mL). After a 30 min. incubation at 60 ^{0}C, absorbance was measured at 562 nm and the concentration of the protein in the samples was calculated from the standard curve.

For Western Blot analysis of HIP-1 expression, the procedure was similar to the above protocol. The supernatant from the original centrifugation containing the cytoplasmic fraction, was saved along with the membrane fraction for further use.

For either analysis, usually 5 and 10 μg of sample were added to dithiothreitol (DTT)-containing loading buffer and separated by 8 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run at 100 mV for 1.5 hours. Gels were transferred overnight onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in transfer buffer containing: 12.5 mM Tris, 100 mM glycine, 20 % methanol in H$_2$O with stirring on ice at 0.02-0.03 amps. After transfer, blots underwent a 30 min incubation with 5 % milk blot solution (5 % dry milk, 0.5 % polyoxyethylene-sorbitan monolaurate, 10 % Tris-buffered saline (TBS)) and then a 5 min incubation with 0.5 % milk blot solution with shaking. Blots were incubated in 0.5 % milk blot solution with rabbit polyclonal NR2A/NR2B (1:100; Chemicon) applied overnight, or with antibody against NR1A (1 μg/mL; Upstate) applied for 1.5 hr, or with polyclonal HIP-1 (HIP-1-526) antibody (1:50 for a 2 hour incubation – a gift from Dr. M.R Hayden, CMMT, Vancouver, BC, Canada). Blots were washed 3 times with 0.5 % milk blot solution, 10 min each with shaking. HRP-labelled secondary antibody--anti-rabbit Ig HRP-linked whole antibody from donkey (Amersham; 1:5000)--in 0.5 % milk blot solution was applied for about 1-h. Blots were washed three times with 0.5 % milk blot solution followed by two washes with TBS solution, each for 10 min. Blots were incubated for 1 min in equal proportions of enhanced chemiluminescence system (ECL) reagents 1 and 2 (Amersham). Bands were visualized by exposure of blots to film (usually a 2, 5 and 10 min exposure) in the dark, which was developed

and processed immediately by a 100 Plus automatic x-ray film processor (All-Pro Imaging Corp., Hicksville, NY, USA).

2.6 Materials

NR1A and NR2B cDNAs from rat brain (gifts from S. Nakanishi, Kyoto University, Kyoto, Japan) and NR2A cDNA from mouse brain ($\epsilon 1$) (a gift from M. Mishina, University of Tokyo, Tokyo) were previously subcloned into the mammalian expression vector, pRK5, containing the cytomegalovirus promoter (Raymond et al., 1996; Chen et al., 1997). cDNA constructs for the full-length htt with 15 or 138 glutamine repeats (htt-15Q or htt-138Q), and the N-terminal fragment of htt with 15 or 128 glutamine repeats (htt-N548-15Q or htt-N548-128Q, respectively) were generated and subcloned into an expression vector containing the cytomegalovirus promoter, as previously described (Goldberg et al., 1996). HIP-1 (containing the point mutation F398G) was cloned into the pCI vector (a gift from Dr. M.R. Hayden). The *Escherichia coli* β-gal cDNA (pCMVβ) was from Clontech Laboratories, Inc. (Palo Alto, CA). All chemicals, unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2.7 Data Analysis and Presentation

Figures were generated using Origin or Adobe Photoshop or Corel Draw software. Data are presented as the mean $\pm$ S.E.M. Significance was determined by Student's *t* test (paired or unpaired, as appropriate) or one-way ANOVA using a 95 % confidence interval.

YAC Transgenic Mice:

2.8 YAC Transgenic Mice

Neuronal cultures were generated postnatal day 0-1 using heterozygous YAC72 mice from line 2511 (as described by Hodgson et al., 1999) derived from a pure FVBN/N strain background. The control mice were pure FVBN/N. The transgenic mice expressing the human HD gene were generated using two well characterized YACs, YGA2 and 353G6 (Hodgson et al., 1996). YGA2 is the larger YAC at 600 kb and extends ~350 kb 5' and 50 kb 3' of the HD gene, and 353G6 is 350 kb, extending 25 kb 5' and 120 kb 3' of the HD gene (Hodgson et al., 1996). After homologous recombination in yeast to introduce the expanded CAG repeats (Hodgson et al., 1999), the YAC46 mice and YAC72 mice were generated. In addition to the human HD gene, the YACs contained the Trpl gene, ARS and CEN4 on the left YAC arm and the URA3 gene on the right YAC arm (Hodgson et al., 1996, 1996). Only crosses between two homozygous YAC transgenic mice, designed as described previously by Hodgson et al., 1999, or between homozygous YAC and WT mice, were used to make neostriatal cultures. YAC 46 (46Q; 668 line) or YAC 72 (72Q; 2511 line) mice were used as models expressing full-length mutant htt and compared to FVB WT mice.

2.9 Neuronal Culture

2.9.1 Primary Striatal Culture

Primary striatal cultures have been extensively used to study characteristics of striatal neurons (Kovacs et al., 2001; Leveque et al., 2000; Martin-Negrier et al., 2000; Moldrich et al., 2001; Parak et al., 2001; Petersen and Brundin, 1999; Petersen et al., 2000; Mao and Wang, 2001). Generally, methods that utilize a serum-free growth medium for striatal neurons contain ~1-7 % glial presence (Moldrich et al., 2001; Kovacs et al., 2001; Petersen et al., 2000) shown by immunohistochemical staining for glial fibrillary acidic protein (GFAP). Striatal cultures using this medium are found to express >95 % DARPP-32 positive neurons, deemed to be the

marker for MSNs (Petersen and Brundin, 1999). Furthermore, striatal cultures are found to be physiologically mature after 8 DIV (Petersen, et al., 2000; Moldrich et al., 2001; Kovacs et al., 2001; Koroshetz et al., 1990). Maximal cell death induced with NMDA application was ~50-60 % more than MSNs exposed to control conditions (Petersen and Brundin, 1999; Moldrich et al., 2001). At 8 DIV, Kovacs et al., 2001 have shown there is more NR2B subunit mRNA expression in striatal cultures than NR2A mRNA expression.

Anterior striata were dissected, using landmarks previously described (Howe and Surmeier, 1995), from postnatal day 0-1 (P0-1) mice in ice-cold divalent-free Hank's Balanced Salt Solution (HBSS) (Gibco), then diced and dissociated with 0.25 % trypsin-EDTA (Gibco) followed by enzymatic inhibition with 10 % heat-inactivated fetal bovine serum (Gibco) in Neurobasal medium (Gibco), or else dissociated with papain solution containing: 18 U/mL papain, 1.1 M L-cysteine, 1 mM $CaCl_2$, 500 μM EDTA in HBSS and enzymatically inhibited with solution containing: 2.5 mg/mL trypsin inhibitor, 2.5 mg/mL BSA, 200 μg/mL DNAase I in Neurobasal media. Cells were further dissociated using a series of reducing bore-size Pasteur pipettes and then plated at a density of ~1 x 10^6 cells/mL on poly-D-lysine (M.W. 30-70 g/mol; 250 μg/mL final)-coated dishes with or without nitric acid-treated 12 mm round coverslips in plating medium containing: Neurobasal media, B27 (Gibco), 100 units/mL penicillin-streptomycin (Gibco), and 2 mM α-glutamine and maintained at 37 °C, 5 % CO_2 with humidity. Cells were fed ~3 days after plating with medium lacking α-glutamine. Cells were used for experiments at 9-12 DIV.

2.9.2 Primary Cerebellar Culture

We elected to use primary cerebellar neuronal cultures as control cultures since the cerebellum is largely unaffected in HD and these neurons express different NMDAR subunit

composition than striatal neurons. Primary cerebellar neuronal cultures have been used extensively to study glutamate receptor-mediated signal transduction and cell death (Balazs et al., 1992; Brandoli et al., 1998; Cebere et al., 1999; Didier et al., 1997; Kovacs et al., 2000; 2001 2001; Moldrich et al., 2001; Weller et al., 1993). Cerebellar neurons are largely composed of cerebellar granule neurons, are almost completely glutamatergic and express high levels of various glutamate receptors (Hack and Balazs, 1995; Resink et al., 1995) and especially NR1 with NR2A and/or NR2C but not NR2B NMDAR subunits in mature cerebellar cultures and *in vivo* (Kovacs et al., 2001; Thompson et al., 2000; Vallano et al., 1996). Cerebellar neurons can be successfully cultured with or without serum (Kovacs et al., 2001; Moldrich et al., 2001) in the growth media, but do require 25 mM KCl to be present in order for cell survival (Moldrich et al, 2001, reviewed by Ikeuchi et al., 1998). Utilizing a serum-free growth medium for cerebellar neurons or adding DNA synthesis inhibitor, cytosine-β-arabinofuranoside, cultures contain <5 % glial presence (Moldrich et al., 2001; Kovacs et al., 2001), as shown by immunohistochemical staining for glial fibrillary acidic protein (GFAP). Cerebellar cultures are physiologically mature after 8 DIV (Moldrich et al., 2001; Kovacs et al., 2001). Additionally, maximal stimulation with NMDA in CGNs showed >40 % cell death (Daniels and Brown, 2001) which could be increased to 80 % when CGNs had been co-cultured with a glial layer (Daniels and Brown, 2001).

Cerebella were dissected from P8 mice following a similar protocol as for the MSNs with a few changes. Trypsin action was ended with plating medium containing: Basal Medium Eagle with Earle's Salts and $NaHCO_3$, 10 % heat inactivated fetal calf serum (Gibco), 100 units/mL penicillin-streptomycin, 2 mM α-glutamine, 17 mM D-glucose, and 25 mM KCl. Cerebellar granule neurons (CGNs) were plated at 2.0 x 10^6 cells/mL and examined 8-9 DIV.

2.10 Induction of Cell Death

After 9-12 DIV, cultured MSNs were exposed for 10 min to varying NMDA concentrations (30, 100, and 3000 μM) in triplicate or greater, in the presence of 50 μM glycine in a physiological salt solution: 139 mM NaCl, 3.5 mM KCl, 2 mM NaHCO$_3$, 5 mM HEPES, 3 mM Na$_2$HPO$_4$-7H$_2$O, 2.3 mM CaCl$_2$, 11 mM D-glucose, and phenol red, pH 7.35 or exposed to salt solution alone (control). After NMDA treatment, the cells were rinsed with plating medium, the original (conditioned) plating medium was replaced, and cells were maintained in humidified 5 % CO$_2$ at 37 °C. An additional set of sister cultures that did not undergo the procedure (i.e., they were removed from the incubator for the same length of time as the treated cells but did not undergo changes to the medium) were also maintained under the same conditions (untreated). Some cultures underwent exposure to 20 μM MK-801, a NMDAR use-dependent antagonist, or to 10 μM ifenprodil, an NR2B-NMDAR subtype-specific antagonist, in conditioned medium for 30 min. before agonist exposure, in the agonist solution, as well as after exposure. Other experiments utilized 10 μM caspase inhibitor z-DEVD-fmk (Calbiochem) or 1 μM cyclosporin A (Petersen et al., 2000), requiring a 1-h pretreatment, with additions during and after agonist exposure. Other tests utilized 10 μM staurosporine, a potent protein kinase C inhibitor which has been widely used to activate programmed cell death in neuronal and non-neuronal cells (Bertrand et al., 1994; Koh et al., 1995; Prehn et al., 1997; Krohn et al., 1999) for a 6-h exposure, added directly to the media. In experiments examining non-NMDAR-mediated cell death, MSNs underwent a 2-h exposure to 1 mM AMPA (Tocris) and 50 μM cyclothiazide with or without 10 μM ifenprodil and were examined for cell death 24 h later. At 8-9 DIV CGNs underwent exposure to NMDA for 30 min., and then were refreshed with serum-omitted medium and examined for cell death 24 h later.

2.11 Toxicity Assay: Trypan Blue Inclusion Assay

In striatal cultures, the trypan blue inclusion assay was done 24-h after exposure to NMDA. Viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be discriminated by differential staining. Cells with damaged plasma membrane permeability are stained, whereas undamaged cells are not stained with dyes that do not penetrate the plasma membrane ('exclusion dyes'). The trypan blue dye binds to intracellular proteins of leaky cells. Trypan blue dye (0.4 %) was added to the cultures and 10 random photographs of different brightfield (250X) images per dish were taken (photographer was not blinded). The photographs of the 10 fields were blind-coded and quantified for total cell numbers and number of trypan blue-positive cells; only cells that met the morphological criteria for MSNs were scored. Values were averaged per condition and total cell numbers were compared within a condition group and between conditions. The percentage trypan blue-positive cells in cultures that underwent exposure to salt solution alone were subtracted from the percentage trypan blue positive cells measured after NMDA treatment. Neuronal loss in the control (salt solution alone) was minimal (~5 %), and comparable with that of the untreated group.

2.12 Assessment of Apoptosis: TUNEL Assay

For the fluorescent TUNEL (Terminal Deoxynucleotidyltransferase-mediated dUTP-FITC Labelling) assay, 24-h after the MSN cultures grown on coverslips were exposed to the aforementioned NMDA concentrations, they were fixed for 1-h in a 4 % paraformaldehyde solution, pH 7.3. Cells were stained with the TUNEL mixture, following the protocol as described by Roche Diagnostics (TUNEL Enzymatic Labelling Assay-*In Situ* Cell Detection Kit; Fluorescein), or to label solution alone (negative control), counterstained with 4 μM propidium iodide (PI) (Molecular Probes) and incubated with SlowFade from the Antifade Kit (Molecular Probes), according to manufacturer's instructions. The number of TUNEL-positive cells was compared to the total number (>500) of PI-positive cells in randomly chosen microscopic fields

after blindly counting only cells showing condensed nuclei and resembling MSNs. The percentages of apoptotic cells in the control solution group were subtracted from the percentage of apoptotic cells after exposure to NMDA, for each respective genotype.

2.13 Caspase Activity Assay

As described by Wellington et al., 2000, we measured DEVD-ase activity with the ApoAlert fluorometric kit (Clontech) at different time points (3, 6, 12, 18, 24-h) after exposure to NMDA in MSN primary cultures. At the various time points after agonist exposure, we harvested the cells, lysed them with Cell Lysis Buffer (Clontech) and collected the supernatant. Protein concentration in the supernatant was measured using the Lowry assay. The supernatant was incubated with reaction mixture containing the substrate, DEVD-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) (Clontech) and compared to control samples that were inhibited with DEVD-CHO and to substrate blanks, which lacked DEVD-AFC. Caspase activity was measured fluorometrically every 5 min. for 1-h at 37 °C at excitation wavelength 390 nm and emission wavelength 510 nm. Values were expressed as relative levels of DEVD-ase activity (linear range of rate of change in fluorescence) normalized to the concentration of protein of the samples. Samples that underwent exposure to NMDA were compared to measurements from cells that underwent exposure to salt solution alone.

2.14 Immunohistochemical Staining

MSNs cultured on coverslips were treated with NMDA or salt solution as a control. After the stimulus, cells recovered for 24-h and were rinsed with PBS and fixed in freshly prepared 4 % paraformaldehyde in PBS, rinsed in PBS, permeabilized with 0.2 % Triton-X100 in PBS, washed in PBS, and blocked with 10 % normal goat serum (NGS) in PBS for 30 min. Coverslips were then incubated with a polyclonal antibody specific for the NR2A or NR2B

NMDAR subtypes (1:2000; a kind gift of Dr. R. Huganir) in 2 % NGS in PBS or polyclonal antibody to DARPP-32 (1:2000; Chemicon) or polyclonal NR1A antibody (1 μg/ml; Upstate) overnight at 4 ^{0}C. After washing three times in PBS, coverslips were then incubated with biotinylated anti-rabbit secondary antibody (1:500; Vector) in 2 % NGS in PBS for 2 hours at room temperature, followed by 3 washes in PBS. Coverslips were incubated with shaking for 60 min at room temperature with ABC Kit (avidin-biotin-HRP complex) and washed 3 times with PBS. Coverslips were stained for 10-30 min with DAB Substrate Kit for Peroxidase (Vector). After development, coverslips were kept in PBS until mounted on slides. Control neurons were exposed to secondary antibody alone and underwent subsequent incubation with ABC kit and stained with DAB Substrate Kit (data not shown). Images were captured using a CCD camera using Northern Eclipse software.

MSNs were cultured on coverslips and treated for caspase activity measurements. After recovery for 6-h, cells were rinsed with PBS and fixed in freshly prepared 4 % paraformaldehyde in PBS, rinsed in PBS, permeabilized with 0.5 % Triton-X100 in PBS, washed in PBS, and blocked with 4 % normal goat serum (NGS) in PBS for 30 min. Coverslips were then incubated with a polyclonal antibody (1:5000) specific for the cleaved and activated form of caspase-3 in 4 % NGS in PBS (a kind gift of Drs. Donald Nicholson and Sophie Roy) overnight at 4 ^{0}C in a humidified chamber. After washing three times in PBS, coverslips were then incubated with Alexa 594-conjugated goat-anti-rabbit secondary antibody (1:800) in 4 % NGS in PBS for 30 min. at room temperature, followed by 3 washes in PBS. Cells were counterstained with Hoechst dye (Molecular Probes) and mounted on glass slides. >500 MSNs were counted per condition. Images were captured using a CCD camera using Prince (Northern Eclipse) software.

After a 6-h recovery period from a brief exposure to NMDA or salt solution, cells were rinsed with PBS and fixed in freshly prepared 4 % paraformaldehyde in PBS, rinsed in PBS, permeabilized with 0.5 % Triton-X100 in PBS, washed in PBS, and blocked with 4 % normal

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goat serum (NGS) in PBS for 30 min. Coverslips were then incubated with antibodies specific

for the C-terminal ends of htt caspase cleavage products ending at aa 513 or 552, (Htt513 and

Htt552) as primary antibodies (1:1500) (A kind gift of Dr. Donald Nicholson) [C.L. Wellington

and M.R. Hayden (personal communication) for description of preparation] in 4 % NGS in PBS

overnight at 4 ^{0}C in a humidified chamber. After washing three times in PBS, coverslips were

then incubated with Alexa 594-conjugated goat-anti-rabbit secondary antibody (1:800) in 4 %

NGS in PBS for 30 min. at room temperature, followed by 3 washes in PBS. Cells were

counterstained with Hoechst dye (Molecular Probes) and mounted on glass slides. Images were

captured using a CCD camera using Prince (Northern Eclipse) software.

2.15 Western Blot Analysis

Cultured MSNs received a toxic stimulus and were allowed to recover. The medium was

aspirated and 75 uL Sucrose Buffer containing: 5 mM PMSF, COMPLETE protease inhibitor

tablet (Roche), 10 μM z-VAD-fmk and z-DEVD-fmk (Calbiochem), 0.25 M sucrose, 20 mM

Tris-HCl, 1 mM $MgCl_2$, 0.5 mM EDTA in H_2O was added to each culture plate. Cells were

harvested and underwent 5 sec sonication and then spun for 5 min at 6000 rpm at 4 ^{0}C. The

pellet was discarded as debris and the supernatant (whole cell lysate) was used as the sample to

be tested for htt cleavage. As described previously, a protein assay was run on the samples.

Standardly, 30 and 60 μg of sample were loaded in 8 % SDS-PAGE gels run at 150 mV for 1-1.5

hours. Gels were transferred overnight and purified primary antibodies to polyclonal N-terminal

htt [(BKP1 at 7.5 μg/mL-a kind gift of M.R. Hayden) or (Chemicon mAB2166 at 1:200)] were

applied overnight. For use with the mAB2166 antibody the HRP-labelled secondary antibody--

anti-rabbit Ig HRP-linked whole antibody from donkey (Amersham; 1:5000)--in 0.5 % Blot

Solution was applied for about 1-h. Use with the BKP1 antibody required an HRP-conjugated

goat-anti-mouse secondary (1:2000; Jackson Laboratories) incubated for 30 min. Blots were

washed three times with 0.5 % Blot Solution followed by two washes with TBS solution, each 10 min. Blots were incubated for 1 min in equal proportions of ECL reagents 1 and 2 (Amersham), and exposed to film (usually 2, 5 and 10 min, or longer), which was developed and processed and bands visualized. Blots for re-analysis with another antibody were stripped with buffer containing: 100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl, pH 6.7 in distilled H_2O for 30 min at 50°C with shaking. Blots were then rinsed twice with 1x Tris-Buffered Saline solution, and blocked and reprobed with primary antibody as before.

2.16 Intracellular Calcium Imaging

MSNs were cultured on coverslips for at least 9 days and measurements were taken from age-matched cultures expressing mutant htt or normal htt on the same day at room temperature. Coverslips were incubated in the dark at room temperature for 30 min in 25 μg Mag-Fura-2 acetoxymethyl ester (AM) (final concentration was 5 μM) (Molecular Probes) dissolved in 35 μL anhydrous DMSO and 35 mg BSA in 7 mL Balanced Salt solution (BSS) containing: 139 mM NaCl, 3.5 mM KCl, 2 mM $NaHCO_3$, 5 mM HEPES, 3 mM Na_2HPO_4-$7H_2O$, 2.3 mM $CaCl_2$, 11 mM D-glucose, 50 μM glycine, pH 7.35. Mag-Fura2 was used as a low-affinity calcium indicator to avoid underestimation of the calcium response (Stout and Reynolds, 1999). Although Mag-Fura2 also has some affinity for magnesium (K_d for Mg^{2+} is 1.9 mM; K_d for Ca^{2+} is 25 μM), we thought that a magnesium-free solution would eliminate this variable; this was confirmed using Fura-FF (data not shown). The coverslips were then transferred to BSS alone for 20 min before each experiment. Coverslips were loaded into a chamber that permitted fast perfusion at 2.4 mL/min of the BSS over the cells. Cells were viewed on a Zeiss Axiophot fluorescence microscope. This fluorescent ratiometric dye is dually excited at 334 and 380 nm (fluorescence increases at 334 nm with increasing free calcium levels and fluorescence decreases

at 380 nm with increasing free calcium levels) and emitted at >510 nm). The ratio of fluorescence of Mag-Fura-2 at 334/380 nm excitation (corrected for background fluorescence) was determined at a rate of 1 per sec during the exposure to NMDA and a rate of 1 per 10 sec during exposure to control BSS. Imaging used a 40X objective, a CCD camera and digital Attofluor RatioVision imaging software system. The camera gain was set at a level in order to minimize camera saturation while at the same time maximizing image intensity. The data were analyzed using AttoGraph and Microsoft Excel programs. Regions of interest (ROIs) were sized and placed over the soma of cells that morphologically resembled MSNs. Responses were subtracted from a ROI that was placed over an area lacking cells, representative of the background fluorescence (all values are corrected for background fluorescence). The number of cells measured at one time ranged from less than ten to 99, depending on the density of the cells on one coverslip. The cells that responded to stimulus application were used in the analysis, and the number of cells that responded to stimulus varied between coverslips. The baseline response to BSS flow was measured for 10 minutes to assure cellular homeostasis before the stimulus was applied. A 10 min application of 3 mM NMDA in BSS was perfused on the coverslips and recovery followed with reapplication of BSS alone. Cells were allowed to recover for at least 30 min or longer. After recording, cells were fixed in 4 % paraformaldehyde and could be used for immunohistochemistry. Data conversion calculations and analysis were performed using Microsoft Excel Macros created in Microsoft Visual Basic and described by Dr. Gord Rintoul (2000), as found in his doctoral thesis. Control cells were also measured using 2 μM Fura-FF (Molecular Probes), a low affinity calcium indicator with no affinity for magnesium, producing similar results (data not shown).

2.17 Mitochondrial Membrane Potential Imaging

Mitochondrial membrane potential ($\Delta\psi_m$) was assessed by single wavelength imaging of rhodamine-123 fluorescence. This dye is preferentially sequestered in mitochondria where its fluorescence is quenched. A reduction in $\Delta\psi_m$ results in the release of rhodamine-123 into the cytoplasm and an increase in fluorescence (Brorson et al., 1997; Schuchmann et al., 1998). MSNs were cultured on coverslips for at least 9 days and measurements were taken from age-matched cultures expressing mutant htt or normal htt on the same day at room temperature. Coverslips were incubated in the dark at room temperature for 15 min in 2.5 µL Rhodamine-123 (Molecular Probes; 10 mg/mL in ethanol) stock solution in 5 mL BSS, as described above. The coverslips were then transferred to BSS alone for 5 min before the experiment. Coverslips were loaded into a chamber that permits fast perfusion at 2.4 mL/min of the BSS over the cells. Cells were viewed on a Zeiss Axiophot fluorescence microscope and rhodamine-123 fluorescence was excited at 488 nm and measured above 510 nm using a 495 nm dichroic mirror and a 510 nm long-pass filter. Imaging used a 40X objective, CCD camera and digital Attofluor RatioVision imaging software system. Data were analyzed using AttoGraph and Microsoft Excel programs. Regions of interest (ROIs) were sized and placed over the soma of cells that morphologically resembled MSNs. Responses were subtracted from a ROI that was placed over an area lacking cells, representative of the background fluorescence. The number of cells measured at one time ranged from less than ten to 99. The cells that responded to stimulus application were used in the analysis, and the number of cells that responded to stimulus varied between coverslips. The baseline response to BSS flow was measured for 10 minutes to ensure cellular homeostasis before the stimulus was applied. A 10 minute application of 3 mM NMDA in BSS was perfused on the coverslips and recovery followed reapplication of BSS alone. Cells were allowed to recover for at least 30 min or longer. After recording, cells were fixed in 4 % paraformaldehyde and could be used for immunocytochemistry. Data were expressed as background corrected delta F/F values since rhodamine-123 is a single wavelength dye and its initial fluorescence intensity

will vary from cell to cell depending upon the efficiency of loading and the volume of the cell in the field of view. Data collection and analysis were similar to the Mag-Fura2 experiments. Data conversion calculations and analysis were performed using macros created and described by Dr. Gord Rintoul (2000), as found in his doctoral thesis.

2.18 Data Analysis and Presentation

Figures were generated using Origin or Adobe Photoshop or Corel Draw software. Data are presented as the mean $\pm$ S.E.M. Significant differences were determined using the two factor ANOVA followed by Bonferroni-Dunn's post hoc test for pairwise comparisons, unless otherwise noted.

CHAPTER 3

Selective Enhancement of NR1A/NR2B-Mediated Apoptotic Cell Death By Mutant Full-Length Htt in HEK293 Cells

Many recent studies have been utilizing mammalian expression systems to explore the properties of a foreign protein when it is overexpressed. The transfer of foreign genes into cell lines, such as human embryonic kidney cells (HEK293 cells), has been essential to our understanding of the functional significance of genes and regulatory sequences, and the resulting protein characteristics. We decided to use HEK293 cells as a good place to begin to test whether NMDARs may functionally interact with mutant htt. Heteromeric NMDAR complexes of NR1A and NR2A or NR2B expressed in HEK293 cells show agonist-evoked current amplitudes and biophysical properties similar to those found in neurons (Chen et al., 1997; reviewed by Dingledine et al., 1999). Therefore, this expression system has been widely used as a model to determine links between NMDAR ion channel properties and excitotoxicity (Cik et al., 1993, 1994; Anegawa et al., 1995; Boeckman and Aizenman, 1996; Raymond et al., 1996). First, we reported that the expression of full-length mutant htt in HEK293 cells resulted in enhanced current amplitude for NR1A/NR2B but not NR1A/NR2A (Chen et al., 1999b; see Figure 1). We elected to use this model system to determine whether mutant htt would similarly enhance excitotoxic cell death selectively in NR1A/NR2B-transfected cells.

It was also a useful model because we could control the expression of desired proteins and could restrict cell death analysis to only transfected cells (Chen et al., 1999a,b; Raymond et al., 1996). We did this by co-transfecting cDNA encoding the marker protein β-galactosidase (β-gal) and measured activity of this enzyme spectrophotometrically in viable, adherent cells

relative to the activity found in the incubation media (released by ruptured, non-viable cells), in a manner analogous to the lactate dehydrogenase assay typically used in other cell death studies (Koh and Choi, 1987). Furthermore, to determine cell death mediated strictly by NMDAR activation, we normalized survival of NMDA/glycine-treated cells to that of cells treated with supersaturating concentrations of NMDAR antagonist (see Methods). Cell death was evaluated after a 6-h exposure to 1 mM NMDA. These conditions were selected based on our previously published data showing that 1 mM NMDA results in near maximal death (~60%) of cells expressing NR1A/NR2A, assessed by a β-gal staining assay (Raymond et al., 1996). Although we did not perform a dose-response curve for NMDAR-mediated cell death in cells expressing NR1A/NR2B, this subunit has been shown to be more sensitive to agonist than NR1A/NR2A (EC_{50} for glutamate-evoked peak current of ~1 μM for NR1A/NR2B versus ~8 μM for NR1A/NR2A -- Chen et al., 1999b) and others have found that death of NR1A/NR2B-transfected CHO cells is maximal for NMDA concentrations above 30 μM (Boeckman and Aizenman, 1996). Therefore, we used 1 mM NMDA as a maximal stimulus for both NMDAR subtypes. For reasons cited above, this cellular expression system seemed like a logical place to begin testing whether NMDARs may play a role in HD.

3.1 Full-Length Mutant Htt Enhances NMDAR-Mediated Cell Death in HEK293 Cells

To determine whether expression of full-length mutant htt could increase NR1A/NR2B or NR1A/NR2A-mediated cytotoxicity, we compared NMDA-induced cell death among sister cultures of HEK293 cells co-expressing β-gal; NR1A; NR2B or NR2A; and GFP, or htt-15Q, or htt-138Q. When these transfections were done in parallel, plates of cells transfected with htt-15Q, or htt-138Q showed equivalent NMDAR expression levels, as indicated by western blot analysis with NR1-specific antibodies (data shown for NR1A/NR2B in Chen et al., 1999b, and

unpublished data for NR1A/NR2A-transfected cells). All three sets of transfected cells (i.e., NR1/NR2 with GFP, htt-15Q or htt-138Q) were exposed for 6-h to agonist or antagonist in a physiological salt solution. β-gal assays showed that coexpression of htt-138Q with NR1A/NR2B resulted in a significant increase in excitotoxic death compared to the htt-15Q or GFP controls (Figure 3A). Specifically, we observed 42% agonist-induced death for NR1A/NR2B-transfected cells expressing htt-138Q versus 28% and 23% for htt-15Q and GFP-expressing cells, respectively. Comparison among these sister cultures, therefore, showed that the polyQ expansion in htt is associated with an increase in NR1A/NR2B-mediated excitotoxic cell death of 50% and 82% above that observed for htt-15Q and GFP, respectively (Figure 3B). By comparison, the overall level of agonist-induced death was higher in cells expressing NR1A/NR2A, but there was a smaller relative increase upon co-expression of htt-138Q. For example, NMDA induced 78% cell death for htt-138Q-expressing cells versus 68% and 61% for htt-15Q and GFP, respectively, yielding a 14% and 28% increase in excitotoxic death for htt-138Q compared with controls (Figures 3A, B). It should be noted that the relative difference between mutant htt-enhanced NR1A/NR2A- and NR1A/NR2B-dependent excitotoxicity is not observed when the values of cell death for GFP-expressing cells are subtracted from those for htt-138Q-transfected cells. Therefore, it is important to regard the NMDAR-subunit dependent differences in excitotoxicity, found using our ratio analysis in this section, as preliminary; these differences become more apparent upon examination of specific modes of cell death (Sections 3.2 and 3.3). Although there was a trend toward increased excitotoxic cell death in those cells co-expressing NMDARs and htt-15Q compared with GFP, this difference was not significant (p=0.15, N=10 for NR1A/NR2B and p=0.19, N=6 for NR1A/NR2A, by paired t test). Importantly, when NMDARs were blocked by antagonist, death of NR1/NR2-transfected cells was similar for co-transfections with GFP, htt-15Q, and htt-138Q, in spite of the 6-h withdrawal of growth factors (Figure 3C).

Our results demonstrate for the first time that expression of mutant huntingtin enhances cell death mediated by NMDAR activation, supporting a role for excitotoxicity in the pathogenesis of HD. It is interesting to note that in spite of selective enhancement of NR1A/NR2B and not NR1A/NR2A currents by full-length mutant htt in the HEK293 cell line (Chen et al., 1999b; see Figure 1), early (6-h) overall cell death in response to incubation with a saturating concentration of agonist was enhanced for both NMDAR subtypes by full-length mutant htt. Even though the relative increase in 1 mM NMDA-induced death of NR1A/NR2A/htt-138Q-transfected cells compared with controls was considerably smaller than that of NR1A/NR2B/htt-138Q-transfected cells (see Figure 3B), it was still significant ($p<0.05$). This increase in excitotoxic cell death without a correlative increase in peak current observed for NR1A/NR2A with full-length mutant htt could not be attributed to an additive toxic effect of NMDAR activation and the presence of full length mutant htt, since there was no increase in death of cells transfected with htt-138Q compared with htt-15Q or GFP in the absence of NMDAR activation (i.e., the antagonist condition). It is possible that expression of mutant htt increases sensitivity to NMDAR activation-induced cell stress by altering downstream processes such as calcium buffering, ion homeostasis and/or energy production. Further experiments are required to elucidate the mechanism(s) by which mutant full-length htt enhances excitotoxicity independently of its effect on NMDAR channel activity.

FIGURE 3. HEK293 cells co-expressing NR1A/NR2B or NR1A/NR2A and full-length mutant htt show a significant increase in excitotoxic cell death compared with control conditions. Survival of transfected cells was assessed using the β-gal activity assay. (A) 36 h after co-transfection with NMDAR subunits, β-gal, and either GFP, or htt-15Q, or htt-138Q, cells were subjected to 6-h incubation with 1 mM NMDA or 1 mM APV with 250 μM 5,7 dichlorokynurenic acid and relative cell death calculated as described (see Methods 2.3). Bars represent mean ± SEM from 10 (NR1A/NR2B) or 6 (NR1A/NR2A) different batches of transfected cells. Cells co-expressing NR1A/NR2B and htt-138Q show a significant increase in excitotoxic cell death when compared to control conditions ($p<0.001$ compared with GFP and $p=0.01$ compared with htt-15Q, by paired t test). NR1A/NR2A and htt-138Q co-transfected cells also show a significant increase in excitotoxic cell death when compared to control conditions ($p=0.027$ and $p=0.047$, compared with GFP and htt-15Q, respectively, by paired t test). (B) Increase in relative death of htt-138Q-expressing cells compared to GFP and htt-15Q expressing cells co-transfected with either NR1A/NR2B or NR1A/NR2A after 6-h exposure to 1 mM NMDA. Means are calculated from raw data shown in Figure 3A. (C) Expression of full-length mutant htt does not increase cell death in the absence of NMDAR activation. Cells undergo exposure to 1 mM APV with 250 μM 5,7 dichlorokynurenic acid alone. N=5 and 13 different batches of transfected cells for NR1A/NR2B and NR1A/NR2A, respectively. * indicates $p<0.05$, ** $p<0.001$ vs. GFP; # indicates $p<0.05$ vs. htt-15Q.
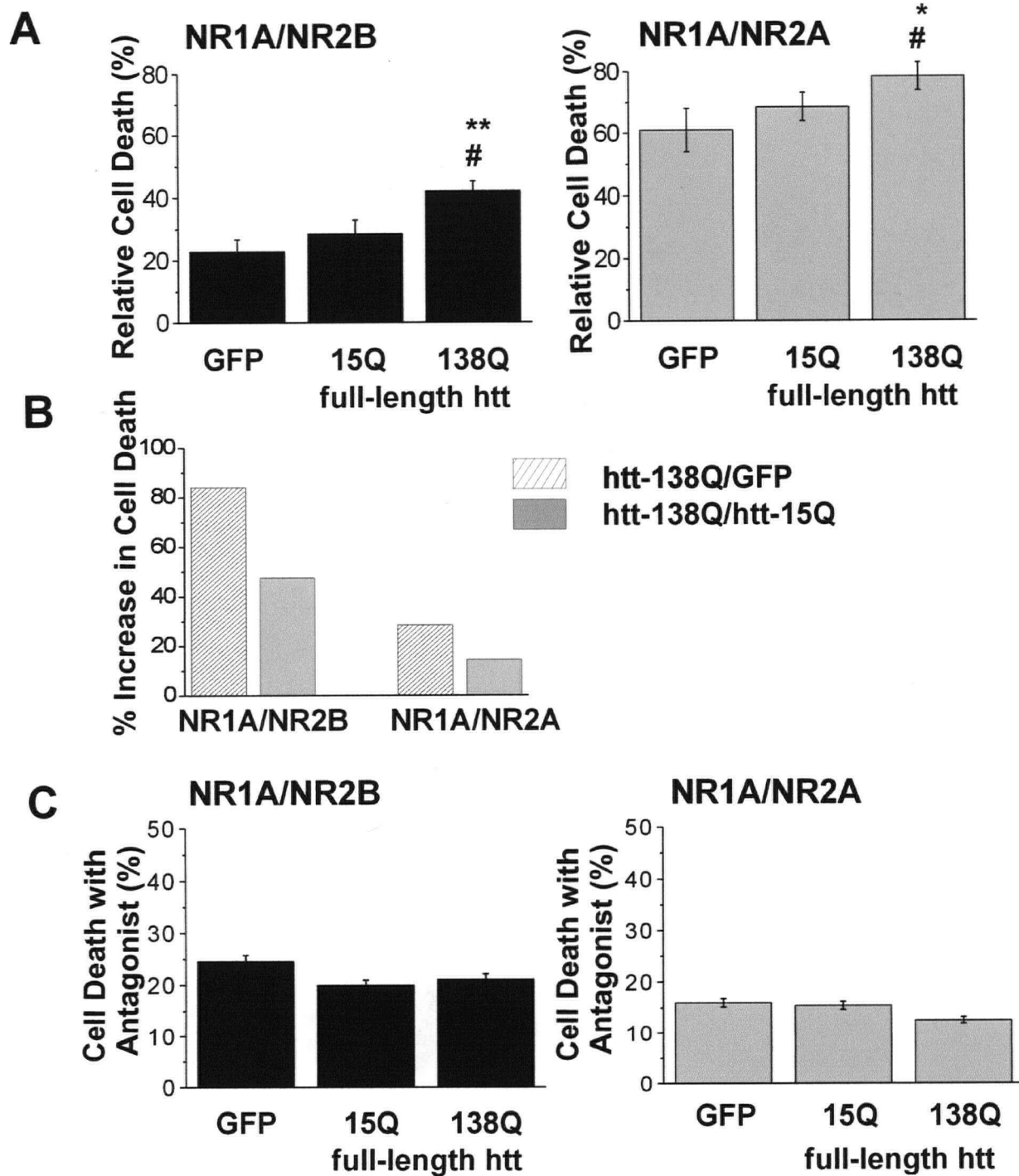
3.2 NR1A/NR2B-Mediated Apoptotic Cell Death Enhanced by Full-Length Mutant Htt in HEK293 Cells

To determine whether there was a difference between the modes of NR1A/NR2A- and NR1A/NR2B-mediated cell death associated with expression of full-length mutant htt, we used the Hoechst 33258 dye to identify apoptotic cells (Saudou et al., 1998). Only cells displaying globular or blebbed nuclei (see Figure 4B) were scored as apoptotic, even though a much larger number showed the small, brightly fluorescent nucleus typical of chromatin condensation (Figure 4A,B). Although some microscopic fields showed clusters of apoptotic cells, we blindly scored >1000 cells from 5-10 different (400X) microscopic fields for each condition in order to obtain a representative sample from each slide. Using these criteria, we observed a significant increase in the number of cells displaying apoptotic features among those cells co-expressing NR1A/NR2B and full-length mutant htt, compared to controls after a 6-h incubation with agonist (Figure 4A,B,C). The fraction of apoptotic cells was 4.7 $\pm$ 0.4 % for those cells treated with NMDA co-expressing htt-138Q and NR1A/NR2B, whereas only ~1 % of cells co-expressing GFP or htt-15Q were apoptotic (p<0.001, N=3; one-way ANOVA, Bonferroni post-hoc test). This increase in agonist-dependent apoptosis was not observed in cells co-expressing NR1A/NR2A and full-length mutant htt (Figure 4A,C). Furthermore, those cells expressing full-length mutant htt that were exposed to NMDAR antagonist only did not exhibit an increase in apoptosis compared with controls, in spite of the 6-h withdrawal of growth factors (Figure 4C).

Thus, upon examination of the mode of cell death, we found that cells co-expressing full-length mutant htt and NR1A/NR2B subunits exhibited a significant increase in NMDAR-mediated apoptosis, which was not seen for cells co-expressing full-length mutant htt and NR1A/NR2A. Our results suggest a selective interaction of full-length mutant htt and

NR1A/NR2B that augments NMDAR-triggered apoptotic cell death. This selective enhancement by full-length mutant htt of NR1A/NR2B-mediated apoptotic death correlates with the specific increase in glutamate-evoked current previously reported for NR1A/NR2B- and not NR1A/NR2A-transfected cells (Chen et al., 1999b; see Figure 1).

Evidence suggests an apoptotic pathway underlies neuronal death in HD (Portera-Cailliau et al., 1995, reviewed by Wellington et al., 1997). A variety of factors may determine whether excitotoxic insults result in necrotic (also known as oncotic) or apoptotic neuronal death, including the severity of the insult and resulting levels of intracellular Ca^{2+} concentration and reactive oxygen species (reviewed by Choi, 1996; Lee et al., 1999). Apoptosis triggered by excitotoxicity is likely due to chronic, mild insults over prolonged time periods (reviewed by Coyle and Puttfarcken, 1993; Ankarcrona et al., 1995), causing moderate elevations of intracellular Ca^{2+} and levels of oxidative stress (Bonfoco et al., 1995; McConkey and Orrenius, 1996; Keller et al., 1998; Gwag et al., 1999). In our model system, we found that early death of NMDA-treated transfected HEK293 cells was 2-3 fold larger for NR1A/NR2A than NR1A/NR2B (Figure 3A), whereas others have shown that delayed death of NMDA-treated NR1A/NR2B-transfected cells is similar to that observed for NR1A/NR2A (Boeckman and Aizenman, 1996). Furthermore, our previous results suggest oncosis makes a large contribution to NR1A/NR2A cell death under these conditions (Raymond et al,, 1996). Although we did not fully characterize the mechanism of cell death in this study, we postulate that in response to a maximal stimulus, NR1A/NR2A-mediated death occurs largely by oncosis, whereas NR1A/NR2B-mediated death may require a longer period to fully manifest and show a larger contribution from apoptosis, at least in transfected HEK293 cells. Further experiments are necessary to determine the time course of the progression of apoptotic cell death seen in our system. Interestingly, we have previously reported that peak NMDAR-mediated currents are 3- to 5-fold larger for NR1A/NR2A- than NR1A/NR2B-transfected HEK293 cells, but the

integrated current response to brief application of a saturating glutamate concentration is nearly identical since deactivation is much slower for NR1A/NR2B than NR1A/NR2A (Chen et al., 1999a). Although, our experiments employed a 6 hr exposure to NMDA, it may possible that this difference in the temporal characteristics of initial current influx render NR1A/NR2B-containing cells more vulnerable to apoptosis and resistant to oncosis. Further experiments are required to test this hypothesis, and to investigate the possibility that neurons predominantly expressing NR1/NR2B versus NR1/NR2A are more likely to undergo apoptosis in response to an excitotoxic stimulus.

Although the apoptosis seen for cells co-expressing NR1A/NR2B and htt-138Q following 6-h exposure to NMDA is only 5 %, we believe the numbers are underestimated. First, our criteria for identifying apoptotic cells are very strict, choosing only those with globular or blebbed nuclei, but as shown in Figure 4A, the majority of agonist-treated cells transfected with NR1A/NR2B and htt-138Q exhibited a largely apoptotic phenotype, with condensed nuclei. Furthermore, by assaying cells after an acute stimulation with agonist, this model system is designed to evaluate rapid NMDAR-mediated excitotoxic death, and apoptotic death is more likely to occur at later time points. Finally, we have observed that most of the dead HEK293 cells detach from the culture dish (see Methods), and therefore would not be detected by staining the remaining adherent cells with the Hoechst dye. However, following detachment, the large majority are apparently degraded since very few can be observed as "intact" cells floating in the medium at the end of the 6 hour incubation. Our results, showing that significant NMDA-triggered apoptotic cell death is restricted by htt polyQ size and NMDAR subtype, support the notion that excitotoxic apoptotic death of NR1A/NR2B-expressing medium spiny striatal neurons may contribute importantly to the pathogenesis of HD.

FIGURE 4. An increase in percentage of apoptotic cells after NMDA incubation is observed selectively for cells co-expressing NR1A/NR2B and full-length mutant htt. (A) Transfected HEK293 cells were stained with Hoechst dye at the end of 6-h incubation with 1 mM NMDA. Nuclear staining reveals more apoptotic cells, with small, brightly fluorescent, globular nuclei, in a 400X microscopic field for cells co-transfected with NR1A/NR2B and htt-138Q compared with htt-15Q or cells co-transfected with NR1A/NR2A and htt-138Q or htt-15Q. (B) Image of NR1A/NR2B/htt-138Q-transfected cells shown at higher gain; arrows indicate cells scored as positive for apoptosis. (C) Blindly scoring >1000 cells per slide for apoptotic features revealed significantly higher percentage of apoptotic cells after 6-h incubation with 1 mM NMDA for NR1A/NR2B/htt-138Q-transfected cells compared to all other groups (*$p<0.001$ by one-way ANOVA against all other groups; N=3 different transfections). APV = cells incubated for 6 h with 1 mM APV and 250 μM 5,7 dichlorokynurenic acid instead of 1 mM NMDA. Calibration bars = 20 μM.

A

htt-15Q   htt-138Q

NR1A/NR2A

NR1A/NR2B

B

C
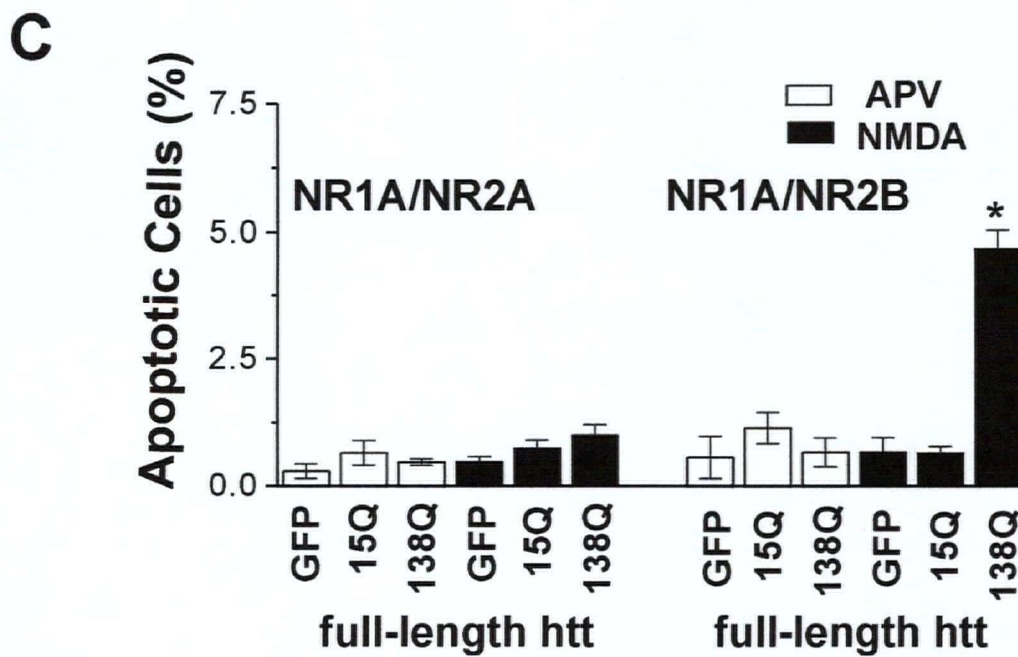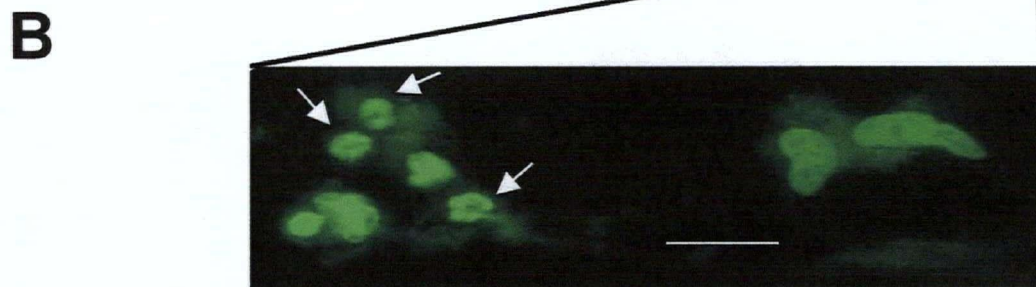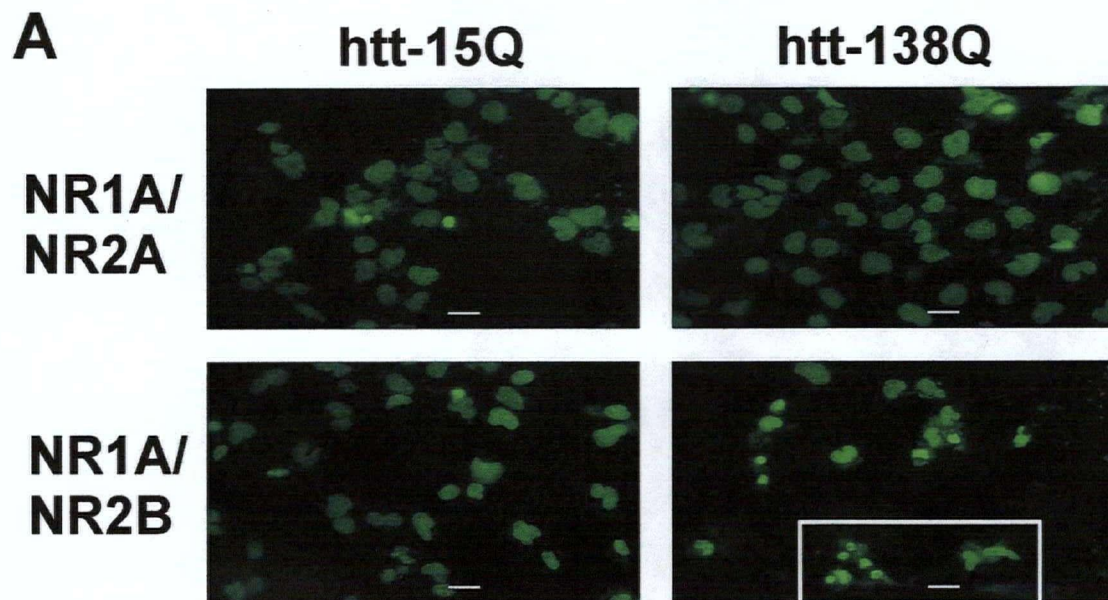
3.3 Reduced NR1A/NR2A Stimulation Increases Full-Length Mutant Htt-Enhanced

Excitotoxicity, But Not Apoptosis in HEK293 Cells

The larger relative increase we observed in cell death associated with htt-138Q for NR1A/NR2B compared with NR1A/NR2A might have been due, at least in part, to the relatively high (60-70%) NR1A/NR2A-mediated cell death evoked by saturating concentrations of agonist observed under control conditions (GFP and htt-15Q). Such high levels of cell death under control conditions might effectively limit sensitivity for detecting an increase in cell death associated with full-length mutant htt. As well, evidence suggests that milder excitotoxic insults increase apoptotic vs. necrotic cell death (see discussion above), and therefore we may have missed an increase in apoptotic death for NR1A/NR2A/htt-138Q-transfected cells due to the intensity of the insult associated with exposure to 1 mM NMDA. To test this possibility, we repeated the cell death analysis following 6-h exposure to lower concentrations of NMDA (ranging from 3 μM to 100 μM) for cells transfected with NR1A/NR2A and either GFP, htt-15Q or htt-138Q. As shown in Figure 5A, NMDA-induced death of NR1A/NR2A cells co-expressing GFP showed a clear concentration-dependence, ranging from a low of 26 $\pm$ 2.7 % for 3 μM to a high of 61 $\pm$ 7 % for 1 mM. Cell death at the lowest concentration of NMDA tested (3 μM) was similar to that found for NR1A/NR2B/GFP-transfected cells following exposure to 1 mM NMDA (compare Figure 3A with Figure 5A). However, as shown in Figure 5B, the increase in 3 μM NMDA-induced NR1A/NR2A-transfected cell death associated with htt-138Q -- 28 % and 62 % compared with htt-15Q and GFP-transfected cells, respectively -- although larger than that found for 1 mM NMDA, was still not as large as that shown for NR1A/NR2B-transfected cells treated with 1 mM NMDA (compare Figure 5B with 3B). Furthermore, there was no significant difference in NR1A/NR2A-transfected cell death between cells co-transfected

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with htt-15Q versus htt-138Q after exposure to 3 μM NMDA (p=0.067, paired *t* test, N=6). Moreover, analysis of apoptosis for NR1A/NR2A/htt-138Q-transfected cells after exposure to 3 μM and 10 μM NMDA showed no significant increase in the percentage of apoptotic cells compared to controls (p>0.05, one-way ANOVA, Table 1). Despite the milder insult and lower amount of NR1A/NR2A-mediated cell death associated with lower NMDA concentrations, the percentage of apoptotic cells found immediately following a 6-h exposure remained quite low and was not different from that observed for high concentrations of NMDA (compare Table 1 with Figure 4C). We speculate that these 6 hour experiments may not be long enough to observe an increase in apoptosis with lower NMDA agonist concentrations and experiments examining apoptosis at later time periods might discern this.

These results confirm for NR1A/NR2A-transfected cells that the magnitude of NMDA-induced cell death is dose-dependent, as previously reported (Raymond et al. 1996), indicating that the NMDA-mediated peak current amplitude is an important factor contributing to excitotoxic cell death. As predicted, the lower magnitude of cell death induced by low concentrations (3 – 10 μM) of NMDA in NR1A/NR2A-transfected cells was associated with some enhancement of the relative increase in cell death for htt-138Q co-transfected cells, although the difference between htt-15Q and htt-138Q was not significant. It is interesting that under these conditions, there is still no increase in apoptotic cell death for NR1A/NR2A/htt-138Q transfected cells compared to controls. These data provide further support for the notion that there is a specific interaction between mutant full-length htt and NR1A/NR2B that selectively enhances NMDAR-triggered apoptosis. Selective enhancement of current amplitude in combination with the unique macroscopic kinetic properties of NR1A/NR2B-type NMDARs (see above) may be sufficient to explain the increase in apoptosis seen with htt-138Q. On the other hand, NMDARs have been shown to interact with a variety of cytoskeletal-associated

proteins, many of which are expressed in HEK293 cells. In many cases, such interactions are NMDAR subunit- and/or splice variant-specific (Kornau et al., 1995; Wyszynski et al., 1997; Ehlers et al., 1998; Sans et al., 2000) and modulate NMDAR subcellular distribution or activity, or else signaling downstream of NMDAR activation (Krupp et al., 1999; Mori et al., 1998; O'Brien et al., 1998; Sprengel et al., 1998; Zhang et al., 1998). Thus, it is possible that htt-138Q alters NMDA-induced apoptosis by influencing NR1A/NR2B-mediated signal transduction, via interactions with NR1A/NR2B-associated proteins.

FIGURE 5. (A) NMDA dose-response for NR1A/NR2A-transfected cell death. 36 h after co-transfection with NR1A/NR2A-NMDAR subunits, β-gal, and either GFP, or htt-15Q, or htt-138Q, cells were subjected to 6-h incubation with NMDA at concentrations ranging from 3 μM to 100 μM or to 1 mM APV with 250 μM 5,7 dichlorokynurenic acid. Cell survival was assessed using the β-gal activity assay and relative cell death calculated as described (see Methods 2.3). Data for 1 mM NMDA shown in Figure 3A are also included. N = 6, 7, 5, 3, and 6 different batches of transfected cells for 3, 10, 30, 100 and 1000 μM NMDA, respectively. * indicates $p < 0.05$ vs. GFP; # indicates $p < 0.05$ vs. htt-15Q. (B) Increase in relative death of htt-138Q-expressing cells compared to GFP and htt-15Q expressing cells co-transfected with NR1A/NR2A after 6-h NMDA exposure. Means are calculated from raw data shown in Figure 5A. Note that although the relative increase in htt-138Q compared with GFP and htt-15Q in NR1A/NR2A-transfected cells death is enhanced at low NMDA concentrations (compare with Fig. 1B), this difference is not significant for htt-138Q vs. htt-15Q.

A NR1A/NR2A

Legend:
- GFP
- htt-15Q
- htt-138Q

y-axis: Relative Cell Death (%)
x-axis: [NMDA] μM (3, 10, 30, 100, 1000)

B NR1A/NR2A

Legend:
- htt-138Q/GFP
- htt-138Q/htt-15Q

y-axis: % Increase in Cell Death
x-axis: [NMDA] μM (3, 10)

77
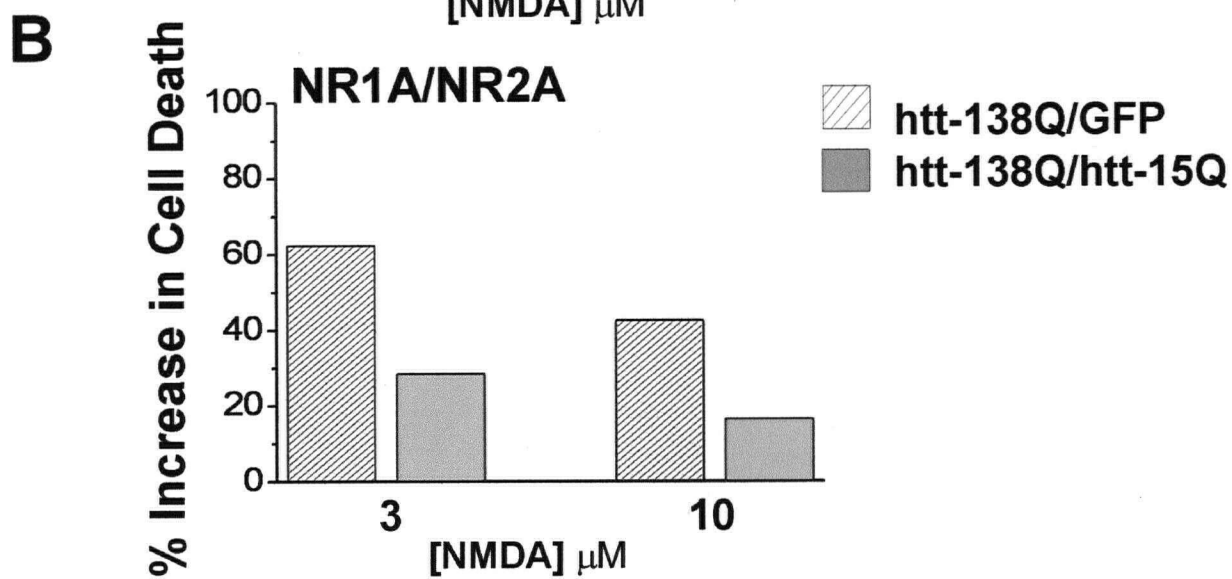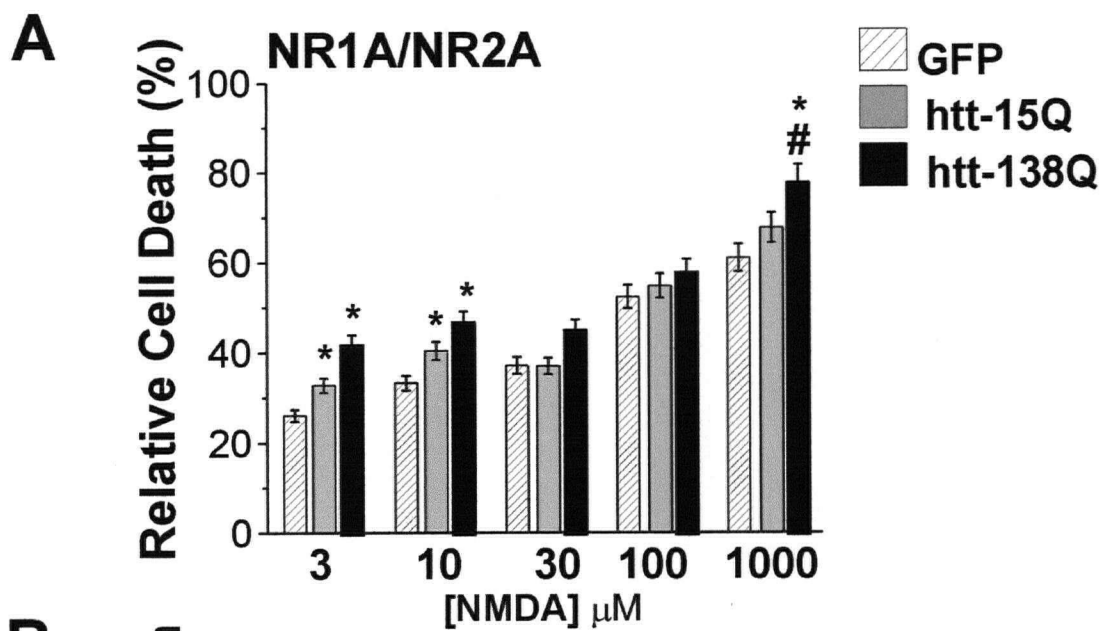
TABLE 1. HEK293 Cell Apoptosis With and Without NMDAR Activation

Transfection	[NMDA] (μM)	Apoptotic Cells (%)	Apoptotic Cells (%) in 1 mM APV and 250 μM 5,7-dichlorokynurenic acid
N=5			
NR1/2A/GFP	10	0.59 ±0.03	0.59 ±0.04
NR1/2A/htt-15Q	10	0.68 ±0.07	0.50 ±0.05
NR1/2A/htt-138Q	10	0.67 ±0.13	0.56 ±0.19
N=4			
NR1/2A/GFP	3	1.17 ±0.09	0.87 ±0.13
NR1/2A/htt-15Q	3	1.27 ±0.23	1.05 ±0.26
NR1/2A/htt-138Q	3	1.29 ±0.18	1.23 ±0.15
N=3			
NR1/2B/GFP	1000	0.38 ±0.06	0.21 ±0.02
NR1/2B/htt-N548-15Q	1000	0.47 ±0.02	0.54 ±0.07
NR1/2B/htt-N548-128Q	1000	0.42 ±0.09	0.68 ±0.08

Note. N denotes number of different batches of cells that were transfected, treated with NMDA or APV for 6 h, and analyzed for apoptosis (all in parallel). Analysis of percentage apoptotic cells was done as described for Figure 4.

3.4 N-Terminal Fragment of Htt Marginally Increases Cell Death and Has No Effect on

Apoptosis Mediated by NR1A/NR2B in HEK293 Cells

Recent studies in cell lines and transgenic mice indicate that expression of N-terminal fragments of htt containing the polyQ expansion results in increased vulnerability to apoptotic cell death or neuronal dysfunction, respectively (Mangiarini et al., 1996; Davies et al., 1997; Sapp et al., 1997; Cooper et al., 1998; Hackam et al., 1998). Therefore, we tested whether co-expression of an N-terminal fragment of htt containing either 15Q or 128Q (htt-N548-15Q or htt-N548-128Q, respectively) with NR1A/NR2B could enhance NMDA-induced cytotoxicity in HEK293 cells. As shown in Figure 6A, although we found a significant ($p<0.05$, paired t test; $N=7$) increase in NR1A/NR2B-mediated cell death in htt-N548-128Q-transfected cells compared to controls (13 % and 28 % relative to htt-N548-15Q and GFP-transfected cells, respectively), this difference was quite small compared with that observed for full-length mutant htt (see Figure 3B). Again, it should be noted that the difference between NMDAR-mediated excitotoxicity in cells expressing full-length vs. truncated mutant htt is smaller when the values of cell death for GFP-expressing cells are subtracted from those for htt-N548-138Q or htt138Q-expressing cells (see Section 3.1). Therefore, it is important to regard this difference as preliminary, and examination of specific modes of cell death makes the difference more apparent (see below). As for full-length htt, cells expressing truncated mutant htt that were exposed to NMDAR antagonist showed no significant difference in cell death compared with controls (Figure 6B). As well, we observed no significant increase in the percentage of cells displaying apoptotic features among those cells co-expressing NR1A/NR2B subunits and truncated mutant htt compared to controls after a 6-h incubation with agonist ($p>0.6$, one-way ANOVA; $N=3$; Table 1). Taken together, these results suggest that the region of htt C-terminal to amino acid ~540 plays an important role

in mediating the increase in NR1A/NR2B-dependent apoptosis. Our data also suggest that the enhanced toxicity reported for expression of truncated htt in a variety of models is independent, or downstream, of NMDAR activation.

FIGURE 6. HEK293 cells co-expressing NR1A/NR2B and truncated mutant htt show very small increases in excitotoxic cell death compared with control conditions. (A, B) Survival of transfected cells was assessed using the β-gal activity assay. (A) 40-42 hr after co-transfection with NMDAR subunits, β-gal, and either GFP, or htt-N548-15Q, or htt-N548-128Q, cells were subjected to 6-h incubation with NMDAR agonist (1 mM NMDA with 50 μM glycine) or antagonist (1 mM APV and 250 μM 5,7 dichlorokynurenic acid) and relative cell death was calculated as in Figure 3. NR1A/NR2B/htt-N548-128Q-transfected cells show a very small but significant increase in excitotoxic cell death compared to control conditions (p=0.027 and p=0.032 compared to GFP and htt-N548-15Q, respectively, by paired t test, N=7). (B) Expression of truncated mutant htt does not increase cell death in the absence of NMDAR activation (p>0.33 by paired t test comparing htt-N548-128Q with two other groups; N=7 different batches of transfected cells).

A **NR1A/NR2B**
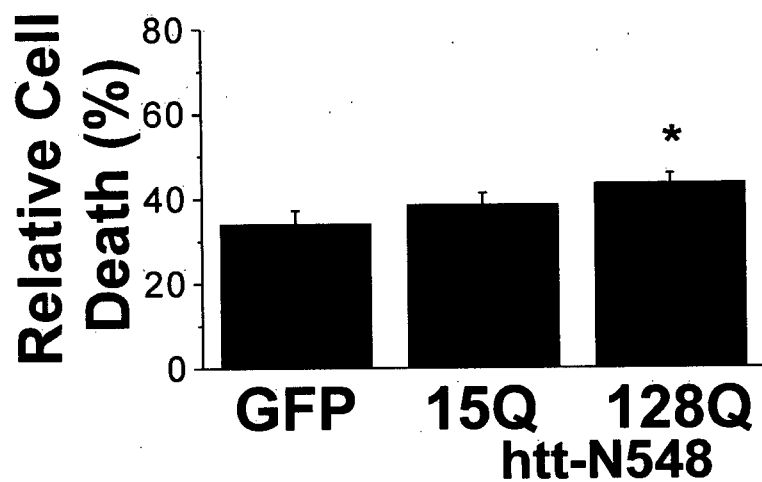
B
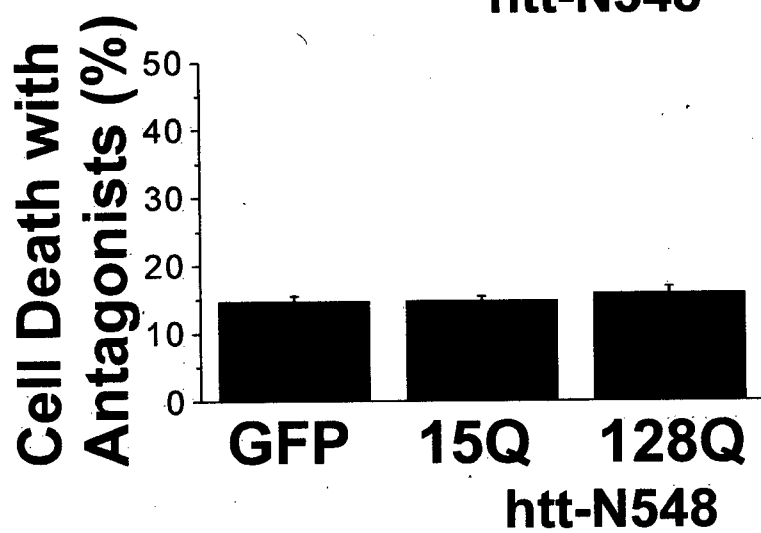
3.5 Protein Expression in Nontransfected and Transfected HEK293 cells

After we found an enhancement in NMDAR-mediated apoptosis in HEK293 cells expressing mutant htt and the NR1/NR2B NMDAR, we wanted to begin to examine the protein interaction taking place directly or indirectly in our model. We hypothesized that in MSNs the interaction between mutant htt and NMDARs may be indirect through cytoskeletal proteins, such as PSD-95 family members or α-actinin with HIP-1. It was interesting that we found a non-neuronal model system that facilitated this interaction between mutant htt and NMDARs, so we first wanted to explore the cytoskeletal proteins that may be expressed endogenously in HEK293 cells. Using western blot techniques, I found that HIP-1 is expressed in HEK293 cells (Figure 7A) in moderate levels. It is interesting that after transfection HEK293 cells appear to express relatively larger amounts in the cytoplasmic fraction than the membrane fraction compared to endogenous levels (Figure 7A). These differences have not yet been quantified. Since HIP-1 is a cytoskeletal membrane-associated protein (Kalchman, 1997), its increased expression in the cytosolic fraction may indicate that the processes that associate HIP-1 with the membrane may be a limiting factor in our overexpression model. Further characterization of HEK293 cells was done by C. Icton in our laboratory, and her unpublished findings show that HEK293 cells also highly express endogenous α-actinin, but no PSD-95 family members (PSD-95, SAP102 and chapsyn 110 were probed). Other laboratories have also shown that HEK293 cells express endogenous HIP-1 or α-actinin (Kalchman et al., 1997; Wanker et al., 1997; Zhang et al., 1998; Krupp et al., 1999). These initial experiments provided the basis for which C. Icton further examined and discovered that HIP-1 and α-actinin interact in a co-immunoprecipitation assay in HEK293 cells (unpublished data), suggesting a possible mechanism by which htt and NMDARs could be interacting in our system.

Furthermore, I examined expression levels of NMDARs in the HEK293 cells after transfection with the NR1A/NR2A or NR1A/NR2B NMDAR cDNAs co-transfected with β-galactosidase and GFP or htt-15Q or htt-138Q cDNAs. Using a polyclonal antibody to the NR1A subunit (Figure 7C) or polyclonal antibodies that recognize either NR2B (Figure 7B) or NR2A (data not shown), I found that NMDAR subunits appear to show robust expression levels in the membrane fraction (Figure 7B, C) and at the surface (data not shown) even with transfection of additional cDNAs (for β-galactosidase and GFP or htt-15Q or htt-138Q). Expression levels of NR2A or NR2B show comparable levels in the membrane fraction or at the cell surface (data not shown). Importantly, we have not observed any difference in NMDAR expression levels in the membrane fraction or at the cell surface when co-expressing mutant or normal htt (Figure 7B), supporting the idea that it is not a change in overall numbers of receptors at the surface but more an alteration in the function of the NMDARs that may cause enhanced current amplitude and apoptosis. This had been shown previously by our laboratory (Chen et al., 1999a,b). The NR1A expression levels appear as a double band (Figure 7C); the upper band may be due to cross-reactivity of the NR1A antibody with a protein other than NR1A (this has been found previously by our laboratory). These results confirm that in our experiments, adequate amounts of NMDAR protein are expressed, in cell membranes and at the cell surface in HEK293 cells, which may be available to form functional recombinant receptors and potentially interact with htt, important to the outcome of our *in vitro* assays. Since we transfected double the amount of cDNA encoding for GFP or htt-15 or htt-138 as that used for NMDARs, we assumed that there would a high level of expression of these proteins and therefore did not directly examine those expression levels. Furthermore, our laboratory has already shown them to be overexpressed in the HEK293 cell model (Chen 1999b; see Figure 1). Overall, amounts of NMDAR protein have not yet been quantified in the blots shown. In all, we can say that most of the transfected cells appear to express proteins at levels sufficient to observe an interaction.

FIGURE 7. Expression of HIP-1 in non-transfected HEK293 cells and HIP-1 and NMDARs in transfected HEK293 cells. (A) Cytoplasmic or membrane fraction expression of HIP-1 found in non-transfected (endogenous) and transfected HEK293 cells, using anti-HIP-1 antibody. (B) Total membrane fraction expression of the NR2B NMDAR subunit, probed with anti-NR2B antibody, in HEK293 cells transfected with cDNAs encoding β-gal/NR1A/NR2B/htt-15 or htt-138. (C) Total membrane fraction expression of the NR1A NMDAR subunit, probed with anti-NR1A antibody, in HEK293 cells transfected with cDNAs encoding β-gal/NR1A/NR2A or NR2B/GFP. Protein samples were loaded as indicated and separated by SDS-PAGE. N = 2. Upper band of molecular weight markers indicates 193 kDa; lower band represents 112 kDa.

A

cyto mem cyto mem
endogenous overexpressed

HIP-1→

5 μg 10 μg 5 μg 10 μg 5 μg 10 μg 5 μg 10 μg

B

htt-15

NR2B→

1 μg 2 μg 4 μg

htt-138

NR2B→

1 μg 2 μg 4 μg

C

NR2A

NR1A→

2 μg 4 μg

NR2B

NR1A→

2 μg 4 μg

86
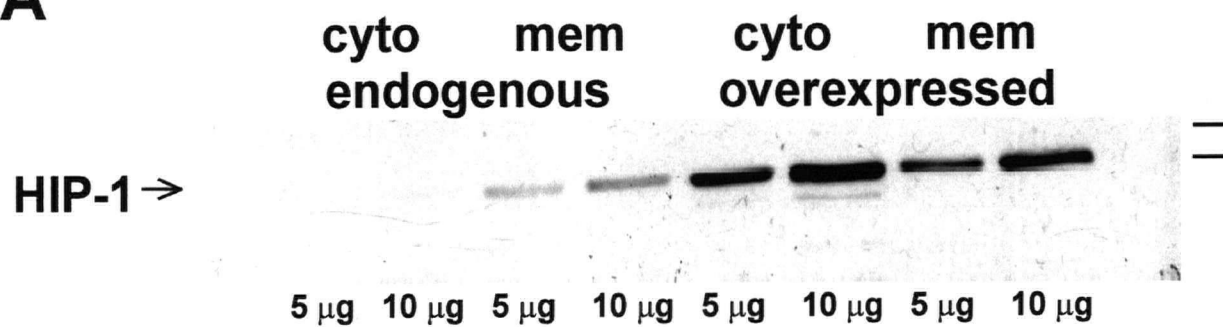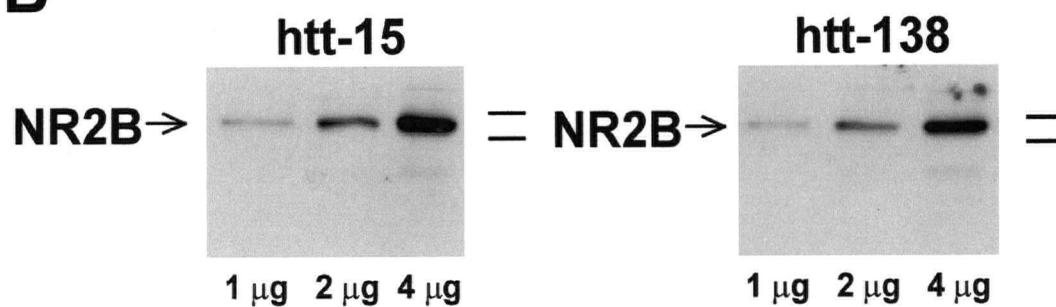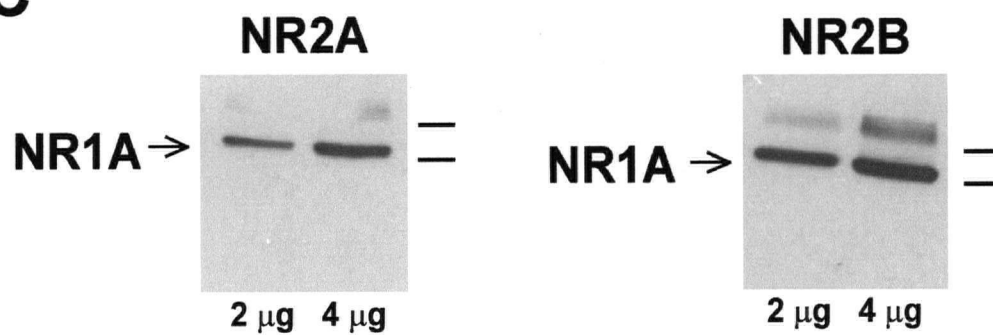
3.6 Summary

The transfected HEK293 cells proved to be a useful system in which we could test our hypothesis whether mutant htt interacts with the NR1/NR2B NMDAR to cause a change in NMDAR function. My study, together with our previous findings (Chen et al., 1999b), show that it is the full-length mutant htt protein that selectively interacts with the NR1/NR2B NMDAR subunit to cause an increase in current amplitude and apoptosis. Our findings in HEK293 cells support the idea that MSNs may be selectively targeted for neurodegeneration via a specific relationship between NR1/NR2B NMDARs and mutant htt.

A previous report demonstrates that mice transgenic for exon 1 of the human HD gene containing a large polyQ expansion (R6/1 and R6/2 mice – Mangiarini et al., 1996) are resistant to neuronal loss resulting from striatal injection of the NMDAR agonist quinolinic acid at relatively older ages, an effect that increases with age presymptomatically (Hansson et al., 2001; Hansson et al., 1999). One possible explanation is that enhanced vulnerability of neostriatal MSNs to NMDAR-mediated toxicity in HD may require expression of full-length htt. In support of this possibility, results by N. Chen in our laboratory using patch clamp recording suggest that the C-terminal portion of htt is required for functional interaction with NMDARs (Zeron et al., 2001). Whereas our previous study showed that expression of htt-138Q resulted in increased NR1A/NR2B-mediated peak current (Chen et al., 1999b; see Figure 1), data by N. Chen (Zeron et al., 2001) reveal no significant difference in NR1A/NR2B peak current amplitude in cells co-expressing htt-N548-128Q compared with those expressing htt-N548-15Q or a control protein, which agrees with the absence of observable enhanced NR1A/NR2B-mediated apoptosis in cells co-expressing truncated htt (Table 1). On the other hand, a report indicates that NMDA-induced swelling of striatal neurons is enhanced in R6/2 mice (identical to R6/1 except for a larger polyQ

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expansion – Mangiarini et al., 1996) compared with wild-type controls (Levine et al., 1999). However, R6/2 striatal neurons also showed significantly more depolarized resting membrane potentials than wild type neurons, which would lead to increased NMDAR activation owing to decreased voltage-dependent Mg^{2+} block (ibid). More recently, the authors directly measured NMDA-evoked currents in presymptomatic and symptomatic R6/2 mice and found there to be enhanced NMDAR-mediated current amplitude and calcium influx in striatal slices (Cepeda et al., 2001a) compared to WT MSNs, although these mice show increased resistance to NMDAR-mediated toxicity *in vivo* (Hansson et al., 1999; 2001). These findings paralleled similar measurements by the same group (Cepeda et al., 2001a) on the YAC HD mice (Hodgson et al., 1999), expressing the full-length htt protein.

Why do the R6/2 and R6/1 mice shows no striatal degeneration, increased resistance to NMDAR-mediated striatal toxicity *in vivo* but enhanced current and calcium response to NMDA *in vitro* striatal slices? One likely explanation is the widespread intranuclear aggregation of htt in up to 90 % of these neurons (Davies et al., 1997), since it has recently been correlated the development of nuclear inclusions and more efficient handling of Ca^{2+} increases with the appearance of resistance to NMDAR-induced toxicity in R6 mice (Hansson et al., 2001). These data suggest that R6 neurons are under chronic stress and have developed compensatory mechanisms that diminish the damage of excitotoxic insults (Hansson et al., 2001). YAC72 mice and patients with advanced HD exhibit intranuclear inclusions in a small percentage of striatal neurons (Hodgson et al., 1999; Sapp et al., 1999) and therefore these mice may constitute a more relevant disease model.

These findings together with the results reported in my thesis strongly support the notion that neuronal degeneration in HD may be triggered by the novel interaction of mutant full-length htt with protein(s) expressed selectively in vulnerable neurons, and with NR1A/NR2B-type NMDA receptors in particular. In this regard, NMDARs are functionally modulated by

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interactions with cytoskeletal-associated proteins (Rosenmund and Westbrook 1993; Paoletti et al, 1994; Korau et al, 1995; Kim et al, 1996; Muller et al, 1996; Wyszynski et al, 1997; Zhang et al., 1998; Krupp et al., 1999). Huntingtin also interacts with proteins such as HIP-1 and HAP-1 that are proposed to mediate vesicle transport, membrane trafficking, and cytoskeletal functions (Li et al, 1995, 1998; Kalchman et al, 1997; Wanker et al, 1997). We propose that an increase in NR1A/NR2B function, mediated by interaction with full-length mutant htt via cytoskeletal proteins, in neostriatal MSNs may result in an increase in calcium-dependent oxidative stress, eventually triggering an apoptotic pathway resulting in cell death.

However, conclusions from the HEK293 cell system must be drawn cautiously. These cells are not neurons and therefore may lack some of the key proteins expressed in MSNs required for the full pathogenic pathway in HD. Moreover, we have overexpressed NMDARs and htt in this model system, and thus may have induced interactions not found in neurons expressing lower levels of the protein. Therefore, to test the validity of our findings in the neuronal population most vulnerable in HD, we examine NMDAR-induced excitotoxicity in MSNs cultured from a mouse model of HD. These experiments and results are outlined in Chapters 4-6.

CHAPTER 4

Enhanced Excitotoxicity in Cultured Neonatal Medium Spiny Striatal Neurons Expressing

Mutant Huntingtin

We showed previously that HEK293 cells expressing full-length mutant htt only display an enhancement in NMDAR-mediated current amplitude and apoptosis when coexpressing the NR1A/NR2B NMDAR subtype (Chen et al., 1999b; see Figure 1; Zeron et al., 2001; see Chapter 3). We also discovered that the region of htt C-terminal to amino acid ~540 was important for this interaction; without this region there was no selective increase in NMDAR-mediated current amplitude and apoptosis in cells co-expressing truncated mutant htt and the NR1A/NR2B subtype compared to controls (Zeron et al., 2001; see Chapter 3). Furthermore, N. Chen in our laboratory has used whole-cell patch clamp recordings from acutely dissociated MSNs expressing mutant htt (72Q) from 6-11 week old transgenic HD mice (Hodgson et al., 1999). Consistent with the HEK293 cell findings, N. Chen has shown that these neurons display larger NMDAR peak current amplitudes and current density (amplitude normalized to membrane capacitance to take into account variability in cell size) compared to MSNs from wild type mice (see Figure 2; Zeron et al., 2002). N. Chen was also able to show that >50 % of the peak current evoked by application of NMDA could be blocked by 10 μM ifenprodil (a NR1/NR2B subtype-specific antagonist) (see Figure 2). These findings support the idea that in a mouse model of HD, MSNs display a selective interaction between the NR1A/NR2B NMDAR subtype and mutant htt, causing enhanced current influx that could lead to enhanced vulnerability to cell death. It was our goal in the next series of experiments to test whether primary cultured MSNs expressing mutant htt from YAC transgenic HD mice are more vulnerable to NMDAR-dependent excitotoxic cell death, correlating with enhanced current amplitude.

We elected to use YAC transgenic mice in our studies because, one, they were readily available to us by the M.R. Hayden laboratory on campus, and two, this particular mouse model shows exceptional similarity to the human HD condition, including similar behavioural and striatal degeneration. We tested our hypothesis in this transgenic mouse model, described by Hodgson and coworkers (Hodgson et al., 1999), in which FVB/N mice express a yeast artificial chromosome (YAC) containing the full-length human HD gene including all endogenous regulatory elements with an expanded (46 or 72) CAG repeat (YAC46 or YAC72 mice). This model optimizes normal developmental and cell-specific regulation of full-length htt expression, and YAC72 mice show progressive neurobehavioral and selective striatal neurodegeneration similar to those changes seen in HD (Hodgson et al., 1999).

We used cultured striatal neurons from mice transgenic for HD to examine whether NMDARs have a role in the pathogenesis of HD. We elected to examine NMDAR-mediated excitotoxicity in the striatum of the HD mice since it is the region primarily affected in HD and since our laboratory had previously found enhanced current amplitudes in acutely dissociated striatal neurons from 6-11 week old HD mice. We used primary striatal cultures from the transgenic mice as striatal culture techniques had already been established in our laboratory (Price et al., 1999) and the technique allowed for examination of a large population of neurons at one time, necessary for most cell death assays.

The striatum is composed primarily of MSNs (Gerfen, 1992; Jiang and North, 1991), making up greater than 95 % of the total striatal neurons. Other striatal neuronal subtypes include medium- and large-sized interneurons, such as the large cholinergic interneuron. *In vitro* primary cultures of MSNs show preservation of the neuronal subtypes (Petersen and Brundin, 1999). The particular characteristics of neuronal subtypes found within the striatum support the idea that NMDAR expression plays a role in selectivity for neurodegeneration within the striatum. A recent study has shown that large-sized striatal interneurons are less responsive to

NMDA receptor activation than MSNs, while kainate induced-currents were enhanced in large-sized interneurons compared to MSNs (Cepeda et al 2001b). These findings could be explained by the fact that large-sized cholinergic interneurons express lower levels of NR1A and NR2B NMDAR subunit mRNA than MSNs (Landwehrmeyer et al, 1995), no NR2A subunits (Standaert et al, 1996), and some NR2D subunits (Standaert et al, 1996). MSNs express predominantly NR1A, NR2B and some NR2A but no NR2D NMDAR subunits. Therefore, we tested whether NR1A/NR2B-expressing MSNs showed enhanced vulnerability to excitotoxic cell death when co-expressing mutant htt.

4.1 Characterization of MSNs *in vitro*

Very commonly, different culture techniques and conditions for making primary neuronal cultures can affect the distribution of cell populations or even properties or physiology of a given cell type (Kovacs et al., 2001). Therefore, it is important when one performs primary neuronal culture to characterize the mature cultures to ensure that the cell population and neuronal properties are similar to those in mature neurons *in vivo*. It was our intent to use morphological criteria in parallel with immunocytochemical analysis to determine the identity of MSNs and characterize their endogenous NMDAR composition *in vitro*.

As mentioned before *in vivo*, the striatum is composed primarily of MSNs (Jiang and North, 1991; Gerfen, 1992), making up greater than 95 % of the total striatal neurons. Other striatal neuronal subtypes include medium- and large-sized interneurons, such as the large cholinergic interneurons. *In vitro* primary cultures of MSNs show preservation of the neuronal subtypes (Petersen and Brundin, 1999). Dopamine- and cyclic adenosine 3', 5'-monophosphate-regulated phosphoprotein, 32 kDa (DARPP-32) plays an important role in the biology of dopaminoceptive neurons, such as MSNs, by regulating the phosphorylation state and activity of many downstream proteins, including neurotransmitter receptors, such as NMDARs and

AMPARs (see Greengard et al., 1999 for a comprehensive review). DARPP-32 is highly expressed in virtually all MSNs (Ouimet et al., 1989; 1984) and is used as a molecular marker for adult MSNs, apart from the morphological appearance of MSNs. Striatal cultures performed by other groups are found to express >95 % DARPP-32 positive neurons (Petersen and Brundin, 1999). We used immunohistochemical techniques with a polyclonal antibody to DARPP-32 to confirm that our cultures show comparable numbers of DARPP-32-stained neurons, believed to be MSNs. Controls consisted of neurons that underwent exposure to secondary antibody alone to confirm specificity of positive staining (data not shown). In Figure 8, we showed that the majority (>95 %) of the neurons are stained positive for DARPP-32, regardless of the genotype of the mouse [i.e. WT vs 46Q vs 72Q (data not shown)]. These neurons also displayed the morphological characteristics that have been previously described for MSNs, such as ovoid somata 7-15 μM in size with scant cytoplasm and few to moderate numbers of dendritic processes (Figure 8) (Surmeier et al, 1988). Therefore, we can conclude that our mature primary striatal cultures are primarily composed of MSNs as found *in vivo* (Jiang and North, 1991; Gerfen, 1992) and as other groups have produced *in vitro* (Petersen and Brundin, 1999).

Next, it was important to analyze the NMDAR expression in our striatal cultures. MSNs have been found *in vivo* to predominantly express NR1A, NR2B and some NR2A but no NR2D NMDAR subunits (Landwehrmeyer et al., 1995; Ghasemzadeh et al., 1996; Rigby et al., 1996; Kuppenbender et al., 1999). In mature cultures, at 8 DIV, Kovacs et al., 2001 have shown there is more NR2B subunit mRNA expression in striatal cultures than NR2A mRNA expression. Using immunohistochemical staining with polyclonal antibodies to the NR1A or NR2A or the NR2B NMDAR subunits, we showed that most of the MSNs express the NR1A subunit, with some staining in some cells that may be interneurons or glia (Figure 8). There was only weak staining overall for the NR2A subunit, and little to no staining in MSNs (Figure 8). In contrast, robust staining for the NR2B subunit was observed in the vast majority of MSNs, with little

staining in other cell types (Figure 8). The pattern of expression of NMDAR subtypes does not appear to depend on the genotype of the striatal cultures [WT vs 46Q vs 72Q (not shown)] (Figure 8). The immunocytochemical labelling technique employed was not intended to demonstrate, nor quantify, the relative levels of NR2A and NR2B present in MSNs. Instead, we demonstrated that the majority of MSNs showed staining for the NR2B subunit whereas a small minority of MSNs stained for the NR2A subunit. Further examination with Western Blot analysis is necessary to quantify the overall expression levels of NR2A or NR2B in MSNs. Furthermore, Western Blot analysis is necessary to determine whether the presence of htt affects the overall membrane or surface expression of NMDARs in MSNs *in vitro*. However, our results agree with unpublished data by C. Icton and L. Raymond in striatal tissue lysates from 6-11 week old mice showing a higher ratio of NR2B to NR2A expression in the striatum compared with cortex or hippocampus, and that there was no influence of genotype (WT vs 46Q or YAC72) on total striatal NR2B or NR2A expression. In summary, our immunocytochemical analysis on striatal cultures indicates that most MSNs express DARPP-32, and NR2B and NR1A NMDAR subunits, with little NR2A, as found previously for MSNs *in vivo* and *in vitro*.

FIGURE 8. Immunocytochemical analysis of MSNs expressing normal (WT) or mutant htt (46Q) *in vitro*. After 9-12 DIV, cultured neurons that morphologically resemble MSNs predominantly express DARPP-32, NR2B and NR1A, but little NR2A, as assessed with histochemical staining with respective polyclonal antibodies. [Bar=25 μm]

WT　　　　**46Q**

DARPP-32
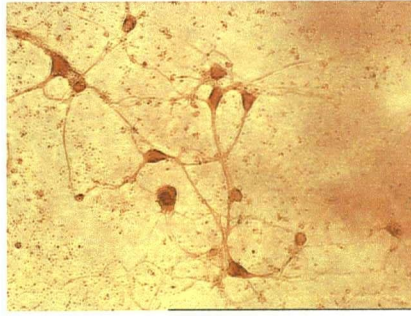
NR2B
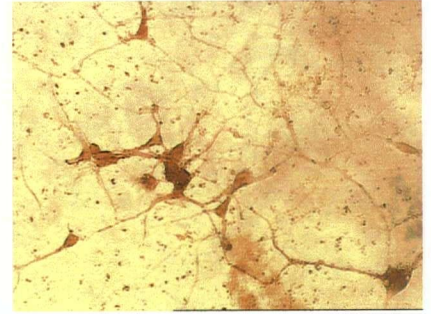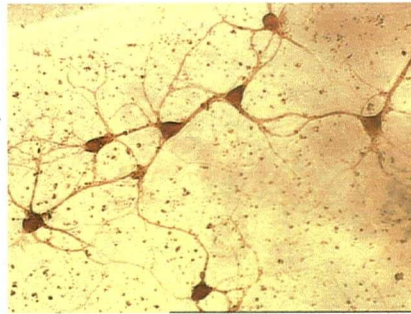
NR2A
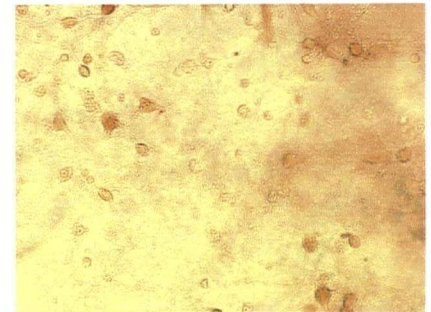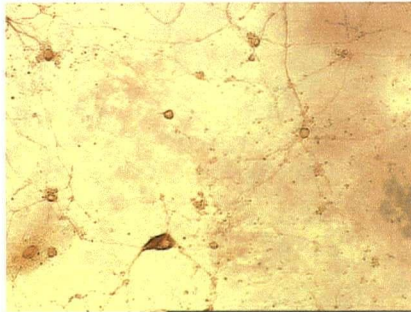
NR1A
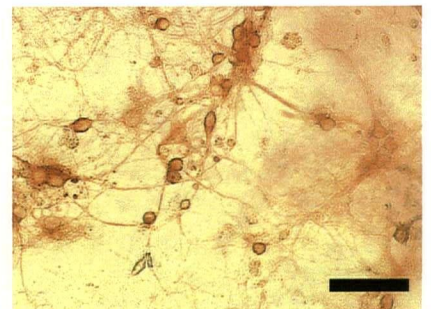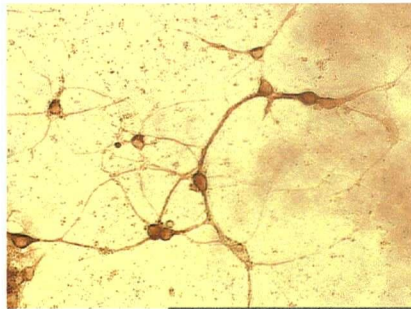
4.2 MSNs Expressing Mutant Htt Show Increased NMDAR-Dependent Excitotoxicity

Our first experiments to perform on the primary striatal cultures were to determine if our results obtained in the HEK293 cell system (Chen et al. 1999b; see Figure 1; Zeron et al., 2001; see Chapter 3) and the enhanced NMDAR-mediated current amplitude found in acutely dissociated MSNs expressing mutant htt in older mice found previously by N. Chen (see Figure B; Zeron et al., 2002) correlated with an enhanced vulnerability to NMDAR-mediated cell death in MSNs *in vitro*. My collaborators, Brundin and Hansson, showed enhanced vulnerability to excitotoxicity after quinolinic acid injection in the striatum of YAC transgenic HD mice that are 6 and 10 months of age compared to WT mice (Zeron et al., 2002). However, the increased vulnerability to NMDAR-mediated striatal cell death in the YAC72 mice might be a consequence of other cellular changes resulting from mutant htt expression, since around this age the mice show electrophysiological abnormalities and an HD-like phenotype (Hodgson et al., 1999).

To help determine whether augmented sensitivity to NMDAR activation is a primary mechanism underlying increased striatal neuronal degeneration *in vivo*, we tested whether MSNs from young (early postnatal) YAC72 mice also exhibit enhanced vulnerability to NMDAR agonists. We used an *in vitro* model of excitotoxicity, which involves treating primary neostriatal cultures with NMDA and glycine for 10 minutes and assessing cell survival 24 hours later, similar to models used by others to investigate mechanisms of excitotoxicity (reviewed by Choi, 1988). Neuronal loss in the control (salt solution alone) was minimal (~5 %), and comparable with that of the untreated group. Numbers of MSNs (assessed by morphological criteria) remaining attached to the dish were similar between the cultures from YAC72 and WT mice (data not shown). NMDA-induced cell death, as measured by percentage of MSNs with trypan blue inclusion, was concentration-dependent (2-factor ANOVA, effect of concentration

F(2,29)=41.2, *P*<0.0001): it was nearly 2-fold larger after a supramaximal concentration of 3 mM than after 100 µM (Figure 9A). Further data are necessary to obtain a concentration-response curve of excitotoxic cell death from which we could derive the EC_{50} for excitotoxic cell death to determine whether this is similar to the measured EC_{50} for NMDAR currents recorded from murine cortical and diencephalic neurons (Sather et al., 1992). As well, brief (20 min) exposures to NMDA concentrations in this range have been shown previously to induce apoptotic death in cultured cortical neurons (Budd et al., 2000). Similar to *in vivo* results, MSNs expressing mutant htt were found to be more sensitive to NMDA-mediated cell death *in vitro*, where a larger percentage of MSNs were dead 24 hours after NMDA exposure in cultures from YAC72 compared to WT mice (2-factor ANOVA, effect of genotype F(1,29)=6.9, *P*<0.05) (Figure 9A). These *in vitro* data indicate that MSNs expressing mutant htt show an increased sensitivity to NMDAR activation even from birth, providing support for the idea that cellular dysfunction could begin early and that NMDAR activation may be a trigger of cell death in HD.

FIGURE 9. Enhancement of NMDAR-mediated cell death in MSNs expressing mutant htt compared to WT control MSNs. (A) Survival of MSNs was assessed using the trypan blue exclusion assay 24 h after exposure to NMDA compared to survival of MSNs that underwent exposure to salt solution alone. $*P<0.05$ vs WT at same NMDA concentration. N=4-6 different batches of cultured neurons for each treatment. (B) No trend of enhanced NMDAR-mediated cell death in primary cultured CGNs expressing mutant htt compared to WT CGNs. Survival of CGNs was assessed using the trypan blue exclusion assay 24 h after exposure to NMDA compared to survival of CGNs that underwent exposure to salt solution alone. N=3 different batches of cultured neurons for each treatment. Bars represent mean $\pm$ S.E.M

A
MSNs

B
CGNs
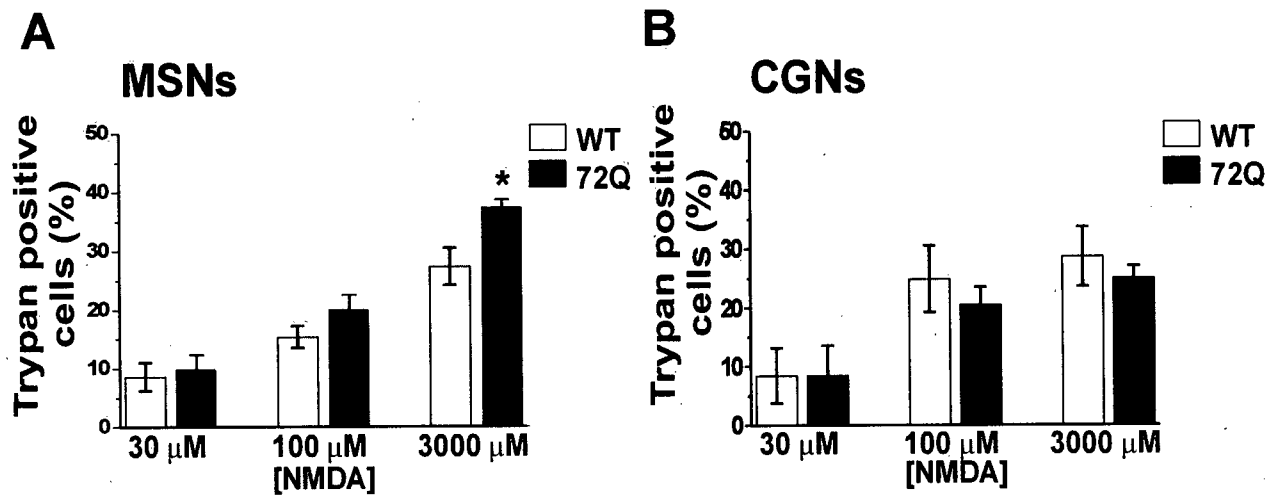
4.3 Enhanced NMDAR-Dependent Excitotoxicity is Not Found in CGNs Expressing Mutant Htt

To examine whether this enhanced cell death effect was selective for neurons affected in HD we repeated these experiments in cultured cerebellar granule neurons (CGNs). We elected to use primary cerebellar neuronal cultures as controls since the cerebellum is largely unaffected in HD and they express different NMDAR composition than striatal neurons. Primary cerebellar neuronal cultures have been used extensively to study glutamate receptor-mediated signal transduction and cell death (Balazs, et al., 1992; Brandoli et al., 1998; Cebere et al., 1999; Didier et al., 1997; Kovacs et al, 2000; 2001; Weller et al., 1993; Moldrich et al., 2001). Cerebellar neurons are largely composed of cerebellar granule neurons, are almost completely glutamatergic and express high levels of different glutamate receptors (Hack et al., 1995; Resink et al., 1995) and especially NR1 with NR2A and/or NR2C but not NR2B NMDAR subunits in mature cerebellar cultures and *in vivo* (Vallano et al., 1996; Thompson et al., 2000; Kovacs et al., 2001). As found for MSNs, NMDAR-mediated CGN cell death was concentration-dependent (2-factor ANOVA, effect of concentration , $F_{(2,17)} = 28.6$, $P<0.0001$) (Figure 9B). In contrast to MSNs, however, primary CGNs showed no mutant htt-dependent enhancement in NMDAR-mediated cell death measured by the trypan blue assay (2-factor ANOVA, effect of genotype $F_{(1,17)} = 1.6$; $P>0.05$; genotype x concentration interaction $F_{(2,17)} = 0.4$; $P>0.05$) (Figure 9B). It is interesting that developing CGNs express NR2B, but with maturation *in vivo* and *in vitro* (i.e., by ~9 DIV) these neurons express predominantly the NR2A and NR2C NMDAR subtypes (Hollmann and Heinemann, 1994; Monyer et al., 1994; Vallano et al., 1996; Thompson et al., 2000; Kovacs et al., 2001). Thus, our data are consistent with the pathology of HD, in which the

cerebellum is largely spared, and support the idea that excitotoxic enhancement seen in the mutant mice is NMDAR subtype-dependent.

4.4 MSNs Expressing Mutant Htt Show Enhanced NMDAR-Mediated Apoptosis Which is Also PolyQ Length-Dependent

Next, we used the terminal deoxynucleotidyl transferase-mediated dUTP (TUNEL) fluorescein-tagged staining technique to examine NMDAR-induced apoptosis in our cultures, since previous work has suggested that apoptosis may be the primary mode of cell death in HD (reviewed by Wellington and Hayden, 2000). In some cases, advanced stages of degraded necrotic cells can also stain using the TUNEL technique (Trump et al., 1997); therefore, we only considered as apoptotic TUNEL-positive cells that also had small, condensed nuclei as visualized with propidium iodide. Furthermore, we only counted cells that appeared to be MSNs by morphological criteria under brightfield. We employed this technique, as opposed to the Hoechst dye assay used in the HEK293 experiments (see Section 3.2), since the nuclei of the MSNs were smaller and more difficult to analyze using the Hoechst dye staining alone. By combining morphology of condensed and/or globular nuclei using propidium iodide staining with TUNEL staining we could eliminate many false positive neurons (necrotic and TUNEL-positive) and more selectively count apoptotic MSNs. As seen in Figure 10 A and B, there was a marked enhancement of NMDAR-induced apoptosis among MSNs cultured from YAC mice expressing mutant htt compared with WT mice (2-factor ANOVA, effect of genotype $F(2,40) =$ 14.9; $P<0.001$), and this enhancement was NMDA concentration-dependent (genotype x concentration, $F(4,40) = 3.4$; $P<0.05$). Further analysis indicated that at 3 mM NMDA treatment both the mutant htt groups displayed significantly more apoptosis than WT cultures (Bonferroni post-hoc test; WT vs 46Q $P<0.05$; WT vs 72Q $P<0.001$) and the 72Q group exhibited

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significantly more apoptosis than the 46Q group (Bonferroni post-hoc test, $P<0.05$) (Figure 10 A,B). These *in vitro* data indicate that MSNs expressing mutant htt show an increased sensitivity to NMDAR activation that is polyQ length-dependent and our results provide support for the idea that cellular dysfunction could begin early and that NMDAR activation may be a trigger of apoptotic cell death in HD.

FIGURE 10. Trend in enhancement of NMDAR-mediated apoptosis in MSNs expressing mutant htt compared to WT MSNs is polyQ length-dependent. (A) Representative fields of striatal neurons are shown 24 h after exposure to salt solution alone (control) or 3 mM NMDA followed by TUNEL staining and propidium iodide (PI) counterstaining. [Bar=25 μm]. In (B) apoptotic MSNs (>500) were assessed using TUNEL staining and morphological criteria as shown by propidium iodide (PI) counterstaining 24 h after exposure to NMDA, compared to apoptosis observed in salt solution alone-exposed MSNs. *$P<0.05$, ***$P<0.001$ vs WT at same NMDA concentration. #$P<0.05$ vs 46Q at same concentration. N=3-6 different batches of cultured neurons for each treatment. Bars represent mean ± S.E.M.

A

Control | 3 mM NMDA

TUNEL | PI | TUNEL | PI

WT

46Q

72Q

B

105
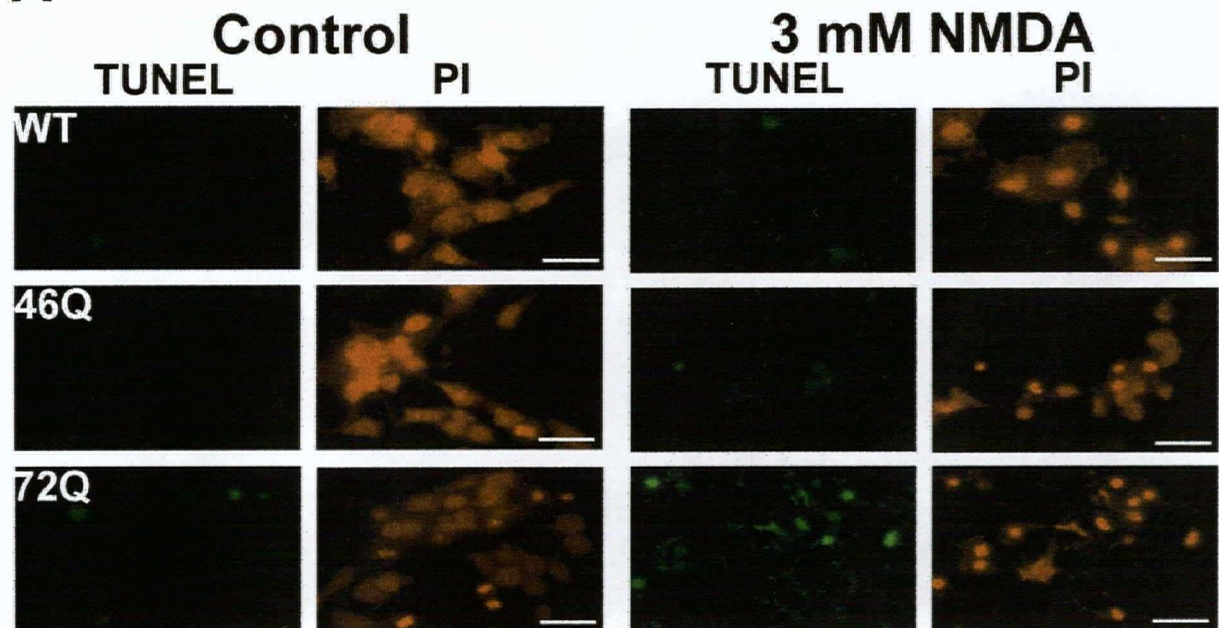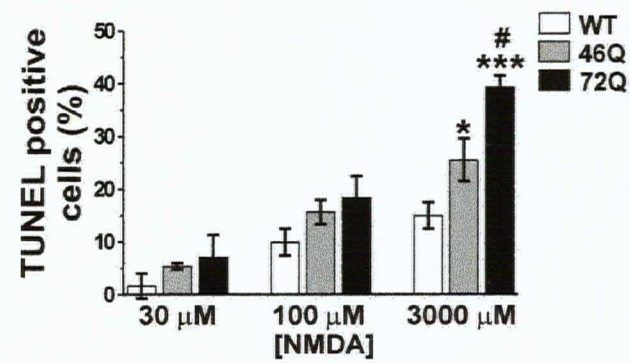
4.5 NMDAR-Dependent Increase in Excitotoxicity in MSNs Expressing Mutant Htt May Be Inhibited By NMDAR Antagonists

To ensure that the high NMDA concentration we used was not activating other lower affinity receptors, we treated MSNs with the use-dependent NMDAR antagonist MK-801 together with 3 mM NMDA to specifically eliminate the contribution of NMDAR activation to cell death. The plating medium was pretreated with 20 μM MK-801 for >30 min before exposure to 3 mM NMDA. 20 μM MK-801 was also added during the 10 min exposure to NMDA, and then the preconditioned medium containing 20 μM MK-801 was reapplied to the MSNs. We used 20 μM MK-801 to maximize the binding rate of this antagonist and ensure complete inhibition of any channels that opened during NMDA application. Under these conditions cell death was reduced nearly to baseline levels (i.e., similar to cultures treated with MK-801 alone) confirming that >80% of the cell death observed in response to 3 mM NMDA resulted from NMDAR activation in our system (Table 2). Moreover, we found that a similar proportion (>80 %) of 3 mM NMDA-induced cell death was inhibited by the NR1/NR2B subtype-specific antagonist ifenprodil (IFN) (Williams, 1993) for cultured striatal neurons from both YAC72 and WT mice (Table 2). The plating medium was pretreated with 10 μM ifenprodil for >30 min before exposure to 3 mM NMDA. 10 μM ifenprodil was also added during the 10 min exposure to NMDA, and then the preconditioned medium containing 10 μM ifenprodil was reapplied to the MSNs. We used 10 μM ifenprodil in order to saturate inhibition of NR2B-type NMDARs while minimally inhibiting NR2A-type receptors (Williams, 1993). The effects that MK-801 and ifenprodil have in our system may not wholly be due to their effects on NMDARs. It has been shown that high concentrations of MK-801, such as that employed in our

experiments, may block voltage-gated potassium and sodium channels (Rothman, 1988; Wooltorton and Mathie, 1995), thus confounding our conclusion that the effect of MK-801 in our model is due to inhibition of NMDAR-mediated excitotoxicity alone. Furthermore, 10 μM ifenprodil used in our experiments may not be inhibiting the NR1/NR2B-NMDAR component alone, since it has been shown that >3 μM ifenprodil may have some affinity for the NR1/NR2A subtype as well (Williams 1993). Also, ifenprodil has also been shown to inhibit high voltage-gated calcium channels (Bath et al., 1996); the contribution of these channels to excitotoxicity in our model is largely unexplored. Further tests are necessary to determine the specificity of the NMDAR antagonists employed in our system. Nonetheless, our results suggest that mutant htt may enhance excitotoxicity mediated specifically by the NR1/NR2B subtype of NMDAR.

Table 2. Inhibition of excitotoxicity in MSNs after exposure to NMDAR antagonists.

		3 mM NMDA	3 mM NMDA + 20 µM MK-801	3 mM NMDA + 10 µM IFN	# trials
Trypan positive cells (%)	WT	11.4 ± 0.6	1.4 ± 2.2	1.9 ± 2.3	3
	72Q	29.8 ± 2.8	3.4 ± 2.1	2.3 ± 1.3	3-4
TUNEL positive cells (%)	WT	14.0 ± 0.8	1.9 ± 0.6	1.5 ± 0.6	3
	46Q	28.5 ± 1.9	3.8 ± 1.1	2.8 ± 0.4	3
	72Q	33.5 ± 2.6	4.4 ± 1.2	4.9 ± 1.4	3-4

4.6 Mutant Htt-Enhanced Cell Death is Glutamate Receptor Subtype Selective

Expression of mutant htt might increase MSN vulnerability to a variety of stresses, even though in our experiments cultures were made from early postnatal mice. Therefore, we investigated the effect of another excitotoxic stress – activation of AMPA receptors – to determine whether the increased vulnerability to cell death found for mutant htt-expressing MSNs was specific for NMDAR activation. We treated cultured MSNs with 1 mM AMPA and 50 μM cyclothiazide (to eliminate AMPAR desensitization and thereby increase toxicity – Brorson et al., 1995) in the presence or absence of 10 μM ifenprodil for 2 hours. Cell death was measured 24 hours later by the trypan blue assay and the TUNEL assay. Interestingly, AMPA-stimulated cell death was reduced by 10 μM ifenprodil and therefore partially mediated by NR2B-subtype NMDARs (Table 3). However, there was no increase in AMPAR-mediated cell death in MSNs expressing mutant htt compared to WT either in the presence or absence of ifenprodil (Table 3). NMDAR stimulation likely resulted from glutamate released from dying neurons. It is also possible that ifenprodil may directly inhibit the AMPA-evoked currents. Further testing would be necessary to determine whether this may be true. Notably, the ifenprodil-sensitive component showed a trend toward increased cell death for YAC72 compared with wild type or YAC46 MSNs, but this difference was not significant. These data confirm that mutant htt enhances excitotoxicity mediated by NMDARs but not AMPARs.

Table 3. No trend in enhancement of non-NMDAR-mediated excitotoxicity in MSNs expressing mutant htt compared to WT MSNs.

		1 mM AMPA + cyclothiazide	1 mM AMPA + cyclothiazide + IFN	# trials
Trypan positive cells (%)	WT	29.8 ± 3.0	16.4 ± 1.8	4
	46Q	26.8 ± 4.3	14.0 ± 3.8	4
	72Q	34.2 ± 1.3	14.8 ± 1.3	2
TUNEL positive cells (%)	WT	37.8 ± 10.2	18.8 ± 5.1	4
	46Q	37.5 ± 4.6	21.1 ± 3.1	5
	72Q	47.5 ± 1.1	12.6 ± 6.2	2

Analysis of trypan blue data: 2-factor ANOVA; effect of ifenprodil $F_{(1,19)} = 26.5$, $P < 0.001$; effect of genotype $F_{(2,19)} = 0.7$, $P > 0.05$; genotype x ifenprodil interaction $F = 0.4$, $P > 0.05$.

Analysis of TUNEL data: effect of ifenprodil $F_{(1,21)} = 17.8$, $P < 0.001$; effect of genotype $F_{(2,21)} = 0.03$, $P > 0.05$; genotype x ifenprodil interaction $F = 0.9$, $P > 0.05$.

4.7 Summary

Our findings indicate that MSNs in culture that express mutant htt (46Q or 72Q) show enhanced vulnerability to NMDAR-mediated excitotoxic cell death and apoptosis, which is polyQ-length-dependent as well. We also showed that the excitotoxic cell death and apoptosis could be inhibited by 10 μM ifenprodil, an antagonist with relative selectivity for the NR1/NR2B NMDAR subtype. Also, we showed that NMDAR-mediated enhancement in cell death was restricted to areas affected in HD, and that mutant htt-dependent enhancement in excitotoxic cell death was exclusively due to NMDARs, but not AMPARs.

These results agree with our previous findings that HEK293 cells co-expressing full-length mutant htt and NR1A/NR2B NMDAR subtype display enhanced NMDAR-mediated current amplitude and apoptosis (Chen et al., 1999b; see Figure 1, Zeron et al., 2001; see Chapter 3). As well, these data are consistent with previous findings by N. Chen that acutely dissociated MSNs from YAC transgenic mice expressing mutant htt show enhanced NMDAR-evoked current amplitude, which could mostly be blocked by 10 μM ifenprodil (Zeron et al., 2002). MSNs expressing mutant htt display enhanced NMDAR-mediated current amplitude (Zeron et al., 2002) as well as enhanced excitotoxicity, demonstrating that increased sensitivity to NMDAR-mediated excitotoxicity is not just a secondary effect to other changes induced by mutant htt. However, we cannot rule out that additional processes are involved in the increased excitotoxicity associated with mutant htt (e.g., abnormal mitochondrial function or calcium homeostasis).

Our findings also agree with our collaborators, Brundin and Hansson, who showed enhanced NMDAR-dependent cell death in MSNs from 6 and 10 month old YAC transgenic mice expressing full-length mutant htt *in vivo* (Zeron et al., 2002). This is important, since our *in vitro* model of MSNs excitotoxicity may not duplicate *in vivo* events for many reasons, apart

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from the difference in age of the neurons used for *in vivo* and *in vitro* experiments. Our *in vitro* model may be too simple or too altered for comparison with the *in vivo* model, since, for example, it has been shown that the properties or physiology of neurons can change *in vitro* (Kovacs et al., 2001). Furthermore, the MSNs in our culture model do not receive the same innervation they would *in vivo* (i.e. no input from the cortex nor thalamus). This may change the response to stimulus *in vitro* (i.e. lack of depolarizing effect of glutamate release from cortical neurons during maturation of these cultured striatal neurons depresses response to agonist *in vitro* (Chen et al., 1999). It is striking that we have to use a very large concentration of NMDA to attain maximal cell death. It is possible that a lack of extra-glutamatergic stimuli (from cortical innervation or glial release) or depolarization (from a source like KCl) during maturation of MSNs *in vitro* may result in fewer receptors at the surface (Balazs et al, 1992; Resink et al., 1995; Li et al., 1998; Chen et al., 1999; Cebers et al., 2001). On the other hand, in preliminary studies, we have examined the effects of NMDA in mouse organotypic cortico-striatal slice cultures and still see a relative resistance to excitotoxic cell death of the striatal neurons compared to the cortical neurons, suggesting that MSNs may be less sensitive to NMDAR stimulation. Moreover, we only examined the consequence of NMDAR activation without consideration of other converging pathways that could modulate NMDAR response i.e. dopamine receptor activation, which may be a factor *in vivo*. It is unknown whether the results from our acute model of excitotoxicity translates into similar pathogenic results in HD brains over the span of a lifetime. However, taken together, our data suggest NMDARs may play a key role in the pathogenesis of HD, causing increased ion influx, membrane depolarization and excitotoxic cell death in MSNs expressing mutant htt.

CHAPTER 5

NMDAR-Mediated Caspase-3 Activation in Medium Spiny Striatal Neurons Expressing

Mutant Htt *In vitro*

Now that we had established a role for enhanced NMDAR-dependent excitotoxity in MSNs expressing mutant htt we were interested in what downstream effects this may have on MSNs, leading to apoptosis. Since proteases, like caspases, have been implicated in apoptotic cell death (Yuan et al., 1993; Troy et al., 1996, 1997; Du et al., 1997; Tenneti et al., 1998), we elected to examine whether caspase activation takes place in our model. The caspase family consists of 14 members to date, and corresponds to the mammalian homologues of ced-3 from *Caenorhabditis elegans* (Yuan et al., 1993; Humke et al., 1998). Specifically, caspase-3 has been found to be an important mediator of neuronal apoptosis (Kuida et al., 1996; Tenneti and Lipton 2000). Peptide inhibitors of caspases have been found to lessen ischemic and excitotoxic neuronal damage *in vivo* (Hara et al., 1997; Chen et al., 1998) in addition to traumatic brain injury (Yakovlev et al., 1997). These data suggest a therapeutic use for caspase inhibitors against neuronal injury and death.

Caspase-3 has been shown to cleave full-length htt into fragments containing the N-terminal ~500 amino acids and including the polyQ (Wellington et al., 2000). Similar htt fragments were found to be toxic when translocated to the nucleus (Hackam et al., 1998; Lunkes and Mandel, 1998). Furthermore, caspase-3 plays an important role as a downstream effector of programmed cell death. NMDAR activation after mild insults has been found to involve the time-dependent activation of caspase-3-like proteases and subsequent apoptosis (Tenneti and Lipton, 2000). Caspase-3 has shown to be activated as a consequence of mitochondrial

dysfunction and/or in concert with other pathways, such as calcium-dependent calpain activation of caspases (Li et al., 1997; Green and Reed, 1998; McGinnis et al., 1999; Wolf et al., 1999). Since caspase-3 has been found to play an important role in programmed cell death, it recognizes htt as a substrate possibly creating toxic N-terminal fragments, and caspase-3 activation has been found to occur after NMDAR activation, we examined caspase-3 activity in the primary striatal cultures after exposure to NMDA.

5.1 Increased Immunocytochemical Staining of Activated Caspase-3 6 Hours After Exposure to NMDA in MSNs Expressing Mutant Htt

We examined caspase-3 activity in the primary striatal cultures after exposure to NMDA. First we utilized immunocytochemistry to detect the active form of caspase-3. Caspases are normally expressed in cells as inactive zymogens, also called pro-caspases. Upon apoptotic induction, the zymogen is activated by proteolytic cleavage at aspartic acid residues creating large and small subunits (Srininvasan et al., 1998). Active caspase-3 is composed of p18 (amino acids 29-175) and p12 (amino acids 176-277) subunits (Nicholson et al., 1995). The use of an antibody to detect activated caspase-3 *in situ* was first demonstrated by Srininvasan et al., 1998, who effectively showed that they were able to recognize only the p18 subunit of cleaved caspase-3. With a similar antibody, we observed a significantly larger increase in the number of MSNs positive for its expression 6 hours after brief application of 3 mM NMDA in YAC46 and 72 MSNs compared to WT controls (2-factor ANOVA, effect of genotype $F(2,47)=7.1$, $P<0.01$) (Figure 11A,B). As well, there was a trend toward a larger increase in NMDAR-induced caspase-3 activity for YAC72 versus YAC46 MSNs, which was not significant because of the variability in absolute percentages of immunopositive neurons between different batches of

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cultured MSNs. In cultures treated with the caspase inhibitor z-DEVD-fmk immediately before, during and after NMDAR stimulation, activation of caspase-3 was not different from that observed in control cultures that were not stimulated with NMDA (Figure 11A,B). It is interesting that the data presented in Figure 9A,B do not show as large a difference between the YAC46 and the YAC72 MSNs as was seen when comparing TUNEL positive cells (Figure 10). This could be due to observer variability in data collection (cell counting) in Figure 11A,B since my collaborator Cheryl Wellington analyzed most of the YAC72 data and I analyzed the YAC46 data. It is possible that we used slightly different criteria for counting caspase-3 positive MSNs. My criterion for counting a neuron as positive for caspase-3 activation was the appearance of any immunofluorescence, not distinguishing between overall levels of fluorescence intensity. Further analysis could be done in the future to determine whether using a stricter criterion (i.e. counting cells as positive for caspase-3 activity if their entire cytoplasm is stained very brightly) would change the outcome of our results. Also it is possible there could be more caspase-3 positive cells than TUNEL positive cells since caspase-3 assays were conducted at 6 hours after exposure to NMDA whereas the TUNEL assays were conducted 24 hours later by which time some cells could have detached from the coverslip after death.

This immunofluorescent analysis provides compelling evidence for a role of NMDAR-dependent enhancement of caspase-3 activation in MSNs expressing mutant htt, correlating with the enhancement of apoptosis observed in MSNs expressing mutant htt after NMDA challenge (Figure 10A,B).

FIGURE 11. Increase in NMDA-mediated caspase-3 activation in MSNs expressing mutant htt compared to WT controls. Cultures were treated 9-12 DIV with the various drugs for 10 minutes and then fixed 6 hr later. Immunocytochemical staining and subsequent analysis was completed equally by Dr. Cheryl Wellington or I. (A) Caspase-3 activation was assessed by *in situ* immunofluorescence 6-h after exposure to salt solution (C) or 3 mM NMDA (A), with or without 10 μM z-DEVD-fmk (I), in MSNs expressing mutant htt (72Q) compared to WT MSNs. DAPI staining in blue represents total nuclei present and red indicates staining for activated caspase-3. MSNs are shown in phase contrast (upper panels) or in fluorescence (lower panels) [Bar=20 μm]. (B) Enhancement of NMDAR-dependent caspase-3 activation is also polyQ length-dependent as assessed by *in situ* immunofluorescence 6 h after exposure to experimental solutions. **$P<0.01$, ***$P<0.001$ vs WT at same NMDA concentration. >500 MSNs were counted per treatment condition per batch of cultured neurons. N=3-5 different batches of cultured neurons for each treatment. Bars represent mean ± S.E.M

A

	C	C+I	A	A+I
72Q				
WT				

B
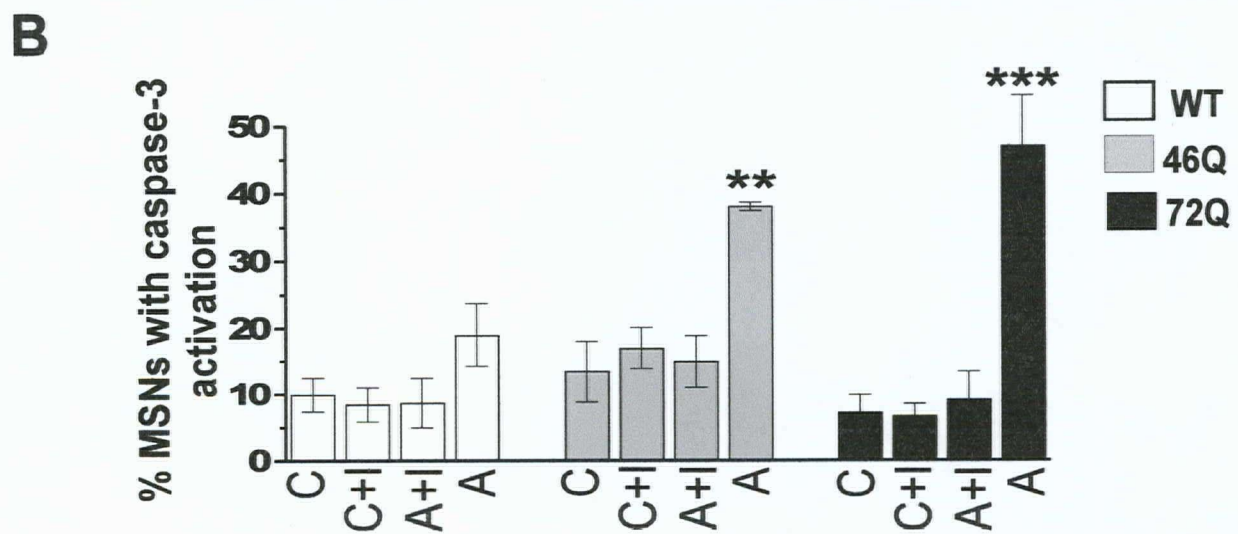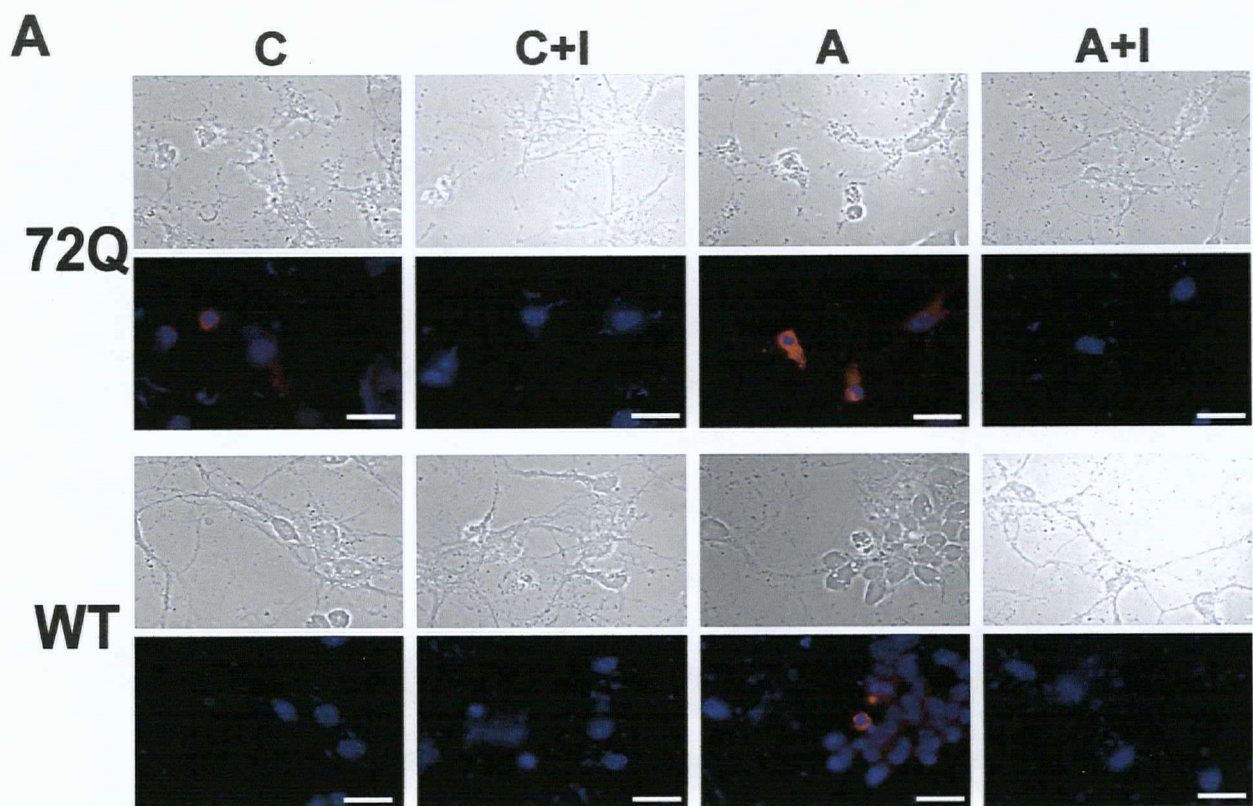
5.2 Enhanced Caspase Proteolysis 3 and 6 Hours After Application of NMDA in Striatal Cells Expressing Mutant Htt

To further confirm our findings obtained using the immunofluorescent analysis of activated caspase-3 we also used an assay that examines caspase-3 like activity from striatal cell lysates. This method is advantageous because activity is easily quantifiable using a plate reader, but disadvantageous as it is not as specific for activated caspase-3 as the antibody assay described in the previous section, and is not specific for MSNs but samples all cells in the culture (although >90 % of these are MSNs). This caspase activity assay also measures activity of any other proteases that recognize the DEVD tetrapeptide sequence, (e.g. caspase-6 and −7). Still, we were able to replicate the difference between WT and YAC72 MSNs using the fluorometric assay for caspase activity. WT MSNs exposed to 3 mM NMDA had >2 times more caspase activity 3 and 6 hours later than those WT MSNs exposed only to physiological salt solution. This agrees with data obtained by other groups (Schulz et al., 1998; Nath et al., 2000; Qin et al., 2000). We found a significant enhancement of caspase activation in cultured striatal cell lysates from YAC72 compared to WT mice 3 hours and 6 hours after exposure to 3 mM NMDA (2-factor ANOVA, effect of genotype $F(1,14)=34.8$, $P<0.001$ at 3 h and $F(1,14)=7.1$, $P<0.05$ at 6 h) (Figure 12). Together, these results (Figure 12) are consistent with the TUNEL staining data (Figure 10A,B), indicating that NMDA-induced apoptotic death is significantly higher for MSNs cultured from YAC72 mice compared with WT mice. Furthermore, increases in caspase activation measured at 6 hours after exposure to NMDA in WT and 72Q MSNs were ~2 times and ~5 times, respectively, that measured for sister cultures treated with salt solution, using either caspase activation analysis (compare Figure 11A,B and Figure 12). Notably, the ratio of caspase activity measurements in WT controls to 72Q controls measured 6 h after exposure to

salt solution alone was 1.01 $\pm$ 0.12 (N=4), indicating that cells expressing mutant htt and those expressing WT htt have similar basal caspase activity. The data are expressed as ratios instead of actual values since absolute values varied between experiments while ratios between the agonist groups versus control salt solutions groups remained relatively constant. It is unclear why there is such a large difference between the 3 and 6 hour time points for the NMDA-treated 72Q-expressing MSNs. Some of the possibilities include: 1) other proteases (non-caspase-3) activated at the 3-h but not 6-h time point are detected by the DEVD-ase activity assay; 2) caspase-3 is maximally activated around 3 h and its ability to proteolyze the DEVD-ase is diminished by 6 h due to binding to other substrates, inactivation by other proteases or to its degradation; 3) cells with earlier activation of caspase-3 have completed apoptosis by 6 hr. Further experiments are required to distinguish among these possibilities.

Taken together, our results demonstrate that cultured MSNs expressing mutant htt show enhanced caspase-3 activity compared with those expressing WT htt after NMDAR activation. These data correlate well with the enhanced apoptosis shown by the TUNEL staining technique (Figure 10A,B).

FIGURE 12. Enhancement of NMDAR-dependent activation of caspase proteolysis in cultured striatal cells expressing mutant htt (72Q) compared to WT. Caspase activation was assessed fluorometrically by cleavage of Ac-DEVD-AFC 3 h and 6 h after exposure to NMDA and normalized to background fluorescence, amount of protein/sample, and compared to sister cultures that underwent exposure to salt solution alone (see Methods). **$P<0.01$, ***$P<0.001$ vs WT at same NMDA concentration. N=3-5 different batches of cultured neurons for each treatment and time point. Bars represent mean $\pm$ S.E.M
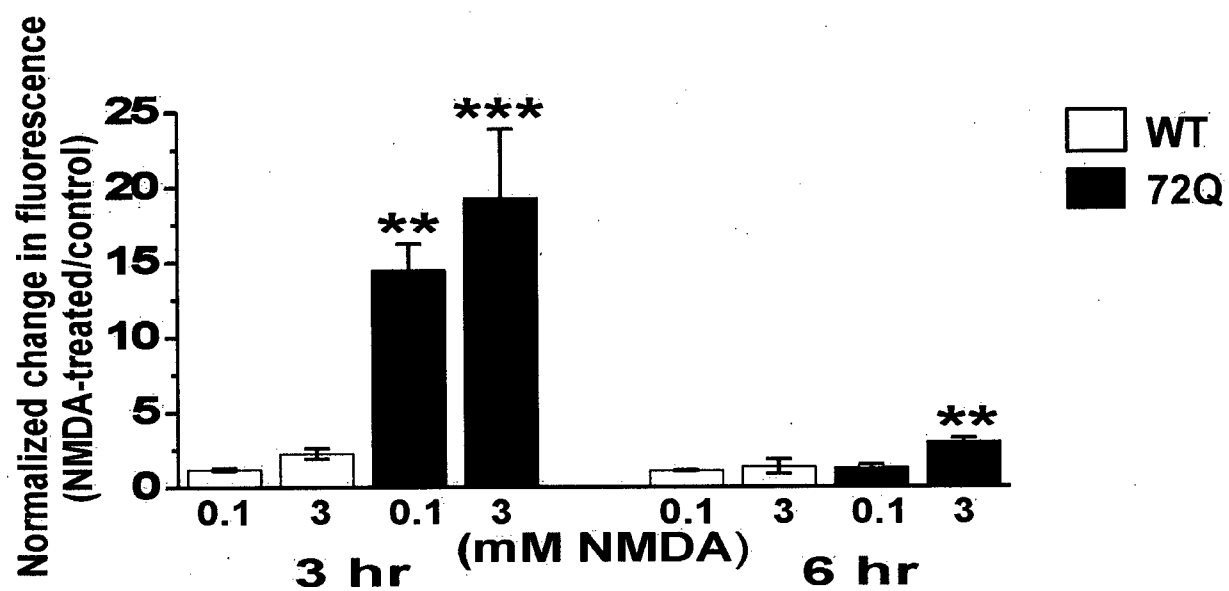
5.3 NMDAR-Dependent Excitotoxicity in MSNs Can Be Blocked By a Caspase-3 Like Inhibitor

Previously we showed that cultures treated with the caspase inhibitor z-DEVD-fmk just before, during and after NMDAR stimulation had significantly depressed caspase-3 activation (Figure 11A,B). We also wanted to determine whether the caspase inhibitor could prevent cell death as well in the NMDA-stimulated MSNs. In sister cultures that underwent treatment with the caspase inhibitor z-DEVD-fmk (antagonist of caspase-3, -6 or −7), we also examined cell death of MSNs in parallel using the trypan blue inclusion assay and the TUNEL assay (Table 4). The plating medium was pretreated with 10 μM z-DEVD-fmk for >30 min before exposure to 3 mM NMDA. 10 μM z-DEVD-fmk was also added during the 10 min exposure to NMDA, and then the preconditioned medium containing 10 μM z-DEVD-fmk was reapplied to the MSNs. We used 10 μM z-DEVD-fmk to maximize the binding rate of this inhibitor to ensure complete inhibition of any caspase-3 like activity that occurred during NMDA application. These experiments were done in parallel with excitotoxic assays shown earlier displaying the effects of NMDAR antagonists (see Table 2) and thus we use the same data for the 3 mM NMDA condition. We show an almost complete depression of NMDAR-mediated cell death in MSNs treated with caspase inhibitor (Table 4). These results indicate that caspase-3-like activation is required for mediating NMDA-induced cell death in the cultured MSNs.

Table 4. Decreased excitotoxicity in MSNs after exposure to a caspase-3 like inhibitor.

		3 mM NMDA	3 mM NMDA + z-DEVD-fmk	# of trials
Trypan positive cells (%)	WT	11.4 ± 0.6	1.7 ± 2.2	3
	72Q	29.8 ± 2.8	2.6 ± 1.2	3-4
TUNEL positive cells (%)	WT	14.0 ± 0.8	1.5 ± 1.9	3
	46Q	28.5 ± 1.9	4.2 ± 0.4	3
	72Q	33.5 ± 2.6	2.0 ± 4.2	3-4

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5.4 Huntingtin Cleavage Analysis

A large body of evidence in *in vitro* models suggests that cellular accumulation of short fragments of the N-terminal region of htt containing the polyQ tract can cause neuronal dysfunction and/or death (reviewed by Wellington and Hayden, 2000). Huntingtin is cleaved by caspase-3 and –6, as well as calpains [Wellington et al., 2000; Kim et al., 2001; Gafni and Ellerby, 2002; C.L. Wellington and M.R. Hayden (personal communication)], in the brains of normal controls as well as patients symptomatic with HD. Our data showing enhanced NMDAR-induced caspase-3 activation in mutant htt-expressing striatal neurons suggest NMDAR activity as a potential trigger for increased htt cleavage.

To test this hypothesis, we used two different htt N-terminal antibodies (Chemicon mAB2166 and BKP1 from M.R. Hayden's laboratory). We found that most of the htt protein is in the cleaved state in cultured striatal neurons (two bands 60-70 kDa in size) (Figure 13A,B,C). There are high levels of htt cleavage product present under all conditions tested, including treatment with 3 mM NMDA, control (salt solution), or 10 μM staurosporine (inhibits protein kinase C and has been found to induce apoptosis in culture) (Figure 13A,B,C). The high level of htt cleavage is seen for all genotypes, including WT, 46Q (not shown) or 72Q-expressing striatal cultures (Figure 13A,B,C). We have done preliminary experiments to attempt to delineate the role of the basal htt cleavage seen in our cultures and this will be discussed in Section 7.2. In all, we have observed high basal levels of htt cleavage products and little difference in the extent of cleavage in cultured murine MSNs across different genotypes [wild-type, YAC46 (not shown) and YAC72] (Figure 13A,B,C) or treatment conditions (NMDA versus buffered salt solution) (Figure 13A,B,C) by Western blot analysis using multiple N-terminal htt antibodies (Chemicon mAB2166 or BKP1) (Figure 13A,B,C).

Importantly, the high basal htt cleavage could not have influenced our excitotoxicity results in culture, because the increase in cell death in mutant htt-expressing MSNs was selective for NMDAR activation and not seen after AMPAR stimulation. It is interesting that regardless of the increased presence of mutant htt fragments there was no enhancement in vulnerability to generalized stress, such as AMPAR-mediated excitotoxicity, as had been suggested by previous studies (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998; Li et al., 1999). In contrast to our data found in HEK293 cells (see Section 3.4; Zeron et al., 2001), our findings in cultured MSNs support the idea that mutant htt, as either full-length or truncated forms, may have interactions with NMDARs. These findings are supported by current research in animal models, whereby NMDAR-mediated abnormalities in evoked current and intracellular calcium levels have been found in animal models expressing full-length mutant htt or truncated mutant htt (Levine et al., 1999; Cepeda et al., 2001a; Laforet et al., 2001). Also, findings by C.L. Wellington and M.R. Hayden (personal communication), showed there is only a low level of constitutive htt cleavage in YAC mouse tissue at ages 2-11 months, the same time that our group has shown enhanced NMDA-mediated MSN death *in vivo* (6 and 10 month old YAC72 mice) and (in acutely dissociated MSNs from 6-11 week old YAC72 mice) showed enhanced current amplitude (Zeron et al., 2002; see Figure 2). Therefore, it seems improbable that cleavage of htt, producing fragments that may subsequently enhance cell death as previous groups have found (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998; Li et al., 1999), plays a large role in modulating NMDA-induced cell death in our model.

Our results suggest that the mutant htt-induced increase in caspase-3 activation following NMDA treatment in our model of acute excitotoxicity may result in cleavage of many additional substrates that together lead to death of MSNs, consistent with the accepted role for caspase-3 as a final executioner of apoptotic death in the CNS. Further tests are needed to decide whether

caspase-3 or other proteases, such as calpains, play a definitive role in the cleavage of htt and htt fragment toxicity.

FIGURE 13. Western blot analysis of htt cleavage in mouse primary striatal cultures expressing normal or mutant htt. (A) Cytoplasmic fraction from striatal cultures expressing mutant htt (72Q) probed with N-terminal htt mAB2166 primary antibody (left panel) and then reprobed with another N-terminal htt primary antibody, BKP1 (right panel). Cultures underwent exposure to 10 μM staurosporine (stauro) for 6 hr and were harvested, or were exposed for 10 min to 3 mM NMDA (NMDA) or control salt solution (control) and collected 6 hours later. N=2 for probing the same blot with both antibodies; N=4 for probing with mAB2166 alone. (B) Cytoplasmic fraction from striatal cultures expressing normal htt (WT) probed with N-terminal htt mAB2166 primary antibody. WT striatal cultures underwent exposure to staurosporine, NMDA or control salt solution. N=4. (C) Whole cell lysate from striatal cultures expressing normal htt analyzed with BKP1 primary antibody. WT striatal cultures underwent exposure to staurosporine, NMDA or control salt solution. N=3. Protein samples loaded are as indicated underneath each blot. Molecular weight bands to the right of the blots are as follows: 250 kDa, 160 kDa, 105 kDa, 75 kDa, 50 kDa. *indicates that sample was not boiled before loading in SDS-PAGE gel.

A 72Q mAB2166 BKP1

stauro NMDA control   stauro NMDA control

full-length htt →

cleaved htt ⇒

10 µg 20 µg 10 µg 20 µg 10 µg 20 µg   10 µg 20 µg 10 µg 20 µg 10 µg 20 µg

B

WT mAB2166

stauro control NMDA

full-length htt →

cleaved htt ⇒

10 µg 17 µg 10 µg 17 µg   10 µg 20 µg

C

WT BKP1

stauro control NMDA

full-length htt →

cleaved htt ⇒

30 µg 60 µg 30 µg 60 µg 60 µg 30 µg 60 µg
*

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5.5 Summary

Our findings show that caspase-3 activation is enhanced in MSNs expressing mutant htt compared to controls 3 and 6 hrs after NMDAR stimulation (Zeron et al., 2002). We also demonstrate that by pretreating with a caspase-3-like antagonist, we could depress caspase-3 activation to control levels as well as eliminate NMDAR-induced excitotoxic cell death and apoptosis. This demonstrates that caspase-3 activation is required for NMDAR-mediated cell death in MSNs expressing mutant htt. These results correlate well with our previous findings (see Chapter 4) that there is also an observable enhancement in apoptosis in MSNs expressing mutant htt after exposure to NMDA. Furthermore, we were unable to determine whether elevated caspase-3 levels in MSNs expressing mutant htt after exposure to NMDA caused increased htt cleavage, by Western blot analysis using htt antibodies that recognize N-terminal fragments. Since caspase-3 is not the only protease that has been shown to act on htt (Kim et al., 2001, Gafni and Ellerby, 2002), we conclude that NMDAR-mediated enhancement of caspase-3 activity in MSNs expressing mutant htt plays a larger role in mediating proteolysis of substrates, other than htt, to induce cell death in our acute model.

Since we only examined caspase-3 activation in our model, we cannot say whether it is the only caspase that is activated after NMDAR stimulation in MSNs expressing mutant htt. Other upstream caspases and proteases, like calpains, that may be activated in the apoptotic pathway remain unexplored. Since inhibition with a caspase-3 like antagonist could inhibit most of the cell death resulting from NMDAR activation in MSNs expressing mutant htt, we can conclude that it appears that caspase-3 plays an important role as the final executioner of the caspase cascade to induce apoptosis. Mechanisms upstream of caspase-3 activation have yet to be delineated.

It is interesting that MSNs from early postnatal cultures display high levels of htt cleavage. This contrasts with the relatively low levels of basal cleavage in adult neurons [Kim et al., 2001; C.L. Wellington and M.R. Hayden (personal communication)]. This may reflect an enhanced activation of proteases in developing cells, resulting in cleavage of full-length htt, perhaps signifying an important function of cleaved htt during development. This remains an interesting area for further exploration. Importantly, the high levels of basal htt cleavage does not affect the outcome of our excitotoxicity experiments since the cell death in mutant htt-expressing MSNs was selective for NMDAR activation and not seen after AMPAR stimulation. It must be emphasized that many of our results regarding cleavage of htt in the cultured MSNs are preliminary and need to be repeated in order to discern the role that htt cleavage may play in our acute model.

The data presented here identify NMDAR activation as a trigger for enhanced caspase activation and cell death in striatal MSNs expressing mutant versus wild type htt. In this regard, a variety of studies suggest a strong role for caspase activation in neuronal degeneration in HD (Ona et al., 1999; Sanchez et al., 1999; Wellington and Hayden, 2000). Furthermore, minocycline, an inhibitor of caspase-1 and –3, delays death in the R6 mice (expressing N-terminal htt with an expanded polyQ) (Chen et al., 2000), and intracerebral infusion of caspase inhibitors or crossing R6/2 mice with a mutant mouse dominant negative for caspase-1 have both been reported to inhibit development of the R6 phenotype (Ona et al., 1999). Our results indicate that increased NMDA-induced caspase-3 activation and cell death, as well as increased NMDAR-mediated currents (Zeron et al., 2002; see Figure 2), occur in mutant htt-expressing MSNs as early as the first few weeks postnatal, strongly supporting a primary role for excessive NMDAR stimulation in MSN degeneration in HD.

CHAPTER 6

NMDAR-Dependent Response of Mitochondria in Medium Spiny Striatal Neurons

Expressing Mutant Htt

Early experiments in animal models using mitochondrial toxins injected systemically or intrastriatally displayed similar pathology to animal models injected intrastriatally with NMDAR agonists, and mitochodrial toxin effects could be blocked with MK-801 (Beal et al., 1993a; Greene et al., 1993). These experiments demonstrated that impaired energy metabolism may play a role in the degeneration of MSNs in HD and that mitochondria may be involved downstream of NMDAR activation, in secondary excitotoxicity. Impairment of mitochondrial function results in decreases in high-energy phosphate stores and a deterioration of membrane potential leading to the relief of the voltage-dependent Mg^{2+} block of NMDAR activation by glutamate and therefore increasing the total amount of NMDAR activity (reviewed by Vonsattel and DiFiglia, 1998). Another study showed that polyQ-induced cell death in primary rat neurons could be blocked by coexpression with Bcl-2 or Bcl-x_L also supporting a role for mitochondria in HD (Sanchez et al., 1999).

A.V. Panov and J.T. Greenamyre (personal communication) have recently shown that mitochondrial calcium homeostasis is compromised in HD. Lymphoblast mitochondria from HD patients are shown to have a lower membrane potential and depolarize at lower calcium loads than controls [A.V. Panov and J.T. Greenamyre (personal communication)] Similarly, brain mitochondria from the same type of mice we have used in our studies, the YAC transgenic HD mice (Hodgson et al., 1999), show a mitochondrial defect preceding the onset of known physiological, pathological or behavioral abnormalities [A.V. Panov and J.T. Greenamyre (personal communication)]. This suggests that mitochondrial calcium abnormalities occur early

in HD pathogenesis and may be a direct effect of mutant htt on the mitochondria [A.V. Panov and J.T. Greenamyre (personal communication)]. This event may work in concert with NMDAR overactivation in MSNs in HD, resulting in increased quantities of calcium intracellularly that originates from overstimulated NMDARs allowing enhanced calcium influx and dysfunctional mitochondria unable to sequester calcium normally. In addition, it has been shown that NMDAR activation can also influence mitochondrial function, causing mitochondrial membrane depolarization, and altered homeostasis of intracellular free calcium (reviewed by Budd et al., 2000). Also, studies have shown that dysfunctional mitochondria can cause caspase-3 activation (Li et al, 1997; Green and Reed, 1998; see Introduction 1.6.3.2). We have demonstrated enhanced caspase-3 activation in MSNs expressing mutant htt after exposure to NMDA (Zeron et al., 2002; see Chapter 5). Therefore, we wanted to determine whether MSNs cultured from YAC transgenic HD mice display mitochondrial dysfunction that may play a role in the downstream effects seen after NMDAR activation.

6.1 NMDAR-Mediated Enhancement of Intracellular Calcium Levels in MSNs Expressing Mutant Htt

Intracellular Ca^{2+} concentration is thought to be important in determining cell health, and neuronal survival is inversely correlated with total Ca^{2+} load (Dessi et al., 1994; Hartley et al., 1993; Eimerl and Schramm, 1994) by triggering downstream Ca^{2+}-dependent events that lead to cellular dysfunction and death. Evidence suggests that the bulk of the Ca^{2+} load is taken up by mitochondria. If mitochondrial Ca^{2+} concentration reaches a certain threshold, mitochondria can have difficulties sequestering it, organelle function fails and Ca^{2+} is released to the cytoplasm (Budd and Nicholls, 1996). Glutamate-induced excitotoxicity has been shown to trigger the rapid intracellular uptake of Ca^{2+}, mainly via NMDARs. It has been shown that mitochondrial

uptake of calcium after NMDAR stimulation is necessary to mediate excitotoxic cell death in cultured forebrain neurons (Stout et al., 1998). Thus the study of the effects of NMDAR-induced mitochondrial stress may prove to illuminate downstream triggers of cell death in HD.

Monitoring the cells' response to NMDA-dependent intracellular free Ca^{2+} changes with Fura-2, a ratiometric and high Ca^{2+} affinity fluorescent indicator (Grynkiewicz et al., 1985), can help elucidate the cell's vulnerability to death. Using the MSNs at 9 DIV, we loaded the cells at room temperature with Mag-Fura-2, a low-affinity free calcium indicator that has been shown to be more effective in measuring intracellular free Ca^{2+} concentrations in glutamate-stimulated forebrain neurons than Fura-2 (Stout and Reynolds, 1998; Cheng et al., 1999). Mag-Fura-2 can also measure Ca^{2+} bound in subcellular compartments (e.g. can be used to monitor IP_3-sensitive Ca^{2+} stores) (Kirkland and Frankline, 2001); it remains to be explored whether this would affect interpretation of our data. We compared the mean of the initial peak response (the initial, and usually rapid, increase in fluorescence 334/380 values that normally results in the first peak during the application to NMDA) to NMDA application (Figure 14A,B), in MSNs from YAC46 compared with WT mice. Our data showed, that with a 10 min exposure to 3 mM NMDA there was a trend towards a larger increase in intracellular free Ca^{2+} in MSNs expressing mutant htt (46Q) compared to WT MSNs (Figure 14A,B). As well, there appeared to be a trend towards a difference in basal intracellular calcium levels (Figure 14A) in MSNs expressing mutant htt (46Q). 72Q MSNs also showed these trends, but data are preliminary and not shown here. This may be indicative of a large amount of calcium entering the cell during NMDAR activation and/or failure of mitochondrial buffering or other Ca^{2+} buffering and transport mechanisms in HD. Other preliminary data (not shown here) demonstrated a trend towards an increase in the mean of the second peak response during application of NMDA in MSNs expressing mutant htt (46Q).

Our findings agree with data obtained examining acute slices from older YAC transgenic mice (symptomatic), finding NMDAR-dependent enhancement in current amplitude and intracellular calcium flux in MSNs expressing the expanded polyQ (72Q) (Cepeda et al., 2001a). Essential control experiments still to be performed are: 1) to calibrate the maximum ratio possible with Mag-Fura-2 in our system using an ionophore, such as A23187, in the presence of 2.3 mM $CaCl_2$; 2) add an uncoupler, such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), (to release Ca^{2+} stored within mitochondria) after recovery from application to NMDA to determine the efficiency of mitochondrial calcium sequestration. Also, the differences measured between groups appear to be large but are not statistically significant; more experiments must be conducted. To date, our results suggest that an NMDAR-induced imbalance of calcium homeostasis in MSNs expressing mutant htt may play a role in HD.

FIGURE 14. Calcium imaging with Mag-Fura 2 in primary cultured MSNs from YAC transgenic HD mice during NMDAR stimulation. (A) A representative trace of cells loaded with Mag-Fura 2 reveals enhanced intracellular calcium levels in MSNs expressing mutant htt (46Q) upon exposure to 3 mM NMDA compared to control MSNs (WT) as shown by increasing 334/380 (334 nm and 380 nm) ratiometric fluorescence values. (B) Mean initial peak response values of fluorescence (334/380) for intracellular calcium are higher in MSNs expressing mutant htt (46Q) with exposure to 3 mM NMDA compared to control MSNs (WT). $P=0.07$, using unpaired t test. N=3 experiments conducted with different batches of WT MSNs with 68 responding MSNs in total. N=5 experiments conducted with different batches of 46Q expressing MSNs with 108 responding MSNs in total. Bars represent mean $\pm$ S.E.M.
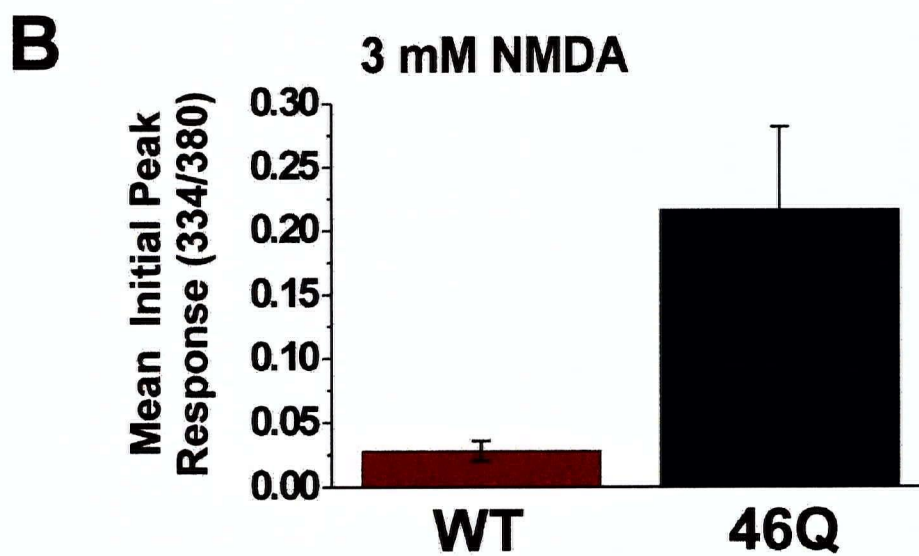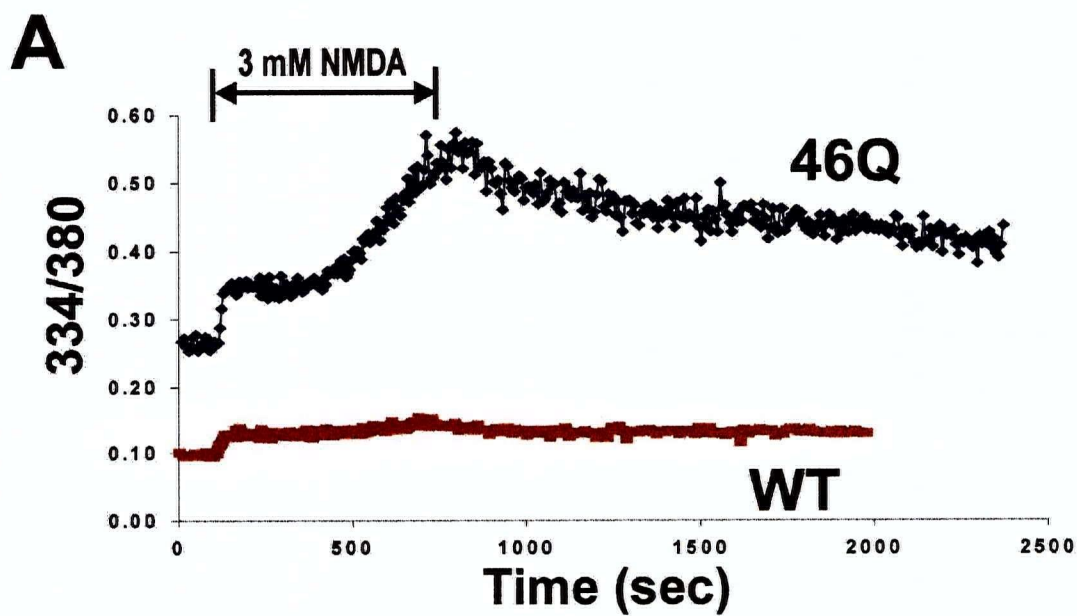
6.2 NMDAR-Mediated Enhancement of Mitochondrial Membrane Depolarization in MSNs Expressing Mutant Htt

The mitochondrial membrane potential (MMP) is also important in predicting cell health and recovery from a cell stress (reviewed by Nicholls and Ward, 2000). If cellular stress causes the mitochondrial membrane to become greatly depolarized or prevents recovery from depolarization, then the ability of the mitochondria to function normally--i.e. produce ATP and sequester calcium--is inhibited. Apoptotic or necrotic death following glutamate exposure has been associated with recovery or sustained collapse, respectively, of the MMP (Ankarcrona et al, 1995; Bonfoco et al., 1995; Ayata et al., 1997; Larm et al., 1997). The dysfunction of the mitochondria can lead to cellular dysfunction and cell death. Therefore, we wanted to determine whether the MMP was altered in MSNs expressing mutant htt after exposure to NMDA.

MSNs at 9 DIV were loaded for 15 min at room temperature with rhodamine-123 (rho-123) in BSS. The indicator concentrates within the mitochondria (due to the pH gradient relative to the cytoplasm) while the MMP is intact. When the mitochondrial membrane is depolarized, rho-123 diffuses to the cytoplasm causing a large increase in fluorescence (reviewed by Nicholls and Budd, 2000). Cells were loaded at room temperature and exposed for 10 min to NMDA in BSS.

We compared the mean of the peak response during NMDA application (Figure 15A,B), in MSNs from YAC46 compared with WT mice. Our data showed that NMDAR activation with 3 mM NMDA for 10 min the mitochondrial membrane depolarization was significantly larger for MSNs expressing mutant htt (46Q) (72Q MSNs also showed this trend but data are preliminary and not shown here) compared to control MSNs (Figure 15A,B) as shown with increased fluorescence (delta F/F) in rho-123 dye. There was no significant loss of recovery of

mitochondrial membrane depolarization in most of the MSNs analyzed (data not shown). This supports the idea that apoptosis would be more likely to occur, since findings indicate that mild excitotoxic insults lead to recoverable mitochondrial membrane depolarization and reversible energy compromise causing ATP to be temporarily available to stimulate the apoptotic pathway (Ankarcrona et al., 1995; Bonfoco et al., 1995; Ayata et al., 1997; Larm et al., 1997). Appropriate controls utilizing an uncoupler to cause maximal depolarization of the mitochondrial membrane potential and provide calibration for rho-123 are essential to correlate mitochondrial membrane depolarization with calcium loading.

A.V. Panov and J.T. Greenamyre (personal communication) have shown that mitochondria from lymphocytes from HD patients and from brain of YAC transgenic mice (the same that we use for our studies) have depolarized mitochondria membrane potentials, agreeing with our findings. They suggest that mitochondrial dysfunction results in calcium loading abnormalities seen in HD patient lymphocyte mitochondria and the YAC transgenic mice brain mitochondria. Our data indicate enhanced intracellular calcium levels may in part reflect increased levels of NMDAR activity. However, the magnitude of the effect on intracellular free calcium is much larger than the increase in NMDAR current that we found previously (Zeron et al., 2002), suggesting intracellular calcium buffering and homeostatic mechanisms may also be altered by mutant htt. Therefore, NMDARs may work in concert with dysfunctional mitochondrial calcium buffering ability, leading to enhanced intracellular free calcium levels that could activate downstream proteases and cell death effectors in MSNs expressing mutant htt. Taken together, our data support the idea that overactivation of NMDARs may cause enhanced mitochondrial membrane depolarization via enhanced calcium overload and may work in concert with already dysfunctional mitochondria, leading to enhanced vulnerability to apoptotic cell death in MSNs expressing mutant htt.

FIGURE 15. Cellular imaging of changes in rhodamine-123 fluorescence in primary cultured MSNs from YAC transgenic HD mice during NMDAR stimulation. (A) A representative trace of cells loaded with rho-123 reveals enhanced mitochondrial membrane depolarization in MSNs expressing mutant htt (46Q) upon exposure to 3 mM NMDA compared to control MSNs (WT) as shown by increasing Delta F/F fluorescent values. (B) Mean peak response values of fluorescence (Delta F/F) for mitochondrial membrane potential are significantly higher in MSNs expressing mutant htt (46Q) during exposure to 3 mM NMDA compared to control MSNs (WT). ***$P<0.001$, using unpaired t test. N=4 experiments conducted with different batches of WT MSNs with 150 responding MSNs in total. N=10 experiments conducted with different batches of 46Q expressing MSNs with 373 responding MSNs in total. Bars represent mean $\pm$ S.E.M.
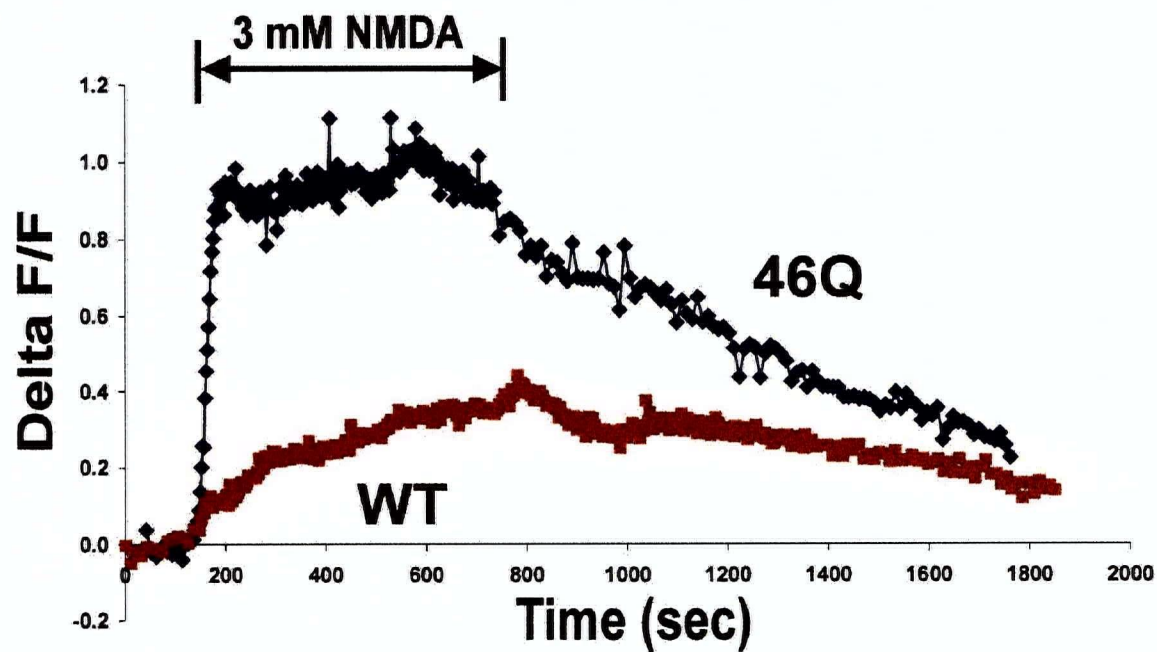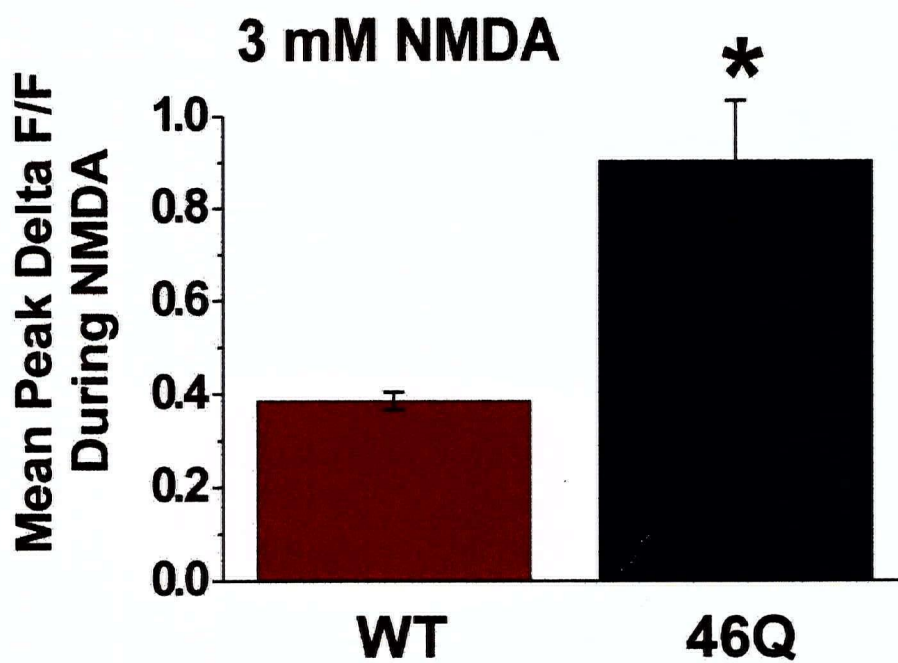
6.3 Enhanced Inhibition by Cyclosporin A of NMDAR-Mediated Excitotoxicity in MSNs from Transgenic Mice

Since our data suggest that an NMDAR-dependent increase in intracellular calcium and mitochondrial membrane depolarization in MSNs expressing mutant htt may play a role in the pathogenesis of HD, we wanted to determine if we could rescue the MSNs from excitotoxic cell death by inhibiting the mitochondria from exerting apoptogenic effects. We elected to use 1 μM cyclosporin A, which is proposed to inhibit the opening of the PTP [A.V. Panov and J.T. Greenamyre (personal communication); Nicholls and Budd, 2000]. Presumably, cyclosporin A addition to our cultures would decrease the protein permeability of the mitochondrial outer membrane and the subsequent release of factors such as cytochrome c, which have been shown to activate caspase-3 (Li et al., 1997; Green and Reed, 1998).

Our findings showed that cultures that underwent exposure to cyclosporin A before, during and after stimulation with 3 mM NMDA and examined for cell death using trypan blue inclusion or TUNEL assays 24 hr later showed only a partial inhibition--but significantly different from the agonist alone condition--of NMDAR-dependent cell death in MSNs expressing normal (WT) or mutant (46Q) htt (Figure 16). The percent inhibition was greater for those MSNs expressing mutant htt (~53 % decrease from agonist condition obtained by the trypan blue inclusion assay and a ~60 % decrease from the agonist condition obtained by the TUNEL assay) compared to the percent inhibition observed for MSNs expressing normal htt (~34 % decrease from agonist condition obtained by the trypan blue inclusion assay and a ~25 % decrease from the agonist condition obtained by the TUNEL assay). Also, the amount of cell death for MSNs expressing normal or mutant htt after exposure to NMDA with cyclosporin A was not significantly different (P>0.64 for WT vs 46Q with inhibitor obtained by the trypan blue inclusion assay; P>0.11 for

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WT vs 46Q with inhibitor obtained by the TUNEL assay). As found previously, 46Q-expressing MSNs after agonist condition had significantly different enhancement of cell death compared to WT-expressing MSNs exposed to agonist conditions ($P<0.05$ obtained by trypan blue inclusion assay and $P<0.001$ obtained by TUNEL assay). Our data support the idea that increased mitochondria membrane permeability may play a role in the cell death of MSNs exposed to NMDA, and a larger role in the death of mutant htt-expressing MSNs. Our data suggest that inhibition of the PTP after NMDAR stimulation may eliminate the difference in cell death between normal and mutant htt-expressing MSNs.

Our findings also correlate with results showing that after cyclosporin A addition to brain mitochondria from YAC transgenic HD mice, mitochondria showed increased calcium retention capacity but still had calcium handling defects even in the presence of PTP inhibitors [A.V. Panov and J.T. Greenamyre (personal communication)], suggesting that cyclosporin A only partially prevents mitochondrial dysfunction. Furthermore, our results suggest that apoptotic cell death pathways, other than those resulting from dysfunctional mitochondria, such as calpain activation of caspase-3, may be involved in NMDAR-mediated cell death in MSNs expressing mutant htt. Cyclosporin A has been shown to inhibit calcium-calmodulin-dependent protein phosphatase 2B, or calcineurin, (Jovanovic et al., 2001; Miyata et al., 2001) which could affect the results seen here; therefore, further tests need to be performed with other PTP inhibitors that act independently of calcineurin, such as bongkrekic acid (blocks the ANT believed to play a role in the PTP; Cao et al., 2001; reviewed by Fiore et al., 1998) to confirm our results. Furthermore, it has been suggested that cyclosporin A may also act as a protein synthesis inhibitor (Kirkland and Franklin, 2001), which could contribute to the anti-apoptotic effects seen in our system. In all, we can say that NMDAR-mediated cell death in MSNs expressing mutant htt can be partially inhibited by cyclosporin A, which supports a role for dysfunctional mitochondria in the pathogenesis of HD.

FIGURE 16. Partial protection of NMDAR-mediated cell death in primary cultured MSNs expressing normal or mutant htt with addition of cyclosporin A. MSNs that expressed normal (WT) or (46Q) mutant htt underwent exposure to 3 mM NMDA (A) with or without addition of 1 μM cyclosporin A (I), before, during and after the 10 min exposure to NMDA. Excitotoxic cell death was analyzed 24 hr later using the trypan blue inclusion assay, shown in the top diagram, and the TUNEL assay, shown in the bottom diagram. *$P<0.01$ vs agonist alone within same genotype, using an unpaired t test. ***$P<0.001$ vs agonist alone within same genotype, using an unpaired t test. N=2-3 different batches of cultured neurons for each treatment.
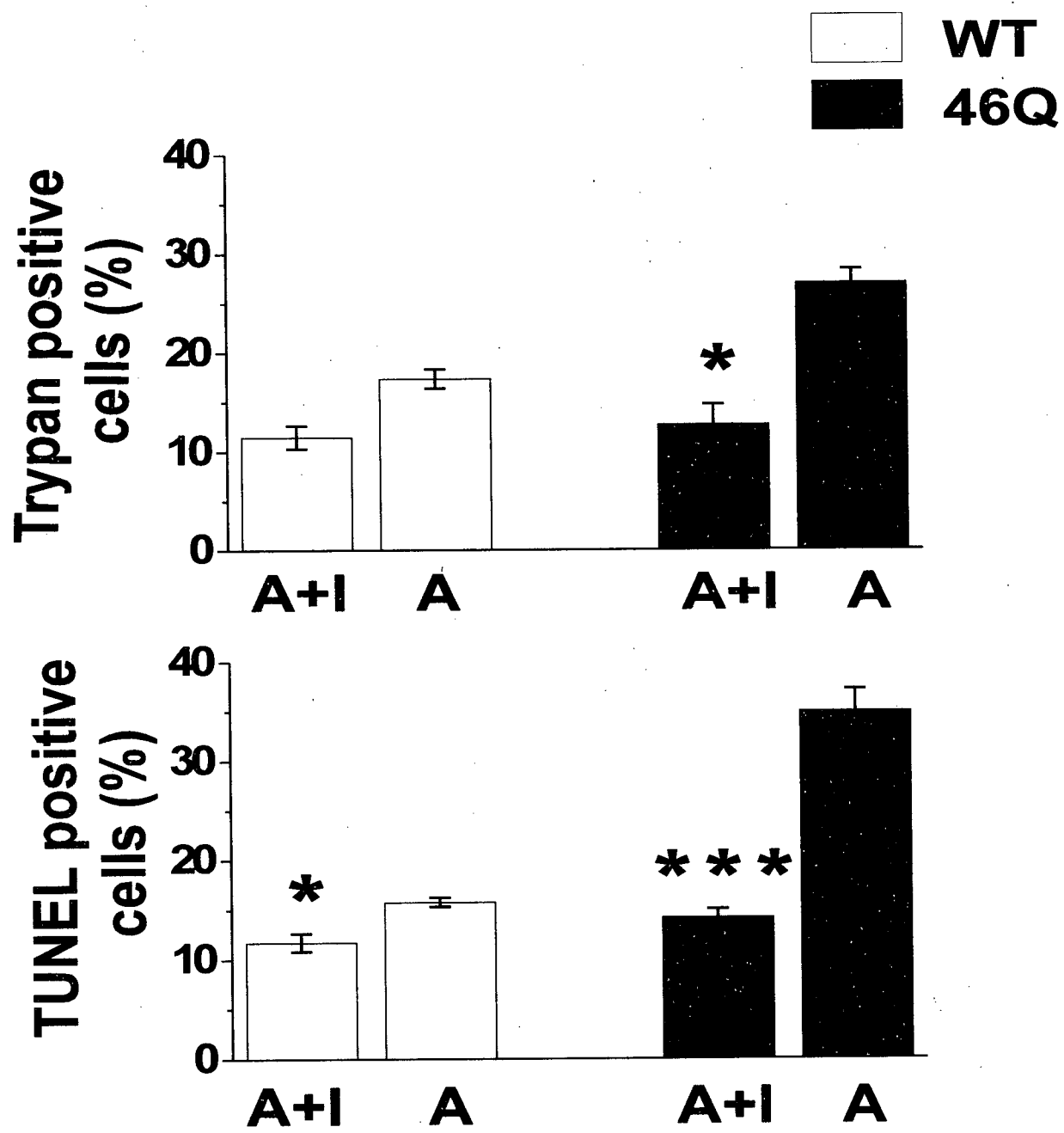
6.4 Summary

Our findings suggest there may be NMDAR-dependent enhancement of intracellular free calcium levels, mitochondrial membrane depolarization and permeabilization in MSNs expressing mutant htt compared to controls. Excitotoxicity could be inhibited by addition of a PTP antagonist, cyclosporin A, almost twice as effectively in MSNs expressing mutant htt than MSNs expressing normal htt. Our findings, suggest a role for NMDAR-stimulated mitochondrial dysfunction in the pathogenesis of HD. It is interesting that increased mitochondrial permeability plays a larger role in the death of mutant htt-expressing MSNs, suggesting that inhibition of the PTP after NMDAR stimulation may eliminate the selective difference in cell death between mutant and normal htt-expressing MSNs. Further studies need to be done to determine the extent of the contribution of the mitochondrial dysfunction to the caspase activation cascade in the pathogenesis of HD, and whether free radical production by dysfunctional mitochondria contribute to excitotoxic cell death in HD. Results indicate that mitochondria from both neuronal and non-neuronal sources expressing mutant htt display enhanced dysfunction compared to control mitochondria [A.V. Panov and J.T. Greenamyre (personal communication)]. Therefore, one should test whether cultured CGNs, which failed to show enhanced NMDAR-induced cell death when expressing mutant htt, also display increased NMDAR-induced mitochondrial dysfunction.

The enhancement in NMDAR-dependent intracellular free calcium levels has to be examined further to determine which factors contribute, such as: 1) an increase in calcium influx through the receptors; 2) an inability for mitochondria to adequately buffer calcium; 3) an alteration in calcium efflux routes through the calcium/ATPase pump or sodium/calcium exchangers; 4) altered ability of additional intracellular calcium storage organelles, such as the endoplasmic reticulum; 5) altered calcium buffering ability of calcium binding proteins, such as

calbindin. Also of interest, is whether the enhanced intracellular calcium levels alone are enough to replicate the increase in excitotoxicity or whether a special relationship, spatial and/or temporal, exists between the NR1A/NR2B NMDARs and mitochondria and the mutant htt protein in MSNs. This could be tested using a calcium ionophore or inducing calcium influx via voltage-gated calcium channels with application of high KCl.

In all, our findings indicate that NMDAR-dependent mitochondrial dysfunction may play a role in HD pathogenesis, but the extent of the mitochondria functional changes are still to be explored.

CHAPTER 7

General Discussion

For the first time, we show mutant htt-enhanced NMDAR-dependent cellular dysfunction, excitotoxicity, and apoptosis, that is polyQ-length dependent, in MSNs *in vitro*. Our data directly support a role for NMDAR overactivation in the pathogenesis in HD. Previously, our laboratory has demonstrated enhanced NMDAR-mediated current amplitude in cells co-expressing mutant htt in cell lines and acutely dissociated MSNs (Chen et al., 1999b; see Figure 1; Zeron et al., 2002). This correlates with the present findings demonstrating enhanced NMDAR-mediated intracellular free calcium levels, excitotoxicity and apoptosis in MSNs expressing mutant htt. These observations were specific to NMDAR activation and neurons affected in HD—as there was no difference between YAC72 and WT mice in cerebellar granule neurons or after AMPAR stimulation in MSNs. The enhanced current amplitude, excitotoxicity and apoptosis appear to be largely NR1A/NR2B NMDAR subtype-selective. Furthermore, early after NMDAR stimulation caspase-3 activity is elevated in MSNs expressing mutant htt, with a polyQ-length-dependent trend. There is no detectable NMDAR-dependent enhancement in caspase-3-related htt cleavage products in MSNs expressing htt, suggesting that htt cleavage is not necessary for enhanced cell death in our acute model of excitotoxicity, but whether it plays a role in the pathogenesis of HD remains to be determined. Moreover, NMDAR-dependent mitochondrial dysfunction may be involved in cell death in MSNs expressing mutant htt, as seen by an increase in recoverable mitochondrial membrane depolarization, and partial rescue of excitotoxicity and apoptosis by a permeability transition pore inhibitor. These findings are outlined in a model shown in Figure 17. Figure 17 describes some plausible and demonstrated

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downstream effects that may be consequences of NMDAR overstimulation in MSNs expressing mutant htt.

We have demonstrated that mutant htt increases sensitivity of striatal MSNs to excitotoxic cell death induced by NMDAR activation in a transgenic mouse model of HD, addressing a long-standing question regarding the role of excitotoxicity in the pathogenesis of HD. One potential mechanism underlying this enhanced susceptibility of MSNs to NMDA is the increase in NMDAR current we observed in MSNs expressing mutant htt (Zeron et al., 2002). YAC72 neurons would be expected to be as healthy as wild type neurons in the young (6-11 week old) mice used for these studies (Zeron et al., 2002), since neuronal degeneration does not occur in this line until >6 months of age (Hodgson et al., 1999). Therefore, our results are unlikely to be caused by secondary effects of cell stress due to other pathways. In the R6/2 mice, reduced membrane area and truncated dendritic trees and spines have been observed for medium-sized spiny striatal neurons (Levine et al. 1999; Laforet et al., 2001). In contrast, we found no significant difference in mean cell capacitance measured for wild type versus YAC72 neurons, indicating that the total membrane area recorded from was equivalent (Zeron et al., 2002), and we observed that the dendritic trees remaining after acute dissociation appeared similar for the two groups. Moreover, we did not observe any morphological differences in primary cultured MSNs expressing normal or mutant htt (see Chapter 4). This indicates that in MSNs expressing mutant htt, the enhanced NMDAR-evoked current amplitude in 6-11 week old mice (Zeron et al., 2002) or enhanced NMDAR-evoked excitotoxicity, apoptosis and cellular dysfunction in primary cultured MSNs, are probably not due to some prior mutant htt-dependent cell stress causing enhanced vulnerability to NMDAR activation. This is further verified by the inability of AMPAR stimulation to produce enhanced excitotoxic effects in mutant htt-expressing MSNs (see Chapter 4).

In previous studies, electrophysiological recordings from MSNs in acute striatal slices have suggested only a very minor role for NMDARs in routine cortico-striatal synaptic activity, due to the hyperpolarized resting membrane potential measured for these neurons (Calabresi et al., 1987) and voltage-dependent Mg^{2+} block of NMDARs (Hollmann and Heinemann, 1994). On the other hand, measurements made *in vivo* indicate that MSNs show spontaneous depolarizations of membrane potential, and that the role of NMDARs in cortico-striatal MSN synaptic transmission may be substantial during such times (Cepeda and Levine, 1998; Calabresi et al., 2000). Therefore, it is plausible that an increase in NMDAR-mediated current in response to synaptic stimulation, resulting in increased intracellular calcium load, may chronically stress MSNs expressing mutant htt (Figure 17). The increase in cell stress resulting from the mutant htt-induced increase in NMDAR activity would be one candidate mechanism for contributing to the "mutant steady-state" in human HD (Clark et al., 1997). As well, this increased stress from the earliest ages could be consistent with late onset neurodegeneration, or 'cumulative-damage hypothesis', since the aging process is associated with a progressive decrease in the ability of cells to compensate for oxidative stress (reviewed by Beal, 1992).

It has been proposed that presynaptic mechanisms, such as aberrant activity and/or increased release of glutamate from cortical afferents, might contribute to degeneration of striatal neurons in mice expressing mutant htt (Gutekunst et al., 1999; Li et al., 2000). Recent data indicate mitochondrial dysfunction in YAC72 mice [A.V. Panov and J.T. Greenamyre (personal communication)], and MSNs from another model of HD, the R6 mouse, tend to have depolarized resting membrane potentials (Levine et al., 1999), which would facilitate activation of NMDARs. As well, previous studies in late-stage symptomatic mice expressing N-terminal fragments of mutant htt, and in older (1-2 years) YAC72 mice, have also shown a trend toward increased NMDAR currents recorded from striatal neurons (Cepeda et al., 2001a; Laforet et al., 2001). Our data strongly support a role for mutant htt in enhancing responsiveness of

postsynaptic NMDARs in MSNs, even in very young, presymptomatic animals; this mechanism could act synergistically with presynaptic processes, mitochondrial dysfunction and postsynaptic membrane depolarization to facilitate degeneration of MSNs.

Figure 17. Diagram of possible downstream effects of NMDAR activation in MSNs expressing mutant htt. In this schematic, we propose that mutant htt indirectly interacts with NMDARs through cytoskeletal-associated proteins. PSD-95 interactions with htt (Sun et al., 2001), or interactions between α-actinin and HIP-1 (C. Icton and L. Raymond, unpublished data), are potential links between NMDARs and mutant htt. Cytoskeletal-associated proteins and actin have been shown to modulate NMDAR function (see review by McBain and Mayer, 1994; Sattler and Tymianski, 2000); thus, interactions between htt and such proteins may explain how NMDARs could be overactivated with expression of mutant htt (Chen et al., 1999b; Hodgson et al., 1999; Levine et al., 1999; Zeron et al., 2001, 2002). Possible consequences of enhanced calcium load through overactivated NMDARs include mitochondrial dysfunction, with subsequent mitochondrial membrane depolarization, decreased calcium sequestration, increased free radical production, and activation of the permeability transition pore (reviewed by Nicholls and Budd, 2000). These events can result in protease activation leading to apoptosis (reviewed by Nicholls and Budd, 2000). Calcium itself can function to activate proteases, as well as many other calcium-dependent enzymes (for review see Sattler and Tymianski, 2000). Caspase-3 activation appears to be important for NMDAR-mediated cell death in our system. As well, caspases have been shown (Wellington et al., 2000) to promote cell death by cleaving full-length huntingtin into smaller N-terminal fragments that can be toxic when translocated to the nucleus (Hackam et al., 1998; Lunkes and Mandel, 1998). Cleavage of full-length huntingtin by caspase-3 does not appear to play a role in MSNs after exposure to NMDA in our acute excitotoxicity model. In all, NMDAR activation may be an upstream trigger in the pathogenesis of HD.
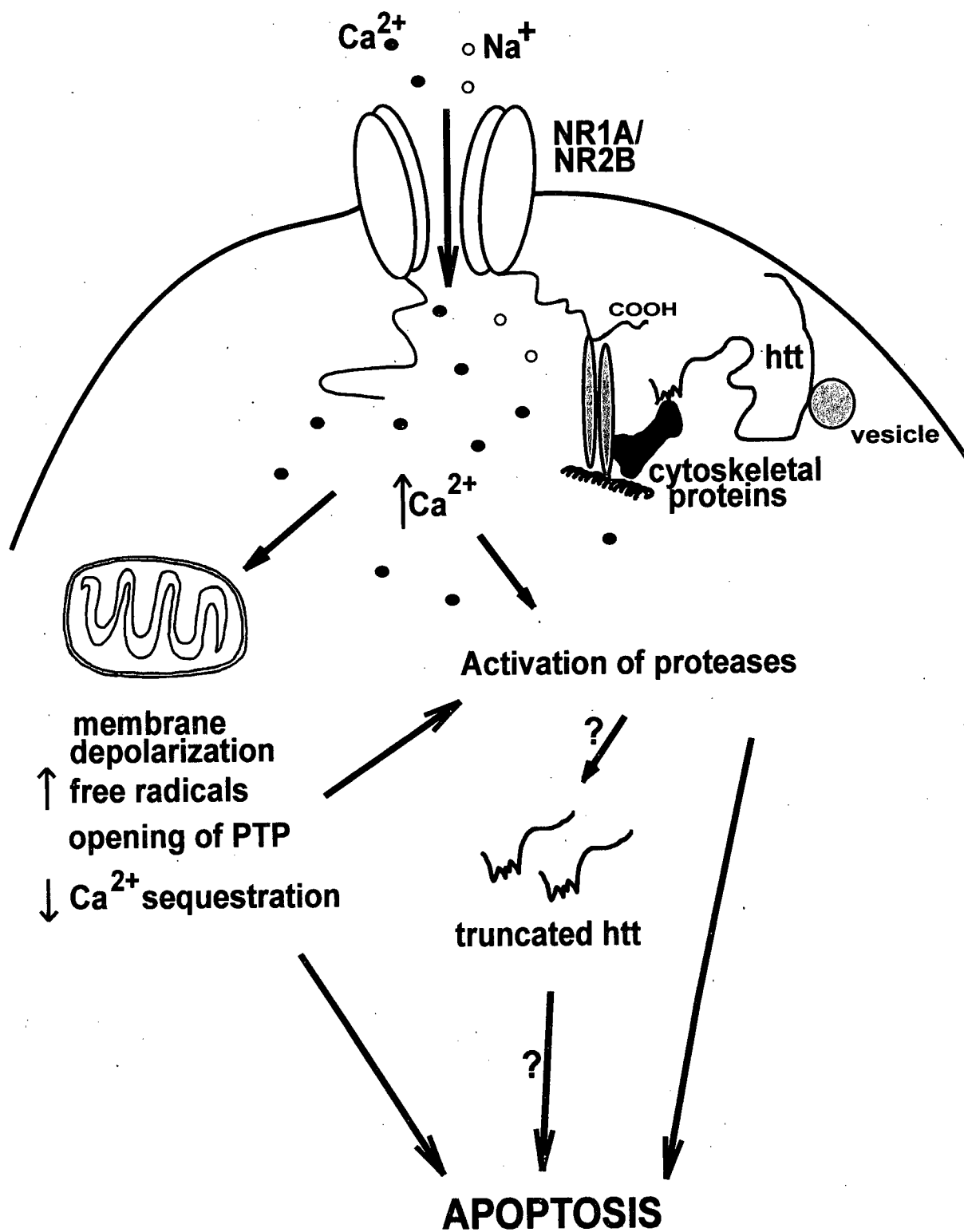
7.1 Potential Molecular Interactions Between NMDARs and Huntingtin

Further data is necessary to determine whether our results (see Figure 9) suggest that mutant htt effects little, if any, change in the EC_{50} for NMDA-induced cell death but a robust increase in maximal toxicity. These results would then parallel studies of modulation of glutamate receptor currents by protein kinases, which also induce a large potentiation of the maximal current response with little or no change in EC_{50} (Wang et al., 1993; 1994). The mechanism of NMDAR current potentiation by mutant htt has not been addressed in the present studies; however, preliminary results suggest that expression levels of NR1 and NR2B are not increased in striatal tissue from 6-12 week old YAC46 or YAC72 mice compared with wild type (C. Icton and L. Raymond, unpublished data). On the other hand, recent data indicate that htt may associate with NMDARs within a larger complex, via direct interaction with postsynaptic density protein-95 (PSD-95; Sun et al., 2001) or through interactions with huntingtin-interacting protein 1 (HIP-1) and α-actinin (C. Icton and L. Raymond., unpublished data). This is represented in our model (Figure 17) as hypothetical cytoskeletal proteins that may link mutant htt with NMDARs, thereby altering NMDAR function.

As previously mentioned, Sun and associates (2001) have examined PSD-95, a NMDAR interacting cytoskeletal protein, and suggest a potential role in HD. They showed that normal full-length htt is associated with NMDARs and KARs via PSD-95, the SH3 domain of PSD-95 mediates binding to htt, and an expanded polyQ interferes with htt binding to PSD-95 (Sun et al., 2001). They hypothesized that as polyQ length increases, htt's direct interaction with PSD-95 decreases, allowing more PSD-95 to be free to interact with the C-terminus of NMDARs, leading to enhanced clustering of the NMDARs at the synapse, thereby increasing the total number of receptors receiving transmitter. This could fit in our model shown in Figure 17 as one possible way interaction with cytoskeletal proteins could alter NMDAR function: efficacy of glutamate

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transmission could be increased by potentiating postsynaptic current without altering the total number of NMDARs at the surface. Other candidate proteins, yet unexplored, are other members of the PSD-95 family and calmodulin, which has been shown to interact with htt within a large complex in the presence of calcium (Bao et al., 1996).

The interaction of actin and NMDAR is thought to be indirect through several bridging proteins, including α-actinin (Allison et al., 1998, Wyszynski et al., 1998; Dunah et al., 2000). Alpha-actinin is part of the spectrin superfamily of actin-binding and crosslinking proteins (Chan et al., 1998; Viel, 1999) and α-actinin subtypes 1 and 2 have been shown to directly bind to the C-terminus of NR1 and NR2B subunits *in vitro* (Wyszynski et al., 1998; Vallenius et al., 2000). Since both actin and α-actinin interact, and have been shown to influence NMDAR function and interact with NMDARs, then htt could influence this interaction in a polyQ length-dependent manner if it were found that any htt interacting proteins directly or indirectly interacted with NMDARs or any NMDAR-associated cytoskeletal proteins, such as α-actinin or actin. Increasing polyQ length of htt could cause an altered cytoskeletal protein interaction with NMDARs, leading to changes in NMDAR function or clustering. Such changes might enhance NMDAR function, causing overactivation of the receptor and enhanced calcium influx. In this regard, our laboratory has found an interaction between HIP-1 and α-actinin in cell lines (C. Icton and L. Raymond, unpublished data) using coimmunoprecipitation. Possibly, binding occurs through the talin-like domain found on HIP-1 and the spectrin-like domain found on α-actinin. In this interaction, we would hypothesize that with increasing polyQ length of htt there is less interaction with HIP-1, perhaps allowing more HIP-1 to interact with α-actinin, leading to an enhanced stabilization or clustering of the NMDARs, resulting in enhanced NMDAR function. Further experiments are necessary to confirm this interaction in MSNs and to explore the possibility of additional binding partners. This interaction represents a candidate pathway

linking htt and NMDARs within a larger complex, which may facilitate the overstimulation of NMDARs by mutant htt, which could also fit into our model shown in Figure 17.

Interestingly, recent data show that HIP-1 can interact with a protein named Hippi in the presence of htt containing an expanded polyQ (Gervais et al., 2002). They interact through unique death effector domains (DED) forming pro-apoptotic Hippi-HIP-1 heterodimers recruiting procaspase-8 into the complex and launching apoptosis through a caspase-dependent pathway (Gervais et al., 2002), capable of activating downstream caspase-3 (Nicholson, 1999). Taken together, these data indicate that HIP-1 may have dual roles in the augmentation of cell death in MSNs in HD: one, by augmenting NMDAR function and stimulation of mitochondrial dysfunction and caspase-3-dependent cell death, and two, by stimulating caspase-8 activation initiating an additional pathway activating caspase-3. In this case, our model proposed in Figure 17 would have to be altered to accommodate this alternative caspase cascade leading to apoptosis.

In all, the exact mechanisms by which mutant htt may alter NMDAR function have yet to be delineated. It is possible that mutant htt increases NMDAR current and sensitivity to NMDAR-mediated excitotoxicity by interacting with these cytoskeletal-associated proteins (Figure 17), which are known to modulate NMDAR channel function and/or subcellular localization (Scannevin and Huganir, 2000).

7.2 Huntingtin Cleavage

As we showed indirectly in Section 5.4 enhanced NMDAR-induced caspase-3 activation in mutant htt-expressing striatal neurons did not result in increased htt cleavage. Regardless of genotype and of type of stimulus, cultured MSNs showed little full-length htt protein and high levels of htt cleavage products. In spite of high levels of cleaved htt in striatal cultures from wild

type as well as YAC transgenic mice, there was no increase in baseline neuronal death or cell death in response to stressors other than NMDAR activation in the cultures expressing the mutant htt fragments. Our findings appear to oppose a model in which htt cleavage can lead to increased vulnerability to cell death (Cooper et al., 1998; Hackam et al., 1998; Li et al., 1999; Martindale et al., 1998). Therefore a role for htt cleavage should not be considered to play a part in our model (Figure 17).

In spite of this, we were concerned as to the source of the high basal htt cleavage levels since there is a larger proportion of full-length htt found *in vivo* in adult HD brains and YAC transgenic mice [Kim et al., 2001, C.L Wellington and M.R. Hayden (personal communication)]. Preliminary data (not shown in this dissertation) showed that lysates of striatal neuronal cultures collected at earlier days *in vitro* still showed high levels of htt cleavage. Moreover, in preliminary experiments, lysates from cultured embryonic rat hippocampal neurons, as well as cultured human embryonic kidney cells transfected with wild-type htt, also showed high basal levels of htt cleavage. Therefore, we wondered whether cleavage of htt was a characteristic of cells in culture, or if htt cleavage was a feature of embryonic and/or early postnatal cells. To distinguish these two possibilities, in preliminary studies we compared htt cleavage *in vivo* in homogenized striatal tissue from postnatal day 0 (P0) mice and 4-month old mice. Homogenized striatal tissue from P0 WT or 46Q or 72Q mice showed high basal htt cleavage relative to the full-length htt protein — although the cleaved htt product was larger (~100 kDa) — even before MSNs underwent the culturing technique. On the other hand, the P0 striatal tissue displayed greater full-length htt protein levels than those seen in cultured MSNs. The striatal tissue from 4-month old wild-type mice appeared to have more full-length htt product relative to cleaved htt product. These preliminary results suggested that the low ratio of full-length to cleaved htt in early postnatal mouse neurons may be due to a high level of protease activity associated with developmental processes. It also appeared there may be an increased level of protease activity

during the culturing process, causing an increased amount of cleavage of the full-length htt protein in cultured cells. In all, we have observed high basal levels of htt cleavage products and little difference in the extent of cleavage in cultured murine MSNs across different genotypes or treatment conditions by Western blot analysis using multiple N-terminal htt antibodies.

Because the cleavage sites for many of the proteases that cleave htt are tightly clustered and some are indistinguishable (i.e., caspase-3 and caspase-2), it may not be possible to use htt as the sole marker to detect a shift from one proteolytic pathway to another upon NMDAR activation using currently available antibodies. Recent studies [Kim et al., 2001; C.L. Wellington and M.R. Hayden (personal communication)] show constitutive cleavage of htt at amino acid 552 in brains of normal controls and asymptomatic HD gene carriers, supporting the idea that cleavage of htt cannot be used as a simple marker for caspase-3 activation and imminent cell death. Rather, our results suggest that the mutant htt-induced increase in caspase-3 activation following NMDA treatment in our model of acute excitotoxicity may result in cleavage of many additional substrates that together lead to death of MSNs, consistent with the accepted role for caspase-3 as a final executioner of apoptotic death in the CNS. Further tests are needed to determine whether caspase-3 or other proteases, such as calpains, play a definitive role in the cleavage of htt and/or the toxicity of htt fragments.

7.3 An Oversimplified Pathway

GABAergic MSNs are a somewhat unique population of neurons since they possess many special types of receptors (reviewed by Greengard et al., 1999), such as dopamine receptors, glutamate receptors (AMPA, kainate, metabotropic glutamate receptors, NMDARs), adenosine (A_{2A}) receptors, opiate receptors, as well as receptors for neuropeptides, to name but a few. These other receptors have also been shown to influence the activity of MSNs (reviewed by Greengard et al., 1999), so we can not rule out the possibility that NMDARs do not work alone

to trigger the degeneration of MSNs but may be working synergistically or in parallel through other receptors, such as the dopamine-DARPP-32 pathway described by Greengard et al., 1999, to enhance the striatal excitotoxic vulnerability. Previously, a positive interaction between NMDARs and group I mGluRs, via PKC interaction, had been shown in MSNs and not striatal interneurons, suggesting that this may partially contribute to the selective vulnerability of MSNs (Calabresi et al., 1999). Although the mechanisms of dopamine-mediated striatal toxicity *in vivo* are unclear, these data suggest there may be an interaction of NMDARs and dopamine receptors in the pathogenesis of HD (Peterson et al., 2001). Our excitotoxicity model in cultured MSNs only examined the role of the NMDARs may be playing in HD pathogenesis as shown in Figure 17. Examination of how other receptors, such as mGluRs or dopaminergic receptors, may be acting to modulate the activity of NMDARs may prove to be vital to the complete understanding of the selective degeneration of MSNs. We have also ruled out that AMPARs may play a role in the pathogenesis of HD. Further testing is needed to discern whether other pathways could be modulating NR1/NR2B-NMDAR-selective striatal degeneration in HD.

7.4 Increased NR2B-Subtype NMDAR Activity May Explain Selective Neuronal Degeneration

Our results shed light on the issue of how a family of autosomal dominant hereditary neurodegenerative disorders, each caused by a (different) gene encoding an expanded polyglutamine repeat within a protein of wide CNS distribution, may show selective neuronal vulnerability in distinct brain regions (for review see Tobin and Signer, 2000). We propose that the upstream events triggering the probable final common pathway in these diseases may be unique for each disease and related to interactions between the mutant protein and other proteins that are selectively expressed in vulnerable neuronal populations. By using the YAC transgenic

mouse model of HD, in which selective neuronal degeneration so closely parallels that found in the human disease (Hodgson et al., 1999), we have been able to address this issue.

Within the striatum, the vulnerable MSNs display important differences in NMDAR subunit composition and electrophysiological characteristics compared to the population of aspiny cholinergic neurons, which are relatively resistant to degeneration in HD. MSNs express mainly NR1A and NR2B, a receptor complex previously shown to be modulated by mutant htt, whereas cholinergic interneurons express mainly NR2D with NR1 (Landwehrmeyer et al., 1995; Chen et al., 1999; Kuppenbender et al., 1999). As well, MSNs respond with markedly larger amplitude membrane depolarization to exogenous application of AMPA, kainate, and NMDA receptor agonists compared with striatal cholinergic neurons; this increased responsiveness may be one mechanism for increased MSN vulnerability to cell death in ischemia or neurodegenerative diseases (reviewed by Calabresi et al., 2000; Cepeda et al., 2001b).

It is interesting that the regional variation in severity of neuronal degeneration in HD – striatum > cortex >> cerebellum and brainstem (Vonsattel and DiFiglia 1998) – correlates well with the relative expression levels of NR2B compared with other NR2 subunits (Hollmann and Heinemann, 1994; Monyer et al., 1994; Portera-Cailliau et al., 1996; Thompson et al., 2000). It is noteworthy that the medial striatum degenerates first in HD, progressing laterally (Vonsattel et al., 1985). This correlates with the predominant expression of NR1/NR2B-NMDARs medially, in contrast to the lateral striatum, which is thought to express trimeric combinations of NR1/NR2B/NR2A subunits (Buller et al., 1994; Christie et al., 2000; Watanabe et al., 1993). On the other hand, cortical pyramidal layers III, V and VI are most affected in HD (Vonsattel and DiFiglia, 1998), but only layers V and VI display slightly increased ratios of NR2B expression compared with NR2A subunits (Charton et al., 1999; Scherzer et al., 1998). The CA1 pyramidal layer is another area vulnerable in HD (Spargo et al., 1993), and expresses large amounts of both NR2B and NR2A subunit (Scherzer et al., 1998; Charton et al., 1999). The CA1 region of

YAC46 mice does not display any significant degeneration up until 20 months of age, but does show some electrophysiological abnormalities (Hodgson et al., 1999). It is our prediction that neurons expressing NR2B at lower ratios to other NR2 subunits than found in MSNs, such as pyramidal neurons of the cortex and hippocampus, might show a smaller increase in NMDAR-mediated current and excitotoxicity in the YAC72 mice. Further experiments are required to fully test this prediction, and the results would help determine whether NR2B expression is sufficient or if other downstream factors contribute to selective neuronal vulnerability in HD. One brain region that does not support our hypothesis that high NR2B expression predicts neuronal vulnerability in HD is the CA3 region of the hippocampus, which has high levels of NR2B (Scherzer et al., 1998; Charton et al., 1999) but is largely unaffected in HD (Spargo et al., 1993).

We propose that mutant htt selectively enhances activity and toxicity of NR1/NR2B-type NMDARs, as supported by our data in neuronal cultures showing: 1) no difference between wild type and YAC72 striatal medium spiny neuron death upon selective activation of AMPARs; 2) no difference between wild type and YAC72 cerebellar granule neuron death upon stimulation of NMDARs, which are composed of NR1 with NR2A and/or NR2C but do not include NR2B (Vallano et al., 1996; Thompson et al., 2000; Kovacs et al., 2001); 3) the majority of NMDA-evoked current in MSNs was sensitive to the NR2B-selective antagonist ifenprodil (Zeron et al., 2002; see Figure 2) and NMDA-mediated excitotoxic cell death was nearly eliminated by treatment with this antagonist. Selective enhancement of NR1/NR2B-type NMDARs by mutant htt may, in part, explain selective vulnerability of striatal MSNs to neurodegeneration in HD. Moreover, our results suggest that NMDAR antagonists specific for the NR1/NR2B subtype may show higher efficacy in slowing progression of this disease than the less specific inhibitors of glutamatergic transmission tested thus far.

7.5 Potential Therapeutics

Currently, cellular transplantation therapy is being tested as a therapeutic option in HD. This area is being explored because fetal cell transplantation in striata of patients with Parkinson Disease (PD) and in animal models of PD, have shown some promising results, particularly when combined with the addition of trophic factors, antioxidants or anti-apoptotic factors (Alexi et al., 2000). To date, cellular transplantation therapies for HD have proven less successful since it appears that HD requires the reconstruction of the striatal network as opposed to the replacement of dopaminergic cells in PD (Alexi et al., 2000). Transplantation therapies are highly invasive and run the risk of causing increased damages and therefore are clinically less attractive as a treatment option.

Drug therapies are a more attractive solution to clinical treatment of HD. Previous clinical drug trials in patients affected with HD used less specific inhibitors of glutamatergic transmission - working directly to block NMDAR activation or inhibit glutamate release or inhibit neuronal depolarization (Alexi et al., 2000). Lamotrigine, which inhibits voltage-gated sodium channels and thus inhibits glutamate release, was tried in HD patients for 30 months without clear evidence that it slowed the progression of HD (Kremer et al., 1999). Patients did report an increase in symptomatic improvements and there was a trend towards a decrease in chorea in those that took lamotrigine (Kremer et al., 1999). Remacemide, recently used in a large clinical trial, has a low affinity for NMDAR and may have other modulatory effects on glutamatergic neurotransmission (Alexi et al., 2000). Treatment with remacemide showed no significant slowing of functional decline in early HD over a 30 month period (Kieburtz et al., 1996; Huntington Study Group, 2001). Ketamine, a non-competitive NMDAR antagonist, was also tried clinically on HD patients, resulting in decline in cognitive abilities, increase in psychiatric symptoms and impaired eye movement (Murman et al., 1997). Similar effects have been observed previously, and are one of the drawbacks to the use of general NMDAR

antagonists (MK-801, CGS 19755, D-CPP-ene) since they can cause undesirable psychological side effects (Lippek et al., 1988; Sveinbjornsdottir et al., 1993; Yenari et al., 1998).

Highly directed targeting of the MSNs for drug therapy seems to be the key in treating persons affected with HD. Our data may prove to be very valuable with regard to possible therapies for HD. Since the effect seen in our experiments appears to be NMDAR subunit-specific, selective antagonism of the NR1/NR2B-subtype in drug treatment of HD could show higher efficacy for targeting for those neurons affected in HD, slow disease progression, and help reduce global drug affects in the nervous system and subsequent side effects. Selective antagonism of NMDARs in the MSNs would be a very important breakthrough in HD therapeutics. A NMDAR antagonist, memantine, that is well tolerated in humans has already been used in clinical treatment for a variety of neurological disorders, focused on the treatment of dementia (reviewed by Parsons et al., 1999). Memantine is unique, as it is a non-competitive channel blocker of NMDAR and believed to be 2-3 times more potent in antagonizing receptors composed of NR1A/NR2B than those consisting of NR1A/NR2A (Grimwood et al., 1996). The non-competitive, voltage-independent, activity-dependent NMDAR antagonist, ifenprodil (Chizh et al., 2001), as used in our studies, or a specific ifenprodil derivative, CP101,606 could also be used to target the NR1A/NR2B NMDAR subtype. Currently, ifenprodil is being tested as an analgesic in the treatment of neuropathic pain in humans and appears to be well tolerated and have few side effects (Chizh et al., 2001). Furthermore, a useful property of ifenprodil and its derivative, CP101,606, is their insensitivity to inhibit receptors composed of NR1/NR2B/NR2A (Brimecombe et al., 1997). This may enhance their antagonist specificity for MSNs, which are largely composed of NR1A/NR2B receptors, and decrease the possibility of side effects. The use of these NMDAR antagonists may be useful in the targeting of NR1A/NR2B receptors in the MSNs to prevent the onset of HD pathogenesis in presymptomatic HD patients and may have less psychological side effects, and this remains to be tested.

Also, still largely unexplored is the potential therapeutic administration of inhibitors of downstream events of NMDARs or mitochondrial dysfunction in HD. Post-mortem HD brains show an increase in oxidized DNA, indicative of damage by free radicals (Browne et al., 1997). Therefore, anti-oxidant drug therapy in HD may also prove effective (Alexi et al., 2000). A clinical trial of the anti-oxidant α-tocopherol showed a small decrease in the rate of neurological decline in patients in the early stages of HD (Peyser et al., 1995). Also, metabolic supplements such as coenzyme Q(10), showed a trend towards slowing the functional decline in motor abilities in HD patients over a 30 month period (Huntington Study Group, 2001), suggesting that metabolic energy compromise in HD may also be a potential target for therapy. Our findings suggest that inhibition of the opening of the permeability transition pore by cyclosporin A may be effective against slowing disease progression in HD. Our results using a caspase-3 like inhibitor support the idea that caspase inhibition could be a potential therapy for treating HD (Wellington and Hayden, 2000), which has seen some success in other HD mouse models (Ona et al., 1999; Chen et al., 2000). The difficulty in these potential therapeutic drugs is that their administration may not be selective enough in the striatum to prevent cell death in MSNs and/or they are too general and cause global and undesirable side effects. Nonetheless, inhibiting downstream events of NMDAR activation, such as free radical production or caspase inhibition (reviewed by Wellington and Hayden, 2000), could also be a possible therapeutic strategy in the treatment of HD and remains to be tested.

7.6 Differential Sensitivity to NMDAR-Induced Excitotoxicity of R6 and YAC72 Mice

In contrast to the present results obtained in YAC72 mice, intrastriatal injection of quinolinic acid in the transgenic R6 mouse models of HD (Mangiarini et al., 1996) resulted in significantly less neuronal degeneration and apoptosis compared with wild-type littermates (Hansson et al., 1999), as briefly mentioned earlier. The resistance developed gradually with age

and more rapidly in R6/2 (150 CAG repeats) than R6/1 mice (115 CAG repeats) (Hansson et al., 2001). R6 mice and YAC 72 mice differ in many respects, including: 1) R6 mice express only a small fragment (~3 % of the total length) of the htt protein whereas YAC transgenics express the full-length protein; 2) htt expression is controlled by some but not all of the regulatory elements contained in the 5' untranslated region of the human gene for R6 mice but all regulatory elements are included in controlling expression of htt in YAC mice; 3) the number of CAG repeats in the HD gene is twice as large for R6/2 mice as for YAC72 transgenics; 4) R6 mice show widespread and abundant intranuclear inclusions, whereas those found in brains of YAC transgenics are more sparse and restricted in distribution; and 5) the R6 mice are hybrids of the CBA and C57Bl/6 strains, while the YAC72 mice are of FVB/N strain.

We have shown previously that while expression of full-length mutant htt with 138Q significantly increased NMDAR-mediated apoptosis in a cell line, an N-terminal fragment (~540 amino acids) of htt showed no enhancement in NMDAR-mediated apoptosis (Zeron et al., 2001). Thus, it is possible that the full context of the mutant htt protein is required to increase NMDAR-mediated cell death, and that these initial steps of pathogenesis in HD are bypassed in the R6 mice. On the other hand, recent data suggest that NMDARs in striatal neurons of R6/2 mice show increased activity, in part due to decreased sensitivity to Mg^{2+} block, at ages when symptoms and htt aggregates are present (Cepeda et al., 2001a). Why then are the R6 striatal neurons resistant to NMDAR-mediated excitotoxicity?

One likely explanation is the widespread intranuclear aggregation of htt in up to 90 % of neurons in the R6/2 mice (Davies et al., 1997), since recent data correlate the development of nuclear inclusions and more efficient handling of Ca^{2+} increases with the appearance of resistance to NMDAR-induced toxicity in R6 mice (Hansson et al., 2001). Accordingly, striatal neurons of 3 week old R6/1 mice have no nuclear inclusions and display a trend towards increased susceptibility to quinolinate (Hansson et al., 2001). These data suggest that R6

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neurons are under chronic stress and have developed compensatory mechanisms that diminish the damage of excitotoxic insults (Hansson et al., 2001). YAC72 mice and patients with advanced HD exhibit intranuclear inclusions in a small percentage of striatal neurons (Hodgson et al., 1999; Sapp et al., 1999) and therefore these mice may constitute a more relevant disease model.

Another explanation for the differential susceptibility of R6 and YAC72 mice to excitotoxicity is the speed of the progression of the disease between the two types of transgenic HD. R6 mice develop neurological symptoms much earlier than YAC72 [8-9 weeks old in R6/2 mice (Mangiarini et al., 1996) compared to 7 months old in YAC72 (line 2511) (Hodgson et al., 1999)]. Therefore, R6 mice may also develop resistance to excitotoxicity much earlier than YAC72 mice. As mentioned previously, striatal neurons from 3 week old R6/1 mice show a trend toward enhanced vulnerability to quinolinate. Resistance to intrastriatal application of quinolinate in older YAC72 mice (>10 months) has not been tested.

Lastly, the genetic backgrounds of the strains in which the R6 and YAC72 were generated are different. Different mouse strains have been shown to display varying susceptibilities to excitotoxicity, demonstrating differential abilities to upregulate cellular defense systems, dependent on the genetic background (Royle et al., 1999; Schauwecker and Steward, 1997). CBA x C57Bl/6 mouse genes may enable these mice to have a better ability to handle excitotoxic stress than the FVB/N mouse strain. This was confirmed with systemic injection of kainic acid or intrastriatal lesions with quinolinate, showing significantly more hippocampal neuronal death or striatal degeneration, respectively, in FVB/N mice compared to C57Bl/6 strains (Schauwecker and Steward, 1997; Royle et al., 1999; Hansson et al., 2001). A deeper examination of the effect of different genetic backgrounds of HD mice on susceptibility to striatal excitotoxicity at various stages of the disease is needed.

7.7 Conclusion

Our research done with non-neuronal models and neurons from mice transgenic for the human huntingtin gene provide strong supportive evidence that NMDARs, specifically the NR1A/NR2B subtype, play a role in the pathogenesis of the HD. Our research suggests that mutant huntingtin enhances NR1A/NR2B-NMDAR-dependent ion currents, excitotoxic cell death, and apoptosis in HEK293 cells and MSNs (Chen et al., 1999b; Zeron et al., 2001, 2002). Furthermore, an NMDAR-dependent enhancement in intracellular free calcium measured in MSNs expressing mutant htt may correlate with caspase-3 activation and changes in mitochondrial function including, enhanced mitochondrial membrane depolarization, and permeabilization. These findings indicate that NMDARs may be the initial trigger for increasing intracellular calcium levels, resulting in cellular dysfunction and apoptotic cell death in the pathogenesis of HD. NMDAR antagonists specific for the NR1/NR2B subtype may show higher efficacy in slowing progression of this disease than the less specific inhibitors of glutamatergic transmission tested thus far.

APPENDIX

I would like to recognize my collaborators and their contributions to this study. First, Cheryl Wellington, contributed to the studies examining activated caspase-3 immunopositivity. For these studies, we both contributed equally to the immunohistochemical staining steps and analysis. M. R. Hayden's laboratory provided invaluable reagents, including; htt, HIP-1 and caspase-3 antibodies (some gifts of Sophie Roy and Donald Nicholson), caspase-3 activity assay substrates, and most importantly, the YAC transgenic HD mice. Tao Luo carried out the protein assay, gel electrophoresis and Western Blot steps of the htt cleavage analysis of the striatal cultures. In the last 6 months, Jackie Shehadeh performed most of the primary striatal cultures and assisted with the preparation of some experiments. Nansheng Chen had provided the data for the electrophysiological studies included in my publications and mentioned in this thesis (see Figures 1 and 2). Oskar Hansson and Patrik Brundin provided the data for the *in vivo* intrastriatal quinolinate studies included in my latest publication, as well as guiding our data analysis for that paper. Claudia Krebs, Ken Baimbridge, and John Church provided reagents and technical support for the mitochondrial potential and calcium imaging studies. Bo Li generously shared a sample of his cultured rat hippocampal neurons. Mannie Fan had donated a sample of 4 month old whole homogenized mouse striatal tissue. Allen Ting-Chun Lee contributed to the examination of cell death or apoptosis in the HEK293 cells co-expressing truncated mutant htt and the NR1/NR2B subunit. Lynn Raymond's contributions are too innumerable to record ☺

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