PROBING THE INTERACTION BETWEEN HUNTINGTIN AND NMDA RECEPTORS

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ABSTRACT

Huntington's Disease (HD) is caused by an expansion (>35) of a polyglutamine (polyQ) tract, in the N-terminus of the protein, huntingtin (htt). Evidence suggests that the selective degeneration of striatal neurons seen in HD is, in part, caused by overactivation of N-methyl-D-aspartate (NMDA) type glutamate receptors (NMDARs). Previously, our lab has shown that co-expression of NMDARs and full-length htt (138Q), in HEK 293 cells, results in a significant increase in glutamate-evoked current amplitude for NR1/NR2B but not NR1/NR2A-type NMDARs. Since channel function and/or receptor distribution of NMDARs may be modulated by interactions with cytoskeletal proteins, we postulate that the polyQ expansion in mutant htt permits the indirect interaction of htt with NMDARs through cytoskeletal proteins, contributing to changes in NMDAR properties and resulting in overactivation of the receptors. We began by characterizing protein expression of htt, htt interacting proteins (i.e. HIP-1), PSD-95 family members and components of the actin cytoskeleton (actin and α-actinin) in HEK 293 cells and striatal, hippocampal and cortical tissue from wildtype and transgenic mice (18Q and 46Q). HEK 293 cells were shown to endogenously express HIP-1, α-actinin and actin. The wildtype and transgenic mice expressed all proteins of interest with relatively high expression of PSD-95 and SAP-102. In addition, NR2A vs. NR2B expression in the mouse striatal, hippocampal and cortical tissues was compared. Densitometric analysis revealed expression levels of NR2B in the striatum, relative to the cortex, is higher as compared to NR2A. The expression of α-actinin was further examined in both HEK cells and mouse tissues using antibodies specific for two isoforms of α-actinin, α-actinin-2 and α-actinin-4. Alpha-actinin-4 was present in HEK 293

cells and striatal, hippocampal and cortical tissue from both wildtype and transgenic mice. In contrast to α-actinin-4, no α-actinin-2 was detected.

Following completion of protein characterization we began to address our hypothesis by investigating a potential interaction between HIP-1 and α-actinin-4 in transfected HEK 293 cells. We have established that there is an interaction between HIP-1 and α-actinin-4. This interaction could prove to be a mechanism by which htt indirectly binds to NMDARs.

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LIST OF ABBREVIATIONS

β-gal	β-galactosidase
^{o}C	Degrees centigrade
Ca^{2+}	Calcium
CaM	Calmodulin
CBS	Cystathionine beta-synthase
cDNA	Complementary DNA
Chapsyn 110	Channel-associated proteins of the synapses-110
CO_2	Carbon dioxide
CNS	Central nervous system
DED	Death effector domain
dlg	Discs-large tumor suppressor gene
EAA	Excitatory amino acids
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis[β-aminoethyl ether]-N,N,N'N'-tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GluR	Glutamate receptor
Grb2	Growth factor receptor-binding protein
HAP-1	Huntingtin-associated protein-1

HD	Huntington's Disease
HEK 293 cells	Human embryonic kidney 293 cells
HIP-1	Huntingtin-interacting protein-1
htt	Huntingtin
iGluR	Ionotropic glutamate receptor
K^+	Potassium
LB broth	Luria-Bertani broth
LYA	Left YAC arm
M	Molar
MAGUK proteins	Membrane-associated guanylate kinases proteins
MAP1A	Microtubule-associated protein 1A
MEM	Minimum essential medium
mGluR	Metabotropic glutamate receptor
mM	millimolar
MSNs	Medium spiny neurons
Na^+	Sodium
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
nNOS	Neuronal nitric oxide synthase
NR1	NMDA receptor subunit-1
NR2	NMDA receptor subunit-2
PBS	Phosphate-buffered saline
PDZ domain	**PSD-95/dlg/ZO**-1 domain

PH	Pleckstrin homology
PMSF	Phenylmethylsulfonyl fluoride
PSD-95	Postsynaptic density-95
PTB	Phosphotyrosine binding
PVDF	Polyvinylidene difluoride
RasGAP	Ras-GTPase-activating protein
rpm	Revolutions per minute
RYA	Right YAC arm
SAP-102	Synapse-associated protein-102
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2 domain	Src homology 2 domain
SH3 domain	Src homology 3 domain
TB	Terrific broth
TBS	Tris-Buffered saline
TE	Tris-EDTA
tS/TXV	Terminal S/TXV
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP
YAC	Yeast artificial chromosome

ACKNOWLEDGMENTS

CHAPTER 1

Introduction

1.1 Huntington's Disease

Huntington's Disease (HD) is a progressive neurodegenerative disorder which is inherited in an autosomal dominant manner (Walling et al., 1998). Typically, onset of symptoms commences midlife (late 4th to 5th decade), however, cases which begin in childhood or extremely late in life have been recorded (Walling et al., 1998; Petersen et al., 1999). Symptoms develop gradually, resulting in motor impairment, cognitive decline and psychiatric manifestations (Rosenblatt and Leroi, 2000). As a result of a lack of treatment for alleviating an inexorable progression, death usually results within 20 years of the onset of symptoms (Gusella and MacDonald, 1995).

HD is caused by an expansion of a CAG trinucleotide (>35) in the coding region of the HD gene, which encodes an expanded polyglutamine (polyQ) repeat in the N-terminus of the protein, huntingtin (htt) (HDCRG, 1993). Despite the fact that htt is widely expressed in both central and peripheral tissues (Sharp et al., 1995; Nance, 1997) and does not display increased levels of expression in disease affected brain regions, selective neuropathology is evident in HD.

The characteristic neuronal loss in HD involves atrophy of the basal ganglia, specifically the caudate and putamen nuclei which constitute the striatum (Glass et al., 2000). Within the striatum, the medium spiny neurons (MSNs), which release the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and account for ~95% of all striatal neurons, undergo the most severe damage and degeneration in HD (Walling et al., 1998;

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Petersen et al., 1999). These projection neurons contain enkephalin or dynorphin and substance P, and primarily innervate the substantia nigra and globus pallidus (Petersen et al., 1999). In contrast to the MSNs which display a vulnerability in HD, other neurons in the striatum, including: cholinergic interneurons; medium-sized aspiny neurons that co-localize somatostatin, neuropeptide Y and nitric oxide synthase; and a subset of GABAergic neurons containing parvalbumin, are relatively preserved (Walling et al., 1998; Petersen et al., 1999; Zoghbi and Orr, 2000). In addition to the striatum, neurodegeneration extends to layers of the cerebral cortex (layers III, IV and VI) and a portion of subcortical structures including the hypothalamic lateral nucleus, amygdala and thalamus (Vonsattel et al., 1985; Lunkes et al., 1998; Petersen et al., 1999).

Substantial progress has been made with regards to understanding the clinical features and genetic component of HD, however, the fundamental biochemical deficiency of the disease is not known (Beal, 1994). Although the mechanism underlying the pathophysiology of the selective neurodegeneration in HD remains a mystery, a hypothesis which attempts to partially explain the etiology of HD implicates excitotoxicity (Dure et al., 1991; Beal, 1994).

1.2 The Excitotoxic Hypothesis of Huntington's Disease

Excitotoxicity is defined as a process in which neuronal death is the result of overstimulation of receptors by excitatory amino acid (EAA) neurotransmitters (Choi, 1992; Doble, 1999). The EAA neurotransmitters consist of glutamate, aspartate and exogenous compounds of either natural (quisqualic, kainic and domoic acid) or synthetic derivation (N-methyl-D-aspartate (NMDA)) and constitute the primary afferent transmitters from the

cortex to the striatum (Dure et al., 1991; Doble, 1999). Glutamate and related EAAs account for a vast majority of excitatory synaptic functioning in the mammalian central nervous system (CNS) and are released by approximately 40% of all synapses (Coyle and Puttfarcken, 1993).

Support for the principle of excitotoxicity and its potential role in neuronal death is derived from numerous experiments, both *in vitro* and *in vivo*. An awareness of the potential toxic effects of EAA neurotransmitters was discovered in the 1950s when systemic administration of high doses of glutamate to mice resulted in the degeneration of retinal neurons (Lucas and Newhouse, 1957). Since this initial report, additional evidence has accumulated which convincingly argues for a role of endogenous EAA neurotransmitters in cell death (Meldrum and Garthwaite, 1990). Experiments supporting a link between EAA neurotransmitters and excitotoxicity include: systemic infusion of glutamate and other EAAs into young animals results in the degeneration of certain vulnerable brain areas, namely the arcuate nucleus in the hypothalamus; local injections of glutamate analogues into specific brain regions of adult animals cause a similar pattern of damage to that observed in the immature animals and use of receptor specific antagonists blocks the toxic effects of the EAAs and protect the neurons (Watson et al., 1989; Meldrum and Garthwaite, 1990).

Considerable evidence has revealed that excitotoxicity can play a significant role in neuronal injury instigated from acute insult, such as stroke and traumatic brain injury, to the CNS (Weiss and Choi, 1991). Involvement of excitotoxicity in ischaemic stroke was corroborated from data collected from studies in which rats where injected with antisense oligonucleotides coding for the NMDAR (Wahlstedt et al., 1993). This led to the decrease in expression of the NMDAR, and consequently the severity of brain lesions following

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experimental focal ischaemic stroke was lessened. Interestingly, numerous studies have duplicated these results, consistently illustrating that agents which inhibit the expression or functioning of the EAA receptors are shown to attenuate the extent of the lesion in laboratory animals following experimentally produced ischaemic stroke (Doble, 1999). Experimental traumatic brain injury models have also implicated a role for excitotoxicity in an acute setting. Utilizing similar techniques to those used in animal stroke models, administration of antisense oligonucleotides to the NMDA receptor or injections of NMDA and/or AMPA receptor antagonists have been shown to improve the outcome and recovery from experimental traumatic brain injury in rodents (Faden and Simon, 1988; Wrathall et al., 1992; Myseros and Bullock, 1995; Sun and Faden, 1995). Therefore, it is now hypothesized that an excitotoxic process could contribute to the etiology and progression in a more chronic setting, including several human neurodegenerative disorders, of particular interest, HD (Dure et al., 1991; Weiss and Choi, 1991; Coyle and Puttfarcken, 1993).

The proposal that excitotoxicity could contribute to the development of HD was advanced following several observations. Initially, kainic acid, a cyclic glutamate analogue, which activates α-amino-3-dydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors, was injected into the striatum. This resulted in the degeneration of intrinsic striatal neurons, along with astroglial proliferation, however a preservation of striatal afferents was observed (McGeer and McGeer, 1976; Coyle, 1983). Subsequent studies utilized an alternative EAA analogue, quinolinic acid, which contrary to kainic acid is both present in the mammalian CNS and selectively activates the NMDAR. Administration of this compound into the striatum of both rats and primates produced an improved model of HD. It generated a lesion which histologically and pathologically resembles the degeneration in

HD, with a relative preservation of somatostatin, neuropeptide Y and cholinergic interneurons (Greenamyre, 1986; Dure et al, 1991; Beal, 1994). Secondly several clinical manifestations which are evident in HD, including impaired movement, learning and memory and weight loss, were mimicked in rats following bilateral excitotoxic striatal lesions (Greenamyre, 1986).

As previously mentioned, glutamate and related EAAs are believed to participate in excitotoxicity leading to neuronal death in both acute and chronic contexts. EAAs exert their neurotoxic effects through the prolonged stimulation of a class of postsynaptic receptors, termed glutamate receptors.

1.3 Glutamate Receptors

Glutamate receptors (GluRs) can be subdivided into two subclasses, namely metabotropic (mGluR) and ionotropic (iGluR) receptors (Rothman and Olney, 1987). iGluRs can further be classified into two additional groups, NMDA and non-NMDA (AMPA and kainate receptors), deriving their nomenclature from their preferred agonists (Seeburg, 1996; Doble, 1999). The ionotropic receptors are able to be distinguished both pharmacologically and electrophysiologically, differing with regards to time courses of activation and deactivation, subunit composition, biophysical traits of ion permeabilities and ion conductances (Seeburg, 1993; Dingledine et al., 1999; Doble, 1999). The structure and function of the GluRs will be further described and any potential roles in excitotoxicity will be highlighted.

1.3.1 Metabotropic Receptors

mGluRs are coupled to the G-protein second messenger system and classified into three broad classes (Calabresi et al., 1999). The binding of a neurotransmitter to Group I (mGluR1, 5) receptors results in the activation of G proteins and phospholipase C, producing the second messengers $InsP_3$ and DAG. $InsP_3$ acts as the ligand for a ligand-gated calcium (Ca^{2+}) channel on the endoplasmic reticulum (ER) which results in the release of Ca^{2+} from the ER into the cytoplasm (Calabresi et al., 1999). The remaining classes, group II (mGluR2, 3) and group III (mGluR4, 6, 7, 8) upon activation inhibit adenylate cyclase activity (Calabresi et al., 1999; Doble, 1999).

In contrast to the ionotropic receptors, a comprehension of the physiological contributions of mGluRs has been difficult to discern due to the lack of potent or receptor-subtype specific agonists and antagonists (Doble, 1999). While several roles have been attributed to mGluRs, due to their heterogeneity and coupling to several transduction pathways, it is believed that mGluRs do not contribute to fast synaptic neurotransmission because they do not contain ion channels (Contractor et al., 1998; Doble, 1999). Additionally, mGluRs are not direct mediators of the toxic consequences of glutamate, although their activation has been suggested to enhance or attenuate neurotoxicity (Coyle and Puttfarcken, 1993). Findings have implicated mGluRs in both the modulation of NMDA receptors and NMDA-receptor mediated excitotoxicity (Contractor et al., 1998; Doble, 1999). Evidence has demonstrated a link between NMDA receptors and group I mGluRs which may, in part, be responsible for the selective neurodegeneration seen in the striatum in HD (Calabresi et al., 1999). Specifically, research has provided evidence that stimulation of group I mGluRs intensifies NMDA-mediated responses in striatal MSNs but not in the large aspiny cholinergic interneurons (Calabresi et al., 1999).

1.3.2 Non-N-methyl-D-aspartate Receptors

The AMPA and kainite receptors were initially identified through electrophysiological means as receptors which were preferentially stimulated by quisqualic or kainic acid. The original name, quisqualate receptor, was changed to AMPA receptor after it became known that quisqualic acid, contrary to AMPA, also activates mGluRs (Doble, 1999). Originally, the pharmacological differentiation between the two receptors proved challenging as selective antagonists used to distinguish them did not exist. However, with the advancement of cloning techniques the specific receptor subtypes of both AMPA and kainate receptors were identified (Doble, 1999).

It was determined that there are nine subunits, GluR1-7 and KA1-2, which constitute the non-NMDARs. The subunits which form kainate receptors are GluR5, GluR6, GluR7, KA-1 and KA-2 (Seeburg, 1996; Doble, 1999). *In vitro* experiments have suggested that homooligomeric (ie. GluR5, GluR6) and heteromeric (ie. GluR6/KA-2) receptors may exist *in vivo* (Ruano et al., 1995; Seeburg, 1996). The remaining four subunits, GluR1-4, whose structural homology is ~70%, assemble as either homo or heterooligomers to form functional AMPA channels (Seeburg, 1996; Doble, 1999).

Activation of both AMPA and kainate receptors can occur after exposure to glutamate, AMPA or kainate. These cation channels permit the influx of sodium (Na^+) ions, the efflux of potassium (K^+) ions, and generally exhibit extremely low calcium (Ca^{2+}) permeability, although Ca^{2+} permeable AMPA receptors do exist (Seeburg, 1993; Doble, 1999). Contrary to the well-defined physiological functions of AMPA and NMDARs, the significance of the kainate receptors in the CNS still remains to be elucidated (Seeburg, 1993, 1996). Presently, their trademark, fast desensitization in the presence of kainate, has

been recorded from peripheral neurons and hippocampal neurons, however this activity remains to be seen in other neurons of the CNS (Lerma et al., 1993; Seeburg, 1993, 1996). AMPA receptors, located in a significant portion of excitatory synapses, mediate rapid excitatory neurotransmission (Seeburg, 1993; Doble, 1999). The involvement of AMPA receptors in glutamatergic transmission has been witnessed in several instances including spinal cord monosynaptic reflexes, sensory pathways in the cochlear nucleus, olfactory cortex and lateral geniculate nucleus, the main excitatory circuits in the cerebellum and hippocampus and corticofugal projections to the striatum and thalamus (Doble, 1999).

Although a significant portion of research findings implicate NMDARs as the prominent mediators in excitotoxic cell death, evidence has suggested that non-NMDA receptors may contribute to neuronal toxicity under certain circumstances (Brorson et al., 1995; Choi, 1994; Doble, 1999). Firstly, *in vivo* experiments which involve the injection of AMPA/kainate receptor agonists directly into the brain result in excitotoxic lesions (Coyle, 1983). Additionally, administration of AMPA/kainate receptor antagonists provide a defense against glutamate-induced neurotoxicity, as observed in a variety of experimental systems (Prehn et al., 1995). Finally, the small fraction of AMPA/kainate receptors which are Ca^{2+} permeable may contribute to the elevation in intracellular Ca^{2+} concentrations in neurons, leading to cellular death (Doble, 1999).

1.3.3 N-methyl-D-aspartate Receptors

The final class of ionotropic receptors and the one which is the central focus of this research, is the NMDARs. NMDARs are heteromeric structures composed of two NR1 subunits which are paired with at least two NR2 subunits: NR2A; NR2B; NR2C and/or

NR2D (Arzberger et al., 1997). Immunoprecipitation experiments have illustrated that it may be possible for two different NR2 subunit isoforms to simultaneously be contained within certain NMDARs (Sheng et al.,1994). Refer to figures 1 and 2 for the primary sequences of the NR1 and NR2B subunits of the NMDARs.

The NR1 subunit exists as eight different splice variants and displays ubiquitous expression, with detection in certain cell types, for example adult cerebellar Purkinje cells, which do not even express functional NMDARs (Doble, 1999). The creation of transgenic mice, expressing a disrupted murine NR1 (ξ) gene, led to the realization that the NR1 subunit is a common element in all NMDARs. These mice lacked functional NMDARs and homozygous expression of the disrupted NR1 gene resulted in death during the neonate stage (Forrest et al., 1994).

The NR2 subunits are believed to be the modulatory subunits since NMDARs expressing different NR2 subunits exhibit differences with regards to macroscopic kinetics, single-channel properties, pharmacology, and regulation by a variety of endogenous modulators and second messenger systems (Seeburg, 1993; Dingledine et al., 1999). In contrast to the ubiquitous expression of the NR1 subunit, the NR2 subunits exhibit a more discrete spatial and temporal expression pattern (Seeburg, 1993; Rigby et al., 1996). Neurons in the forebrain display high levels of the NR2A and NR2B subunits, the cerebellum predominantly contains NR2C, whereas NR2D is found in brain stem and spinal cord (Hollmann and Heinemann, 1994). Interestingly, the MSNs of the striatum express primarily NR2B (Rigby et al., 1996).

Activation of NMDARs requires three processes: the binding of two molecules of glutamate, the binding of two molecules of the co-agonist, glycine; and the removal of

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voltage–dependent magnesium (Mg^{2+}) block (Westbrook, 1993). At normal neuronal resting membrane potentials the Mg^{2+} block exists, therefore the membrane must first be depolarized by another excitatory mechanism, in order to activate NMDARs after glutamate binding (Mayer et al., 1984). In comparison to AMPA receptors, NMDARs have much slower kinetics (Seeburg 1993; Doble, 1999). This has been confirmed in the neocortex, where it is apparent that the stimulation of NMDARs accounts for the slow portion of the excitatory response between glutamatergic interneurons and pyramidal cells (Thomson et al., 1985). Once activated, NMDARs are highly permeable to Ca^{2+}, in addition to monovalent cations (Seeburg, 1993; Westbrook 1993).

An abundance of experimental evidence has suggested that NMDARs may function as the primary vehicle through which excitotoxic injury occurs (Doble, 1999). NMDARs are the principal subtype of GluRs which are highly Ca^{2+} permeable and the activation of this channel is a major source of calcium influx into the cell. Additionally, the stimulation of NMDARs facilitates, through a mechanism which remains to be understood, the movement of calcium from intracellular storage sites (Mody and MacDonald, 1995). The link between NMDARs and excitotoxicity is consistent with the knowledge that an elevation in intracellular Ca^{2+} levels is thought to trigger a cascade of events, including oxidative stress and activation of caspases, which leads to cellular dysfunction and ultimately ends in cell death (Coyle and Puttfarcken, 1993).

Further support for involvement of NMDARs in the pathogenesis of HD has been accumulated from several experiments over the past two decades. The administration of NMDAR agonists directly into the striata of rats and monkeys has produced lesions which parallel the neurochemical, neuropathological, and behavioural alterations evident in HD

(Dawbarn et al., 1985; Beal et al., 1986, 1989; Sanberg et al., 1989). Also, investigation in postmortem brains of HD patients indicates a loss of striatal neurons expressing high levels of NMDARs, demonstrating a selective susceptibility to deterioration (Albin et al., 1990; Arzberger et al., 1997). More recently it has been shown that co-expression of NMDARs and full length mutant htt containing an expanded polyQ tract (138Q), in human embryonic kidney cells (HEK), results in a significant increase in glutamate-evoked current amplitude for NR1/NR2B but not NR1/NR2A-type NMDARs (Chen et al., 1999). Corroborating this result, HEK cells co-transfected with mutant htt (138Q) and NR1/NR2B showed a significant increase in agonist-dependent apoptosis, the mode of cell death believed to occur in HD (Zeron et al., 2001).

As previously mentioned, due to their highly Ca^{2+} permeable channels, NMDARs are thought to be the most effective class of GluRs to facilitate the excitotoxic damage evident in HD. Additionally, the predominant expression of one subclass of NMDAR in the striatum, namely the NR1/NR2B-type NMDAR, raised the possibility that in HD a novel protein-protein interaction could be initiated in the striatum between the NR1/NR2B-type NMDAR and the protein associated with HD (either directly or indirectly), resulting in an enhancement of NMDA receptor function and an increase in excitotoxic death (Chen et al., 1999). This interaction could potentially be instigated through the expression of mutant htt (Zeron et al., 2001).

1.4 Huntingtin

As previously mentioned, the neuropathology of HD is the result of an elongation of

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CAG trinucleotide repeats in exon 1 of the HD gene (Ross, 1995). The HD gene, located on the short arm of chromosome 4, encodes for a ~350 kDa protein, huntingtin (htt) (HDCRG, 1993; Kosinski et al., 1997; Sieradzan et al.,1999). Huntingtin is generated from a gene containing 67 exons and to date no evidence of alternatively spliced isoforms exists (Gusella and MacDonald, 1998). Additionally, htt displays extreme conservation across animal species but despite its large size does not contain any significant homology with known proteins (HDCRG, 1993; Gusella and MacDonald, 1998; Holzmann et al., 1998).

Once htt was identified its localization and distribution were examined. In direct contradiction to the selective neurodegeneration in HD, htt is widespread in brain and peripheral tissues, including liver, kidney, heart, lung and pancreas (Sharp et al., 1995; Wilkinson et al., 1999). Interestingly, although highest levels of htt exist in neurons, htt shows no enrichment in the striatum, creating more confusion as to the mechanism behind the vulnerability of this brain region in HD (Aronin et al., 1995; Sharp et al., 1995). Utilization of immunohistochemical and subcellular fractionation techniques, helped determine that in the normal brain the majority of htt is distributed in the soluble cytoplasmic compartment with a small proportion detected in the nucleus (Bhide et al., 1996; Gusella and MacDonald, 1998; Wilkinson et al, 1999). Contrary to normal htt which is generally found in the cytoplasm, proteolytic fragments of mutant htt, containing the N-terminal polyQ tract, are evident in the nucleus (DiFiglia et al., 1997; Peters et al., 1999). This finding was confirmed using cell culture where it became obvious that truncation of htt enhanced its nuclear inclusion (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998). Analysis of the sequence of htt reveals that it does not contain any domain which would permit it to span the membrane, however htt has been observed to loosely associate

with membranes or cytoskeleton, in cell bodies, dendrites and axons (Sharp et al., 1995). Additionally, htt has been found to be concentrated in nerve terminals, including those found in the striatum (Sharp et al., 1995).

Although htt and the genetic mutation in HD have been characterized, the normal the embryo (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). In contrast function of htt and the consequences of the mutation on its function remain a mystery (Gourfinkel-An et al., 1997; Kim et al., 1999). However, wildtype htt is believed to execute an essential function. This is supported by knowledge that inactivation of Hdh (-/-), the murine homologue of the HD gene, in mice proves to be lethal in heterozygous knockout mice (-/+) seem unaffected, display small irregularities or develop neuronal degeneration leading to premature death (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995; Holzmann et al., 1998). It is also for this reason that it is a common consensus that htt exerts its effects in HD through a toxic gain-of-function as opposed to a loss-of-function (Ross, 1995; Holzmann et al., 1998). In an attempt to decipher the biological function, the htt sequence was analyzed for specific domains. It was determined that htt lacks any kinase, Src homology 2 (SH2), Src homology 3 (SH3), pleckstrin homology (PH), or phosphotyrosine binding (PTB) domains, however it does contain several proline-rich motifs within its sequence which are comparable to SH3 domain binding motifs (Liu et al., 1997). Several potential functions of wildtype htt have been put forward based on htt's association with a plethora of proteins with known activities within the cell. The possible functions of htt include intracellular organelle transport, endocytosis, gene transcription and protein metabolism or axonal transport (Tukamoto et al., 1997; Velier et al., 1998; Boutell et al., 1999; Kim et al., 1999)

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An attempt to elucidate the molecular mechanism by which mutated htt, a widely expressed protein, leads to highly selective neuronal death, suggests an involvement of protein-protein interactions (Ross, 1995; Velier et al., 1998; Boutell et al., 1999; Passani et al., 2000). Recent data supports the notion that the expanded repeat in htt may instigate a toxic gain-of-function, through altered interactions with NMDARs (Chen et al., 1999; Zeron et al., 2001). Similar to NMDARS, it has been shown that htt can interact with numerous proteins, including huntingtin-associated protein-1 (HAP-1), huntingtin-interacting protein-1 (HIP-1), and huntingtin yeast partners (HYPA-C), some of which function in vesicle trafficking, membrane transport and endocytosis (Kalchman et al., 1997; Tukamoto et al., 1997; Wanker et al., 1997; Gusella and MacDonald, 1998; Velier et al., 1998). A selection of cytoskeletal proteins, which serve as potential candidates that may bind htt and/or NMDARs and ultimately indirectly link these proteins, will now be examined further.

1.5 Cytoskeletal Proteins

A plethora of cytoskeletal proteins exist which are responsible for a variety of cellular functions, ranging from synaptogenesis to the coupling of receptors to intracellular signaling pathways. It is conceivable that a variety of these proteins, which are known to bind htt or NMDARs, could be involved in a novel protein interaction which has implications for the pathogenesis of HD. For the purpose of this research, the cytoskeletal proteins which were examined were limited to three broad categories, consisting of huntingtin-interacting proteins, actin cytoskeletal components and postsynaptic density-95 family members.

1.5.1 Huntingtin-interacting proteins

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Recently, yeast two hybrid screens have identified several proteins which are able to bind to the N-terminal repeat-containing section of htt (Gusella and MacDonald, 1998; Boutell et al., 1999). Interestingly, to date, no proteins have been identified as interacting with the remaining regions, which accounts for more than 80% of the length of htt (Gusella and MacDonald, 1998). Although, it is believed that due to the highly conserved nature of this region among animal species, the utilization of techniques other than yeast two hybrid screens may allow for the identification of novel interacting proteins in the remainder of htt (Andrade and Bork, 1995).

A selection of proteins which have been shown to bind htt include HAP-1, HIP-1, HIP-2, HYPA-C, HYPE, HYPH-M, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), growth factor receptor-binding protein (Grb2), Ras-GTPase-activating protein (RasGAP) and cystathionine beta-synthase (CBS) (Li et al., 1995; Kalchman et al., 1997; Liu et al., 1997; Wanker et al., 1997; Gusella and MacDonald, 1998; Boutell et al., 1998, 1999; Passani et al., 2000). The interaction of these proteins with htt can either be enhanced, inhibited or unaffected by the lengthening of the polyQ stretch (Gusella and MacDonald, 1998). Additionally, some of these proteins (ie. HYPA-C) have been shown to bind to htt via its proline-rich region located downstream of the polyQ repeat (Boutell et al., 1999).

Due to the obvious constraints and inability to examine all of the huntingtin-interacting proteins, one protein, HIP-1, was chosen to be further investigated.

1.5.1.1 Huntingtin-interacting protein-1

HIP-1 was isolated by the yeast two hybrid system, from a human brain library, through its binding to the N-terminus of htt (Kalchman et al., 1997; Wanker et al.,

1997). Despite the ubiquitous expression of HIP-1 mRNA, HIP-1 protein was only found to be expressed in the brain, with greatest quantities in the cortex and slightly lower levels in the cerebellum, caudate and putamen (Kalchman et al., 1997; Wanker et al., 1997). Intriguingly, subcellular localization revealed that in the membrane fractions of brain cells, mainly neurons, HIP-1 and huntingtin are seen to co-localize (Kalchman et al., 1997). ·

Examination of the primary sequence of this 116 kDa protein revealed three structural motifs. Firstly, a leucine zipper, encompassing amino acids 412-433, was identified, although the functional importance remains to be determined (Wanker et al., 1997). Secondly, the C-terminus shares homology with talin, a mammalian membrane cytoskeletal-associated protein which is purported to be involved in cell-cell interactions (Kalchman et al., 1997; Ross and Gilliland, 1999). Finally, a novel death effector domain (DED) was recently identified in HIP-1 which is presumed to potentially provide the molecular basis of HIP-1 toxicity (Hackam et al., 2000).

The function of HIP-1 in mammalian cells is unknown (Ross and Gilliland, 1999). To gain an insight into the potential function of HIP-1, an examination of its homologues has been undertaken. HIP-1 shares considerable homology to Sla2p (45% sequence similarity) in *Saccharomyces cerevisiae* and to a *Caenorhaditis elegans* gene of unknown function, ZK370.3 (Kalchman et al., 1997; Hackam et al., 2000). Sla2p, a member of a family of actin-binding proteins, is a peripheral membrane protein which is involved with actin organization, as well as endocytosis, and is essential for the proper functioning of the cytoskeleton in yeast (Engqvist-Goldstein et al., 1999; Yang et al., 1999). Applying the knowledge of the functions of Sla2p to its human homologue HIP-1, it is reasonable to assume that the interaction of HIP-1 and htt may be essential for the normal activity of the

membrane cytoskeleton of neurons (Kalchman et al., 1997). Furthermore, the affinity of HIP-1 for htt is inversely correlated to the length of the polyQ tract, therefore in HD this interaction may be impaired, leading to a deficiency in function of membrane cytoskeleton (Kalchman et al., 1997).

1.5.2 Actin Cytoskeletal Components

Constituents of the neuronal cytoskeleton are the second class of proteins which will be examined for a potential contribution to the etiology of HD. The cytoskeleton is a complicated network composed of microtubules, actin microfilaments, intermediate filaments and many associated proteins, including α-actinin (Allison et al., 2000). Alterations in the cytoskeletal protein architecture are believed to be responsible for several cellular functions encompassing neurite outgrowth, cell migration, axonal transport, growth cone advance/collapse and neurotransmitter release (Shorte, 1997) Recognizing the enormous contribution of the cytoskeleton to essential biological functions of the neuron, it is both reasonable and logical to infer that any change in the normal function of a member of the cytoskeleton may play a role in HD. A subclass of the neuronal cytoskeleton, the actin cytoskeleton, specifically actin and α-actinin, will be examined in greater detail.

1.5.2.1 Actin

Actin, a 42 kDa protein, exists as one of the major cytoskeletal proteins, found in plentitude in neurons (Shorte, 1997). This protein occurs in a dynamic equilibrium containing two fundamental states: a filamentous polymeric form (F-actin) and a globular monomeric form (G-actin) (Shorte, 1997). *In vitro* experiments have demonstrated that G-

actin spontaneously combines to generate F-actin through noncovalent bonds, and a steady state is formed once the concentration of G-actin reaches a critical concentration (Schafer and Cooper, 1995). However, the critical concentration elucidated from *in vitro* work fails to accurately estimate G-actin concentration. In actuality, G-actin concentration is ~10-fold higher due to a variety of actin-binding proteins which affect actin polymerization, depolymerization, capping, nucleation, and bundling activities (Schafer and Cooper, 1995). Moreover, these regulatory activities are mediated by several physiological elements including cytosolic ions, second messenger metabolites and protein phosphorylation (Schafer and Cooper, 1995).

A reason for exploring a potential connection between actin and HD is derived from the recognized interplay of actin and NMDARs. Firstly and most simply, constituents of glutamate synapses, including GluRs, are thought to be possibly anchored to actin-based cytoskeletal networks (Allison et al., 2000). This is consistent with the hypothesis that the mechanism behind the localization of neurotransmitter receptors to their appropriate site of function occurs through interactions with the cytoskeleton (Allison et al., 1998). This is supported by the finding that use of actin-perturbing agents led to a 40% decrease in the number of synaptic NMDAR clusters (Allison et al., 1998). Additionally, stimulation of NMDARs results in depolarization of neurons, culminating in alterations in cytosolic concentrations of intracellular Ca^{2+} and also changing the phosphorylation of several proteins (Scheetz and Constantine-Paton, 1996; Shorte, 1997). These events could directly alter the state of actin polymerization in neurons (Shorte, 1997). The reverse is also true in that the functional state of NMDARs is also mechanosensitive and therefore can be modulated by the actin cytoskeleton in a calcium-dependent fashion (Rosenmund and

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Westbrook, 1993; Allison et al., 1998). Specifically, if the NMDARs shift the actin equilibrium in favour of depolymerization, the binding of actin filament bundling proteins to NMDARs will be impaired, altering the ability of calmodulin to bind and modulate NMDAR functions (Ehlers et al., 1996). Finally, an intriguing hypothesis implies that the ability of the NMDAR activation to affect actin depolymerization, ultimately blocking overstimulation of these receptors may offer a means by which NMDAR activity produces autoinhibitory feedback, potentially protecting cells from glutamate-induced excitotoxicity (Damschrader-Williams et al., 1995). This hypothesis is supported by recent data showing that stabilization of the actin cytoskeleton increases, whereas actin depolymerization decreases, NMDAR-mediated neurotoxicity (Furukawa et al., 1997; Sattler et al., 2000). It is suggested that the relationship between NMDARs and actin be targeted as a potential phenomenon involved in pathophysiology (Shorte, 1997).

While several transmembrane proteins, including NMDAR, are anchored to actin the interaction is an indirect one, mediated by several bridging proteins, including α-actinin (Allison et al., 1998).

1.5.2.2 α-Actinin

Alpha-actinin belongs to the spectrin superfamily of actin-binding and crosslinking proteins (Chan et al., 1998; Viel, 1999). The most significant function of α-actinin entails the bundling of F-actin into parallel formations and fixation of actin filaments at certain locations within the cell (Chan et al., 1998).

Presently, there are four known human α-actinin genes which encode for two skeletal muscle isoforms (α-actinin-2 and –3) and two non-muscle isoforms (α-actinin-

1 and –4) (Chan et al., 1998). An extensive examination of their molecular structure reveals that these proteins share expansive sequence homology (> 70%) and are all ~100 kDa in molecular weight (Chan et al., 1998). Structurally, all α-actinin isoforms possess three conserved domains: an N-terminal actin-binding domain, a central rod domain containing four spectrin-like repeats and two C-terminal EF-hand calcium binding motifs which are only functional in the non-muscle isoforms (Wyszynski et al., 1997; Chan et al., 1998; Honda et al., 1998). Interestingly, the only known functional difference between the muscle and non-muscle α-actinin isoforms resides in their calcium sensitivity (Chan et al., 1998).

The muscle-specific isoforms, α-actinin-2 and –3, are expressed in skeletal and cardiac muscle (Chan et al., 1998). As a result of their extreme homology (80% identity, 90% similarity) heterodimers, mediated by the central rod domain, are able to form between these two isoforms *in vivo*, creating the possibility of a unique functional trait (Chan et al., 1998).

Alpha-actinin-1 and –4, although both non-muscle isoforms and demonstrating a significant amount of homology (80% identity, 87% similarity), display quite different localization patterns (Honda et al., 1998). Alpha-actinin-1 is found along stress fibres and associates with cell adhesion molecules. Conversely, α-actinin-4 is absent from focal adhesion plaques or adherens junctions, instead its expression is induced in migrating cells, seen at the periphery of cell clusters and sometimes displays nuclear staining (Honda et al., 1998; Vallenius et al., 2000).

As mentioned earlier, α-actinin links the NMDARs to the actin cytoskeleton (Wyszynski et al., 1998; Dunah et al., 2000). Alpha-actinin-1 and –2 have been experimentally shown to directly bind to the NMDA receptor subunits NR1 and NR2B

(Wyszynski et al., 1998; Vallenius et al., 2000). These observations paired with the known interaction of α-actinin with actin provides a foundation from which it can be speculated that the actin cytoskeleton, including α-actinin, can modulate channel activity (Shorte, 1997). Hence, a disruption in this latter function could result in a neuropathological state.

1.5.3 Postsynaptic Density-95 Family Members

The Postsynaptic Density-95 (PSD-95) family members are a subfamily of a larger class of proteins called membrane-associated guanylate kinases (MAGUKs) (Kim et al., 1996; Hsueh et al., 1997). MAGUKs are widely expressed in the brain and their importance in synaptic function, including synaptic clustering of neuronal ion channels, has been demonstrated through both biochemical and genetic means (Hsueh et al., 1997; Brenman et al., 1998). In particular, comparison of PSD-95 family members to its Drosophila homologue, discs large (dlg), lends support to the postulated role of PSD-95 family members. *In vitro* experiments have shown dlg to both bind and cluster Shaker K^+ channels (Hsueh et al., 1997). Furthermore, in dlg loss-of-function mutants, the normal synaptic structure is disrupted, including elimination of normal clustering of the Shaker K^+ channels. This provides support for a role of PSD-95 family members in the molecular organization of synapses (Kim et al., 1996; Hsueh et al., 1997). On the other hand, a knock-out mouse for PSD-95 shows normal synaptic clustering and functioning of NMDARs (Migaud et al., 1998). This demonstrates an apparent redundancy in the mammalian system implying that other molecules may play a significant role in the localization of NMDAR subunits to the synapse.

The ability of MAGUKs to regulate synaptic function is believed to be mediated through protein-protein interactions (Brenman et al., 1998). Examination of the structure of MAGUKs reveals three conserved motifs which would facilitate these interactions. The N-terminus contains three PSD-95/dlg/ZO-1 (PDZ) domains (Sheng and Kim, 1996; Hsueh et al., 1997). PDZ domains are modular protein-protein interacting domains which can bind other PDZ domains or certain C-terminal peptide sequences (Hsueh and Sheng, 1999). The N-terminal two PDZ domains of the PSD-95 family members recognize and bind directly to a conserved four amino acid sequence (tS/TXV) located at the C-terminus of the cytoplasmic tails of certain ion channels, including Shaker K^+ channels and NMDARs (NR2B) (Kim et al., 1996; Hsueh et al., 1997). Additionally, MAGUKs contain a SH3 domain. Although the molecular function remains elusive, traditionally SH3 domains are understood to mediate protein interactions by binding to proline-rich sequences (McGee and Bredt, 1999). Interestingly, evidence has suggested an intramolecular interaction between the SH3 domain and the third domain, a guanylate kinase (GK) domain (McGee and Bredt, 1999). In contrast to the homology of the GK domain with the enzyme guanylate kinase, no kinase activity has been detected in MAGUKs (McGee and Bredt, 1999). However, synaptic binding partners for the GK domain, including microtubule-associated protein 1A (MAP1A) and guanylate kinase associated proteins (GKAP) have been isolated (Takeuchi et al., 1997; Kim et al., 1997).

The PSD-95 family encompasses four highly related mammalian proteins: PSD-95, Channel-associated proteins of the synapses-110 (Chapsyn-110), Synaptic associated protein-102 (SAP-102) and Synaptic associated protein-97 (SAP-97) (Hsueh et al., 1997). Only three of these proteins, PSD-95, Chapsyn-110 and SAP-102, will be focused on due to

their predominant postsynaptic localization and their ability to directly interact with NMDARs (Muller et al., 1996; Brenman et al., 1996; Hsueh et al., 1997).

We chose to investigate the members of the PSD-95 family because of the potential interactions with which they could be involved. Firstly, as already mentioned PSD-95 family members can directly bind NMDARs (Muller et al., 1996; Brenman et al., 1996; Hsueh et al., 1997). Additionally, NMDAR associated proteins, including PSD-95 family members, possess the ability to bind to the actin cytoskeleton (Allison et al., 1998). Moreover, HIP-1, which binds htt, contains a talin domain which could mediate a direct interaction with the spectrin repeats of α-actinin, and subsequently indirectly bind actin (Kalchman et al., 1997; Wanker et al., 1997; Engqvist-Goldstein et al., 1999; Hackam et al., 2000) Therefore, through these interactions the PSD-95, in part, could facilitate the indirect interaction of NMDARs and htt, implying a possible involvement of PSD-95 family members in HD.

1.5.3.1 Postsynaptic Density-95

PSD-95, also called SAP-90, was principally identified as a significant protein component of the PSD (Cho et al., 1992). It is the prototypic member of the MAGUKs, sharing extreme homology to two junctional proteins, mammalian zonula occludens 1 (ZO-1) and Drosophila discs large (dlg) (Woods and Bryant, 1991; Willott et al., 1993). PSD-95, a 95 kDa scaffolding protein, participates in both the clustering of NMDARs and attachment of these receptors to internal signaling molecules at the vertebrate glutamatergic synapse (Firestein et al., 1999).

PSD-95 is most abundant in the brain, however expression is also observed in several other tissues including heart, skeletal muscle, liver, testis, ovary and spleen (Stathakis et al., 1997). Within the brain PSD-95 staining demonstrates a somatodendritic pattern in the postsynaptic membrane, with high expression in striatum, hippocampus and cortex (Cho et al., 1992). Paralleling the suggested role of PSD-95 in synaptic organization, examination of the developmental expression of this protein illustrates a rapid increase in levels during postnatal days 8 to 18, a period during which prodigious synaptogenesis occurs in the CNS (Cho et al., 1992).

Several proteins have been discovered to bind to the protein-binding domains in PSD-95. These comprise NR2 subunits, GKAP, neuroligin, CRIPT , SynGAP, neuronal nitric oxide synthase (nNOS), microtubule-associated protein 1A (MAP1A) and citron which bind to the PDZ domains found within PSD-95 (Zhang et al., 1998). To avoid future redundancy, it should be stated that both Chapsyn-110 and SAP-102 are also involved in protein-protein interactions, including several of the above mentioned proteins (Brenman et al., 1996; Muller et al., 1996). Moreover, PSD-95 has been observed to form homodimers or heterodimers with its closely related family member, Chapsyn-110 (Hsueh et al., 1997; Hsueh and Sheng, 1999). The multimerization of PSD-95 and Chapsyn-110 is thought to occur via either a head-to-head or back-to-back mechanism which utilize the first or third PDZ domains, respectively (Hsueh et al., 1997). This heteromultimerization is further mediated by a conserved 64 amino acid stretch in the N-terminus of the protein, called the N-segment (Hsueh et al., 1997). The N-segment contains two cysteine residues and mutations in either prevents heteromultimerization (Hsueh et al., 1997).

1.5.3.2 Channel-associated protein at the synapse-110

Chapsyn-110, also referred to as PSD-93, is a 110 kDa protein which was the third member of the PSD-95 family of proteins to be isolated, following the discovery of PSD-95 and SAP-97 (Hsueh et al., 1997). It derives its name by analogy with the acetylcholine receptor clustering molecule of neuromuscular junctions, 43K/rapsyn (Kim et al., 1996). Comparison of Chapsyn- 110 to PSD-95, with regards to primary structure, reveals a close relationship with 71% identity and 85% similarity (Kim et al., 1996). The most significant differences between the amino acid sequence in Chapsyn 110, as related to PSD-95, occurs outside the highly conserved motifs, specifically, prior to the first PDZ domain, between the second and third PDZ domains and in a region between the SH3 and GK domains (Kim et al., 1996).

Chapsyn-110 displays a somatodendritic expression pattern which somewhat overlaps the distribution seen by PSD-95 (Kim et al., 1996). Northern blot analysis of Chapsyn-110 mRNA expression determined that Chapsyn-110 mRNA is primarily found in the brain as no signal was detected in the heart, skeletal muscle, kidney, lung, liver or spleen (Kim et al., 1996). The cortex, hippocampus and basal ganglia contain substantially high Chapsyn-110 protein levels, with lower expression levels evident in the cerebellum (Kim et al., 1996). With respect to the cerebellum, the soma and dendritic trees of the Purkinje cells are exclusively endowed with Chapsyn-110 (Kim et al., 1996; Brenman et al., 1998).

1.5.3.3 Synaptic Associated Protein-102

The fourth and final member of the PSD-95 family of proteins is SAP-102. Several years after the discovery of SAP-102, a rat postsynaptic protein, its human

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homologue, neuronal and endocrine dlg (NE-dlg), which shares 86% identity was located (Kuwahara et al., 1999). For simplicity, the two proteins will be collectively referred to as SAP-102.

At least three isoforms of SAP-102 have been identified having the apparent molecular weight of ~102 kDa (Muller et al., 1996). SAP-102 mRNA was only seen in brain tissue and was absent from additional tissues including, heart, muscle and liver (Muller et al., 1996). Within the brain, SAP-102 is most prominently expressed in cerebral cortex, hippocampus, cerebellum and the olfactory bulb (Muller et al., 1996). Moreover, it has been observed to be localized to dendritic shafts and synaptic spines and is evenly distributed throughout the cytoplasm in neuronal cell bodies (Muller et al., 1996). Comparable to PSD-95 protein expression, both SAP-102 mRNA and protein levels are most prominent during synaptogenesis (Blue and Parnavelas, 1983; Muller et al., 1996). Examination of cerebral cortex reveals that SAP-102 transcript quantities rise during the first two postnatal weeks, peak around P15 and decrease moderately in aged animals (Muller et al., 1996). Intriguingly, this phenomenon is seen in proteins known to contribute to synaptogenesis, including the α subunit of the Ca^{2+}/calmodulin-dependent protein kinase II and synapsin I, providing evidence of the involvement of SAP-102 in synaptic organization (Sahyoun et al., 1985; Sudhof et al., 1989).

1.6 Research Hypothesis

A plethora of evidence, collected over the past 20 years, has suggested that the selective and progressive degeneration of the medium spiny striatal neurons (MSNs), evident in HD is, in part, due to NMDA receptor overactivation (excitotoxicity) (Coyle and

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Puttfarcken, 1993). However, an understanding of the molecular mechanism underlying the overactivation of these receptors remains to be elucidated.

Several groups have demonstrated that both NMDA receptors and htt can interact with numerous proteins, including some cytoskeletal-associated proteins (Kornau et al., 1995; Kim et al., 1996; Muller et al., 1996; Kalchman et al., 1997; Tukamoto et al., 1997; Wanker et al., 1997; Allison et al., 1998; Gusella and MacDonald, 1998; Velier et al., 1998). In addition, data from our lab demonstrate both an increase in glutamate-evoked current amplitude and an increase in agonist-dependent apoptosis for HEK 293 cells co-expressing NR1/NR2B receptors and full length mutant htt (138Q) (Chen et al., 1999; Zeron et al., 2001), providing further evidence for the role of NMDA receptors in the pathogenesis of HD. The documentation of interactions with cytoskeletal associated proteins for both NMDA receptors and htt, combined with the findings in our lab support our hypothesis that the polyQ expansion in mutant htt permits the indirect interaction of htt with NMDA receptors through cytoskeletal proteins, contributing to changes in NMDA receptor properties and resulting in their overactivation.

The objective of this study is three-fold in nature. Initially, characterization of protein expression of htt, htt interacting proteins (ie. HIP-1), PSD-95 family members and components of the neuronal cytoskeleton (actin and α-actinin) in HEK 293 cells and striatal, hippocampal and cortical tissue from wildtype and transgenic mice (18Q and 46Q) will be performed. Additionally, a comparison of expression of NR2A vs. NR2B in striatal, hippocampal and cortical tissues will be completed. Finally, co-immunoprecipitation will be used to investigate any potential interactions which may exist between the characterized

cytoskeletal proteins and htt and/or NMDARs, in an attempt to discover a protein interaction

which may serve as the link between htt and NMDA receptors.

Figure 1. Primary sequence of the rat NR1A subunit of the NMDA receptor. This is the sequence of the most abundant NR1 splice variant (Wyszynski et al., 1997). The membrane segments, M1-M4, are dotted underlined. The C-terminal tail consists of the C0 segment (italics), C1 segment (bold) and C2 segment (underlined). It was determined that α-actinin can bind to the C0 and C1 segments (Wyszynski et al., 1997). This sequence was obtained from the National Center for Biotechnology Information (Genbank accession number: U08261).

5'-MSTMHLLTFALLFSCSFARAACDPKIVNIGAVLSTRKHEQMFREAVNQAN

KRHGSWKIQLNATSVTHKPNAIQMALSVCEDLISSQVYAILVSHPPTPNDHFT

PTPVSYTAGFYRIPVLGLTTRMSIYSDKSIHLSFLRTVPPYSHQSSVWFEMMR

VYNWNHIILLVSDDHEGRAAQKRLETLLEERESKAEKVLQFDPGTKNVTAL

LMEARELEARVIILSASEDDAATVYRAAAMLNMTGSGYVWLVGEREISGNA

LRYAPDGIIGLQLINGKNESAHISDAVGVVAQAVHELLEKENITDPPRGCVG

NTNIWKTGPLFKRVLMSSKYADGVTGRVEFNEDGDRKFANYSIMNLQNRKL

VQVGIYNGTHVIPNDRKIIWPGGETEKPRGYQMSTRLKIVTIHQEPFVYVKPT

MSDGTCKEEFTVNGDPVKKVICTGPNDTSPGSPRHTVPQCCYGFCIDLLIKLA

RTMNFTYEVHLVADGKFGTQERVNNSNKKEWNGMMGELLSGQADMIVAP

LTINNERAQYIEFSKPFKYQGLTILVKKEIPRSTLDSFMQPFQSTLWLLVGLSV

HVVAVMLYLLDRFSPFGRFKVNSEEEEEDALTLSSAMWFSWGVLLNSGIGEG

APRSFSARILGMVWAGFAMIIVASYTANLAAFLVLDRPEERITGINDPRLRNPS

DKFIYATVKQSSVDIYFRRQVELSTMYRHMEKHNYESAAEAIQAVRDNKLHA

FIWDSAVLEFEASQKCDLVTTGELFFRSGFGIGMRKDSPWKQNVSLSILKSHE

NGFMEDLDKTWVRYQECDSRSNAPATLTFEN*MAGVFMLVAGGIVAGIFLIFIE*

*IAYKRHKDARRKQMQLAFAAVNVWRKNLQ***DRKSGRAEPDPKKKATFRAITS**

TLASSFKRRRSSKDTSTGGGRGALQNQKDTVLPRRAIEREEGQLQLCSRHRES-3'

Figure 2. Primary sequence of the human NR2B subunit of the NMDA receptor. The membrane segments, M1-M4, are dotted underlined. The underlined amino acids represents the segment of the C-terminus which interacts with α-actinin. The amino acids in bold represent the terminal S/TXV domain which interacts with PDZ domain containing proteins, including PSD-95, Chapsyn-110 and SAP-102. This sequence was obtained from the National Center for Biotechnology Information (Genbank accession number: NM_000834).

5'-MKPRAECCSPKFWLVLAVLAVSGSRARSQKSPPSIGIAVILVGTSDEVAIKDAHE

KDDFHHLSVVPRVELVAMNETDPKSIITRICDLMSDRKIQGVVFADDTDQEAIAQ

YIFSIVTTYFPGYQDFVNKIRSTIENSFVGWELEEVLLLDMSLDDGDSKIQNQLKK

LQSPIILLYCTKEEATYIFEVANSVGLTGYGYTWIVPSLVAGDTDTVPAEFPTGLI

SVSYDEWDYGLPARVRDGIAIITTAASDMLSEHSFIPEPKSSCYNTHEKRIYQSNM

LNRYLINVTFEGRNLSFSEDGYQMHPKLVIILLNKERKWERVGKWKDKSLQMK

YYVWPRMCPETEEQEDDHLSIVTLEEAPFVIVESVDPLSGTCMRNTVPCQKRIVT

ENKTDEEPGYIKKCCKGFCIDILKKISKSVKFTYDLYLVTNGKHGKKINGTWNG

MIGEVVMKRAYMAVGSLTINEERSEVVDFSVPFIETGISVMVSRSNGTVSPSAFLE

PFSADVWVMMFVMLLIVSAVAVFVFEYFSPVGYNRCLADGREPGGPS FTIGKAW

LLWGLVFNNSVPVQNPKGTTSKIMVSVWAFFAVIFLASYTANLAAFMIQEEYVDQ

VSGLSDKKFQRPNDFSPPFRFGTVPNGSTERNIRNNYAEMHAYMGKFNQRGVDD

ALLSFGTVPNGSTERNIRNNYAEMHAYMGKFNQRGVDDALLSLKTGKLDAFIYD

AAVLNYMAGRDEGCKLVTIGSGKVFASTGYGIAIQKDSGWKRQVDLAILQLFGD

GEMEELEALWLTGICHNEKNEVMSSQLDIDNMAGVFYMLGAAMALSLITFICEH

LFY WQFRHCFMGVCSGKPGMVFSISRGIYSCIHGVAIEERQSVMNSPTATMNNTH

SNILRLLRTAKNMANLSGVNGSPQSALDFIRRESSVYDISEHRRSFTHSDCKSYNNP

PCEENNGSPQSALDFIRRESSVYDISEHRRSFTHSDCKSYNNPPCEENLFSDYISEVER

TFGNLQLKDSNVYQDHYHHHHRPHSIGSASSIDGLYDCDNPPFTTQSRSISKKPLDI

GLPSS KHSQLSDLYGKFSFKSDRYSGHDDLIRSDVSDISTHTVTYGNIEGNAAKRRK

QQYKD SLKKRPASAKSRREFDEIELAYRRRPPRSPDHKRYFRDKEGLRDFYLDQFR

TKENSHWEHVDLTDIYKERSDDFKRDSISGGGPCTNRSHIKHGTGDKHGVVSGVPA

PWEKNLTNVEWEDRSGGNFCRSCPSKLHNYSTTVTGQNSGRQACIRCEACKKAGN

LYDISEDNSLQELDQPAAPVAVTSNASTTKYPQSPTNSKAQKKNRNKLRRQHSYDTF

VDLQKEEAALAPRSVSLKDKGRFMDGSPYAHMFEMSAGESTFANNKSSVPTAGHH

HHNNPGGGYMLSKSLYPDRVTQNPFIPTFGDDQCLLHGSKSYFFRQPTVAGASKAR

VPDFRALVTNKPVSALHGAVPARFQKDICIGNQSNPCVPNNKNPRAFNGSSNGHVYE

KLSSIESDV-3'

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Chapter 2

Materials and Methods

2.1 Cell Culture

Human embryonic kidney (HEK) 293 cells (CRL 1573; American Type Culture Collection, Rockville MD, USA) were cultured as previously described by Chen et al., 1997. In brief, the cells were maintained at $37^{\circ}C$ and humidified 5% CO_2 in minimum essential medium (MEM) containing Earle's salts and supplemented with L-glutamine (2mM), sodium pyruvate (1mM), streptomycin (100 units/ml), and 10% fetal bovine serum (FBS). The cells were passaged every three to four days and plated at a density of $\sim 2 \times 10^6$/ml. To passage the cells, the medium was aspirated and the cells were washed twice with warmed phosphate-buffered saline (PBS). Following the washes, the cells were exposed to 100 µl 10x trypsin (diluted in 1 ml PBS) for 1-2 minutes. Then, 9 ml of fresh medium was directly added onto the cells and they were subsequently agitated and removed to a new plate. The new plate contained a volume of medium which would allow for the appropriate dilution of cells.

2.2 Plasmid cDNA

The plasmid DNA containing the *Escherichia coli* β-galactosidease (β-gal) gene (pCMVβ) was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). cDNA for α-actinin-2 was subcloned into the pcDNA3 vector and was a gift from Dr. D. Fedida. cDNA for α-actinin-4 was subcloned into pcDNA3.1 (Invitrogen, San Diego, CA, USA), a mammalian expression vector containing the CMV promoter, by D. Kwansnicka and A. El-Husseini (a graduate student in S. Vincent's lab, El-Husseini et al., 2000). HIP-1

(containing the point mutation F398G) was cloned into the pCI vector and was a gift from Dr. M. Hayden.

2.3 Preparation of plasmid DNA

2.3.1 Transformation

DH5α-competent cells (Gibco BRL, Burlington, ON, CANADA) were used for the preparation of plasmid DNA. The competent cells were placed on ice, allowed to thaw and then gently mixed. 100 µl of cells was then aliquoted into the appropriate number of pre-chilled eppendorf tubes and placed on ice. 30-50 ηg of plasmid DNA was added to each tube followed by gentle tapping to thoroughly mix. The cells were then incubated for 30 minutes on ice. The cells were then placed in a 37°C water bath for 45 seconds to heat shock and then immediately placed on ice for 2 minutes. 0.95 ml of SOC Medium was added to each tube. The cells were shaken at 225 rpm for 1 hour at 37°C to allow for expression of plasmid DNA in the competent cells. 100 µl of the mixture was then diluted in 900 µl of SOC Medium. Following this 10-fold dilution, 100 µl was spread on Luria-Bertani (LB) plates containing 100 µg/ml ampicillin. The plates were incubated overnight at 37°C.

2.3.2 Growth of Transformant for Plasmid Preparation

Single bacteria colonies, transformed with plasmids, were selected from plates and grown for ~6 hours at 37°C in a shaking incubator at 250 RPM in LB broth, containing 100 µg/ml ampicillin. The 5 ml cultures were then added to 500 ml of terrific broth (TB) (12 g trypton, 24 g yeast extract, 4 ml glycerol in 900 ml of water mixed with 100 ml of

phosphate buffer containing 2.3 g KH_2PO_4 and 12.5 g K_2HPO_4) containing 100 mg of ampicillin.

2.3.3 Maxiprep of Plasmid DNA

For purification of plasmid DNA, a QIAGEN Plasmid Maxi kit (QIAGEN, Mississauga, ON, CANADA) was used. The procedure outlined in the QIAGEN Plasmid Purification Handbook was followed. The resulting DNA was dissolved in 1X TE (Tris-EDTA) buffer, pH 7.5. The final concentration was calculated by measuring absorbance at 260 and 280 nm, utilizing the Ultrospec 3000 (Pharmacia Biotech, Piscataway, NJ, USA) spectrophotometer.

2.4 Transfections

2.4.1 Calcium Phosphate Precipitation

Cells were transfected by the calcium phosphate method. As previously described (Chen and Okayama, 1987; Chen et al., 1997), cells were plated at a density of ~1 x 10^6 cells/ml in 10 cm culture plates pre-coated with 100 µg/ml poly-D-Lysine (M.W. = ~135,000). The cells were allowed to grow 9-12 hours before the commencement of transfection. The cells were transfected with a ratio of 4:4:4 µg of cDNAs encoding HIP-1, α-actinin 4 or -2 and β-galactosidase (β-gal), using a total of 12 µg of plasmid DNA per 10-cm-diameter-plate. The cells were transfected for ~8 hours in a 3% CO_2 incubator, washed twice with warm PBS, and incubated in fresh medium. The transfection efficiency was assessed by measuring β-gal activity and no further experiments were carried out if the transfection efficiency was less than 50%.

2.4.2 Lipofectamine Transfection

For the Lipofectamine method, as previously described by (Zeron et al., 2001), cells were plated at a density of ~2 x 10^6 cells/ml on 10 cm culture dishes precoated with 100 μg/ml poly-D-Lysine. The cells were grown for ~24 hours before transfection. A 4:4:4 μg ratio of cDNAs encoding HIP-1; α-actinin-4 or -2; and β-gal was used, with a total of 12 μg of plasmid DNA per 10 cm plate. Cells were transfected with a ratio of 1 μg cDNA : 6 μl Lipofectamine (Gibco, Burlington, ON, CANADA) : 100 μl OPTI-MEM (Gibco, Burlington, ON, CANADA) for 5 hours in a 5 % CO_2 incubator. Following the transfection the cells were washed twice with warmed PBS and incubated with fresh medium. For each experiment, staining of β-gal activity was completed to assess the transfection efficiency. If the transfection efficiency was less than 50 % the cells were not used for further experiments.

2.5 Yeast Artificial Chromosome (YAC) Transgenic mice

The transgenic mice which were used in these experiments express human full-length normal (YAC18) or mutant (YAC46) htt, with expression driven by the human promoter of htt, utilizing yeast artificial chromosome (YAC) technology in a FVB/N strain (Hodgson et al., 1999). To generate the transgenic mice expressing the human HD gene, two well characterized YACs, YGA2 and 353G6, were used (Hodgson et al., 1996). These two YACs differ with regards to size and flanking DNA sequences. YGA2 is the larger of the two at 600 kb and extends ~350 kb 5' and 50 kb 3' of the gene as opposed to 353G6 which is only 350 kb, extending 25 kb 5' and 120 kb 3' of the gene (Hodgson et al., 1996). In order to generate the YAC46 mice, homologous recombination in yeast was utilized to

introduce the expanded CAG repeats (Hodgson et al., 1999). In addition to the human HD gene, the YACs contained the Trp1 gene, ARS and CEN4 on the left YAC arm (LYA) and the URA3 gene on the right YAC arm (RYA) (Hodgson et al., 1996).

2.6 Tissue Collection

Mice were anesthetized with halothane prior to decapitation. Following decapitation, an anterior to posterior incision was made along the midline, through the skin layers and skull. The skin and skull were gently pulled back to expose the brain. The optical nerve was severed and the brain was removed into a petri dish containing pre-chilled PBS. Dissections were carried out under a technival 2 dissecting microscope (Germany). A shallow posterior to anterior midline incision was made just deep enough to cut through the corpus callosum without completely separating the two hemispheres. The two hemispheres were then fanned open to expose the underlying structures, including the striatum and hippocampus. The anterior portion of the striatum, the hippocampus and a portion of cortex were removed from each hemisphere and placed into individual glass homogenization tubes. The tubes contained a volume (~500-1000 μl) of pre-chilled 1X RIPA buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate (SDS)) equaling 10X the total volume of tissue. For each brain region, tissue from ~ six animals was combined. The tissues were then homogenized using a Caframo stirrer type R2R1-64 homogenizer (VWR Canlab, Mississauga, ON, CANADA). Following homogenization, protein concentration was determined using the bicinchoninic acid protein assay (BCA kit; Pierce). The homogenized

tissue was then used fresh for experimentation. Any unused tissue was aliquoted and stored at -80°C until future use.

2.7 Western Blot

2.7.1 HEK 293 Cells (total soluble fraction)

Cells were washed twice with 5 ml warm PBS. The cells were then scraped into ice-cold solubilization buffer (1 mM EDTA, 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N'N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 units/ml aprotinin, and 1% Triton-X-100 in PBS), transferred to Eppendorf tubes, sonicated (10 s), and centrifuged (14,000 rpm for 30 mins at 4°C). Supernatants were collected and protein concentrations were determined using the bicinchoninic acid protein assay (BCA kit; Pierce). For the assessment of protein expression levels total soluble protein from each cell lysate was loaded (5 or 10 μg per lane) and separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked for one hour with 5% blot solution (5% dry milk, 0.5% polyoxyethylene-sorbitan monolaurate, 10% Tris-buffered saline (TBS)) and then probed with a specific primary antibody (incubation ranged from two hours to overnight). Concentrations used for the primary antibodies were: 1:1000 (anti-PSD-95, anti-SAP-102, anti-Chapsyn-110, anti-α-actinin-4, anti-NR2A, anti-NR2B), 1:500 (actin, anti-α-actinin-2), 1:200 (anti-α-actinin), 1:100 (anti-htt) and 10 μg/ml (HIP-1). Following primary antibody incubation, the blot was washed three times, each for 10 minutes, with 0.5 % blot solution (0.5% dry milk, 0.5% polyoxyethylene-sorbitan monolaurate, 10% Tris-buffered saline (TBS)) and then the appropriate secondary antibody

(donkey anti-rabbit or sheep anti-mouse each conjugated with horseradish peroxidase at a concentration of 1:5000) (Amersham, Arlington Heights, IL, USA) was added and allowed to incubate for 45 minutes. A repeat of the series of 0.5% blot solution washes was carried out. The blot was then washed two additional times, each for 10 minutes, with TBS. Bands were visualized with Amersham's enhanced chemiluminescence system (ECL) and developed using a 100 Plus automatic x-ray film processor (All-Pro Imaging Corp., Hicksville, NY, USA). Any protein not used was aliquoted and kept at $-80^{o}C$ until further use.

2.7.2 HEK 293 Cells (membrane fraction)

Cells were washed twice with 5 ml warm PBS. Harvest buffer (1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, and PBS) was added to the cells which were then collected into Eppendorf tubes, sonicated (10 s), and centrifuged (14,000 rpm for 30 mins at $4^{o}C$). The supernatant was discarded and 500 µl of solubilization buffer was used to resuspend the pellet. The resuspended solution was sonicated (10s), end-over-end rotated (low speed for 30 mins at $4^{o}C$), and centrifuged (14,000 rpm for 30 mins at $4^{o}C$). The supernatant was collected and the Western blot protocol, as previously described, was continued. Any protein not used was aliquoted and kept at $-80^{o}C$ until further use.

2.7.3 Stripping and Reprobing

Blots were wet with 100% methanol and then washed twice with single distilled water. The blots were then transferred to covered glass containers containing ~50 ml of

stripping buffer (100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl pH 6.7) and incubated for 30 minutes in a 50°C water bath, with constant shaking. Following the incubation the blots were washed twice with TBS at room temperature. The blots were transferred to plastic containers and the previously described Western blot protocol was completed.

2.7.4 Densitometry

Densitometric analysis of Western blots was performed using the program Image Quant3.3, version 3.3 (Molecular Dynamics, Sunnyvale, CA, USA). Protein band intensities were quantified by densitometry to assess any potential cross-reactivity between α-actinin-2 and α-actinin-4 specific antibodies, to compare NR2A vs. NR2B expression among three brain regions (striatum, hippocampus and cortex) and to attempt to quantify the amount of protein (HIP-1) which was able to bind to α-actinin-4 in the co-immunoprecipitation experiments. For determination of cross-reactivity between isoform specific α-actinin antibodies, the band density in the 10 μg lanes of the transfected cells were measured and the density from the α-actinin-2 and α-actinin-4 transfected lanes were calculated relative to the lane containing nontransfected HEK 293 cells. For analysis of relative NR2A and NR2B expression in different forebrain regions, the band density in the 10 μg lanes were measured and the density from the striatum and hippocampus was normalized to that measured from the cortex. For the co-immunoprecipitation blots the percentage of protein able to be pulled down was determined by measuring the band density in the lane containing the resulting product from the co-immunoprecipitation experiments (with primary antibody) and the lane which contained any unbound protein, which was

collected after the first wash. The percentage was then calculated by dividing the band density of the co-immunoprecipitation lane by the combined densities of the two lanes.

2.7.5 Primary Antibodies

The following antibodies were used for Western blotting and co-immunoprecipitation: rabbit polyclonal PSD-95 antibody (a generous gift from Dr. D. Bredt, UCSF, San Francisco, CA, USA), rabbit polyclonal Chapsyn-110 antibody (Synaptic Systems, Göttingen, GERMANY), rabbit polyclonal α-actinin-4 antibody (a generous gift from Dr. T. Yamada, National Cancer Center Research Institute, Tokyo, Japan), rabbit polyclonal HIP1-526 antibody, mouse monoclonal HIP-1 and rabbit polyclonal BKP-1 (huntingtin) antibodies (generous gifts from Dr. M. Hayden, CMMT, Vancouver, BC, CANADA), rabbit polyclonal SAP-102, NR2A and NR2B antibodies (generous gifts from Dr. R. Huganir, Johns Hopkins University, Baltimore, MD, USA), rabbit polyclonal α-actinin-2 antibody (generous gift from Dr. D. Fedida, UBC, Vancouver, BC, CANADA) and rabbit polyclonal actin and mouse monoclonal α-actinin antibodies (Sigma-Aldrich, Oakville, ON, CANADA).

It should be noted that in chapter 6 a comparison of expression of NR2A and NR2B was completed using antibodies specific for NR2A and NR2B. The specificity of these two antibodies were examined by probing HEK 293 cells, transfected with NR1 and either NR2A or NR2B, with both of the antibodies (figure 3). It was determined that the NR2B specific antibody does not cross-react with NR2A. However the NR2A specific antibody does show minimal cross-reactivity with NR2B (see figure 3). Both antibodies were used at a dilution of 1:1000 for experiments shown in chapter 6.

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2.8 Co-immunoprecipitation

Protein A-Sepharose CL4B beads from Amersham Canada Ltd. (Oakville, ON, CANADA) were prepared by resuspending in 50 mM Tris (pH 7.4) by end-over-end rotation (low speed for 15 min at 4°C). Crystallized Bovine Serum Albumin (BSA) (2 mg/ml) was then added followed by an additional end-over-end rotation (30 min at 4°C). The beads were then centrifuged (1000 rpm for 5 min at 4°C), supernatant discarded and resuspended in 50 mM Tris (pH 7.4). The beads were then washed once with 1X Tris wash buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100) and finally resuspended in one volume of 1X Tris wash buffer to make a 1:1 suspension.

Cell homogenate (~200 μl) was precleared with 200 μl each of 2X Tris wash buffer (100 mM Tris (pH 7.4), 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% Triton-X 100) and the 1:1 suspension and allowed to rotate end-over-end (low speed for 1 hour at 4°C). Following preclearing the solution was centrifuged (14,000 rpm for 5 minutes at 4°C), the supernatant was removed to a separate tube and incubated with primary antibody for 60 min at 4°C, with end-over-end rotation. 200 μl of the 1:1 suspension was added and the solution was end-over-end rotated overnight at 4°C. The sample was centrifuged (5000 rpm for 5 min at 4°C), supernatant discarded, and washed with 1X Tris wash buffer. This step was repeated once before repeating two additional times replacing the 1X Tris wash buffer with a high salt Tris wash buffer (50 mM Tris (pH 7.4), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100). Sample buffer was added and mixed gently. The supernatant/sample buffer mixture was then boiled for 2 minutes before loading onto a prepared 8% acrylamide gel for Western blot analysis.

Figure 3. Examination of cross-reactivity of NR2A and NR2B specific antibodies. HEK

293 cells were transfected with NR1 and either NR2A or NR2B. The membrane fraction

of HEK 293 cells was collected and 2, 5 and 10 μg was separated by SDS-PAGE. The blots

were probed with antibodies specific for NR2A and NR2B (1:1000). These experiments

were completed by our lab technician Tao Luo. These results are representative of N=3

independent experiments.
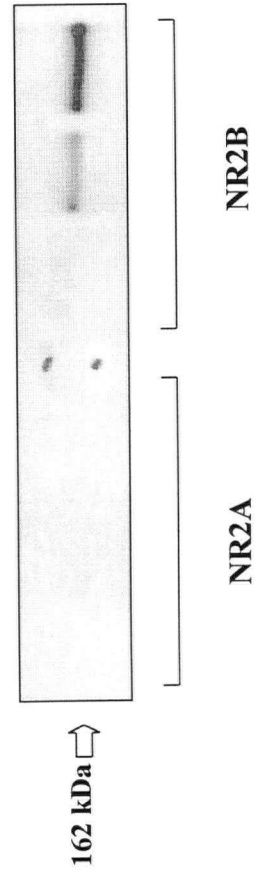
CHAPTER 3

Protein expression in nontransfected HEK 293 cells

The first objective of this research design, as outlined in the research hypothesis, is to characterize protein expression in a variety of cells and tissues, including HEK 293 cells. This chapter focuses on the characterization of proteins in HEK 293 cells as it is both important and necessary to be aware of the endogenous expression of proteins in the system with which one is working. Furthermore, screening the HEK 293 cells for expression of specific proteins of interest may yield results that could provide further insight to the overall research project.

HEK 293 cells have been chosen to be used for several reasons. Firstly, they are a homogenous cell population which can be easily manipulated. In addition, the overexpression of proteins within HEK 293 cells can be controlled in order to simplify biochemical examination. Furthermore, previous findings have demonstrated that NMDARs, expressed in HEK 293 cells, elicit agonist-evoked current amplitudes and biophysical traits which parallel those evident in neurons (Chen et al., 1997; Dingledine et al., 1999). This result promotes the concept that HEK 293 cells are an ideal model system to study the connection between NMDAR ion channel properties and excitotoxicity (Anegawa et al., 1995; Boeckman and Aizenman, 1996; Raymond et al., 1996; Zeron et al., 2001).

HEK 293 cells were maintained as described in chapter 2. The cells were chosen to be harvested when they appeared to be quite confluent (a density of cells which covered ~90% of the plate). Cells were washed twice with warmed PBS, placed on ice with 1 ml solubilization buffer and scraped into Eppendorf tubes. Following sonication and

centrifugation the supernatant (total soluble protein) was isolated. Protein concentrations were determined using the bicinchoninic acid protein assay (BCA kit; Pierce). For the assessment of protein expression levels, total soluble protein from each cell lysate was loaded (5 or 10 μg per lane) and separated by 8% SDS-PAGE. Proteins were transferred to PVDF membranes and the Western blot protocol, as previously described, was carried out with primary antibodies for the following proteins: PSD-95, SAP-102, Chapsyn-110, HIP-1, α-actinin and actin. The significance and reasoning behind the selection of these proteins was outlined in chapter 1. In brief, these proteins have been shown to directly bind either NMDARs or htt (Kim et al., 1996; Hsueh et al., 1997; Kalchman et al., 1997; Wanker et al., 1997; Wyszynski et al., 1998; Dunah et al., 2000) and therefore serve as potential candidates which could indirectly link the NMDAR and htt, possibly contributing to the etiology of HD.

Using the aforementioned antibodies, HEK 293 cells were shown to endogenously express HIP-1, α-actinin and actin (figure 4). It should be noted that previous experiments have shown HEK 293 cells to also endogenously express wildtype human htt (Li et al., 1998; Chen et al., 1999). Antibodies specific for HIP-1, α-actinin and actin detected proteins of the appropriate molecular weights, 130, 110 and 42 kDa, respectively. Based on the intensities of the bands, it appears that α-actinin levels are quite high with more moderate levels of both HIP-1 and actin; however it is difficult to make direct comparisons based on different antibodies. This result confirms previous findings from our lab as well as other labs which have shown HEK 293 cells to endogenously express HIP-1 and α-actinin (Kalchman et al., 1997; Wanker et al., 1997; Zhang et al., 1998; Krupp et al., 1999).

Contrary to the endogenous expression of HIP-1, α-actinin and actin, no members of the PSD-95 family of proteins are present in HEK 293 cells (figure 5). To test for the expression of PSD-95 family members, 10 μg of the total soluble fraction from HEK 293 cell lysate was run along side 10 μg of total protein from three different brain regions (striatum, hippocampus and cortex) harvested from wildtype mice. The PSD-95 family members, including PSD-95, SAP-102 and Chapsyn-110, have all been shown to be present in the brain (Cho et al., 1992; Brenman et al., 1996; Lau et al., 1996). Therefore, the three lanes containing the brain tissue act as controls where we would expect the antibodies to detect their appropriate proteins. Bands corresponding to molecular weights of 95, 102 and 110 kDa were detected by antibodies specific for PSD-95, SAP-102 and Chapsyn-110, respectively. However, these bands were only present in the striatum, hippocampus and cortex and were absent in the HEK 293 cell lysate (figure 5).

Discussion of Results:

As previously mentioned, it was important to characterize the expression of the proteins of interest (PSD-95, SAP-102, Chapsyn-110, HIP-1, α-actinin and actin) in HEK 293 cells. This characterization was necessary to both reveal the endogenous expression of proteins in the system with which you are working and to gain results which could prove to be insightful and beneficial to the overall research project.

The total soluble fraction of HEK 293 cells was probed with several antibodies, specific for each of the six proteins of interest. It was determined that HEK 293 cells endogenously express HIP-1, α-actinin and actin (see figure 4). The existence of HIP-1 and α-actinin in HEK 293 cells is in agreement with previous findings from our lab as well as

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additional labs (Zhang et al., 1998). Additional experiments, outlined in chapter 5, serve to reveal the identity of the α-actinin isoform endogenously found in HEK 293 cells.

HIP-1 and α-actinin have been shown to bind to htt and NMDARs (NR1/NR2B), respectively (Kalchman et al., 1997; Gusella and MacDonald, 1998; Krupp et al., 1999). Furthermore, HIP-1 contains within its carboxy terminus a domain with homology to the protein talin (Hackam et al., 2000). Talin, a mammalian cytoskeletal-associated protein, is able to bind spectrin or proteins which contain spectrin-like repeats, including α-actinin (Rees et al., 1990). Therefore, it is suggested that HIP-1 and α-actinin may be able to directly bind, mediating an indirect interaction between NMDARs and htt. This will be discussed further in chapter 7.

In contrast, PSD-95, SAP-102 and Chapsyn-110 were not present in HEK 293 cells (see figure 5). This finding is in concordance with earlier publications which illustrated that PSD-95, SAP-102 and Chapsyn-110 could only be detected in HEK 293 cells following their overexpression (Tezuka et al., 1999; Sans et al., 2000; Savanenin et al., in press).
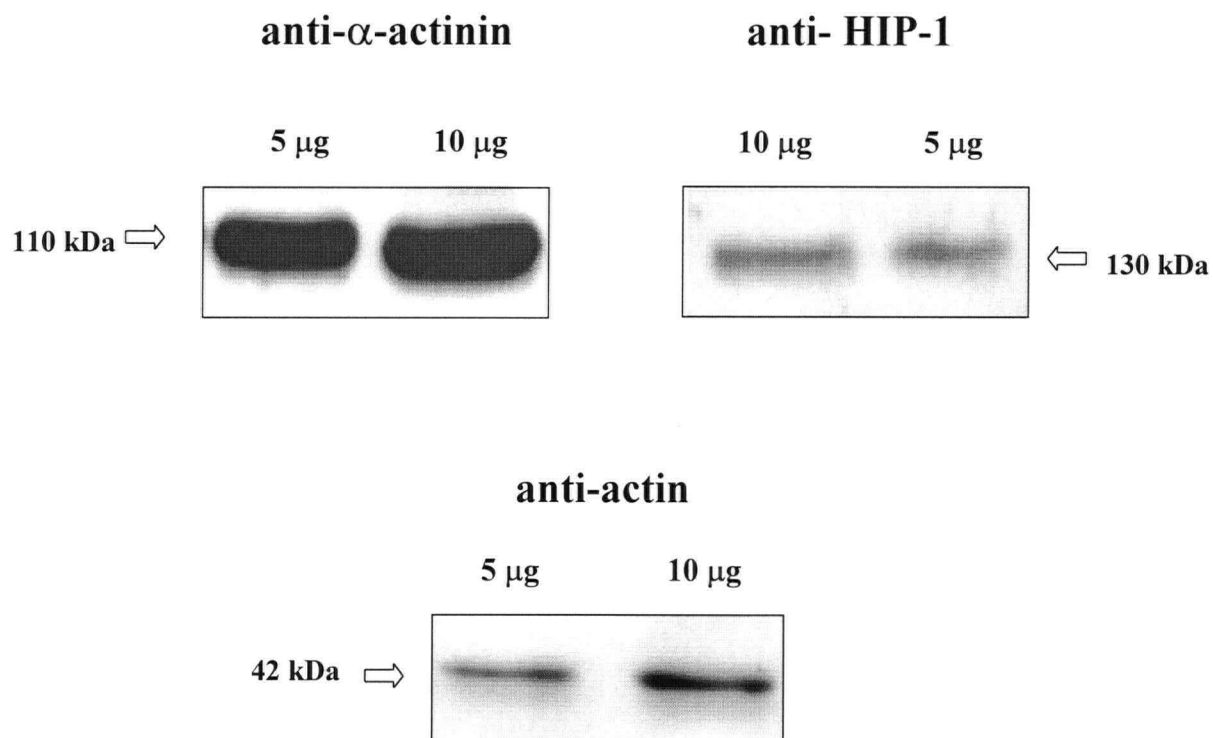
This characterization successfully analyzed protein expression in HEK 293 cells as evident by the consistency with previous findings. Additionally, analysis of the immunoblots was completed to ensure that each antibody recognized a band at the correct molecular weight. Characterization of the antibodies was necessary to ensure their effectiveness in subsequent experiments.

In addition to protein characterization in HEK 293 cells, similar experiments will be completed using mouse brain homogenate in chapter 4. As a result of the absence of PSD-95 family members in HEK 293 cells these proteins are of less interest in investigating functional interactions between NMDARs and htt. However, we remain interested in the

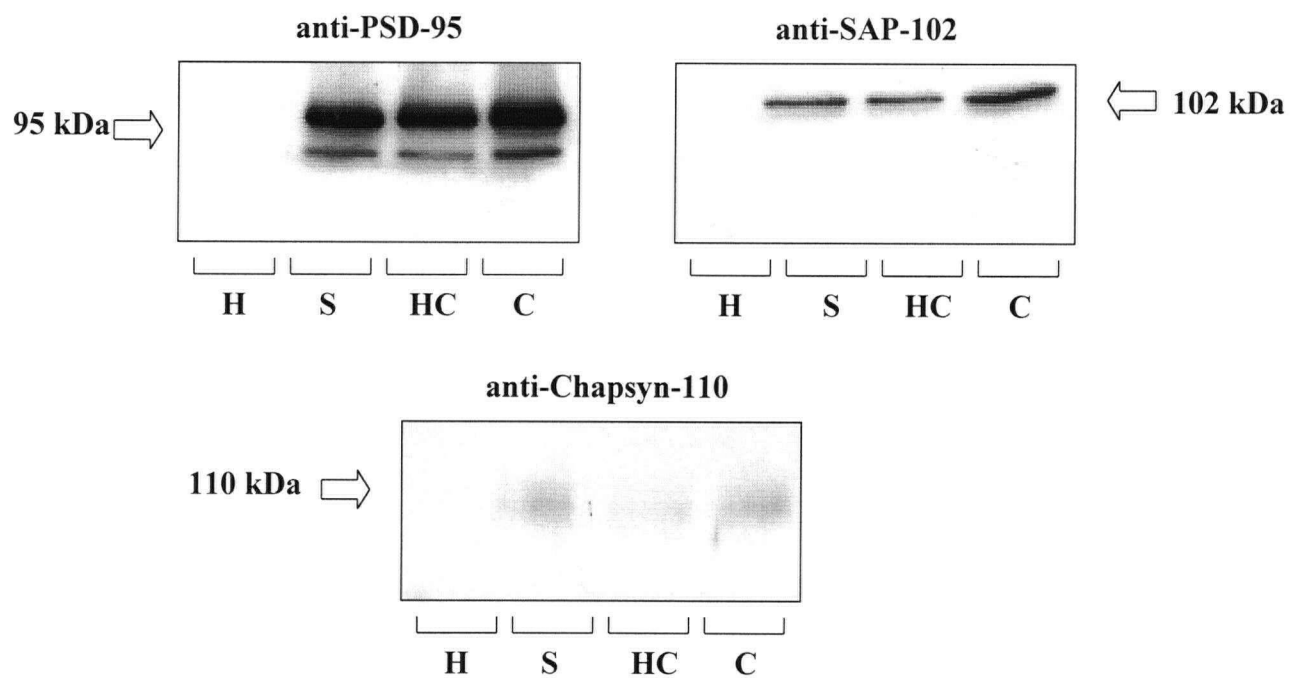distribution of the PSD-95 family members in the striatum relative to the cortex and hippocampus as this has not previously been completed using mouse tissues and the results might prove to be intriguing. The endogenous expression of HIP-1 and α-actinin in HEK 293 cells is of interest and will be further examined in chapters 4, 5 and 7 for the potential of mediating the functional interaction between mutant htt and NMDARs.

Figure 4. Expression of cytoskeletal proteins in nontransfected HEK 293 cells. The total soluble fraction of HEK 293 cells was collected and 5 and 10 μg was separated by SDS-PAGE. The blots were probed with antibodies specific for all of the proteins of interest. α-actinin, HIP-1 and actin specific antibodies revealed endogenous expression of these proteins. All three blots were exposed to film for 30s during development of the blots. This blot is representative of N=3 independent experiments.

anti-α-actinin

anti- HIP-1

5 μg   10 μg

10 μg   5 μg

110 kDa ⇨

⇦ 130 kDa

anti-actin

5 μg   10 μg

42 kDa ⇨

Figure 5. Absence of PSD-95 family members in nontransfected HEK 293 cells. Expression of PSD-95, SAP-102 and Chapsyn-110 in nontransfected HEK 293 cells (H) was compared to expression in striatum (S), hippocampus (HC) and cortex (C). 10 μg of total protein was loaded per lane and separated by SDS-PAGE. Antibodies specific for PSD-95, SAP-102 and Chapsyn-110 detected signals only in the brain regions. All three blots were exposed to film for 30s during development of the blots. This blot is representative of N=3 independent experiments.

anti-PSD-95

95 kDa ⟹

H   S   HC   C

anti-SAP-102

⟸ 102 kDa

H   S   HC   C

anti-Chapsyn-110

110 kDa ⟹

H   S   HC   C

CHAPTER 4

Expression of cytoskeletal proteins in wildtype and transgenic

mouse brain regions

To fulfill the first objective of this research project, characterization of protein expression in wildtype (a FVB/N strain) and transgenic mice (YAC18 and YAC46) was also completed. As already eluded to in chapter 3, six proteins (PSD-95, SAP-102, Chapsyn-110, HIP-1, α-actinin and actin) had been chosen to be characterized based on their ability to either interact with NMDARs or htt. We hypothesize that these cytoskeletal-associated proteins could mediate an association between NMDARs and mutant htt, which could contribute to changes in NMDAR properties and result in their overactivation. The excessive stimulation of NMDARs could, in part, lead to the selective neurodegeneration evident in HD. Therefore, it is initially necessary to determine both the presence and abundance of these proteins in the mouse brain regions before we investigate any hypothesized interactions. Although an inspection of the expression of several of the proteins of interest, including PSD-95, SAP-102, Chapsyn 110, actin and α-actinin (Shorte, 1997; Allison et al., 1998; Wyszynski et al., 1998; Firestein et al., 1999) had already been carried out using rat brain homogenates, similar protein characterization remained to be completed in mouse tissues.

The tissues utilized were harvested from both wildtype and transgenic mice in the age range of 10-12 weeks. Tissues were collected as described in chapter 2. In brief, the mice were anesthetized with halothane and decapitated. Following decapitation the anterior portion of the striatum, the hippocampus and a section of the cortex were removed and

homogenized in 1X RIPA buffer. For each brain region, tissue from ~ 6 animals was pooled. The bicinchoninic acid protein assay (BCA kit; Pierce) was used to measure protein concentrations. For the assessment of protein expression levels, total protein from each brain region was loaded at a concentration of 5 or 10 µg per lane, and separated by 8% SDS-PAGE. Proteins were transferred to PVDF membranes and the Western blot protocol, as previously described, was completed using the following primary antibodies: anti-PSD-95, anti-SAP-102, anti-Chapsyn-110, anti-HIP-1, anti-α-actinin, anti-actin and anti-htt. Protein characterization of the expression levels of all six proteins in wildtype, YAC18 and YAC46 tissue was carried out. The blots were exposed to film for varying times in the range of 10-30s depending on the primary antibody which was used.

All of the proteins which we probed for were present in the striatum, hippocampus and cortex of wildtype and transgenic mice (figures 6-8). Based on the intensities of the bands, PSD-95, SAP-102 and α-actinin appear to be in the highest abundance. Relative to these proteins, there is a more intermediate expression of HIP-1, actin and htt. Furthermore, the Western blots suggest a low expression of Chapsyn 110 in these brain regions. However, it should be noted that this attempt to quantify protein levels is somewhat arbitrary as several different antibodies were used and the differences in band intensities could be partially the result of the quality of the antibody.

There are two trends which are evident and exist for the majority of the proteins in figures 6-8. Firstly, each protein appears to display similar levels of protein expression among wildtype, YAC18 and YAC46 tissues. An accurate comparison would require running tissue from the 3 different groups on the same gel. The exception is htt (compare figure 6 to figures 7 and 8) which shows elevated protein levels in YAC18 and YAC46

tissues. The triviality of this finding will be discussed at the conclusion of this chapter. The second trend concerns protein expression among the striatum, hippocampus and cortex. For any given protein, there is no apparent difference in protein levels among the brain regions. Of the seven proteins examined, none of them is preferentially enhanced in the striatum, which would have been intriguing and of particular importance in proposing a potential mechanism which could contribute to the etiology of HD.

Five of the seven primary antibodies which were utilized produced one band on the Western blots which corresponded to their specific protein of the appropriate molecular weight. However, the anti-PSD-95 antibody generated two bands, one at ~95 kDa and a second band at a slightly lower molecular weight (figures 5, 6 and 8). This finding is consistent with previous data which also detected a lower molecular weight species, using the PSD-95 specific antibody (Kim et al., 1996). The α-actinin-4 antibody would inconsistently produce a second band of ~130 kDa in addition to the one at the appropriate molecular weight of ~110 kDa (figures 7 and 8). Speculation of the identity of these bands will be described in detail in the discussion contained within this chapter.

Lastly, during the process of characterizing cytoskeletal proteins in mouse brain homogenates we were fortunate to gain access to antibodies specific for two isoforms of α-actinin, α-actinin-2 and α-actinin-4. Using these antibodies, it was determined that α-actinin-4 is expressed in the three brain regions and α-actinin-2 is absent. The expression of α-actinin-2 vs. α-actinin-4 within the brain is controversial and warrants more attention (see chapter 5). As a result, the expression of these two isoforms of α-actinin in both HEK 293 cells and mouse brain regions will be examined further in chapter 5.

Discussion of Results:

Supplementing the protein characterization in HEK 293 cells, similar experiments were carried out in wildtype and transgenic mice (YAC18 and YAC46). The reason for completing this task was two-fold in nature. Firstly, characterization of our proteins of interest using mouse brain homogenate had not been reported previously. Hence, it was necessary to investigate the expression levels of these proteins in the brain, in particular the striatum, hippocampus and cortex. Additionally, the third objective of this research project was to attempt to elucidate a novel interaction between cytoskeletal-associated proteins which may facilitate an indirect link between htt and NMDARs. Therefore, this protein characterization in mouse brain regions was completed first, in both normal (wildtype and YAC18) and HD-affected (YAC46) mice, in anticipation that a trend in protein expression levels might be detected which could possibly implicate a role for a select portion of the proteins of interest in the etiology of HD. Their involvement, specifically in a novel protein-protein interaction, could then be further explored.

Contrary to the HEK 293 cells, all proteins of interest (PSD-95, SAP-102, Chapsyn-110, HIP-1, actin, α-actinin and htt) were present in the striatal, hippocampal and cortical tissues from both wildtype and transgenic (YAC18 and YAC46) mice. These results are in accord with data gathered by previous groups which illustrate the presence of these proteins in the rat brain (Kalchman et al., 1997; Shorte, 1997; Wanker et al., 1997; Allison et al., 1998; Wyszynski et al., 1998; Firestein et al., 1999).

Despite the consistency in terms of presence of the proteins, a discrepancy arose with regards to abundance, in particular Chapsyn-110 protein levels. Earlier findings report that the cortex, hippocampus and basal ganglia contain substantially high Chapsyn-110 protein

levels (Kim et al., 1996). Examination of figures 6-8 reveal that relatively low levels of Chapsyn-110 protein is present in striatal, hippocampal and cortical tissues from wildtype and transgenic (YAC18 and YAC46) mice. This variance could be due to several different factors. Firstly, the use of different animals, in particular rat vs. mouse, may partially be responsible for the difference. Additionally and more likely, different Chapsyn-110 primary antibodies were used in the two sets of experiments. A better quality antibody could account for the higher expression levels of Chapsyn-110 reported by Kim et al., 1996.

Unfortunately, regardless of our desire to find a trend in protein expression levels which would help us identify proteins potentially involved in HD, no such trends were observed. Of the seven proteins examined, none of them is selectively elevated in the striatum or HD-affected transgenic mice (YAC46), which would have been interesting and significant in proposing a potential mechanism which could contribute to the neurodegeneration in HD. Comparison of figures 6-8 showed apparent enhanced levels of htt in YAC18 and YAC46 mice vs. wildtype mice. This elevation is most likely due to the increased transgene copy number (Hodgson et al., 1999). The creators of the transgenic mice previously published that the human htt expression levels in the mice were estimated to be 2-3 times that of mouse endogenous protein (Hodgson et al., 1999).

As already mentioned, two of the seven primary antibodies which were used, anti-PSD-95 and anti-α-actinin-4, generated an additional band at a lower (~85 kDa) and higher (~130 kDa) molecular weight, respectively. The existence of a lower molecular weight band, when using the PSD-95 specific antibody, was also found by other groups (Kim et al., 1996; Sans et al., 2000). It is postulated that the lower molecular weight band is indicative of an immaturely processed form of PSD-95 (Kim et al., 1996). The additional band evident

with the α-actinin-4 specific antibody has not previously been reported. One possibility is that the antibody is reacting with a splice variant or post-translationally modified (i.e. phosphorylation, glycosylation) form of α-actinin-4.

At the conclusion of this set of experiments it was determined that all of the proteins of interest were expressed in striatal, hippocampal and cortical tissues of both wildtype and transgenic mice (YAC18 and YAC46). While the majority of these results were consistent with previous findings one exception arose with regards to α-actinin. In contrast to other groups which state α-actinin-2 as the endogenous isoform found in both HEK 293 cells and forebrain structures (Wyszynski et al., 1997; 1998; Zhang et al., 1998; Dunah et al., 2000), our results report α-actinin-4 as the endogenous form in HEK 293 cells, striatal, hippocampal and cortical tissues. In order to address this controversy additional experiments were designed and completed and will be discussed in the following chapter.

Figure 6. Protein Characterization in wildtype mice brain regions. 5 and 10 μg of total protein, per brain region, was separated by SDS-PAGE. Antibodies specific for the proteins of interest (PSD-95, SAP-102, Chapsyn-110, HIP-1, α-actinin-4, actin and htt) were used to probe the different brain regions. The brain regions examined include striatum (S), hippocampus (H) and cortex (C). This blot is representative of N=3 independent experiments.

anti-SAP-102

anti-PSD-95

anti-HIP-1

anti-Chapsyn-110

anti-actin

anti-α-actinin-4

anti-htt

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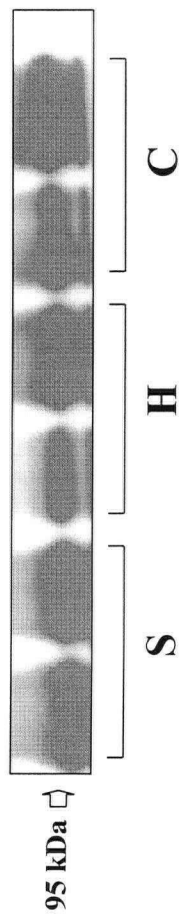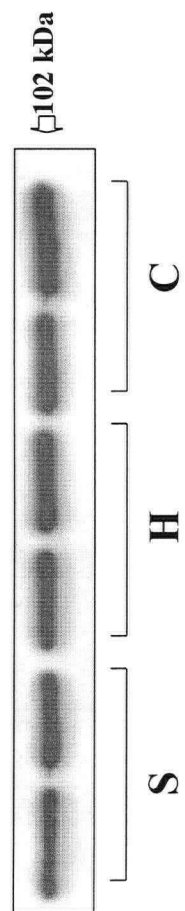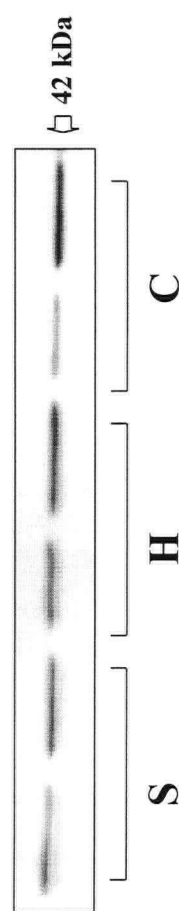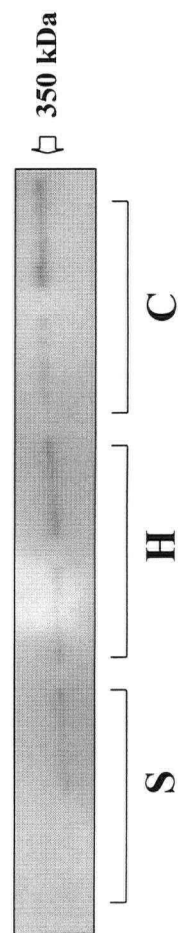
Figure 7. Protein Characterization in YAC18 mice brain regions. 5 and 10 μg of total protein, per brain region, was separated by SDS-PAGE. Antibodies specific for the proteins of interest (PSD-95, SAP-102, Chapsyn-110, HIP-1, α-actinin-4, actin and htt) were used to probe the different brain regions. The brain regions examined include striatum (S), hippocampus (H) and cortex (C). This blot is representative of N=3 independent experiments.
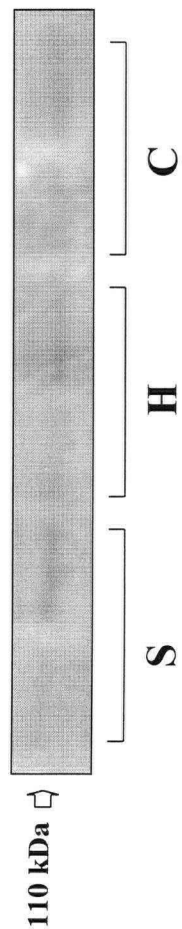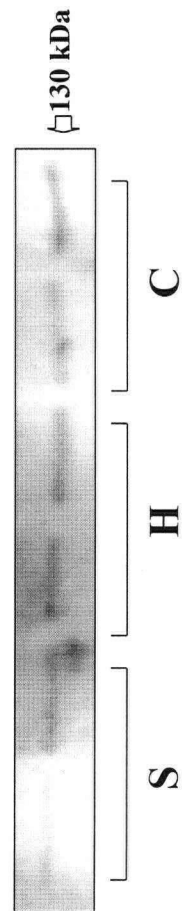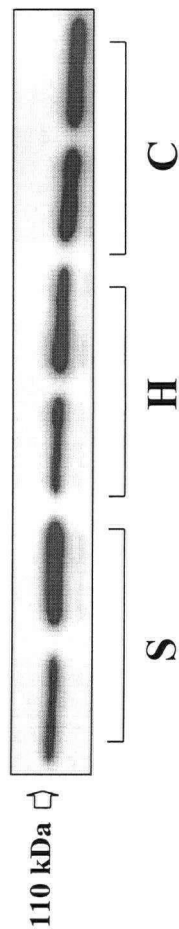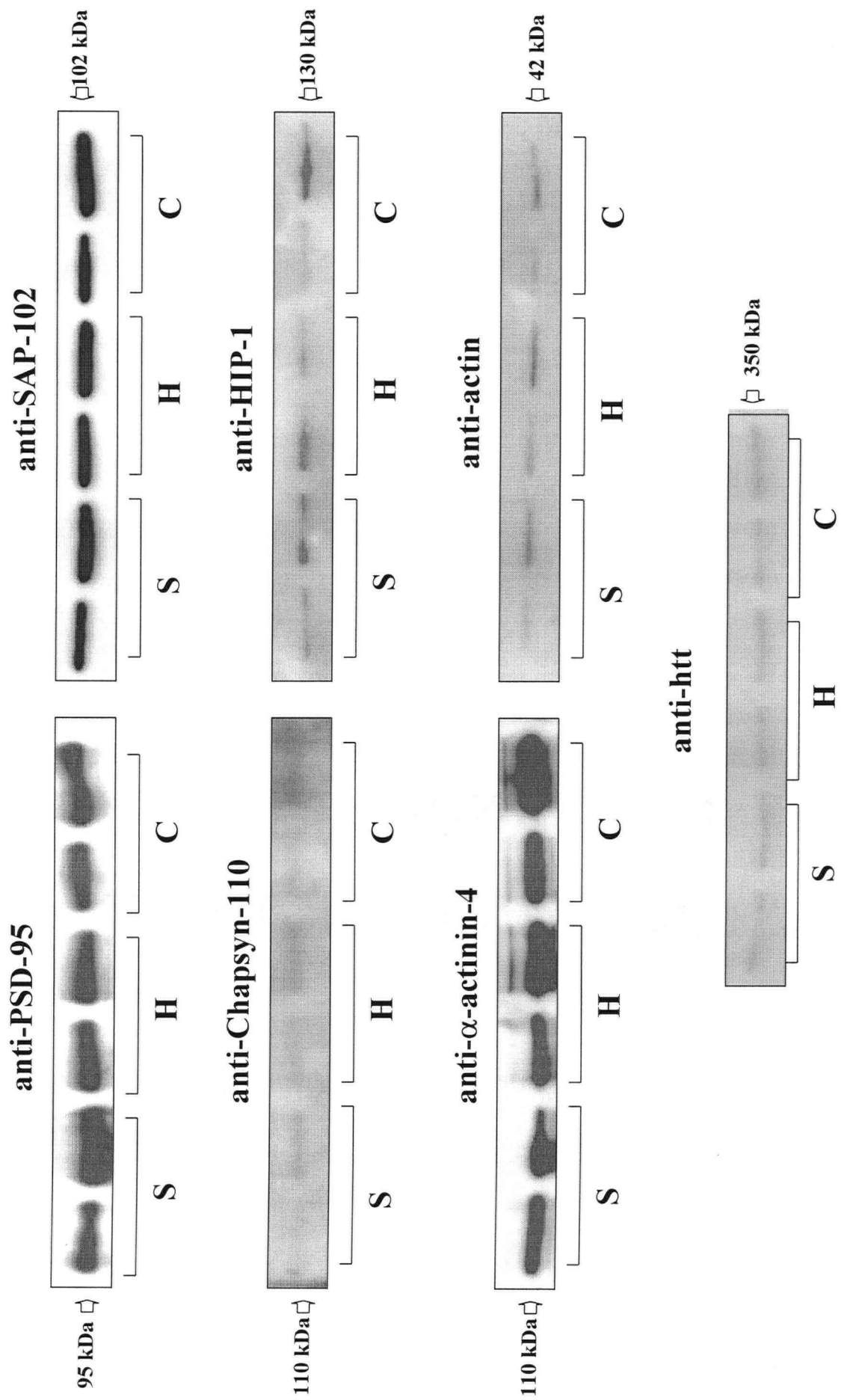
Figure 8. Protein Characterization in YAC46 mice brain regions; striatum (S), hippocampus (H) and cortex (C). 5 and 10 μg of total protein, per brain region, was separated by SDS-PAGE. Antibodies specific for the proteins of interest (PSD-95, SAP-102, Chapsyn-110, HIP-1, α-actinin-4, actin and htt) were used to probe the different brain regions. This blot is representative of N=3 independent experiments.

anti-SAP-102
102 kDa

anti-PSD-95
95 kDa

anti-HIP-1
130 kDa

anti-Chapsyn-110
110 kDa

anti-actin
42 kDa

anti-α-actinin-4
110 kDa

anti-htt
350 kDa

C   H   S
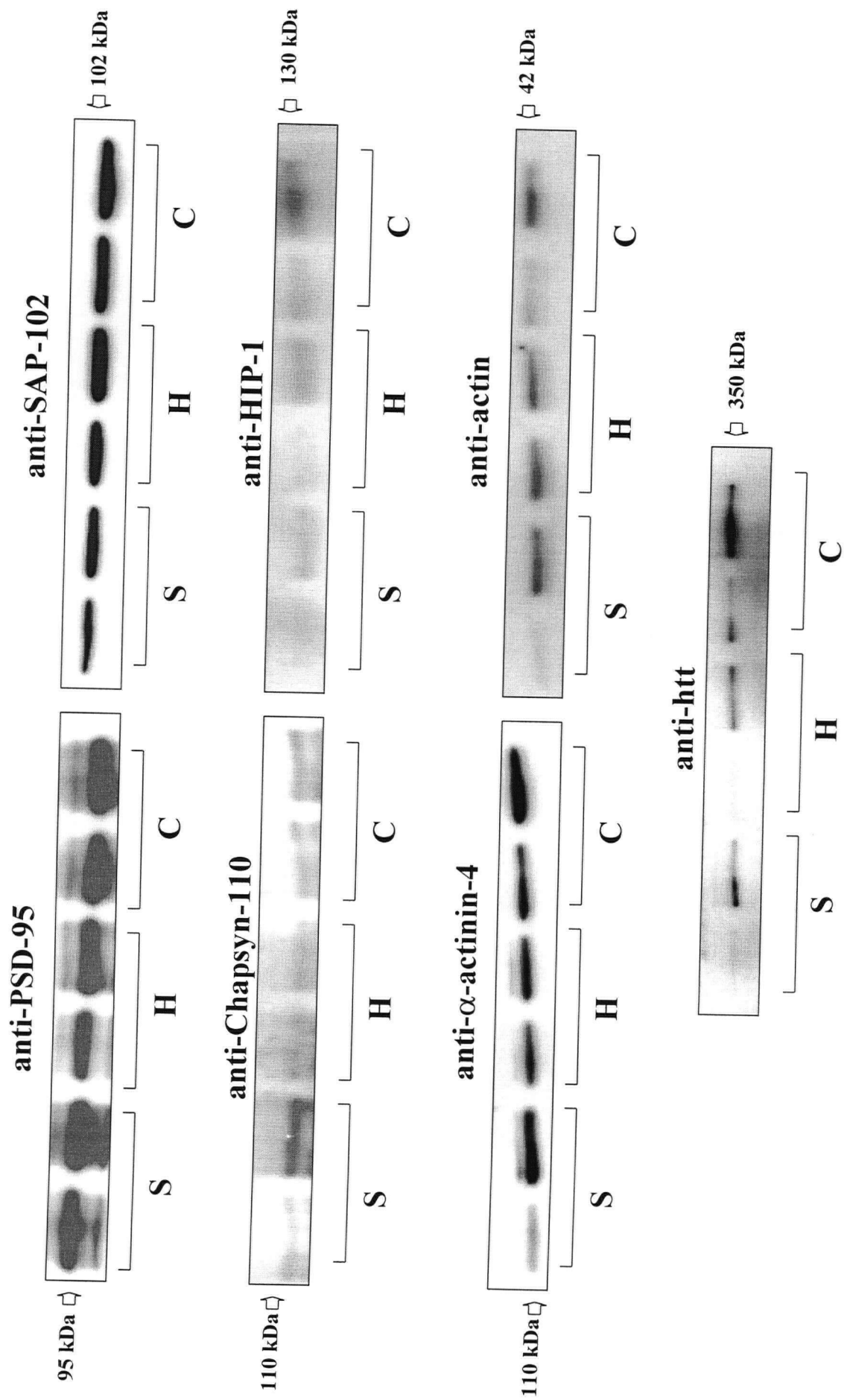
CHAPTER 5

Comparison of α-actinin-2 vs. α-actinin-4 localization

5.1 Expression of α-actinin-2 vs. α-actinin-4 in HEK 293 cells

Previously, to test for the expression of α-actinin in nontransfected HEK 293 cells, we used a commercially available antibody against α-actinin (Sigma-Aldrich, Oakville, ON, CANADA) which is not isoform specific. To compare the expression of α-actinin-2 vs. α-actinin-4 in HEK 293 cells, we designed a supplementary experiment which incorporated the use of two additional antibodies specific for α-actinin-2 and α-actinin-4.

HEK 293 cells were co-transfected with cDNAs encoding either α-actinin-2 or α-actinin-4 and β-gal. In parallel, a plate of HEK 293 cells which were not transfected was maintained. Twenty-four hours following transfection the total soluble fractions from both the transfected and nontransfected HEK 293 cells were collected. The bicinchoninic acid protein assay (BCA kit; Pierce) was used to measure protein concentrations. For the assessment of protein expression levels, the total soluble fraction from α-actinin-2 transfected, α-actinin-4 transfected and nontransfected HEK 293 cells was loaded at a concentration of 5 or 10 μg per lane, and separated by 8% SDS-PAGE. Proteins were transferred to PVDF membranes and the Western blot protocol, as previously described, was completed using the following primary antibodies: anti-α-actinin-2, anti-α-actinin-4 and anti-α-actinin (Sigma).

The α-actinin-2 antibody detected a band at the appropriate molecular weight, 110 kDa, only in the α-actinin-2 transfected HEK 293 cells (figure 9). In the lanes which

contained either the α-actinin-4 transfected or the nontransfected HEK 293 cells no bands were evident. This finding is supported by previous labs which dispute the reports of endogenous expression of α-actinin-2 in HEK 293 cells (Krupp et al., 1999; Anders et al., 2000; Maruoka et al., 2000). In contrast, both the α-actinin-4 specific and the non-specific α-actinin antibodies detected bands of 110 kDa in the lanes containing cells from all three conditions (figure 9). Densitometric analysis of the blot probed with the α-actinin-4 specific antibody reveals an elevation in protein levels of ~1.3 times in the lane containing lysate from α-actinin-4 transfected vs. nontransfected HEK 293 cells (figure 9). Moreover, the α-actinin-4 specific antibody detects protein, of the appropriate molecular weight, in lanes containing the total soluble fraction of protein from α-actinin-2 transfected HEK 293 cells which is roughly comparable to levels seen in the nontransfected HEK 293 cells. The intensity of the band detected in the lane containing α-actinin-2 transfected HEK 293 cells was calculated to be only ~1.05 times that observed in the lane containing the nontransfected HEK 293 cells. These results support the notion that α-actinin-4 is endogenously expressed in HEK 293 cells and that the α-actinin-4 specific antibody shows little, if any, cross-reactivity with α-actinin-2.

Finally, it can be stated that since these results indicate that α-actinin-4 is expressed endogenously in HEK 293 cells and the non-isoform specific α-actinin antibody (Sigma) detects a protein in nontransfected HEK 293 cells, the commercially available antibody appears to be able to detect α-actinin-4. Quantitative analysis illustrates that the intensities of the bands of the lanes containing α-actinin-2 and α-actinin-4 transfected HEK 293 cells, when using the Sigma α-actinin antibody, were ~1.28 and 2.64 times that measured for the

lanes containing the nontransfected cells, respectively. It can therefore be inferred that the Sigma antibody can definitely recognize α-actinin-4 and may be able to also recognize α-actinin-2. This will be discussed further at the conclusion of this chapter.

5.2 Expression of α-actinin-2 vs. α-actinin-4 in wildtype striatal, hippocampal, cortical and heart tissues

Supplementing the experiments completed in HEK 293 cells, expression of α-actinin-2 vs. α-actinin-4 in wildtype striatal, hippocampal, cortical and heart tissues was also examined. This remains a controversial topic as several different groups differ in the opinion of which isoform is the predominant form found in brain (Honda et al., 1998; Wyszynski et al., 1998; Dunah et al., 2000).

The tissues were collected as described in chapter 2. In brief, wildtype mice, aged 10-12 weeks, were anesthetized and decapitated to allow for the harvesting of the anterior portion of the striatum, the hippocampus and a section of the cortex. In addition, a midline incision was made along the chest of the animal to allow for the removal of a section of the heart. Once removed the tissues were homogenized in 1X RIPA buffer. The bicinchoninic acid protein assay (BCA kit; Pierce) was used to measure protein concentrations. For the assessment of protein expression levels, total protein from each brain region and heart was loaded at a concentration of 5 or 10 μg per lane, and separated by 8% SDS-PAGE. Proteins were transferred to PVDF membranes and the Western blot protocol, as previously described, was completed using the following primary antibodies: anti-α-actinin-2, anti-α-actinin-4 and anti-α-actinin (Sigma). The heart tissue was included as a positive control as

both α-actinin-2 and α-actinin-4 have previously been reported to be found in cardiac tissue (Chan et al., 1998; Honda et al., 1998; Maruoka et al., 2000).

Immunoblot analysis revealed that the α-actinin-2 specific antibody reacted with a single protein of ~110 kDa contained only in the heart tissue (figure 10). No equivalent bands were evident in striatal, hippocampal or cortical tissues. Interestingly, this is in direct contrast to other labs which have reported high expression of α-actinin-2 in forebrain structures, particularly the striatum, hippocampus and cortex (Wyszynski et al., 1997, 1998; Dunah et al., 2000).

Contrary to α-actinin-2 expression, the α-actinin-4 specific antibody recognized protein of ~ 110 kDa in all three brain regions and the heart (figure 10). Immunoblot data revealed that highest levels of α-actinin-4 are present in the heart (lanes 7 and 8) and intermediate levels in the striatum, hippocampus and cortex (lanes 1 and 2; 3 and 4; 5 and 6, respectively). The α-actinin-4 levels in the three brain regions are relatively comparable with no one region selectively displaying elevated protein levels.

Similarly, the blot which was probed by the Sigma α-actinin antibody displays an identical pattern to the blot probed with the α-actinin-4 antibody (figure 10). This finding paired with the results in the HEK 293 cells (figure 9) lends support to the notion that this antibody is able to recognize α-actinin-4.

Discussion of Results:

As mentioned earlier, controversy exists regarding the predominant isoform of

α-actinin found in both HEK 293 cells and brain regions. In HEK 293 cells we addressed this concern by designing an experiment which probed α-actinin-2 transfected, α-actinin-4 transfected and nontransfected cells with three antibodies: one commercially available non-isoform specific α-actinin antibody (Sigma) and two antibodies specific for either α-actinin-2 or α-actinin-4. It was shown that α-actinin-2 was detected only in the α-actinin-2 transfected cells. However, α-actinin-4 was expressed in cells from all three conditions, indicating that α-actinin-4 is endogenously expressed in HEK 293 cells.

Several other labs have also found α-actinin-2 to be absent from HEK 293 cells (Krupp et al., 1999; Anders et al., 2000; Maruoka et al., 2000). These labs performed similar experiments to those detailed earlier in this chapter and dispute findings which report a high level of endogenous α-actinin-2 in HEK 293 cells (Zhang et al., 1998). Paralleling our data, their findings state that only after overexpression of α-actinin-2 in HEK 293 cells were they able to detect α-actinin-2 (Krupp et al., 1999, Maruoka et al., 2000).

An essential concern when utilizing the three different α-actinin antibodies was ensuring the absence of cross-reactivity with the isoform specific antibodies (α-actinin-2 and α-actinin-4 specific antibodies) and attempting to determine the isoform specificity of the commercially available α-actinin antibody (Sigma). It was obvious from examination of figure 9 that the α-actinin-2 antibody does not recognize α-actinin-4 as bands were only evident in lanes containing α-actinin-2 transfected HEK 293 cells. On the contrary, the α-actinin-4 specific antibody produced bands in all lanes (see figure 9). Densitometric analysis was then completed to quantify protein levels in the lanes containing each of the three conditions; nontransfected; α-actinin-2 transfected; and α-actinin-4 transfected HEK

293 cells. Analysis demonstrated that protein levels were elevated in the α-actinin-4 transfected HEK 293 cells relative to the nontransfected and α-actinin-2 transfected HEK 293 cell lanes. This is consistent with an endogenous expression of α-actinin-4 in HEK 293 cells. The increase in the lanes containing α-actinin-2 transfected vs. nontranfected HEK 293 cells is minimal (~1.05 times) and can most likely be attributed to slight differences in experimental procedures as opposed to the α-actinin-4 antibody displaying cross-reactivity. Therefore, these results are consistent with the α-actinin-4 antibody being specific for α-actinin-4 and unable to detect α-actinin-2.

Lastly, we attempted to determine the identity of the α-actinin isoform(s) which the commercially available antibody was able to recognize. As already mentioned, we believe that α-actinin-4 is endogenously expressed in HEK 293 cells. Due to the fact that the Sigma α-actinin antibody detects a band in nontranfected HEK 293 cells, we concluded that this antibody is able to detect α-actinin-4. In contrast to this antibody's ability to recognize α-actinin-4, we remain unsure of its ability to detect α-actinin-2. Despite elevated protein levels detected by this antibody in lanes containing α-actinin-4 transfected cells (~2.64 times compared to nontransfected cells) the protein levels were only marginally increased in α-actinin-2 transfected cells (~1.28 times compared to nontransfected cells). This could be indicative of the Sigma antibody's capability to recognize the endogenous isoform (α-actinin-4) and the increase could be the result of the additional α-actinin-2 or experimental error. In order to unambiguously determine the ability of this Sigma antibody to recognize α-actinin-2, it would be necessary to probe a cell type which is known to express only α-

actinin-2 and not α-actinin-4. Until this experiment is completed we will complete our future experiments assuming that this antibody conclusively recognizes only α-actinin-4.

These results indicate that α-actinin-4 and not α-actinin-2 is endogenously found in HEK 293 cells. Further experiments would be necessary to determine if HEK 293 cells endogenously express either of the additional α-actinin isoforms, namely α-actinin-1 and α-actinin-3.

Perhaps the more significant issue concerning the localization of α-actinin-2 vs. α-actinin-4 is with regards to expression in several brain regions, including the striatum, hippocampus and cortex. Techniques which utilized brain homogenate or brain cDNA libraries have shown α-actinin isoforms, including α-actinin-2 and α-actinin-4, to be involved with several cellular functions including: interacting and modulating NMDARs (NR1/NR2B); binding to numerous proteins, including densin-180, BERP and PKN; regulation of the actin cytoskeleton and cellular motility (Mukai et al., 1997; Wyszynski et al., 1997, 1998; Honda et al., 1998; Krupp et al., 1999; Dunah et al., 2000; El-Husseini et al., 2000; Walikonis et al., 2000). Although both α-actinin-2 and α-actinin-4 have been attributed functions within the brain it has not yet been conclusively determined that both of these α-actinin isoforms are in fact present in the brain. Therefore, it is necessary to eliminate any discrepancies concerning the expression of α-actinin isoforms within the brain. Once this problem is rectified a more precise understanding of the physiological role of α-actinin in the brain can be more accurately explored.

In order to analyze the expression of α-actinin-2 vs. α-actinin-4 in mouse brain regions we closely replicated the experimental design which we utilized for the HEK 293 cells. In general, we probed striatal, hippocampal, cortical and heart tissues from wildtype

with three α-actinin antibodies: anti-α-actinin (Sigma), anti-α-actinin-2 and anti-α-actinin-4. Results reveal that α-actinin-4 is expressed in all four tissue types. However, α-actinin-2 is only present in heart tissue.

The expression of α-actinin-4 in the striatum, hippocampus, cortex and heart is consistent with previous findings from the laboratory which isolated this protein three years ago (Honda et al., 1998). In concordance with our data, they detected α-actinin-4 in whole brain and heart tissues (Honda et al., 1998; El-Husseini et al., 2000). Our work served to further explore α-actinin-4 expression in the brain by examining individual structures, namely the striatum, hippocampus and cortex.

In contrast to our α-actinin-4 results which proved to be in accordance with published findings, our α-actinin-2 expression data, with regards to the brain, directly opposes earlier discoveries. It has previously been reported that α-actinin-2 is differentially expressed in several brain regions with prominence in forebrain structures, particularly in the striatum, hippocampus and cortex (Wyszynski et al., 1997, 1998; Dunah et al., 2000). While the discrepancy in these findings cannot be completely understood speculations can be put forward in an attempt to decipher this substantial difference. Firstly, this could be the result of the antibody which was used. The other research groups report the use of a commercially available α-actinin antibody (Sigma) to derive a portion of their results. Earlier in this chapter we discuss the specificity of this antibody. We report that this antibody is able to recognize α-actinin-4 but we cannot definitely state that it also recognizes α-actinin-2 (see figures 9 and 10), and it is certainly not specific for α-actinin-2. Therefore, the use of this antibody in the probing of brain tissue may have led individuals to falsely report expression of α-actinin-2 when in reality they were detecting α-actinin-4. Secondly, in addition to

immunoblot techniques one of the groups used *in situ* hybridization to examine the expression of α-actinin-2 in the brain. Their probes were generated against a region of nucleotides in α-actinin-2 which shares ~70% nucleotide identity with α-actinin-4. Due to this homology between α-actinin-2 and α-actinin-4 it is conceivable that their probes could detect α-actinin-4 and similar to the antibody situation they may be reporting the incorrect α-actinin isoform.

Although it is obvious that our data contradicts others with regards to α-actinin-2 expression in the brain, we do concur with respect to α-actinin-2 expression in the heart. All groups involved have detected α-actinin-2 in cardiac tissue (Chan et al., 1998; Maruoka et al., 2000).

Presently, the debate remains concerning the predominant α-actinin isoform located in the brain. Interestingly, the other laboratories have mentioned using, in addition to the Sigma α-actinin antibody, an α-actinin-2 specific antibody termed 4B2 (Wyszynski et al., 1997). To help resolve the controversy surrounding the expression of α-actinin-2 vs. α-actinin-4 in both HEK 293 cells and brain regions it would be essential to repeat the experiments using this alternative α-actinin-2 antibody. The resulting data could then be used confirm or dispute our previous findings and finally elucidate the true identity of the α-actinin isoform found within the brain.

Determining the identity of the brain α-actinin isoform is necessary because while all isoforms display high homology in the spectrin repeats which would conceivably allow all to interact with NMDARs (NR1 and NR2B), a functional difference between the muscle (α-actinin-2) and non-muscle (α-actinin-4) α-actinin isoforms exists in their calcium sensitivity

(Chan et al., 1998). Although both α-actinin-2 and α-actinin-4 contain two EF-hand calcium binding motifs in their C-terminus, these motifs are only functional in the non-muscle isoforms (Chan et al., 1998). Interestingly, studies examining the interaction of α-actinin-2 and NR1 subunits have shown that Ca^{2+}/calmodulin (CaM) can displace α-actinin-2 from NR1 following postsynaptic Ca^{2+} influx, which may have entered through NMDARs (Wyszynski et al., 1997). However, this same effect was not observed to occur with regards to the interaction between α-actinin-2 and NR2B subunit as Ca^{2+}/CaM is unable to bind to the cytoplasmic tail of NR2B (Wyszynski et al., 1997). Unfortunately, similar experiments have not yet been completed for α-actinin-4, however it can be speculated that Ca^{2+} alone may be sufficient to displace α-actinin-4 from NMDARs and/or actin (Krupp et al., 1999). This functional difference reconfirms the need to identify the α-actinin isoform found within the brain.

Lastly, the aforementioned experiments attempted to determine the identity of the α-actinin isoform found within in the brain. We focused on examining the expression of α-actinin-2 and α-actinin-4 as we only had access to antibodies specific for these two isoforms. However, an additional group has reported the identification of α-actinin-1 in the postsynaptic density fraction, obtained from rat forebrains, by mass spectrometry (Walikonis et al., 2000). They state that the molecular masses of the peptides from the α-actinin band unambiguously identify the isoform in the PSD fraction as a homolog of human α-actinin-1 (Walikonis et al., 2000). Due to these findings it would be important to repeat the experiment in chapter 5 using an additional antibody specific for α-actinin-1. Once the identity of the brain α-actinin isoform is discovered its physiological function within the

brain, including any potential modulating effects of NMDARs and contributions to the

pathogenesis of HD, can be determined.

Figure 9. Comparison of α-actinin-2 vs. α-actinin-4 expression in HEK 293 cells. HEK 293 cells were transfected with either α-actinin-2 or α-actinin-4. 24 hours following transfection, the total soluble fraction of the cells was collected. 5 and 10 μg of total protein was separated by SDS-PAGE for each of the three conditions: α-actinin-2 transfected (α-ac2), α-actinin-4 transfected (α-ac4) and nontransfected (NT) HEK 293 cells. The blots were probed with three different antibodies: anti-α-actinin-2, anti-α-actinin-4 and a Sigma anti-α-actinin. All three blots were exposed to film for 30s during development. This blot is representative of N=3 independent experiments.

anti-α-actinin-2

anti-α-actinin-4

anti-α-actinin (Sigma)
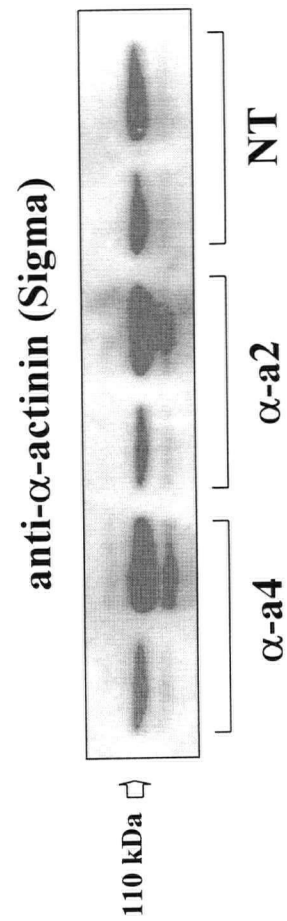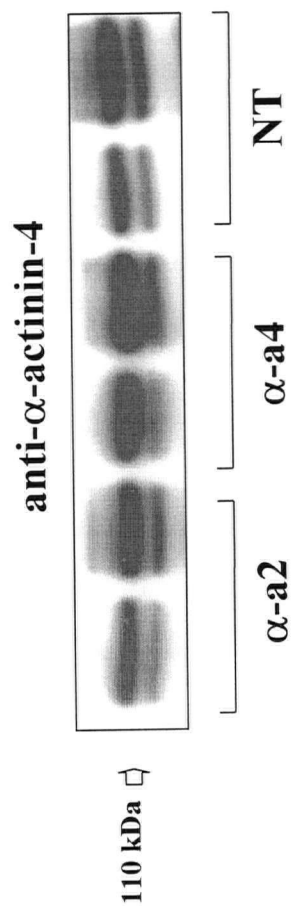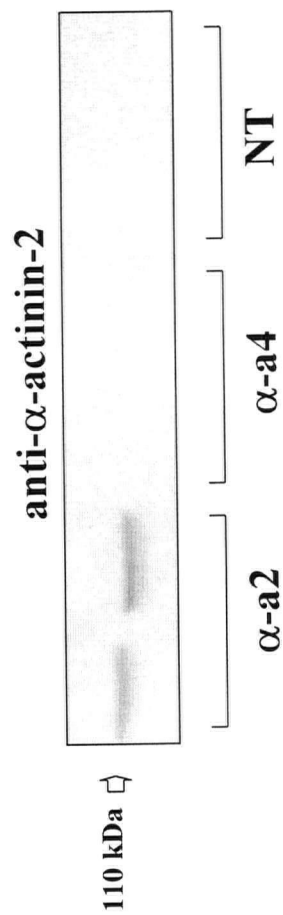
Figure 10. Comparison of α-actinin-2 vs. α-actinin-4 expression in wildtype mouse tissues. The mouse tissues examined include striatum (S), hippocampus (H), cortex (C) and heart (He). 5 and 10 µg of total protein, per tissue, was separated by SDS-PAGE. The blots were probed with three different antibodies: anti-α-actinin-2, anti-α-actinin-4 and a Sigma anti-α-actinin. All three blots were exposed to film for 30s during development. This blot is representative of N=3 independent experiments.
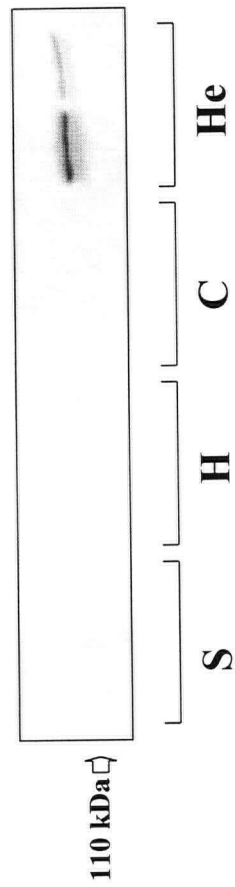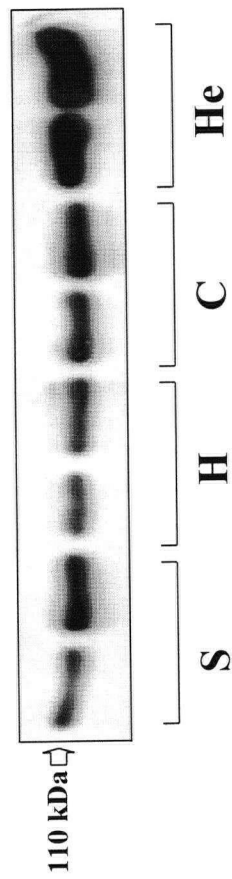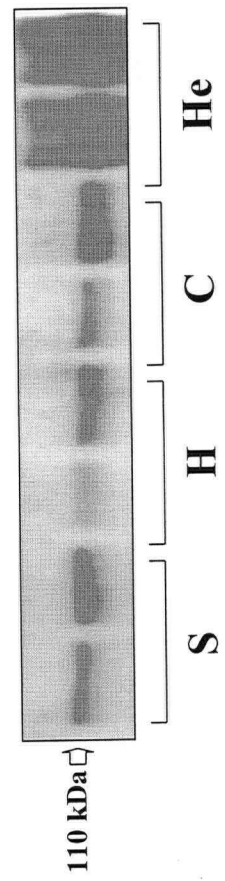
CHAPTER 6

Comparison of NR2A vs. NR2B NMDAR subunit expression in wildtype and transgenic mouse brain regions

The second objective of our research project involves the comparison of NMDAR subunit expression, specifically NR2A and NR2B, in the striatum, hippocampus and cortex of wildtype and transgenic mice (YAC18 and YAC46). Previous data, collected through examination of human and rat brains, has shown that while the NR2A and NR2B subunits are expressed throughout the forebrain, the NR2B subunit is the predominant NR2 subunit found in the neostriatal medium spiny projection neurons (Landwehrmeyer et al., 1995; Ghasemzadeh et al., 1996; Rigby et al., 1996; Kuppenbender et al., 1999). This discrete localization of NR2B, specifically in the brain region most affected by HD, is intriguing as it could potentially help explain the selective neurodegeneration evident in HD. Therefore, the comparison of NR2A vs. NR2B subunit expression will be completed in wildtype and transgenic mice tissues.

The tissues were collected as described in chapter 2. In general, wildtype mice, YAC18 and YAC46, aged 12 weeks, were anesthetized and decapitated to allow for the collection of the anterior portion of the striatum, the hippocampus and a section of the cortex. Once removed the tissues were homogenized in 1X RIPA buffer. The bicinchoninic acid protein assay (BCA kit; Pierce) was used to measure protein concentrations. For the assessment of protein expression levels, total protein from each brain region was loaded at a concentration of 5 or 10 µg per lane, and separated by 8% SDS-PAGE. Proteins were

transferred to PVDF membranes and the Western blot protocol, as previously described, was completed using primary antibodies specific for NR2A and NR2B.

Due to the fact that two separate antibodies were used to detect the NR2A and NR2B subunits, direct comparison of the blots was not an effective means to accurately compare expression levels as the quality of the antibodies may differ. As a result, densitometric analysis was completed which provided a way to quantify protein levels of NR2A and NR2B among the different brain areas. Specifically, protein levels of NR2A and NR2B in the striatum and hippocampus, relative to the cortex, were calculated. These ratios were then used to compare NR2A vs. NR2B protein expression levels in the three brain regions. This was determined to be the most efficient mechanism to assess if these results were consistent with the aforementioned findings which state NR2B as the predominant NR2 subunit found in the MSNs, which comprise ~90% of all neurons in the caudate and putamen nuclei.

Densitometric analysis was performed as described in chapter 2. In brief, protein band intensities were measured from lanes containing 5 and 10 μg of total soluble protein from the striatum, hippocampus and cortex. For the purpose of comparing expression among the different brain regions the numbers generated from lanes containing 10 μg of total soluble protein were used. Once the band intensities were quantified the protein levels in the cortex were arbitrarily designated a value of 100% and relative protein levels in the striatum and hippocampus were calculated (table 1).

Quantitative analysis revealed two apparent trends. The first pattern deals with the distribution of the NR2A and NR2B subunits among the three brain regions. Protein expression for both NR2A and NR2B in wildtype, YAC18 and YAC46 tissues showed

highest levels in hippocampus, intermediate levels in cortex and lowest levels in striatum (table 1). Closer examination of the numeric values associated with this pattern of distribution reveals the second trend and provides a plausible means by which to compare NR2A vs. NR2B protein expression in the striatum. It was determined that the NR2A levels in the striatum, relative to the cortex, were much less in comparison to NR2B levels (table 1). The calculated levels of NR2A in the striatum, normalized to the cortex, were measured to be ~52%, 37% and 32% in wildtype, YAC18 and YAC46 mice, respectively. In contrast, the values for NR2B expression levels in the striatum were determined to be ~80%, 85% and 82% of that observed in the cortex for wildtype, YAC18 and YAC46 mice, respectively. By combining these two sets of numbers, we generated ratios for the levels of NR2B vs. NR2A in the striatum of ~155%, 232% and 251% for wildtype, YAC18 and YAC46 mice, respectively. This data indicates that the NR2B:NR2A expression ratio is markedly higher in the striatum compared with cortex (or hippocampus).

Discussion of Results:

Previous findings have illustrated the discrete spatial and temporal expression pattern of the NR2 subunits (Seeburg, 1993; Rigby et al., 1996). Interestingly, while neurons in the forebrain express high levels of both NR2A and NR2B subunits, NR2B is the predominant NR2 subunit found in the neostriatal medium spiny projection neurons (Hollmann and Heinemann, 1994; Landwehrmeyer et al., 1995; Ghasemzadeh et al., 1996; Rigby et al., 1996; Kuppenbender et al., 1999). This discrete localization of NR2B, specifically in a brain region highly vulnerable in HD, is appealing as it could potentially help explain the selective neurodegeneration seen in HD. As these previous findings were derived from rat

and human tissues, comparison of NR2A vs. NR2B subunit expression was completed in wildtype and transgenic mice tissues.

It is mentioned that striatal, hippocampal and cortical tissues were collected from mice aged 12 weeks. This age was chosen due to the patterns of development of the NR2A and NR2B subunits. NR2A immunoreactivity is initially undetectable at P2 and begins to elevate from P10 to 6 months (Sans et al., 2000). On the contrary, NR2B is highly expressed at P2 and decreases in expression by 6 months (Sans et al., 2000). We sacrificed the mice at 12 weeks of age since there was very little change in subunit expression levels beyond that point (Sans et al., 2000). In addition, this age corresponded to the oldest age at which we carried out electrophysiology to compare NMDAR-mediated peak current density in acutely dissociated MSNs expressing mutant vs. wildtype htt (Zeron et al., submitted).

As previously described, due to the use of two different antibodies to probe for NR2A and NR2B, quantitative analysis was performed. Direct comparison was determined to be an unsatisfactory method by which to compare the expression of NR2A vs. NR2B as a difference in the quality of the antibodies would prevent the recording of accurate results. Densitometric analysis revealed two apparent trends. Firstly, NR2A and NR2B protein levels in wildtype, YAC18 and YAC46 tissues showed highest levels in hippocampus, intermediate levels in cortex and lowest levels in striatum (table 1). This result is consistent with earlier data which reports the presence of both NR2A and NR2B in forebrain structures (Rigby et al., 1996). The second trend, derived though analysis of the precise numeric values associated with the first trend offers a pragmatic method to compare NR2A vs. NR2B protein expression. It was evident that the NR2A levels in the striatum, relative to the cortex, were greatly reduced in comparison to the corresponding NR2B levels in the striatum (table

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1). Hence, although the forebrain contains both NR2A and NR2B, NR2B is more evenly distributed among the striatum, hippocampus and cortex as opposed to NR2A which displays higher abundance in the hippocampus and cortex. Therefore, while these findings do not conclusively show NR2B to be the predominant NR2 subtype found in the striatum, they do illustrate that NR2A appears to be more prominent in brain regions outside the striatum. Moreover, this data demonstrates a higher NR2B:NR2A expression ration in striatum compared to other forebrain structures.

Table 1. Quantitative Analysis of NR2A and NR2B subunit expression levels. The results in this table have been averaged from N=3 independent experiments.

	NMDAR subunit	Striatum	Hippocampus	Cortex
Wildtype	2A	51.7%	111.6%	100%
	2B	80.1%	130.9%	100%
YAC18	2A	36.7%	129.3%	100%
	2B	85.3%	196.8%	100%
YAC46	2A	32.5%	143.6%	100%
	2B	81.5%	151.5%	100%

Figure 11. Comparison of expression of NR2A vs. NR2B in wildtype mice brain regions. 5 and 10 μg of total protein, per brain region, was separated by SDS-PAGE. Antibodies specific for the NR2A and NR2B NMDAR subunits were used to probe the different brain regions. The brain regions examined include striatum (S), hippocampus (H) and cortex (C). This blot is representative of N=3 independent experiments.
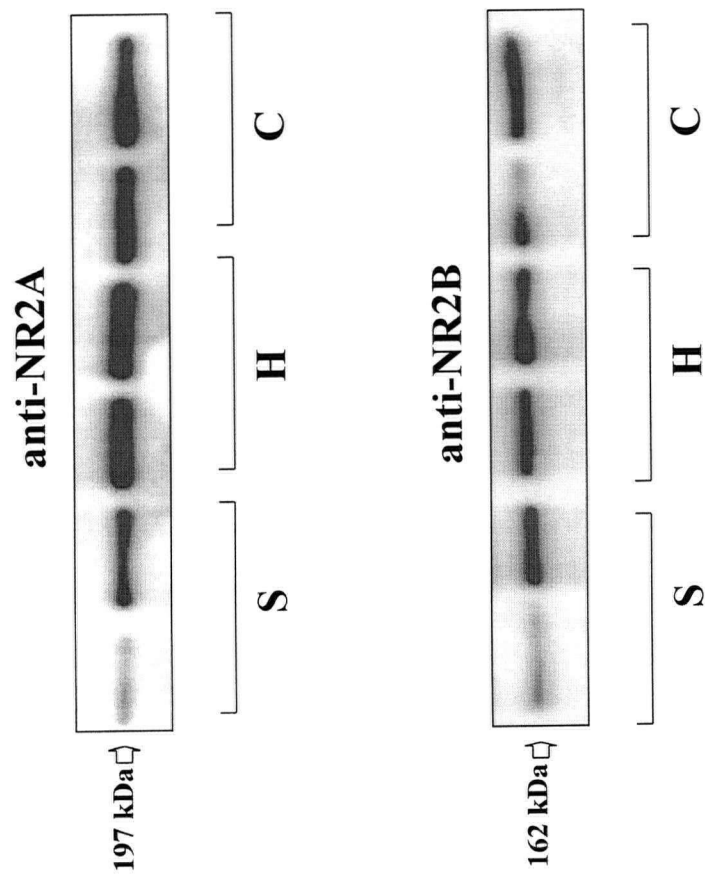
Figure 12. Comparison of expression of NR2A vs. NR2B in YAC18 mice brain regions. 5 and 10 μg of total protein, per brain region, was separated by SDS-PAGE. Antibodies specific for the NR2A and NR2B NMDAR subunits were used to probe the different brain regions. The brain regions examined include striatum (S), hippocampus (H) and cortex (C). This blot is representative of N=3 independent experiments.
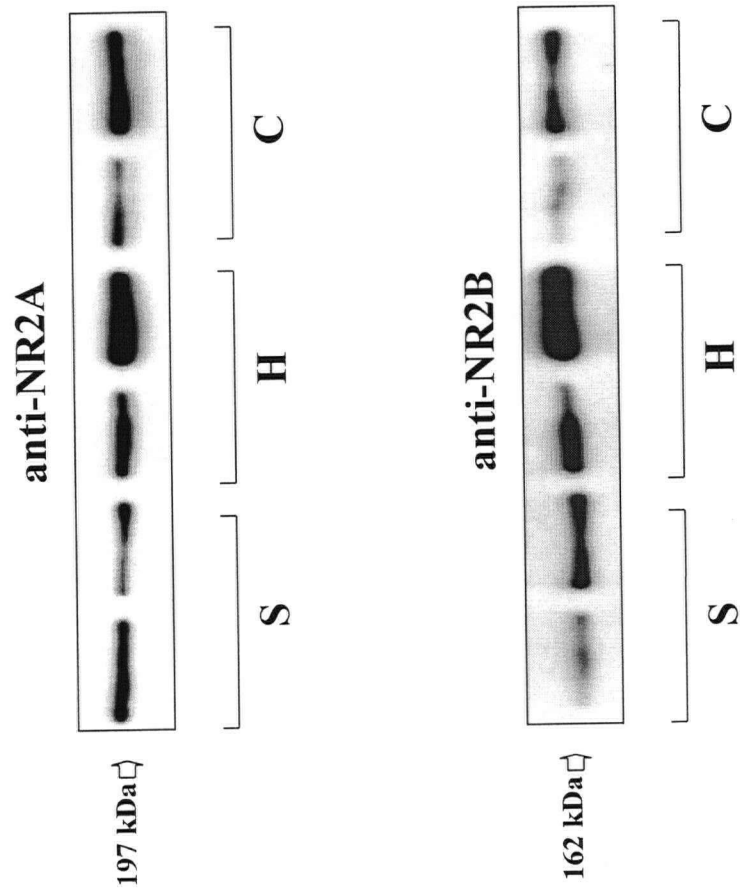
Figure 13. Comparison of expression of NR2A vs. NR2B in YAC46 mice brain regions. 5

and 10 μg of total protein, per brain region, was separated by SDS-PAGE. Antibodies

specific for the NR2A and NR2B NMDAR subunits were used to probe the different brain

regions. The brain regions examined include striatum (S), hippocampus (H) and cortex (C).

This blot is representative of N=3 independent experiments.
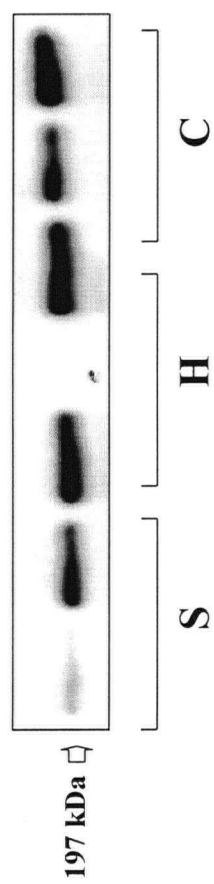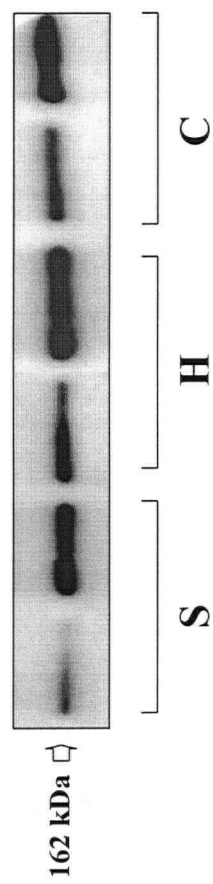
CHAPTER 7

Co-immunoprecipitation of HIP-1 and α-actinin-4 in HEK 293 cells

To achieve our last objective we attempted to elucidate an interaction between two cytoskeletal-associated proteins which could potentially mediate an association between NMDARs and htt. A plethora of evidence has implied that overstimulation of NMDARs is responsible for, at least in part, the selective neurodegeneration evident in HD (Coyle and Puttfarcken, 1993). Moreover, NMDARs and htt have both been shown to interact with cytoskeletal proteins, and for NMDARs, these interactions provide functional modulation (Kornau et al., 1995; Li et al., 1995; Kim et al., 1996; Muller et al., 1996; Kalchman et al., 1997; Wanker et al., 1997; Wyszynski et al., 1997). Therefore, we hypothesize that the polyQ expansion in mutant htt permits the indirect interaction of htt with NMDARs through cytoskeletal proteins, contributing to changes in NMDAR properties and resulting in overactivation of the receptors. As a result, the most significant objective of this research project was to attempt to discover a novel interaction between two cytoskeletal-associated proteins which could mediate an association between NMDARs and htt.

In our attempt to elucidate an interaction between cytoskeletal-associated proteins we limited the potential candidates to three broad classes of proteins including, huntingtin-interacting proteins (HIP-1), actin cytoskeletal components (actin and α-actinin), and PSD-95 family members (PSD-95, SAP-102, Chapsyn-110). Each protein was chosen based on their ability to either bind NMDARs or htt. Due to the fact that there were numerous possible interactions which could occur among these proteins, we initially characterized expression of these proteins in HEK 293 cells, and striatal, hippocampal and cortical tissues

from wildtype and transgenic (YAC18 and YAC46) mice. By characterizing expression patterns of these proteins we had hoped to gain helpful insight which could provide a place to begin with regards to probing for novel protein interactions.

Results from chapters 3 and 5 reveal that HEK 293 cells endogenously express HIP-1, α-actinin-4 and actin but do not contain any members of the PSD-95 family of proteins. In chapters 4 and 5 it was determined that all of the proteins of interest were present in striatal, hippocampal and cortical tissues from both wildtype and transgenic mice. Moreover, of the six proteins examined, none of them was preferentially enhanced in the striatum, relative to the hippocampus and cortex. If this trend had occurred it could have implications for that protein being involved in facilitating an indirect interaction between NMDARs and htt that could potentially explain selective neuronal vulnerability.

After consideration of the characterization of the six proteins of interest, in both HEK 293 cells and mouse brain homogenate, we decided to investigate a potential interaction between HIP-1 and α-actinin-4. There were several reasons for this decision. Firstly, we initially chose to study protein interactions in HEK 293 cells since they are an easy system to manipulate and we first observed a functional interaction between transfected NMDARs and mutant htt in this system (Chen et al., 1997). Therefore, our decision to utilize HEK 293 cells made HIP-1 and α-actinin-4 ideal candidates as they are both endogenously expressed. In contrast, it could be argued that if we had chosen to examine interactions with PSD-95 family members, any potential resulting interactions may be deemed artificial as PSD-95 family members are not native to HEK 293 cells. Secondly, HIP-1 and α-actinin have been shown to bind htt and NMDARs, respectively (Kalchman et al., 1997; Wanker et al., 1997; Gusella and MacDonald, 1998; Wyszynski et al., 1998; Krupp et al., 1999; Dunah et al.,

2000). The N-terminal half of HIP-1 has been shown to bind to the N-terminus of htt, downstream of the polyQ stretch (Wanker et al., 1997). Additionally, α-actinin-2 has been shown to bind by its central rod domain to the cytoplasmic tail of both NR1 and NR2B (refer to figures 1 and 2)(Wyszynski et al., 1997, 1998; Dunah et al., 2000). Controversy exists as to the exact identity of the α-actinin isoform found within the brain (refer to chapter 5). Therefore, the reported interaction involving α-actinin-2 and NMDARs could instead conceivably involve α-actinin-4. Lastly, HIP-1 and α-actinin-4 contain domains which could plausibly allow for their direct interaction. Within the C-terminus of HIP-1 is located a region which shares homology with talin, a mammalian membrane cytoskeletal-associated protein which is suggested to be involved in cell-cell interactions (Kalchman et al., 1997; Ross and Gilliland, 1999). Specifically, talin is able to bind spectrin or spectrin-related proteins, including α-actinin-4, which contains a central rod domain consisting of four spectrin-like repeats (Wyszynski et al., 1997; Chan et al., 1998; Honda et al., 1998). Therefore, an interaction between HIP-1 and α-actinin-4 seems conceivable.

HEK 293 cells were transfected with cDNAs encoding HIP-1, α-actinin-4 and β-gal to increase overall expression of the proteins. The HIP-1 cDNA encodes a HIP-1 which contains a point mutation (F398G) as the nonmutated form of HIP-1 is toxic to the cells (Hackam et al., 2000). Twenty-four hours following the initiation of the transfection, the total soluble fraction of the HEK 293 cell lysate was collected. Following collection of the cells, the co-immunoprecipitation protocol was completed as described in chapter 2. In general, cell homogenate (~200 μl) was precleared with 200 μl each of 2X Tris wash buffer and the 1:1 suspension (Protein A Sepharose beads) and allowed to rotate end-over-end (low speed for 1 hour at 4°C). Following preclearing the solution was centrifuged, the

supernatant was removed to a separate tube and incubated with primary antibody for one hour. 200 μl of the 1:1 suspension was added and the solution incubated overnight. The sample was then exposed to a series of washes with 1X Tris wash buffer and a high salt Tris wash buffer. Sample buffer was added, the supernatant/sample buffer mixture was boiled and then loaded onto an 8% acrylamide gel for Western blot analysis. In addition to the aforementioned conditions, a parallel control condition was also carried out. This sample was exposed to identical conditions with one exception, the primary antibody was not added. This was completed to test for any nonspecific binding which may occur.

To test whether HIP-1 interacts (directly or indirectly) with α-actinin-4 *in vitro*, we initially used antibodies against HIP-1 to precipitate it with associated proteins from the total soluble fraction of the transfected HEK 293 cells. The precipitated proteins were then probed with an antibody specific for α-actinin-4. This experiment revealed an interaction (direct or indirect) between HIP-1 and α-actinin-4 as evident by the protein at ~ 110 kDa, the appropriate molecular weight of α-actinin-4 (figure 14). It appears that this interaction is legitimate as no α-actinin-4 was able to be pulled down when the HIP-1 antibody was omitted during co-immunoprecipitation (figure 14, lane 3). Lanes 2 and 4 of figure 14, represent the protein which did not bind to the beads, illustrating that while α-actinin-4 was present in the lysate of both experimental and control conditions, it was only able to be precipitated when the HIP-1 antibody was used. It should be noted lanes five and six contain 5 and 10 μg of HEK 293 cell lysate not exposed to the co-immunoprecipitation protocol. These two lanes were included as additional controls which could be used to confirm that any bands detected in lanes 1-4 were in fact α-actinin-4 or HIP-1 (figures 14 and 15, respectively).

The biochemical interaction between HIP-1 and α-actinin-4 was further verified by performing a converse experiment in which the total soluble fraction of transfected HEK 293 cells was immunoprecipitated with a Sigma α-actinin antibody (figure 15). This was evident as HIP-1 was only detected when the α-actinin antibody was used (compare lanes 1 and 3, figure 15). Due to the limited supply of the α-actinin-4 specific antibody, the Sigma antibody was substituted. This did not appear to be problematic as our previous experiments had revealed that this antibody is able to detect α-actinin-4 (see figures 9 and 10).

Quantitative analysis of the protein immunoprecipitated by the α-actinin antibody from the total soluble fraction of HEK 293 cells indicated that ~ 40 % of HIP-1 was precipitated. Unfortunately, densitometric analysis was not possible in the converse situation. As evident in figure 14, lane 1 contained an additional band at ~120-130 kDa. The close proximity of this band to the lower band precluded its removal during quantitative analysis. As a result the additional band prevented an accurate measurement of the precipitated protein. However, examination with the naked eye revealed that the quantity of α-actinin-4 precipitated by antibodies against HIP-1 was ~50%, similar to that observed for HIP-1 precipitation by antibodies against α-actinin-4 shown in figure 15.

Discussion of Results:

To test for a possible interaction (direct or indirect) between HIP-1 and α-actinin-4, HEK 293 cells were transfected cDNAs encoding HIP-1 (F398G), α-actinin-4 and β-gal to increase overall expression of the proteins. Co-immunoprecipitation experiments were performed as described in chapter 2. A novel interaction between HIP-1 and α-actinin-4, which could be direct or indirect, was discovered when either a HIP-1 or α-actinin-4

specific antibody was used to immunoprecipitate and the converse antibody was used to probe the precipitated protein (see figures 14 and 15).

The physiological implications for this interaction remains to be determined; however, speculation can be made as to its potential contribution to HD. To begin with, the binding of HIP-1 and α-actinin-4 could serve to indirectly link htt and NMDARs, providing mutant htt with a means to alter the normal functioning of the receptor. This alteration in the NMDAR properties could consequently lead to their overactivation, contributing to the pathology of HD. It is known that the affinity of HIP-1 for htt is inversely related to polyQ length, which would argue against this proposed mechanism (Kalchman et al., 1997). However, it may be that the interaction of α-actinin-4 and HIP-1 serves to stabilize the association of HIP-1 with mutant htt while diminishing the interaction of HIP-1 with wildtype htt. Alternatively, the HIP-1 interaction with α-actinin-4 may result in increased NMDAR activity and the expression of mutant htt may increase availability of HIP-1 for α-actinin-4 binding. Precedence for this hypothesis is found in a recent study reporting a direct interaction between PSD-95 and htt (Savanenin et al., in press). Data from this study show that the ability of htt to bind to PSD-95 declines as the polyQ expands, potentially allowing more PSD-95 protein to be available to cluster NMDARs, resulting in their overactivation and excitotoxicity. Furthermore, it was determined that the expression of mutant htt may cause a redistribution of normal htt within the brain, since the amount of PSD-95 associated with htt, in the brains of HD patients, was found to be ~80% less than in normal subjects. This was a much larger decrease than the expected 50% due to the heterozygous nature of the HD patients (Savanenin et al., in press). Therefore, conceivably a similar relationship could exist between HIP-1 and mutant htt that would provide more

available HIP-1 to interact with α-actinin-4 and subsequently result in an increase in spatial localization of the NMDARs to the synapse. As a result of this increased clustering of the NMDARs at the synapse, via the HIP-1/α-actinin-4 interaction, a larger number of NMDARs would be exposed to glutamate released from the presynaptic terminal. Hence the resulting overactivation of the NMDARs could lead to excitotoxicity and neuronal death. The fact that α-actinin-4 can directly bind actin and indirectly link the NMDARs to actin provides support for the aforementioned hypothesis. Constituents of glutamate synapses, including NMDARs, are believed to be possibly anchored to actin-based cytoskeletal networks (Allison et al., 2000). Previous experiments have demonstrated that although NMDARs can form clusters both synaptically and non-synaptically, only activation of those found at the synapse contribute to a substantial increase in Ca^{2+} accumulation in the neurons and neuronal death (Furukawa et al., 1997; Sattler et al., 2000). Moreover, when actin perturbing agents were used there was a decrease in synaptic NMDAR clusters and as a consequence a decrease in synaptic NMDAR currents and an increase in neuronal survival. This phenomenon indicates that the actin cytoskeleton (actin and α-actinin) can potentially modulate NMDAR-mediated neurotoxicity (Furukawa et al., 1997; Sattler et al., 2000).

Another hypothesis to explain a possible contribution of the HIP-1/α-actinin-4 interaction to the etiology of HD can also be generated. HIP-1 shares significant homology with Sla2p, a yeast protein which is known to be critical for the assembly and function of the cortical cytoskeleton (Holtzman et al., 1993; Kalchman et al., 1997; Wanker et al., 1997). In addition, HIP-1 is found in abundance in the CNS and co-localizes with htt in the membrane fraction of neurons (Kalchman et al., 1997; Wanker et al., 1997). Therefore, the expression and localization of HIP-1 combined with its homology to Sla2p suggests that the HIP-1-htt

interaction may be essential for the normal function of the membrane cytoskeleton of neurons (Kalchman et al., 1997; Wanker et al., 1997). This hypothesized function could be further supported by our reported novel interaction between HIP-1 and α-actinin-4, which could link HIP-1 and htt to the membrane via NMDARs. As a result, in HD the expanded polyQ tract could sever the HIP-1/htt interaction, contributing to an alteration in the membrane cytoskeletal integrity (Kalchman et al., 1997) and potentially affecting NMDAR function (Rosenmund and Westbrook, 1993; see below).

The potential consequences of an altered association of the NMDARs with the actin cytoskeleton are evident when the interplay of actin, α-actinin and NMDARs is explored. First, constituents of glutamate synapses, including NMDARs, are believed to be anchored to actin-based cytoskeletal networks (Allison et al., 2000). This is consistent with reports that use of actin-perturbing agents led to a 40% decrease in the number of synaptic NMDAR clusters (Allison et al., 1998). Furthermore, activation of NMDARs can result in depolarization of neurons, leading to alterations in cytosolic concentrations of intracellular Ca^{2+} and also changing the phosphorylation of several proteins (Scheetz and Constantine-Paton, 1996; Shorte, 1997). The state of actin polymerization in neurons could be directly altered due to these events (Shorte, 1997). Moreover, the functional state of NMDARs is also mechanosensitive and therefore can be modulated, in a calcium-dependent fashion, by the actin cytoskeleton (Rosenmund and Westbrook, 1993; Paoletti and Ascher, 1994; Allison et al., 1998). Specifically, depolymerization of actin has been associated with up to 50% decrease in the NMDAR-mediated peak current amplitude (Rosenmund and Westbrook, 1993). In addition, with a shift in actin equilibrium towards depolymerization, the binding of actin filament bundling proteins to NMDARs will be impaired, altering the

ability of Ca^{2+}/calmodulin to interact with and modulate NMDAR activity (Ehlers et al., 1996). As described in the introduction, α-actinin is responsible for linking the NMDARs to the actin cytoskeleton (Wyszynski et al., 1998; Dunah et al., 2000). Alpha-actinin-1 and –2 have been experimentally shown to directly interact with NMDAR subunits (NR1 and NR2B)(Wyszynski et al., 1998; Vallenius et al., 2000). Additionally, the interaction between NR1-α-actinin is directly antagonized by Ca^{2+}/CaM indicating that α-actinin may contribute to the localization of NMDARs and their modulation by Ca^{2+} (Wyszynski et al., 1997). These findings coupled with the known association of α-actinin with actin provides evidence that the actin cytoskeleton (actin and α-actinin) can modulate channel function (Shorte, 1997). Assimilation of all of these described relationships between the actin cytoskeleton (actin and α-actinin) and NMDARs allows for a logical assumption that a disruption in these interactions could contribute to an overactivation of NMDARs, leading to a neuropathological state.

Interestingly, recent data exists which demonstrates a potential mechanism by which α-actinin may contribute to the selective neurodegeneration evident in HD. Localization studies revealed that α-actinin-2 immunoreactivity is highly expressed in the striatum, displaying cellular specificity with regards to its expression (Dunah et al., 2000). Specifically, α-actinin-2 immunoreactivity is most abundantly expressed in substance-P-containing GABAergic medium spiny projection neurons, a class of neurons which undergo severe degeneration in HD (Dunah et al., 2000). In contrast, α-actinin-2 immunoreactivity is found in very low levels in somatostatin and neuronal nitric oxide synthase interneurons (Dunah et al., 2000). As previously described, controversy surrounds the identity of the α-actinin isoform found within the brain (see chapter 5). Our data (see chapter 5) indicate that

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it is α-actinin-4 which is found in the striatum and α-actinin-2 is not present (see figure 10). Hence, the subcellular localization study by Dunah et al., 2000 could falsely have reported α-actinin-2 instead of the accurate isoform, α-actinin-4. Therefore, if α-actinin-4 was determined to demonstrate cellular specificity in its expression, within the striatum, its interaction with HIP-1 would prove to be intriguing. The interaction of α-actinin-4 and HIP-1 may occur more prominently within a subset of striatal neurons, potentially allowing htt and NMDARs to indirectly interact and contribute to the selectivity of neuronal degeneration in HD. The low levels of α-actinin-4 in somatostatin and neuronal nitric oxide synthase interneurons could interfere with its ability to bind HIP-1 and may, in part, explain why these interneurons are not vulnerable in HD (Dunah et al., 2000).

To investigate the significance of the HIP-1/α-actinin-4 interaction for pathogenesis of HD, further experiments are required. First, we will determine whether co-expression of mutant vs. wildtype htt alters the interaction between HIP-1 and α-actinin-4. In addition, patch clamp recording could be done to determine whether overexpression of HIP-1 and α-actinin-4 alters NMDAR activity. Further experiments designed to interfere with this interaction could be done to assess the effect on NMDAR activity. Lastly, it would be important to repeat all of the previous co-immunoprecipitation experiments in mouse striatal tissue to determine if the interactions are conserved in the brain region most effected in HD.

Figure 14. Co-immunoprecipitation of α-actinin-4 with HIP-1 in transfected HEK 293 cells. HEK 293 cells were transfected with HIP-1 and α-actinin-4 to overexpress these proteins. 24 hours following transfection cells were harvested. Cell lysates were incubated with a monoclonal HIP-1 antibody, followed by Protein-A sepharose beads, and the precipitated protein was probed with a polyclonal α-actinin-4 antibody. The blot was exposed to film for 20s during development. These results are representative of N=3 independent experiments.

10 μg lysate

5 μg lysate

Unbound protein

Co-IP without 1° Ab

Unbound protein

Co-IP with 1° Ab

110 kDa
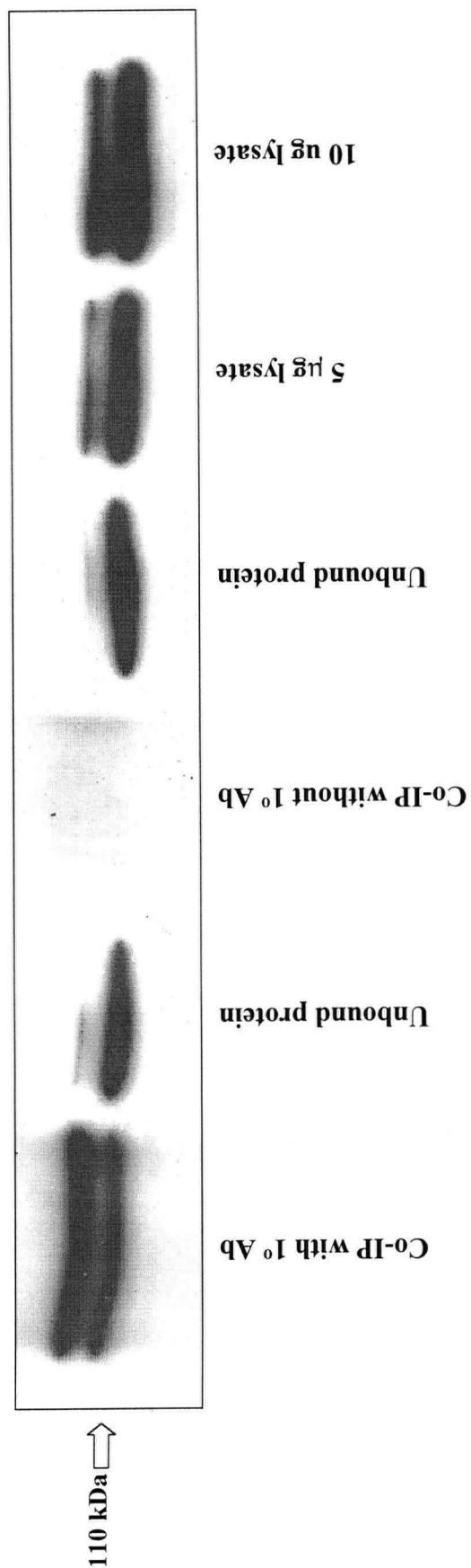
Figure 15. Co-immunoprecipitation of HIP-1 with α-actinin-4 in transfected HEK 293 cells. HEK 293 cells were transfected with HIP-1 and α-actinin-4 to overexpress these proteins. 24 hours following transfection cells were harvested. Cell lysates were incubated with a monoclonal α-actinin antibody (which is known to recognize α-actinin-4), followed by Protein-A sepharose beads and the precipitated protein was probed with a monoclonal HIP-1 antibody. The blot was exposed to film for 30s during development. These results are representative of N=1 experiments.

10 μg lysate

5 μg lysate

Unbound protein

Co-IP without 1° Ab

Unbound protein

Co-IP with 1° Ab

130 kDa

106
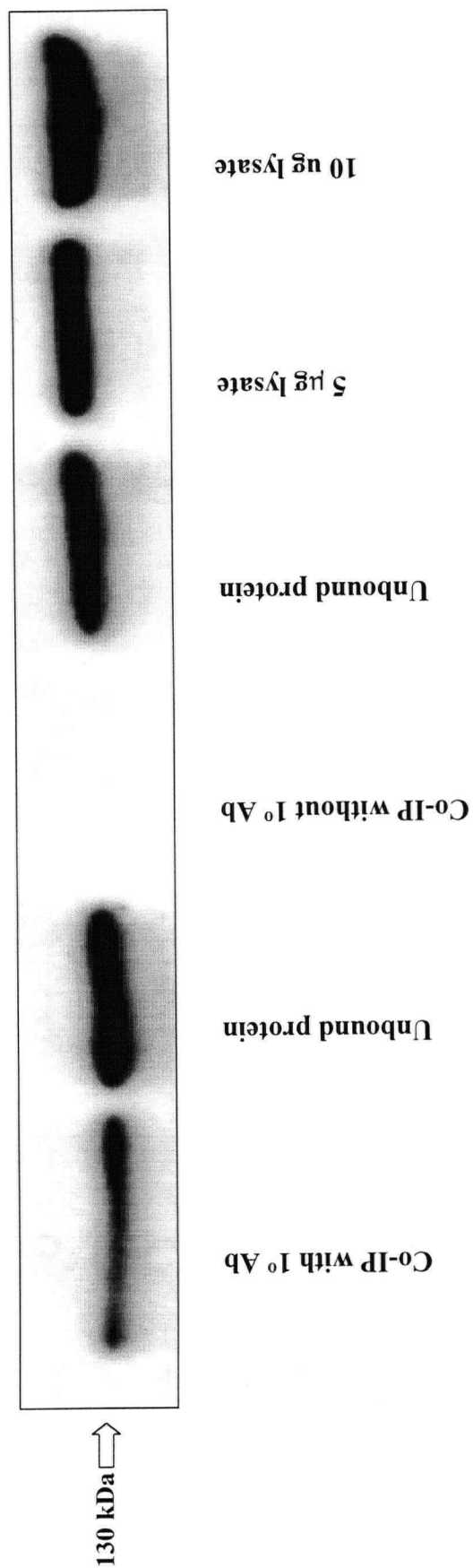
CHAPTER 8

General Discussion

In the initial stages of this project we established three primary objectives: characterization of protein expression of htt, htt interacting proteins (i.e. HIP-1), PSD-95 family members and components of the neuronal cytoskeleton (actin and α-actinin) in HEK 293 cells and striatal, hippocampal and cortical tissue from wildtype and transgenic mice (18Q and 46Q); comparison of expression of NR2A vs. NR2B in striatal, hippocampal and cortical tissues; and investigation of any potential interactions which may exist between the characterized cytoskeletal-associated proteins, in an attempt to discover a protein interaction which may serve as the link between htt and NMDARs. At the conclusion of this project we can state that each objective was successfully completed.

The characterization of the proteins of interest in HEK 293 cells confirmed previous findings by other groups. Additionally, analysis of protein expression in the mouse striatal, hippocampal and cortical tissues proved consistent to earlier results obtained using rat brain homogenate. One interesting result derived from these findings involved the identity of the α-actinin isoform found in both HEK 293 cells and mouse brain regions. It has previously been reported that α-actinin-2 is endogenously expressed in HEK 293 cells and brain tissue (Wyszynski et al., 1997, 1998; Zhang et al.,1998; Dunah et al., 2000). Although this finding has been disputed by several groups no attempts had been made to determine an alternative α-actinin isoform. Hence, our data suggest for the first time that α-actinin-4 is the isoform found within HEK 293 cells and brain tissue. However, since we used a different panel of

antibodies than those used in previous studies, further experiments are required to resolve this controversy.

The comparison of NR2A vs. NR2B expression in both wildtype and transgenic mice brain regions was of particular importance to us. We have postulated that the selective degeneration of striatal neurons seen in HD is, in part, caused by overactivation of NR1/NR2B-type NMDARs, since this subtype, and not NR1/NR2A, was shown to be modulated by mutant htt when coexpressed in HEK 293 cells (Chen et al., 1999). Therefore, we attempted to compare NR2A vs. NR2B levels in striatal, hippocampal and cortical tissues to confirm previous findings which state NR2B is the predominant NR2 subunit found within the striatum (Landwehrmeyer et al., 1995; Ghasemzadeh et al., 1996; Rigby et al., 1996). In contrast to previous experiments which used *in situ* hybridization and RNA amplification technique, we used Western blots to attempt to quantify relative protein levels of NR2A and NR2B. Direct comparison of NR2A and NR2B was hindered by the fact that two different antibodies were used during immunoblotting, as a result densitometric measurement and analysis of NR2B:NR2A ratios were performed which revealed intriguing data. In general, we found that expression levels of NR2B in the striatum, relative to the cortex, are substantially greater as compared to NR2A. Although this finding does not exactly replicate earlier data regarding striatal NR2 protein expression levels, it does suggest that relative to NR2B, NR2A is more prominent in brain regions outside the striatum.

Finally, the most significant discovery in this project was the novel interaction (direct or indirect) between HIP-1 and α-actinin-4 in HEK 293 cells. As outlined in our research hypothesis, one of our goals was to elucidate an interaction between two cytoskeletal-associated proteins which could serve to indirectly link NMDARs and htt. The association

of HIP-1 and α-actinin-4 may play such a role, as HIP-1 and α-actinin are known to bind htt and NMDARs, respectively (Kalchman et al., 1997; Wanker et al., 1997; Wyszynski et al., 1998; Krupp et al., 1999; Dunah et al., 2000). The potential implications of this novel interaction with regards to the etiology of HD remains to be determined. Hopefully, further examination of the interaction between HIP-1 and α-actinin-4, as well as supplementary experiments to explore the possibility of additional binding partners, will shed some light on to the mechanism responsible for the selective neurodegeneration evident in HD.

While the novel association between HIP-1 and α-actinin-4 is intriguing, it is important to note that this interaction was observed in a non-neuronal cell line (HEK 293 cells). Therefore, to better understand any potential implications this interaction may have with regards to HD, it is necessary to determine if this interaction is present in the striatal MSNs. Moreover, the question still remains how an indirect interaction between mutant htt and NMDARs would cause an elevated activity of NMDARs and how this association could contribute to the selective neurodegeneration evident in HD. To attempt to answer these questions our lab examined NMDAR-mediated excitotoxicity in a mouse model of HD.

Previously, we have shown that in HEK 293 cells expressing full-length mutant htt and the NR1A/NR2B but not NR1A/NR2A subtype of NMDARs, there is an increase in NMDAR-mediated current amplitude and apoptosis (Chen et al., 1999; Zeron et al., 2001). These findings are the foundation for our hypothesis that mutant htt functions to increase NMDAR activity, leading to enhanced caspase activation and apoptosis. To test the validity of this proposed mechanism, MSNs from transgenic mice containing an expanded CAG repeat (YAC72) were compared with MSNs from wildtype mice with regards to current influx, caspase activation and apoptosis (Zeron et al., submitted).

Recordings of NMDA-evoked current from striatal neurons obtained from YAC72 vs. wildtype mice revealed substantially increased NMDAR peak current amplitudes and current density in MSNs from YAC72 mice (Zeron et al., submitted). Interestingly, exposure of the MSNs to 10 µM ifenprodil, a dose which selectively blocks current mediated by NR1/NR2B but not NR1/NR2A subtype NMDARs (Williams, 1993), was observed to inhibit more than half of the peak current elicited by application of glutamate or NMDA and glycine (Zeron et al., submitted). Moreover, these results are in accordance with earlier findings obtained from HEK 293 cells and substantiate the claim that enhanced NMDAR-induced ion influx possibly contributes to an increased vulnerability to cell death (Chen et al., 1999; Zeron et al., submitted).

The second parameter which was investigated was caspase activation, specifically caspase-3. Caspase-3 was examined due to its known ability to cleave htt into fragments which when transported into the nucleus prove to be toxic (Hackam et al., 1998; Lunkes and Mandel, 1998; Wellington et al., 2000). Additionally, caspase-3 functions as a downstream effector of programmed cell death. Brief application of 3mM NMDA to YAC72 MSNs demonstrated an increase in expression of the active form of caspase-3 by immunocytochemistry as compared to wildtype controls at 6 hours following NMDAR stimulation (Zeron et al., submitted). Furthermore, using a spectrophotometric assay, caspase-3 activation was determined to be enhanced in cultured striatal cell lysates obtained from YAC72 vs. wildtype mice at 3 and 6 hours following exposure to 3mM NMDA (Zeron et al., submitted). It should be noted that both cells expressing mutant htt and normal htt had similar basal caspase activity. Moreover, exposure to a caspase inhibitor (z-DEVD-fmk) prior to, during and following activation of NMDARs resulted in a substantial decline in

caspase-3 activation and inhibition of cell death (see below). These findings implicate NMDAR activation as an efficient stimulus of caspase-3 activity in cultured MSNs, which consequently is essential for the mediation of NMDA-elicited cell death (Zeron et al., submitted).

Finally, apoptosis, the primary mechanism by which cells are believed to die in HD, was examined (Portera-Cailliau et al., 1995). Primary neostriatal cultures generated from YAC72 mice and wildtype littermates were exposed to NMDA and glycine and cell death was measured 24 hours later. Paralleling the activation of caspase-3, the YAC72 cultures contained a greater percentage of cell death as compared to the wildtype controls (Zeron et al., submitted). Utilizing the terminal deoxynucleotidyl transferase-mediated dUTP (TUNEL) fluorescein tagged staining technique to assess apoptotic cells, it was found that YAC72 cultures displayed an elevated level of NMDAR-induced apoptosis compared with wildtype cultures (Zeron et al., submitted). To further confirm these results MK-801, a use-dependent NMDAR antagonist, and 3mM NMDA were co-applied to the cultures. This resulted in a drastic reduction in cell death, indicating that the majority of the cell death (>80%) resulting from exposure to 3mM NMDA can be attributed to NMDAR activation (Zeron et al., submitted). Additionally, a comparable portion of cell death was inhibited after exposure to ifenprodil, a NR1/NR2B subtype-specific antagonist (Williams, 1993), in both cultured striatal neurons containing mutant and normal htt (Zeron et al., submitted). Therefore, these findings provide evidence for the involvement of NMDARs, in particular the NR1/NR2B subtype, in the mediation of excitotoxicity.

The results presented from the mouse model of HD suggest NMDAR stimulation may

serve to trigger caspase activation, resulting in the cell death of striatal MSNs (Zeron et al., submitted). Furthermore, the data support the notion that mutant htt is discriminative in its augmentation of the activity and toxicity of NR1/NR2B-type NMDARs as demonstrated by using ifenprodil. Although the selective enhancement of this subtype of NMDARs by mutant htt may partially explain the selective neurodegeneration in HD since NR1/NR2B is the predominant NMDAR subtype in striatal MSNs, it is also possible that novel interactions with other proteins selectively in vulnerable neurons may be a factor (Zeron et al., 2001). Incorporating the findings from chapter 7, the interaction between HIP-1 and α-actinin-4 may be another piece in the puzzle. Therefore, we propose that full-length htt indirectly interacts with NMDARs via cytoskeletal-associated proteins (i.e. HIP-1 and α-actinin-4 or PSD-95 family members). Previous findings have demonstrated that cytoskeletal-associated proteins and actin can modulate NMDAR function, therefore associations of htt with these proteins may facilitate the overstimulation of NMDARs by mutant htt (Chen et al., 1999; Sattler and Tymianski, 2000; Zeron et al., 2001). The overactivation of NMDARs results in an influx of calcium which leads to mitochondrial dysfunction, decreased calcium sequestration and increased free radical production (reviewed by Nicholls and Budd, 2000). As a result of these events, proteases are activated, including caspase-3, which lead to the truncation of htt and eventually apoptosis (Sattler and Tymianski, 2000; Wellington et al., 2000). This cascade is depicted in figure 16.

The proposed mechanism (see figure 16) describes plausible downstream effects which are consequences of the overstimulation of NMDAR in MSNs expressing mutant htt. The experiments described in the results sections of this thesis address the initial step in this multi-step cascade. In order to gain additional support for the involvement of the interaction

between HIP-1 and α-actinin-4 in the pathogenesis of HD, further experiments are necessary. Firstly, the interaction of HIP-1 and α-actinin-4 needs to be examined further using striatal tissue to test whether this association can be replicated in the brain region most severely effected by HD. Additionally, while the HIP-1 and htt interaction has been well documented (Kalchman et al., 1997; Wanker et al., 1997), experiments are still required to ensure that similar to α-actinin-2, α-actinin-4 can also bind to the NMDAR (Wyszynski et al., 1998; Dunah et al., 2000). Once these two objectives are met further experiments will be essential to determine the effect mutant htt has on the interaction between HIP-1 and α-actinin-4 and the possible consequences with regards to NMDAR modulation. Hopefully, these experiments will enlighten us on how this novel interaction could potentially be incorporated into our proposed mechanism of HD pathogenesis and bring us one step closer to elucidating the factors responsible for the etiology of HD.

Figure 16. A proposed cascade resulting from the overactivation of NMDARs by an indirect interaction with mutant htt. Cytoskeletal-associated proteins (i.e. HIP-1/α-actinin-4 or PSD-95 family members) indirectly link NMDARs and htt. This results in the overactivation of NMDARS and an substantial influx in Ca^{2+}. This increase in calcium levels leads to mitochondrial dysfunction, decreased calcium sequestration and increased free radical production (reviewed by Nicholls and Budd, 2000). As a result of these events, proteases are activated, including caspase-3, which lead to the truncation of htt and eventually apoptosis (Sattler and Tymianski, 2000; Wellington et al., 2000).

Indirect interaction of NMDARs and mutant htt via cytoskeletal-associated proteins
(i.e. HIP-1/α-actinin-4 or PSD-95 family members)

$\downarrow$

overactivation of NMDARs

$\downarrow$

$\uparrow\uparrow$ influx of Ca^{2+}

$\downarrow$

mitochondrial dysfunction, decreased calcium sequestration and increased
free radical production

$\downarrow$

proteases are activated (including caspase-3)

$\downarrow$ $\downarrow$

truncation of htt into small   apoptosis
fragments and translocation into
nucleus

$\downarrow$

apoptosis

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