HUNTINGTON'S DISEASE IN TURKEY: POSSIBLE EFFECTS OF GLUTAMATE RECEPTOR GENE POLYMORPHISMS ON AGE OF DISEASE ONSET

by

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to my family

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ABSTRACT

HUNTINGTON'S DISEASE IN TURKEY: POSSIBLE EFFECTS OF GLUTAMATE RECEPTOR GENE POLYMORPHISMS ON AGE OF DISEASE ONSET

Huntington's Disease (HD) is a progressive and fatal neurodegenerative disorder that follows an autosomal dominant pattern of inheritance. The clinical symptoms of HD are disability in motor control, cognitive dysfunction and psychiatric disturbances. HD is an adult-onset disease, the onset occurs between the ages of 35 and 50 years. The causative mutation for HD was identified as the expansion of polymorphic CAG repeats in the first exon of the HD gene. It is well-established that there is a strong inverse correlation between the age of onset and expanded CAG repeat length in HD. However, the expanded CAG repeat number in the first exon of the HD gene explains about 42-73 per cent of the variance in the age of onset of the disease. The remaining variation in the age of onset is due to the combination of both environmental and genetic factors beyond the HD gene. An extensive research is conducted on the identification of possible modifiers of age of onset in HD and variations in several genes were found to have modifier effects. It is essential to demonstrate the presence or absence of the modifier effects of previously studied genes in different populations. The aims of this study are to i) investigate the molecular basis of HD in Turkish HD patients, ii) analyze the correlation between the age of onset and CAG repeat genotypes in the HD gene and iii) study the statistical significance of modifier effects of five polymorphisms in three different genes, encoding glutamate receptors, on the age of onset in HD. 108 Turkish HD patients, including an extended kindred, were studied in the framework of this thesis. Our findings did not demonstrate any significant association between the polymorphisms studied and the age of onset in HD. This result does not rule out the roles of these genes in HD. In the literature, there are several contradictions for the polymorphisms studied in this thesis. Analysis of these polymorphisms in a larger study population is apparently needed. We believe that the present study is a first step towards this direction.

ÖZET

TÜRKİYE'DE HUNTINGTON HASTALIĞI: GLUTAMAT RESEPTÖRÜ GEN POLİMORFİZMLERİNİN HASTALIK BAŞLANGIÇ YAŞINA OLASI ETKİLERİ

Huntington Hastalığı (HH), otozomal dominant kalıtım gösteren ilerleyici ve ölümcül bir nörodejeneratif hastalıktır. HH'nin klinik semptomları, motor kontrol yetersizliği, bilişsel işlevlerde azalma, ve psikiyatrik bozukluklardır. HH, geç başlangıçlı bir hastalıktır, 35-50 yaşları arasında ortaya çıkar. HH'ye neden olan mutasyon, genin birinci ekzonundaki polimorfik CAG tekrarlarının artışı olarak tanımlanmıştır. HH'de, CAG tekrar sayısı ile hastalık başlangıç yaşı arasında ters orantılı bir ilişki vardır. Ancak, HH geninin birinci ekzonundaki patolojik CAG tekrar sayısı, hastalık başlangıç yaşını yüzde 42-73 oranında açıklar. Hastalık başlangıç yaşındaki çeşitliliğin geriye kalan kısmı, çevresel etmenler ve HH geninin dışındaki genetik faktörlerin kombinasyonu sonucudur. HH'de, hastalık başlangıç yaşına etki eden olası faktörleri inceleyen birçok kapsamlı çalışma vardır ve bazı genlerdeki polimorfik değişimlerin modifiye edici etkileri kanıtlanmıştır. Modifiye edici etkiler, değişik popülasyonlarda farklılıklar gösterebilir, dolasıyla bu tür çalışmaların her toplum için yapılması gereklidir. Bu tezin amaçları i) HH'nin Türk Huntington hastalarındaki moleküler temelini araştırmak, ii) hastalık başlangıç yaşı ve HH genindeki CAG tekrar sayıları arasındaki etkileşimi incelemek ve iii) glutamat reseptörlerini kodlayan üç farklı gendeki beş polimorfizmin, hastalık başlangıç yaşını modifiye etme etkisinin istatistiksel yöntemlerle araştırılmasıdır. Bu tez çerçevesinde, geniş bir aileyi de içeren 108 Türk Huntington hastası çalışılmıştır. Bulgularımız, incelenen polimorfizmlerle HH başlangıç yaşı arasında istatistiksel olarak anlamlı bir ilişki göstermemekle birlikte bu sonuç, çalışılan genlerin Huntington Hastalığındaki rollerini ekarte etmemektedir. Literatürde, bu polimorfizmlerle ilgili çelişkili bulgular vardır. Söz konusu polimorfizmlerin daha geniş bir hasta popülasyonunda araştırılması gerekir, bu çalışma bu yönde atılmış bir ilk adımdır.

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A	Adenine
AMPA	α-Amino-3-Hydroxy-5-Methyl- 4-Isoxazolepropionic Acid
AO	Age of Onset
ApoE	Apolipoprotein E
APS	Ammonium Persulfate
ATP	Adenosine Triphosphate
BDNF	Brain Derived Neurotrophic Factor
bp	Base pair
BPB	Bromophenol Blue
^{0}C	Degree Celcius
C	Cytosine
Ca	Calcium
CA150	Transcription Elongation Factor 150
cAMP	Cyclic AMP
CBP	cAMP Response Element Binding Protein
CEPH	Centre d'Etude du Polymorphisme Humain
cM	Centimorgan
CNS	Central Nervous System
dH_2O	Distilled Water
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-triphosphate
DRPLA	Dentato-Rubral Pallidoluysian Atrophy
EDTA	Ethylenedinitrilo-tetraacetate
EtBr	Ethidium Bromide
EtOH	Ethanol
FAM	Carboxyfluorescein
G	Guanine
GABA	Gamma Amino Butyric Acid
GluR	Glutamate Receptor

GRIK2	Glutamate Receptor Ionotrophic Kainate 1
GRIN2A	Glutamate Receptor Ionotrophic NMDA 2A
GRIN2B	Glutamate Receptor Ionotrophic NMDA 2B
HAP1	Huntingtin-Associated Protein 1
hCAD	Human Caspase Activated DNase
HD	Huntington's Disease
HEAT	Huntingtin Elongation Factor 3, the PR65/A Subunit of Protein Phosphatase 2A and the Lipid Kinase TOR1
HIP1	Huntingtin-Interacting Protein 1
HIP14	Huntingtin-Interacting Protein 14
HIP1R	HIP1-Related Protein
HIPPI	HIP1 Protein Interactor
HPRT	Hypoxanthine Phosphoribosyltransferase
Hsp40	Heat-Shock Protein 40
Hsp70	Heat-Shock Protein 70
htt	Huntingtin
IP3	Inositol Triphosphate
IT15	Interesting Transcript 15
JHD	Juvenile Huntington's Disease
kb	Kilo base
kDa	Kilo Dalton
Log	Logarithm
M	Methionine
M	Molar
mg	Milli gram
$MgCl_2$	Magnesium Chloride
mGluR	Metabotrophic Glutamate Receptor
ml	Milli liter
mM	Milli molar
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MSN	Medium-sized Spiny Neurons
MSX1	MSH Homeobox Protein 1

MT	Microtubule
MTHFR	Methyltetrahydrofolate Reductase
NaCl	Sodium Chloride
NES	Nuclear Export Signal
ng	Nano gram
NII	Neuronal Intranuclear Inclusions
NLS	Nuclear Localization Signal
nm	Nano meter
NMDA	N-Methyl-D-Aspartate
NMDAR	NMDA Receptor
NR1	NMDA Receptor 1
NR2A	NMDA Receptor 2A
NR2B	NMDA Receptor 2B
NR2C	NMDA Receptor 2C
NR2D	NMDA Receptor 2D
NRSE	Neuron-Restrictive Silencer Element
NRSF	Neuron-Restrictive Silencer Factor
OD	Optical Density
OD_{260}	Optical Density at 260 nm
p53	53 Kilo Dalton Protein
PACSIN1	Protein Kinase C and Casein Kinase Substrate in Neurons-1
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PolyP	Polyproline
PolyQ	Polyglutamine
RBC	Red Blood Cell
RE	Restriction Enzyme
REST	Repressor Element-1 Silencing Transcription Factor
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rs	Reference Sequence
SBMA	Spinal and Bulbar Muscular Atrophy
SCA	Spinocerebellar Ataxia

SDS	Sodiumdodecylsulphate
SN	Substantia Nigra
SNP	Single Nucleotide Polymorphism
Sp1	Specificity Protein 1
SPSS	Statistical Package for the Social Sciences
T	Threonine
T	Thymine
Taq	Thermus aquaticus
TBE	Tris-Boric acid-EDTA
TBP	TATA-box Binding Protein
TE	Tris-EDTA
TEMED	N',N',N',N'-Tetramethylethylenediamine
TFIID	Transcription Factor IID
TUNEL	Transferase-Mediated dUTP Nick-End Labeling
U	Unit
Ub	Ubiquitin
UCHL1	Ubiquitin Carboxy-Terminal Hydrolase L1
UPS	Ubiquitin-Proteasome System
UTR	Untranslated Region
UV	Ultraviolet
V	Volt
YAC	Yeast Artificial Chromosome
µl	Micro liter

1. INTRODUCTION

In the second half of the 19th century, the laws of inheritance were defined by Gregor Mendel, since then, mutations were known to be stably transmitted between generations. This knowledge was revised when a different type of inheritance pattern for some human disorders, such as Fragile X Syndrome, Spinal and Bulbar Muscular Atrophy, Huntington's Disease and Myotonic Dystrophy were described (Mirkin, 2007). The identification of the causative mutations in these diseases, lead a new concept in genetics: Dynamic mutations. Dynamic mutations are unstable expansion of repeating motives, such as CAG, GAA, CTG trinucleotide repeats. Up to date, 16 diseases are described to be caused by unstable triplet repeat expansion mutations, and nine of these result from expanded CAG repeats within the coding regions of the respective genes. These diseases are Huntington's Disease (HD), Dentato-Rubral Pallidoluysian Atrophy (DRPLA), Spinal and Bulbar Muscular Atrophy (SBMA), and Spinocerebellar Ataxia (SCA) Types 1, 2, 3, 6, 7 and 17. Since CAG triplets encode glutamine in the proteins, these nine neurodegenerative disorders are known as "Polyglutamine (polyQ) Diseases", the most prominent representative being Huntington's Disease (Tsuji, 1997) (Table 1.1.).

Table 1.1. Polyglutamine diseases (Shao and Diamond, 2007)

Disease	**Locus**	**Protein**	**Normal CAG Repeat Length**	**Pathogenic CAG Repeat Length**
HD	4p16	Huntingtin	6 – 34	36 – 121
SBMA	Xq11-	Androgen Receptor	9 – 36	38 – 62
DRPLA	12p13	Athrophin-1	7 – 34	49 – 88
SCA 1	6p23	Ataxin-1	6 – 39	40 – 82
SCA 2	12q24	Ataxin-2	15 – 24	32 – 200
SCA 3	14q32	Ataxin-3	13 – 36	61 – 84
SCA 6	19p13	α_{1A}-Ca^{+2} Channel	4 – 20	20 – 29
SCA 7	3p14	Ataxin-7	4 – 35	37 – 306
SCA 17	6p27	TBP	25 – 42	47 – 63

1.1. Historical Background and Epidemiology of Huntington's Disease

In the 19th century, chorea was noted as a hereditary disorder by several physicians, but the distinctive description of the disease was made by the American physician, George Huntington, which led to the eponymous designation of the disorder as Huntington's Disease (Elliotson, 1832; Huntington, 1872; Walker, 2007). This description has stimulated HD research, studies concerning the prevalance of the disease, its worldwide distribution and origin (Figure 1.1.). In most populations of white people, the prevalence of HD is estimated to be 5–7 affected individuals per 100 000, but in exceptional areas, such as Tasmania and the area around Lake Maracaibo in Venezuela, the prevalence is much higher. In Japan, the prevalence of HD is 0,5 per 100 000, and the disease is rare in most parts of Asia. Similarly, African populations show a reduced prevalence. Accordingly, the incidence of Huntington's Disease is thought to be higher in white populations when compared to Africans or Asians (Margolis and Ross, 2003; Cowan and Raymond, 2006; Walker, 2007).

1.2. Clinical Correlates

HD is a progressive and fatal neurodegenerative disorder, that follows an autosomal dominant pattern of inheritance (Bates, 2005). The clinical symptoms of HD can be classified into three main abnormalities: Disability in motor control, cognitive dysfunction and psychiatric disturbances (Borrell-Pagès et al., 2006). HD is considered as an adult-onset disease, since in most cases, the onset of the disease occurs in midlife, between the ages of 35 and 50 years (Gil and Rego, 2008). However, a small number of patients manifests the disease before the age of 20 (Juvenile HD-JHD, less than 10 per cent) (Ribai et al., 2007). Duration of the disease from diagnosis to death is typically 15-20 years (Walker, 2007).

The onset of HD is usually defined by the beginning of motor symptoms, and most often the initial complaint is "clumsiness", "tremor", "balance trouble", or "jerkiness". The primary movement abnormality, and often the earliest symptom, is chorea, continuous and irregular writhing and jerking movements.

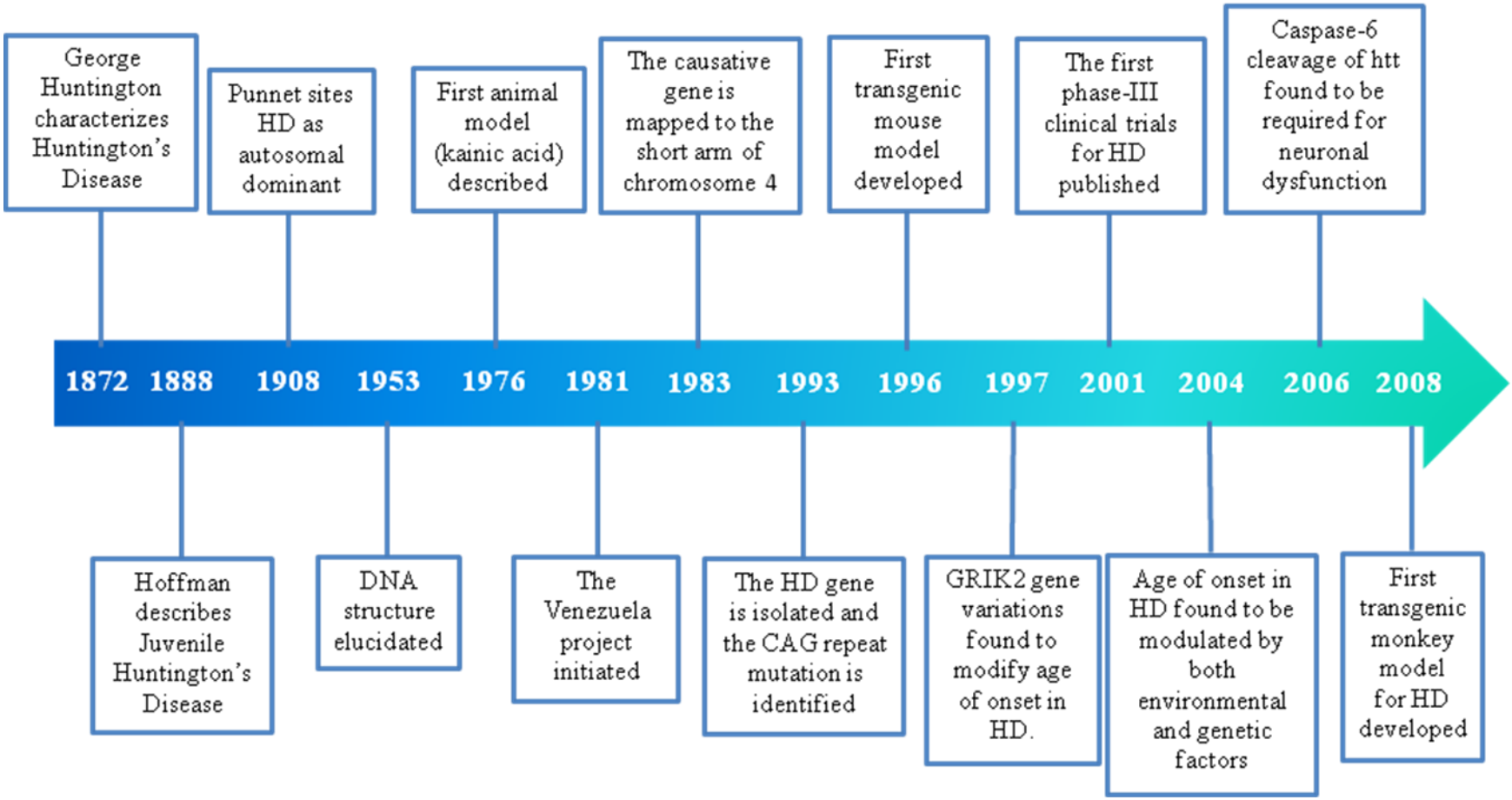


Figure 1.1. Benchmarks in HD research (adapted from Walker, 2007 and Bates, 2005)

The limbs are most prominently affected, but respiratory, laryngeal, pharyngeal, oral and nasal musculature may also be involved. Other motor symptoms include impaired visual tracking, slow, poorly-coordinated, arrhythmic fine motor movements, dysarthria and dysphagia, rigidity, and ataxia (Margolis and Ross, 2003).

In juvenile-onset HD patients, symptoms are somewhat different when compared to adult-onset cases; chorea can be completely absent (Rubinsztein and Carmichael, 2003). The clinical manifestations of juvenile-onset HD are dominated by rigidity, bradykinesia and dystonia (Ribai et al., 2007).

During the course of HD, cognitive function is severely affected. Slowing of the intellectual properties appears as the first sign of cognitive impairment in HD patients, and in some cases, abnormalities in cognitive functions can be detected decades before the onset of motor symptoms. These cognitive impairments worsen over time, and late-stage HD patients develop dementia (Gil and Rego, 2008).

Unlike cognition, psychiatric and behavioural symptoms do not progress in correlation with disease severity. HD patients usually show signs of depression, and suicidal ideation is estimated to be about five to ten times more frequent than the general population (about 5–10 per cent). Manic-psychotic symptoms and personality changes (irritability, apathy and sexual disturbances) are often part of the psychiatric syndrome (Walker, 2007; Gil and Rego, 2008).

1.3. Neuropathology

In Huntington's disease, neuropathological changes are strikingly selective and restricted to the brain, with prominent cell loss and atrophy in the caudate and putamen (Figure 1.2). Striatal GABAergic medium-sized spiny neurons (MSNs) that project to substantia nigra (SN) and globus pallidus are the most vulnerable. Within the striatal spiny neuronal population, enkephalin-containing neurons are more susceptible than substance P-containing neurons. When compared to other neuronal populations, including those from other brain regions and other neurons of the striatum, MSNs are the first to die in early-

stage HD, and they die in the greatest numbers (Gusella and MacDonald, 2006; Cowan and Raymond, 2006).

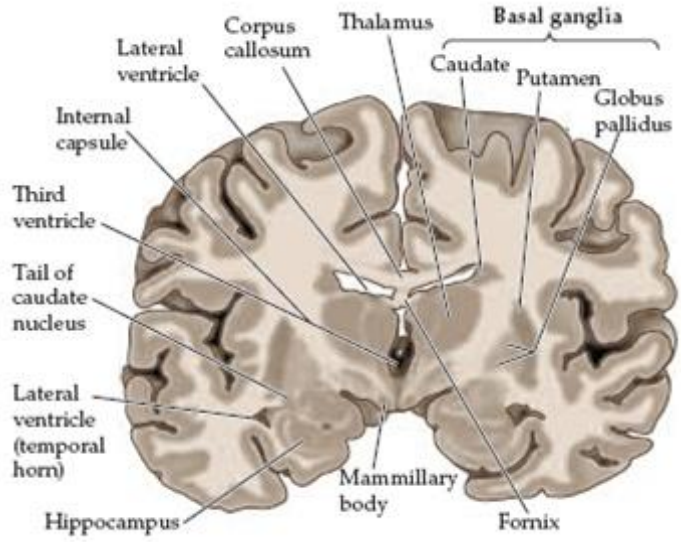


Figure 1.2. Coronal section of the brain showing caudate and putamen: brain parts selectively degenerated in HD (Purves et al., 2004)

There is also significant neuronal loss in the cortex; especially pyramidal neurons in layers III, V, and VI, including those that project directly to the striatum, are degenerated. In the late stages of HD, atrophy is observed in a broad range of brain regions, including globus pallidus, thalamus, subthalamic nuclei, substantia nigra, amygdala, hippocampus, pons and medulla, spinal cord, superior olive, claustrum, and debatably the cerebellum (Cowan and Raymond, 2006).

One of the pathological characteristics of HD is the appearance of neuronal intranuclear inclusions (NIIs) and protein aggregates in dystrophic neurites. Neuronal intranuclear inclusions were shown in the cortex and striatum of transgenic mice, and post-mortem HD brains. The inclusions contain the N-terminal fragment of huntingtin (htt) and ubiquitin (Davies et al., 1997; DiFiglia et al., 1997; Sapp et al., 1999). These data suggest that the mutant huntingtin is targeted for degradation, but cannot be properly degraded. These aggregates are probably responsible for the dysfunction and ultimate degeneration of the neuron. NIIs are thought to be cytotoxic, since in cell lines, transcription factors, such

as cAMP response-element binding protein (CBP) or Sp1 can be recruited to NIIs, which may result in transcriptional dysregulation (McCampbell et al., 2000; Nucifora et al., 2001).

1.4. Molecular Genetics

1.4.1. The HD Gene Structure and Mutation

The HD gene was mapped to chromosome 4p16.3 in 1983, however the identification of the responsible gene remained to be elucidated for ten years (Gusella et al., 1983). In 1993, the disease-causing gene was discovered by the Huntington Disease Collaborative Research Group, using the exon trapping method for the first time (The Huntington's Disease Collaborative Research Group, 1993). The HD gene, also called IT15 (interesting transcript-15), consists of 67 exons, that are spread across 180 kb. The wild-type HD gene contains a stretch of uninterrupted CAG trinucleotide repeats within its first exon, which is translated into a series of consecutive glutamine residues, known as the polyglutamine (polyQ) tract (Rubinsztein and Carmichael, 2003). The CAG trinucleotide repeats in the HD gene are located 17 codons downstream of the initiator ATG codon in exon 1. The number of CAG repeats is 35 or fewer in normal individuals, with 17–20 repeats found most commonly. Repeats between 27 and 35 are rare and are not associated with disease, but are meiotically unstable and can expand into the disease range of 36 and above, especially if transmitted through the paternal line. Most adult-onset cases have 40–50 CAG repeats, whereas expansions of 55 and more repeats frequently cause the juvenile form of the disease. Repeats between 36 and 39 are associated with reduced penetrance, since some individuals with repeats in this range develop HD, and others do not (Imarisio et al., 2008).

The HD gene CAG trinucleotide repeat is followed by an adjacent polymorphic CCG repeat region which is translated into a stretch of prolines (6-12 repeats) (Pecheux et al., 1995). Moreover, in Exon 58, there is a codon-loss polymorphism (Δ2642) which is rare in normal chromosomes, but overrepresented in HD chromosomes (Rubinsztein et al., 1995).

1.4.2. Genotype-Phenotype Relations

1.4.2.1. Age of Onset. It is well-established that there is a strong inverse correlation between the age of onset and expanded CAG repeat length in Huntington's Disease (Langbehn et al., 2004). The expanded CAG repeat number in the first exon of the HD gene explains about 42-73 per cent of the variance in the age of onset of the disease (Metzger et al., 2006). It is likely that the remaining variation in the age of onset of HD is due to the combination of both environmental and genetic factors beyond the HD gene (The U.S.–Venezuela Collaborative Research Project and Wexler, 2004).

1.4.2.2. Penetrance. In Huntington's Disease, if CAG repeats on the expanded allele are in the range of 36–39, the age of onset is either very late, or disease does not occur at all (Quarrell et al., 2007). Repeats between 36 and 39 are rare, and are associated with reduced penetrance. However, since studies are performed with small sample sizes, and since, most of the time, only individuals who develop HD can be studied, and individuals who do not manifest symptoms escape detection, the estimates of penetrance remain poor (Myers, 2004).

1.4.2.3. Anticipation. Generally, 28 or more CAG repeats show instability on replication, that may lead to CAG repeat expansion. On replication, large but not pathogenic expansions of CAG repeats, happen almost exclusively in spermatogenesis than in oogenesis. These findings account for the phenomenon of anticipation in Huntington's Disease. The age of onset decreases in successive generations, and most likely the children with juvenile-onset symptoms inherit the disease from their fathers. Similarly, HD cases, with a negative family history, are results of expansion of an allele in the borderline or normal range (28–35 CAG repeats), most usually on the paternal side (Walker, 2007).

1.5. Huntingtin mRNA and Protein

The human HD gene is expressed as two different mRNA transcripts in all human tissues. Although the HD gene is ubiquitously expressed, the highest levels are found in the brain and testis (Borrell-Pagès et al., 2006).

The HD gene encodes a protein of 3144 amino acids, called huntingtin (htt) (Young, 2003). Huntingtin is a multidomain protein with a molecular mass of 348 kDa that contains a polymorphic glutamine / proline-rich domain at its amino-terminus (Landles and Bates, 2004). Downstream of this polymorphic region, there are multiple HEAT (huntingtin elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase TOR1) repeat sequences (Figure 1.3). A HEAT repeat is a degenerate ~40-amino-acid-long sequence which is composed of two anti-parallel α-helices forming a hairpin structure. HEAT motifs are involved in protein-protein interactions. Huntingtin contains an active carboxy-terminal nuclear export signal (NES) sequence and a less active nuclear localization signal (NLS). Upstream of the polyQ/polyP region, there are several lysine residues which appear to compete for post-translational modifications: SUMOylation and ubiquitination. Finally, htt contains well-characterized protease cleavage sites, and both wild-type and mutant htt are cleaved by various intracellular proteases, including caspase 1, 3, 6, 7 and 8, calpain and an unidentified aspartic protease (Imarisio et al., 2008; Cattaneo et al., 2005).

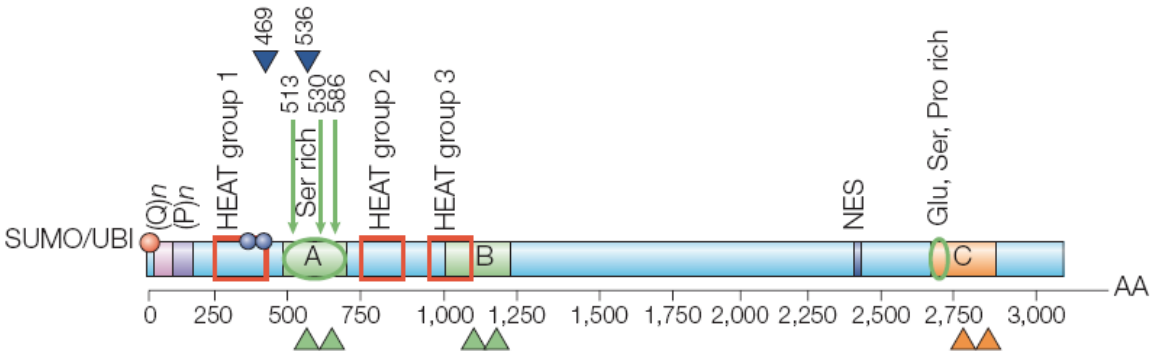


Figure 1.3. Schematic diagram of huntingtin amino acid sequence, showing SUMOylation and ubiquitination sites, polyQ tract, polyP tract, HEAT repeats, protease cleavage sites and nuclear export signal (Cattaneo et al., 2005)

1.6. Function of Wild-Type Huntingtin

It has been difficult to identify the normal function of htt, since it contains very little sequence homology to other known proteins, and since it is localized in many subcellular compartments. Within cells, htt is mainly localized in the cytoplasm, but it is also present

in the nucleus, and is associated with a number of organelles and structures including the Golgi apparatus, endoplasmic reticulum, mitochondria, clathrin-coated vesicles, endosomal and endoplasmic compartments, microtubules and plasma membrane. Considering its subcellular localization, htt appears to be involved in various cellular functions. Consistent with this, htt interacts with several proteins, which are involved in gene expression, intracellular transport, intracellular signalling and metabolism (Li and Li, 2004; Harjes and Wanker, 2003). Among others, roles of htt in transcription and intracellular transport are well established (Charrin et al., 2005; Sugars and Rubinsztein, 2003). Huntingtin is involved in transcriptional regulation by interacting with several transcription factors and other proteins responsible for the regulation of mRNA biosynthesis (Li and Li, 2004; Harjes and Wanker, 2003). For example, the function of htt as a transcriptional regulator in the production of brain-derived neurotrophic factor (BDNF) is well understood. Huntingtin binds and sequesters REST (repressor element-1 silencing transcription factor)/NRSF (neuron-restrictive silencer factor), a transcription factor that binds to NRSE (neuron-restrictive silencer element), an upstream consensus sequence, found in many genes. In this way, huntingtin acts as a positive regulator of transcription of NRSE-regulated genes, such as BDNF (Zuccato et al., 2003).

There is strong support for huntingtin to be also involved in trafficking. Huntingtin interacts with several proteins that have regulatory roles in intracellular transport or endocytosis. These proteins include huntingtin-associated protein 1 (HAP1), huntingtin-interacting protein 1 and 14 (HIP1 and HIP14), HIP1-related protein (HIP1R), protein kinase C and casein kinase substrate in neurons-1 (PACSIN1). Binding of htt with HAP1 subsequently results in its interaction with dynein/dynactin motor complex and kinesin. Through this interaction, huntingtin directly promotes the microtubule-based transport of BDNF in neurons (Borrell-Pagès et al., 2006).

Huntingtin is found to be an essential protein for normal embryonic development, since the loss of htt increases apoptosis and alters transport of maternal nutrients into the fetus, leading to death of mouse embryos. This finding suggests that in addition to its other roles, htt may have anti-apoptotic properties (Nasir et al., 1995; Zeitlin et al., 1995). Several studies reported, that overexpression of wildtype huntingtin protects neurons from apoptotic insults, such as those caused by starvation or mitochondrial toxins. Anti-

apoptotic properties of wildtype huntingtin can be explained by its binding and sequestering of the pro-apoptotic protein HIP1, which together with HIPPI (HIP1 protein interactor) can activate pro-caspase 8 and initiate apoptosis (Gervais et al., 2002).

1.7. Mutant Huntingtin and Huntington's Disease Pathogenesis

Huntingtin with the expanded polyglutamine tract at its N-terminal, leads to dysfunction and finally death of neurons. However, in which way these expanded polyglutamines lead to neuronal degeneration remains to be understood. To explain the pathogenesis of Huntington's Disease, several pathological mechanisms were proposed. It is most likely, that these different pathological mechanisms add together, and subsequently, lead to neurodegeneration (Figure 1.4).

1.7.1. Gain and Loss of Function of the Mutant Huntingtin

The current hypothesis on molecular mechanisms leading to HD is, that gain-of-toxic function of the mutant protein plays an important role in disease pathogenesis, but also loss of the protective functions of wild type huntingtin contributes to neuronal degeneration (Gil and Rego, 2008).

Patients with Wolf–Hirschorn Syndrome, which occurs as a consequence of the terminal deletion of chromosome 4, including the HD gene, do not develop an HD-like phenotype. Yet, most of the time, these patients cannot live long enough to develop HD (Cattaneo, 2003). However, mice with only one functional HD gene do not show features of the disease (Duyao et al., 1995).

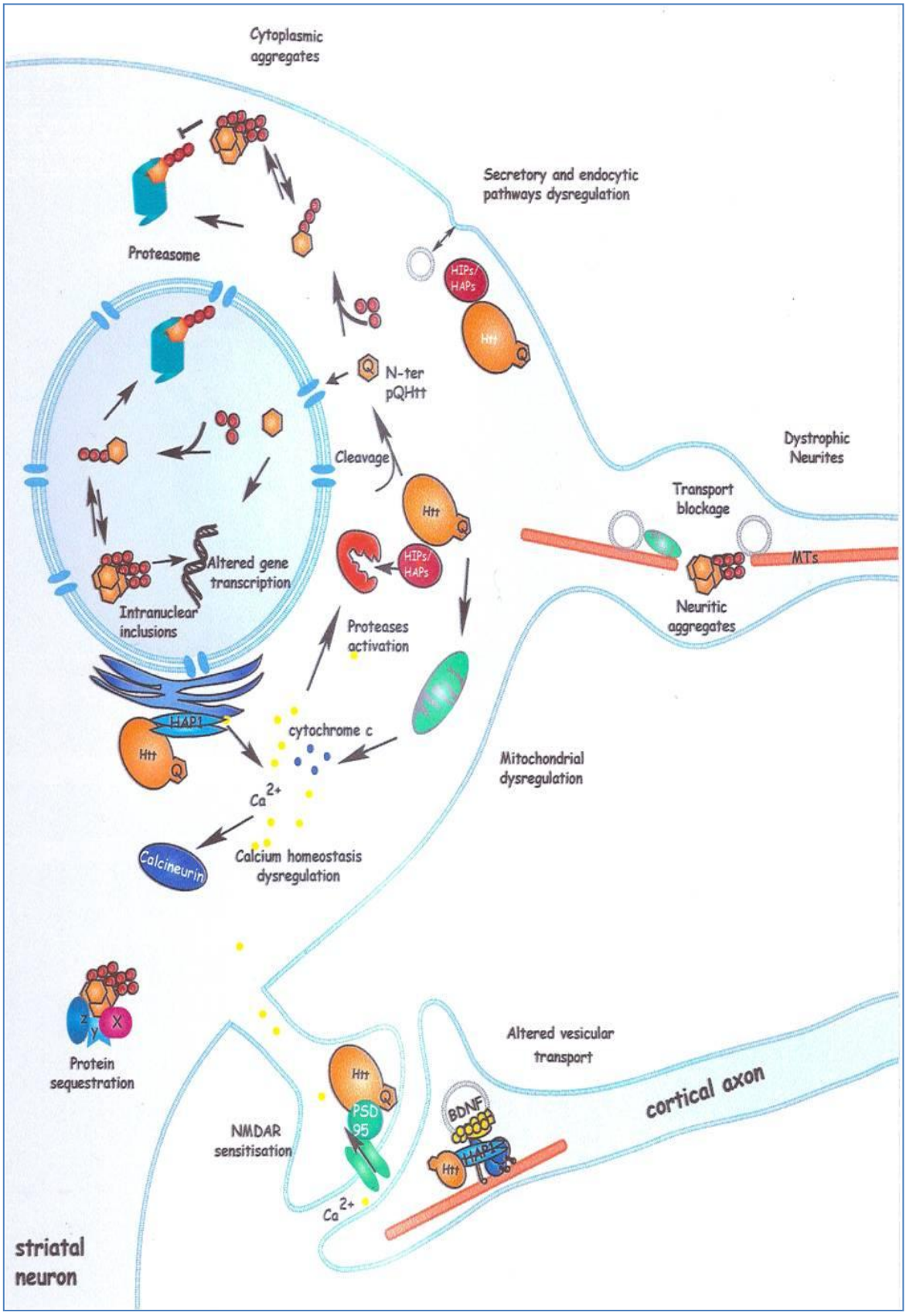


Figure 1.4. Model for HD Pathogenesis (Borrell-Pagès et al., 2006)

The gain-of-toxic-function hypothesis was strongly supported by the study which demonstrated that the expanded CAG repeat is toxic itself. A mouse model with 146 CAGs, inserted into the hypoxanthine phosphoribosyltransferase (HPRT) gene, which is not associated with any polyQ disorder, produced a polyglutamine expanded form of the hypoxanthine phosphoribosyltransferase protein and developed a late onset neurological phenotype that leads to death. However, inactivation of the HPRT gene did not result in the same phenotype; this supports the idea that a specific gene is not required, expanded CAG repeat itself is toxic enough to develop the neurological phenotype (Ordway et al., 1997).

1.7.2. Cleavage and Nuclear Translocation of Mutant Huntingtin

One of the key steps in HD pathogenesis is the proteolytic cleavage of huntingtin into N-terminal fragments, containing the polyQ tract, and their subsequent translocation to the nucleus. To induce neurodegeneration, nuclear translocation was found to be crucial, and reducing cleavage of polyQ-huntingtin was shown to decrease its toxicity slowing disease progression (Wellington et al., 2000). Mutant huntingtin may be cleaved into different fragments by different proteases, including caspases and calpains. The huntingtin protein contains two cleavage sites at residues 513 and 552, which are susceptible to caspase 3 and produce N-terminal fragments of approximately 70 and 75 kDa, respectively. The site at residue 552 is also cleaved by caspase 2. Caspase 6 cleaves htt at residue 586, and produces a slightly larger peptide fragment which is 80 kDa in size (Imarisio et al., 2008). Importantly, a recent study, done with YAC mice, expressing caspase 3- and caspase 6-resistant mutant huntingtin, strongly suggests that cleavage at the caspase 6 site is required for neuronal dysfunction and degeneration in HD (Graham et al., 2006; Warby et al., 2008).

These data indicate that the cleavage of mutant htt may be a rate-limiting step in HD pathogenesis, in such a way that, proteolytic cleavage may be a crucial factor for translocation of polyQ fragments into the nucleus and the formation of intranuclear inclusions.

1.7.3. Mutant Protein Aggregation

Subsequent their cleavage, the N-terminal fragments of mutant huntingtin form neuronal intranuclear and intracytoplasmic inclusions which are pathological hallmarks of HD; these aggregates are a feature of all nine polyglutamine diseases (DiFiglia et al., 1997) (Figure 1.5). However, it is not completely understood, if these inclusions represent toxic effects or protective properties. The formation of neuronal intranuclear inclusions may be a protective mechanism, since the highest percentage of NII-containing neurons are found in the regions which are not degenerated. The formation of NIIs may enable the cell to store the ubiquitinated toxic products temporarily, before they are degraded by the proteasome (Borrell-Pagès et al., 2006).

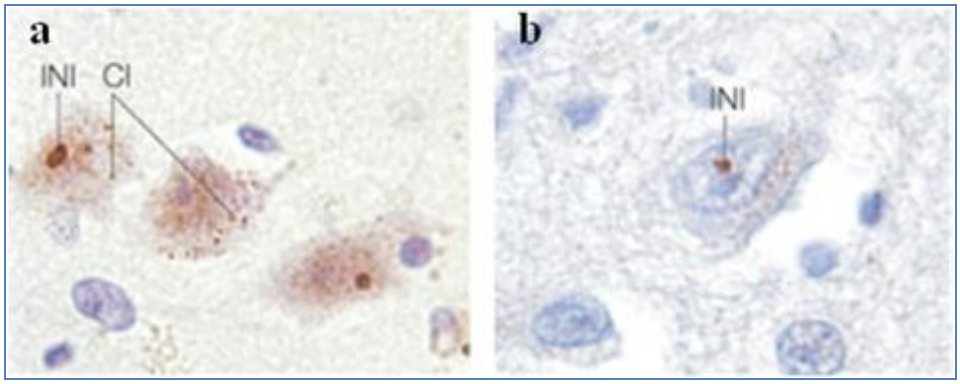


Figure 1.5. Intranuclear inclusions in the HD brain: a. Intranuclear and intracytoplasmic inclusions in the motor cortex of the HD brain, localized with 1C2 antibody to expanded polyglutamine, b. Intranuclear inclusion in the frontal cortex of the HD brain, localized with anti-ubiquitin antibody (Ross and Poirier, 2005)

Intranuclear and intracytoplasmic aggregates also interfere with normal proteins by recruiting some of them into their matrices. Such proteins include those that usually interact with wild-type huntingtin; these are several nuclear and cytoplasmic proteins that regulate transcription, apoptosis, mitochondrial function, tumour suppression, neurotransmitter release and axonal transport (Walker, 2007).

1.7.4. Chaperones and Ubiquitin-Proteasome System

Chaperones are proteins that assist folding of newly-synthesized proteins into their correct conformation. Hsp70 and Hsp40 are the two main classes of molecular chaperones that function in the folding process (Hartl and Hayer-Hartl, 2002). The mutant huntingtin protein has been shown to interact with the chaperone families Hsp70 and Hsp40. These chaperones were colocalized with the mutant htt in aggregates, in both animal models of HD and patient tissue samples. This suggests that the impairments in the protein folding process may contribute to HD pathology (Sakahira et al., 2002) (Figure 1.6).

The Ubiquitin-Proteasome System (UPS) is responsible for the turnover of most soluble proteins present in the cytoplasm and the nucleus of the cell, and functions via degrading short-lived regulatory proteins and damaged or misfolded proteins. The proteasome also has a role in cell signalling, as it degrades many regulatory proteins, such as p53 (Davies et al., 2007). To degrade the substrate proteins, the UPS is composed of several elements. These elements are i) ubiquitin, a highly conserved 76-amino acid protein that has to be added to the substrate to direct it to proteasome for degradation; ii) enzymes that transfer ubiquitin fragments to the substrate; and iii) a multicatalytic protease, called 26S proteasome (Ortega et al., 2007).

The first evidence that supported the hypothesis of an impairment of the UPS in HD, was the staining of neuronal intranuclear and intracytoplasmic inclusions with anti-ubiquitin and anti-proteasome antibodies. These findings were observed both in the brains of HD patients and also in the brains of mouse models of HD. Additionally, pharmacological inhibition of proteasome results in the accumulation of ubiquitin-immunopositive aggregates and increased toxicity (Ortega et al., 2007). Sequestration of the UPS components in aggregates and the alteration of subcellular localization of proteasomes might affect the UPS activity in HD, like in all other neurodegenerative diseases with protein aggregations. It is also suggested that expanded polyglutamine-containing proteins cannot be easily degraded by the proteasome, leading to impairment in the proteasome system (Imarisio et al., 2008) (Figure 1.6).

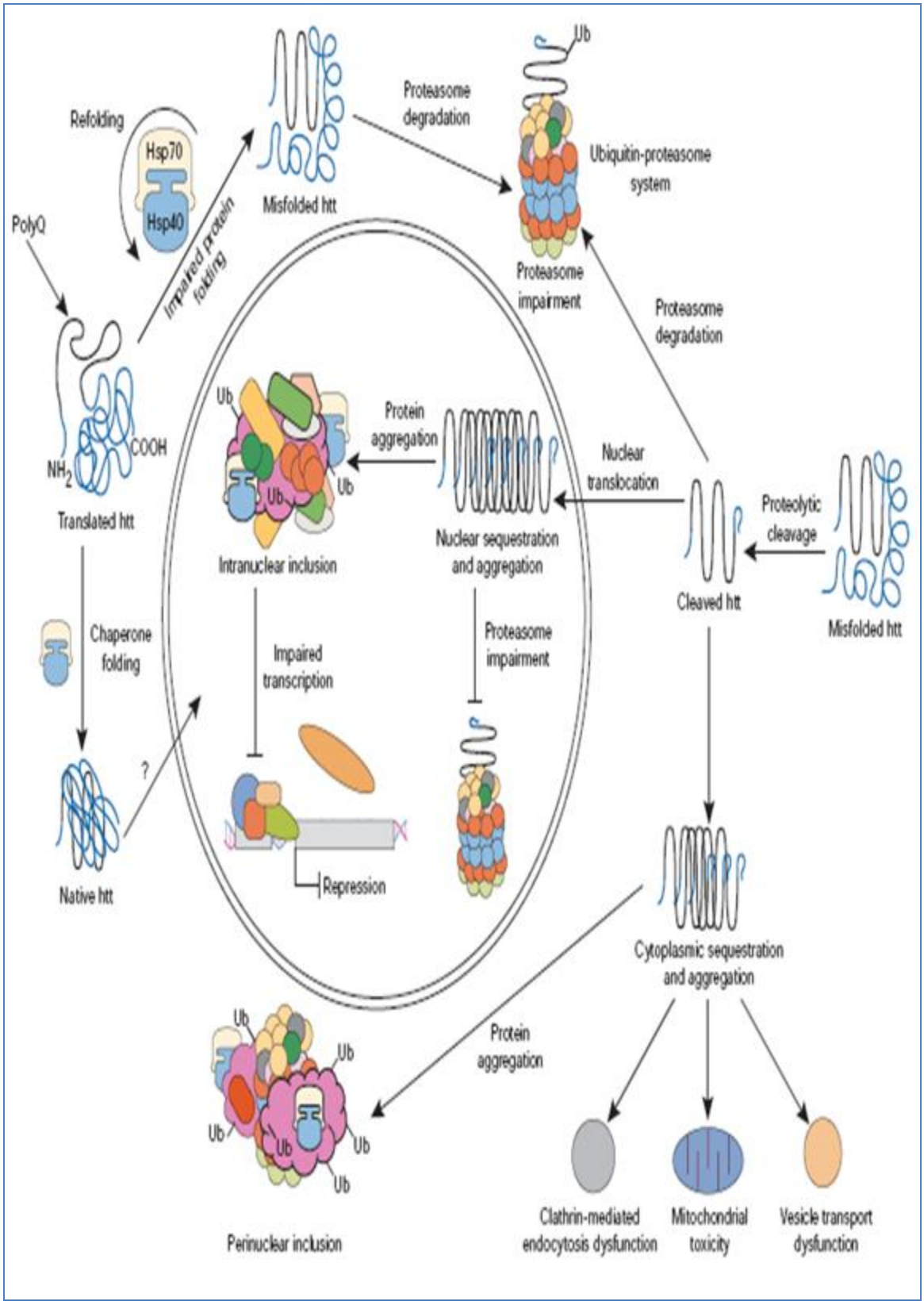


Figure 1. 6. Pathogenesis of HD: Role of Chaperones and the UPS

(Landles and Bates, 2004)

1.7.5. Transcriptional Dysregulation

Cleavage and subsequent nuclear localization of the mutant huntingtin may result in changes in the nuclear functions, such as transcription. Several studies demonstrated that the huntingtin protein, with abnormal polyglutamine tract, sequesters nuclear proteins, including transcription factors, into nuclear aggregates leading to transcriptional dysregulation (Helmlinger, 2006).

Two transcriptional pathways are more extensively implicated in HD; CBP [CREB (cAMP-response element-binding protein)-binding protein] and Sp1 (specificity protein 1) pathways. These transcription factors have vital functions in the expression of many genes. CBP is an important transcription co-activator and is a major mediator of survival signals in neurons. It was demonstrated that huntingtin and CBP interact via their polyglutamine tracts and CBP is sequestered in polyglutamine aggregates (Riley and Orr, 2006). Sp1 is also a transcriptional activator that binds to upstream GC-rich elements in certain promoters. Sp1 selectively binds and directs core components of the general transcriptional complex such as TFIID (transcription factor IID), TBP (TATA-box-binding protein) via its glutamine-rich transactivation domain. The N-terminal fragment of huntingtin has been shown to interact with Sp1 and interferes with Sp1-driven gene regulation (Chen-Plotkin et al., 2006).

Wildtype huntingtin protein is involved in the transcriptional regulation of BDNF, which is an important factor for the survival of striatal neurons and for the activity of corticostriatal synapses. Studies showed that wild type huntingtin modulates BDNF expression in the cortex by regulating its transcription, but the mutated form of huntingtin cannot accomplish this activity. Wild type huntingtin has the ability to bind and sequester the REST/NRSF complex and to prevent its translocation to the nucleus and consequently allow BDNF transcription. However, mutated huntingtin does not bind REST/NRSF effectively, leading to its accumulation in the nucleus. This leads to transcriptional repression of NRSE-sensitive genes, such as BDNF (Zuccato et al., 2003).

1.7.6. Apoptosis

Apoptosis is a conserved cellular mechanism, initiated by diverse stimuli, that lead to cell death. Caspases play an intimate role in apoptosis by directly or indirectly initiating the process of programmed cell death (Pattison et al., 2006). Involvement of caspases in the pathogenesis of Huntington's Disease has been suggested by several studies. In the apoptotic process, caspase-3 cleaves structural and nuclear proteins, as well as other caspases. Caspase-3 also specifically cleaves huntingtin, and if huntingtin contains an expanded polyglutamine tract, it becomes more susceptible to caspase-3 cleavage. N-terminal cleavage products of mutant huntingtin were found to be more toxic and more prone to aggregate formation than the full-length protein (Rubinsztein and Carmichael, 2003).

Early studies with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) identified apoptotic-like cells in the HD striatum. However, after DNA electrophoresis, DNA laddering, which is a characteristic pattern of apoptosis, could not be observed in some of these studies. Although the authors interpreted the presence of TUNEL-positive cells in the HD striatum as indicators of apoptosis, the lack of apoptotic DNA fragmentation argues against it. Furthermore, activated astrocytes are detected in HD brains, suggesting neuroinflammation, which is generally absent in apoptotic conditions. In conclusion, the evidence for a pure apoptotic process contributing to HD cell death is controversial. However, it is likely that the activation of apoptotic pathways contribute to HD pathology to some degree (Gil and Rego, 2008).

1.7.7. Mitochondrial Dysfunction

Various lines of evidence demonstrate the involvement of mitochondrial dysfunction in the pathogenesis of HD. Magnetic resonance imaging spectroscopy showed increased production of lactate in the cerebral cortex and basal ganglia in HD patients, showing a respiratory chain defect (Kwong et al., 2006; Petrozzi et al., 2007). Striatal neurons of mutant huntingtin knock-in mouse embryos showed impaired mitochondrial respiration and ATP production. Decreased activities of mitochondrial complexes II and III of the electron-transport chain in the human HD brain were shown by biochemical studies (Lin

and Beal, 2006). It is likely that involvement of the mitochondrial dysfunction is important in HD pathogenesis, because 3-nitropropionic acid and malonate, which are mitochondrial toxins that selectively inhibit succinate dehydrogenase and complex II, induce a clinical and pathological phenotype that resembles HD (Knott et al., 2008). Huntingtin has been shown to bind to mitochondrial membranes in neurons, using electron microscopy (Panov et al. 2002). One possible mechanism of mitochondrial impairment may be the direct association of mutant huntingtin with the outer mitochondrial membrane (Kwong et al., 2006).

In addition to energy production, mitochondria also play an important role in cellular calcium homoeostasis. Mutant htt may induce calcium-dependent membrane permeability by interacting with mitochondria, and reduce the calcium load, needed to induce the opening. The mechanism of htt-mediated facilitation of mitochondrial permeabilization is still not completely understood, but may involve aberrant interactions between the mutant protein and components of the pore (Kwong et al., 2006).

Mutant huntingtin could also affect mitochondrial function by altering transcription of p53, which is a tumour suppressor protein. p53 is involved in transcriptional regulation of various mitochondrial proteins, which may underlie the mitochondrial abnormalities, especially the vulnerability to mitochondrial depolarization, seen in HD tissues (Sawa, 2001).

1.7.8. Excitotoxicity

The term “excitotoxicity” refers to cell death, resulting from the toxic actions of excitatory neurotransmitters. In the mammalian central nervous system (CNS), the major excitatory neurotransmitter is glutamate. Neuronal excitotoxicity occurs as a result of prolonged exposure to glutamate, which is associated with the excessive influx of ions and water into the cell, leading to death of neurons. Hyperactivation of glutamate receptors causes calcium overload within the cell leading to activation of enzymes that degrade proteins, membranes and nucleic acids (Fan and Raymond, 2007).

One of the hypotheses, that try to explain sensitivity of the medium-sized spiny neurons (MSNs) of striatum to degeneration in HD, is the "excitotoxicity hypothesis". This hypothesis states that, in HD, excessive activation of glutamate receptors in striatal MSNs, may result in dysfunction and death of striatal neurons. This excessive activation of glutamate receptors may be due to increased glutamate release from cortical neurons, reduced uptake of glutamate by glia, or hypersensitivity of post-synaptic glutamate receptors on striatal projection neurons, likely with the contribution of altered intracellular calcium homeostasis and mitochondrial dysfunction (Fan and Raymond, 2007) (Figure 1.7).

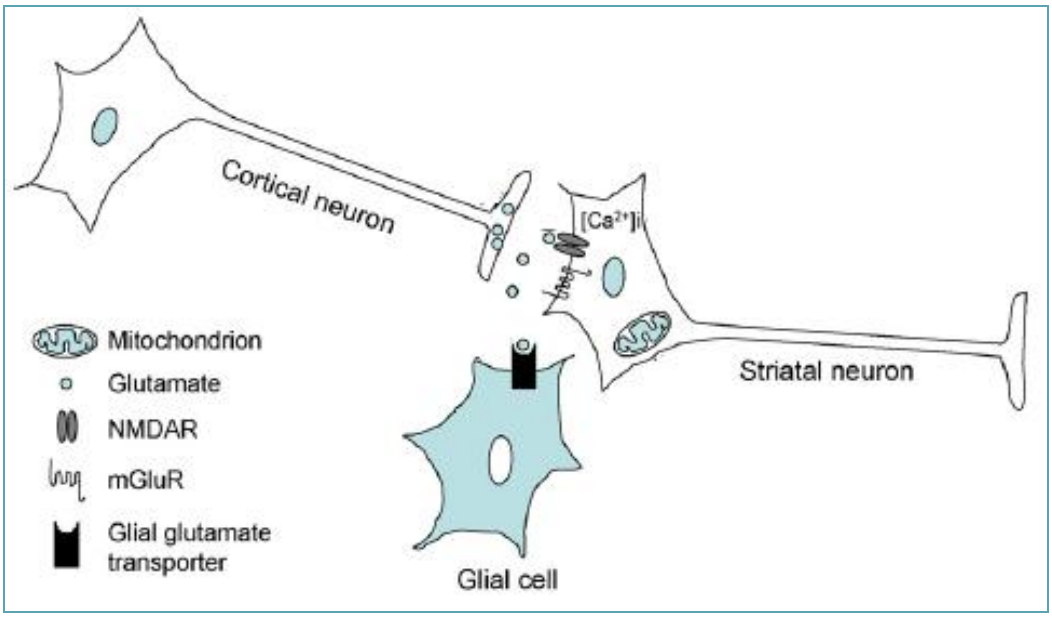


Figure 1.7. Possible points along the corticostriatal pathway which may contribute to neuronal excitotoxicity (Fan and Raymond, 2007)

After its release from synaptic terminals, glutamate activates two families of receptors: Metabotropic receptors and ionotropic receptors. The metabotropic glutamate receptors (mGluR1–7) are coupled to G-proteins and to second messenger signaling. Once activated, mGluR1 and five receptors lead to increased levels of the second messenger inositol triphosphate (IP3), which then binds to the IP3 receptor on endoplasmic reticulum to release intracellular calcium stores. In the YAC128 mouse model of HD, mutant htt has been shown to bind directly to IP3 receptors and make them more sensitive to IP3, which results in increased intracellular calcium release in response to glutamate, and contribute to

excitotoxic neuronal death. Glutamate also activates ionotropic receptors that are ligand-gated ion channels. There are three subclasses of ionotropic glutamate receptors which are named for the agonists that selectively activate them: Kainate receptors activated by kainic acid, AMPA receptors activated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and NMDA receptors activated by N-methyl-D-aspartate. Among these three, NMDA receptors (NMDARs) are especially notable for high permeability to calcium, slow activation and deactivation kinetics, and sensitivity to voltage-dependent block by extracellular magnesium. Consequently, NMDARs play a more prominent role in mediating excitotoxicity than the other glutamate receptors (Cowan and Raymond, 2006).

Functional NMDARs are tetrameric structures composed of two NR1 subunits and two NR2 subunits. NMDARs are co-activated by glutamate and glycine which bind to the NR2 and NR1 subunits, respectively. Eight splice variants of the NR1 subunit have been described, while four different genes encode for the NR2 subunit (NR2A, B, C and D) in a developmentally and regionally regulated manner. Within the striatal MSNs, NR2A and NR2B subunits are predominantly expressed (Sanchez et al., 2008).

Several lines of evidence suggest involvement of NMDA receptor-mediated excitotoxicity in the selective neuronal degeneration process of HD. First, studies with HD transgenic mouse models show increased NMDA receptor activity in neurons. Second, administration of NMDA receptor agonists to the striatum of normal animals causes a selective loss of MSNs and creates HD-like symptoms. Furthermore, NMDA receptor antagonists reduce excitotoxicity in animal models of HD (Shin et al., 2005) Studies of transgenic mouse models of HD also demonstrate changes in NMDAR subunit expression. In the striatum of symptomatic HD mice, the NR2A and NR2B subunits were shown to have significantly decreased intensity with immunohistochemical staining of brain slices, while the NR1 subunit intensity was normal (Cepeda et al., 2001). All these evidences together confirm the involvement of excitotoxicity in the selective neuronal degeneration process seen in HD.

1.8. Huntington's Disease in Turkey

Until today, only a limited number of studies investigated the clinical and genetic characteristics of Huntington's Disease in Turkey. According to these previous reports, incidence of Huntington's Disease in Turkish population is estimated to be 1 in 10000, meaning that approximately 7000 new cases will be diagnosed with HD each year in Turkey. However, since consanquineous marriages are frequent in Turkey, this number may be higher than estimated.

A previous study from our laboratory investigated 130 individuals from 85 families. Out of these 85 families, 98 patients belonging to 71 families had a clinical diagnosis of HD. Using molecular analysis, the HD diagnosis was confirmed in 84 patients. 11 of these 84 patients, did not have an apparent family history of HD. Absence of family history in these patients was speculated to be a result of young parental age, early parental death, variable penetrance of the disease in the family or expansion of the parental repeats in the meiotic instability range into the pathogenic size in the next generation. Patients, investigated in this study, originated from all over Turkey, showing a pattern of homogeneous distribution of Huntington's Disease in Turkey (Ersoy, 1999; Ersoy, 2005).

Another study investigated the HD gene in a cohort of 27 Turkish HD patients from 19 families. In this cohort, CAG repeat numbers varied in the range of 40 to 76 and were found to be inversely correlated with age of onset, explaining 81 per cent of the variance. The CCG repeat polymorphism in the HD gene was also analysed in this study, and all patients were shown to have 7 CCG repeats in this region (Ataç et al., 1999).

Analysis of the genetic structure of the HD gene was performed in a group of 127 Turkish HD patients and 122 Turkish healthy controls. CAG repeats ranged from 38 to 78 in HD patients, and from 10 to 35 in healthy controls. Negative correlation between the age of onset and the expanded CAG repeat size was also confirmed in this study, explaining 67 per cent of the variance (Akbaş and Erginel-Unaltuna, 2003). Studies on Turkish HD patients demonstrated that, the genetic basis of the HD gene, CAG repeat numbers, CCG repeat numbers and age of onset, in the Turkish population was strikingly similar to the populations of Western European descent.

1.9. Genetic Analysis of Candidate Genes Modifying the Age of Onset in HD

Huntington's Disease is a late onset disorder meaning that disease symptoms occur in midlife. Many studies have investigated the relationship between the age of disease onset and the expanded CAG repeats on the HD gene. It is commonly observed that this correlation accounts for 42-73 per cent of the variation in the age of onset (Li et al., 2003). Although expanded CAG repeat number is the strongest determinant of the age of onset, the mechanism by which mutant huntingtin triggers the cascade of HD pathogenesis and eventually produces delayed disease symptoms is not fully understood (Zeng et al., 2006). Under the light of current findings, it is assumed that gain of toxic function of the mutant huntingtin may lead to cumulative damage in vulnerable cells. It seems likely that the level of toxicity is dependent on the number of expanded CAG repeats. Longer repeats are more toxic, causing a faster damage and earlier cell death, which clarifies the condition of late onset (Kaplan et al., 2007). However, this model cannot explain the condition of patients, homozygous for the HD mutation. According to the cumulative damage mechanism, homozygous patients would have significantly earlier age of onset, but recent clinical findings show that homozygousity is not associated with earlier onset (Kaplan et al., 2007).

Currently, it is known that mutant huntingtin is cleaved by several proteases and the N-terminal fragments of the mutant huntingtin form neuronal intranuclear and intracytoplasmic inclusions. However, whether these inclusions represent toxic effects or protective properties is not fully understood yet (Borrell-Pages et al., 2006). One recent study suggests that formation of intranuclear inclusions is associated with the genetic background of mice. In this study, mice with the same number of CAG repeats in the HD gene showed different rates of intranuclear inclusion formation; it was fastest in the C57BL/6 background, slowest in the 129Sv background and intermediate in the FVB/N background (Lloret et al., 2006). Accordingly, several other studies strongly suggest that there are heritable factors other than expanded CAG repeat, that may affect the pathogenic process and subsequently the age of onset (AO) in humans (Djousse et al., 2003). A study with Venezuelan kindreds showed the effect of both environmental and genetic factors as modulators of age of onset. According to the results of this study, after elimination of the effect of CAG repeats, within the remaining variance in the AO, 40 per cent is attributable

to the modifier genes and 60 per cent is environmental (The U.S.–Venezuela Collaborative Research Project and Wexler, 2003).

Prediction of possible age of onset has critical importance in some conditions, however using a particular CAG repeat as the only indicator of AO does not result as precise as it should be, therefore these predictions are not reliable for clinical use (Langbehn et al., 2004). As a result, an extensive research is conducted on the identification of possible modifiers of age of onset; and variations in several genes were found to have modifier effects on onset age of HD.

There are two approaches to identify modifiers of age of onset: Searching for modifier loci with genomewide linkage analysis or analysis of variations in the candidate genes.

1.9.1. Genome-wide Linkage Analysis

One approach to identify modifiers of AO in HD is genome-wide linkage analysis. In order to identify a possible modifier region, a 10 cM density genome-wide scan was performed in 629 sibling pairs. An evidence for linkage was found at 4p16, 6p21-23 and 6q24-26, suggesting that these loci may be useful for investigation of modifier genes. Authors concluded that this genome-wide scan provides a baseline of comparison for other genome-wide scans in other populations in order to define whether there is an overlap in these regions (Li et al., 2003). Soon after these data were published, another study investigated several variations within or close to the HD gene. These were: Δ2462 deletion/insertion polymorphism within the HD gene, BJ56 also known as D4S127, a dinucleotide repeat located 23.4 kb 5' of the HD gene, and the MSX1 gene which is located 1.6 Mb proximal to 3' of the HD gene. Their results indicated that particular genotypes of the MSX1 gene are associated with younger age of onset when controlled for expanded CAG repeat size. On the other hand, variations investigated within the HD gene, did not show evidence of association with the AO (Djousse et al., 2004).

1.9.2. Candidate Gene Approach

The second approach to identify modifiers of age of onset in HD is investigation of variations within candidate genes. Possible candidate genes are the genes that encode for the proteins which interact with or are associated with the wildtype and/or mutant huntingtin protein. Polymorphisms in these genes may have modifier effects on the course of HD (Metzger et al., 2006).

One of the initial studies, using the candidate gene approach, searched for the variations within the mitochondrial DNA, the apolipoprotein E (ApoE) gene, glutamate receptor kainate subtype 6 (GluR6)-coding gene (GRIK2), and also for variations in the HD gene itself. The ApoE gene was investigated, since genotypes of this gene were found to be associated with age of onset in Alzheimer Disease. Mitochondrial DNA was chosen as a candidate with regard to the defects of mitochondrial metabolism seen in HD patients. The polymorphic TAA repeat region in the 3' UTR of the GRIK2 gene was investigated, since this gene resides on chromosome 6, near the region which shows linkage to the age of onset in previous studies, and also because of its possible involvement in the mechanism of excitotoxic cell death. These results indicated that TAA repeat variation within the GRIK2 gene explains 4.1 per cent of the total variance in the age of onset. Individuals with 16 TAA repeats within the GRIK2 gene were shown to have significantly earlier age of onset than expected (Rubinsztein et al., 1997). Consequently, modifier effect of the GRIK2 gene TAA repeat polymorphism was investigated in several other study populations; some of these studies confirmed the modifier effect of the GRIK2 gene (Chattopadhyay et al., 2003; MacDonald et al., 1999), however several other studies could not show this effect within their study group (Andresen et al., 2007; Metzger et al., 2006). In order to address the question of how this polymorphism may lead to earlier age of onset, all variations of the GRIK2 gene were analysed to find a variation in linkage disequilibrium with the TAA repeats. But the results indicated that the TAA repeat itself has the modifier effect, possibly via its role on the GRIK2 mRNA (Zeng et al., 2006).

A number of studies investigated the S18Y polymorphism in the ubiquitin carboxy-terminal hydrolase L1 (UCHL1) gene (Metzger et al., 2006; Naze et al. 2002), the apolipoprotein E ε2ε3 genotype (Kehoe et al., 1999) and the polymorphic (Gln-Ala)38

repeat in the transcriptional coactivator CA150 gene (Chattopadhyay et al., 2003; Holbert et al. 2001). These studies demonstrated that these genes or genotypes also have modifier effects on the age of onset of HD.

A recent study investigated the modifier effects of polymorphisms within the huntingtin-associated protein-1 (HAP1) gene. Their results indicated that the M441 polymorphism in the HAP1 gene is significantly correlated with AO. The M441 polymorphism is a nonsynonymous polymorphism, which replaces the amino acid threonine with methionine in the HAP1 protein. HD patients with less than 60 CAG repeats, and homozygous for the M441 genotype, develop their first symptoms approximately about 8 years later than HD patients with other genotypes (TT or TM). For the first time, in this study, the functional relevance of this variation was also studied. Previously, HAP1 was suggested as a protective protein against mutant htt and polyQ toxicity. This study demonstrated that the M441 form of HAP1 binds soluble mutant htt more tightly than T441, suggesting that it partially inhibits its degradation or its interactions with other proteins, therefore, reducing htt toxicity and delaying the AO of HD (Metzger et al., 2008).

Variations or genes, that could not be shown to have modifier effects on age of HD onset include: the BDNF gene (Metzger et al., 2006; Kishikawa et al., 2006; DiMaria et al., 2006), the methyltetrahydrofolate reductase (MTHFR) gene (Hansen et al., 2005), the huntingtin interacting protein-1 and 14 genes, the TATA binding protein (TBP) gene (Metzger et al., 2006), R72P variation in the TP53 gene and the R196K variation in the gene coding for the human caspase activated DNase (hCAD) (Arning et al., 2005).

Since excitotoxicity has been proposed to play a role in the pathogenesis of HD, and since the GRIK2 gene TAA repeat polymorphism was found to have a modifier effect on age of onset in HD, a number of studies investigated the modifier effects of genes that encode for glutamate receptor subtypes. Consistent with the knowledge, that NMDA receptors play the primary role in the excitotoxic process, variations in the genes encoding the NMDA receptor subunits were investigated. Among NMDA receptor subunits, NR2A and NR2B are abundant in the cortex and striatum, brain parts that are selectively degenerated in HD. Thus, variations of the NR2A and NR2B genes (also known as

GRIN2A and GRIN2B genes) were investigated for the presence of a modifier effect. As a result, particular NR2A and NR2B genotypes were shown to have significant modifier effect on age of onset. In addition to the variance explained by the expanded CAG repeat length, NR2B and NR2A genotype variations added 12.3 per cent and 4.5 per cent explanation to the variance on the age of onset, respectively (Arning et al., 2004). It was also demonstrated, that NR2A and NR2B gene variations modify age of onset in a sex-specific manner, suggesting that female patients, with a particular genotype on the NR2B gene, have later age of onset when compared to individuals with other genotypes (Arning et al., 2007).

Among the polymorphisms of the glutamate receptor-coding genes, studied in the previous studies, polymorphisms which were shown to have significant correlation with the age of onset in HD are compiled in Table 1.2. Among these, the rs1042339 polymorphism was excluded from statistical analysis in the previous study, since all members of the population had the same genotype (Arning et al., 2004, Rubinsztein et al., 1997).

Table 1.2. Variations of the glutamate receptor genes as possible modifiers of AO

SNP	Chromosome	Gene	Region	Variation	Additional Variance Explained	Reference
rs10548788	6q16.3	GRIK2	3' UTR	TAA repeats insertion/deletion	2-4 per cent	Rubinsztein et al., 1997
rs1969060	16p13.2	NR2A	Intron	C / T	4.5 per cent	Arning et al., 2004
rs890	12p13.1	NR2B	3' UTR	T / G	5.2 per cent	Arning et al., 2004
rs1806201	12p13.1	NR2B	Exon 13	C / T Synonymous	12.3 per cent	Arning et al., 2004
rs1042339	12p13.1	NR2B	Exon 13	G / A Missense	-	Arning et al., 2004

2. PURPOSE

Huntington's Disease is a late-onset disorder; this means, the HD gene mutation carriers do usually not develop disease symptoms until mid-life. It is well-established that the age of onset in HD is inversely correlated with expanded CAG repeat number. However, this correlation explains only about 42-73 per cent of the variance in the age of onset. Recent studies indicate, that the remaining variation in the age of onset in HD is also heritable, suggesting that the genetic modifiers affect the pathogenic process prior to onset of HD. To extend the previous findings on modifiers of age of onset in HD and to strengthen the hypothesis of excitotoxicity in HD, additional work is needed. Today, an extensive research is ongoing for the identification of the genetic modifiers of age of onset in Huntington's Disease. It is essential to demonstrate the presence or absence of the modifier effects of previously studied genes in different populations.

In the framework of this thesis, we aim to investigate modifier effects of five polymorphisms in three different genes in the Turkish population. These genes encode for glutamate receptor subtypes. The polymorphisms to be investigated were previously shown to have modifier effects on age of onset in HD in several other populations.

The aims of this study can be summarized in the following points:

- To investigate the molecular basis of HD in Turkish HD patients
    - To identify CAG repeat expansions in the patients
    - To investigate a large Turkish kindred, originating from Tokat, harbouring many individuals affected by HD

- To investigate the correlation between the age of onset in HD and CAG repeat genotypes in the HD gene
    - To investigate the genotype and allele frequency distribution of the following polymorphims in the Turkish population:
        - GRIK2 rs10548788
        - NR2A rs1969060
        - NR2B rs1806201
        - NR2B rs890
        - NR2B rs1042339
    - To study the statistical significance of modifier effects of these polymorphisms on the age of onset in HD

3. MATERIALS

3.1. Blood Samples

Blood samples of patients were provided by the Neurology Departments of the following Medical Schools: İstanbul University, İstanbul and Cerrahpaşa Medical Schools, İstanbul; Celal Bayar University, Manisa; Adnan Menderes University, Aydın; Gazi University, Ankara; İnönü University, Malatya; Hacettepe University, Ankara; Uludağ University, Bursa; Ege University, İzmir; Kocaeli University, Kocaeli; Tepecik Social Security State Hospital, İzmir; Atatürk University, Erzurum; Akdeniz University, Antalya; 19 Mayıs University, Samsun; Dokuz Eylül University, İzmir; Buca Social Security State Hospital, İzmir; Marmara University, İstanbul; Haseki Education and Research Hospital, İstanbul; VKV American Hospital, İstanbul; Gaziosmanpaşa University, Tokat; Yeşilyurt Hospital, İzmir; Erzurum Education and Research Hospital, Erzurum; Manisa Psychiatry Hospital, Manisa; Bakırköy Research Hospital for Psychiatry and Neurology, İstanbul; Erciyes University, Kayseri; Süleyman Demirel University, Isparta.

A total number of 103 HD patients, all of whom originated from Turkey, were investigated in the framework of this thesis. This number includes an affected member of a large kindred originating from Tokat, in the Middle Black Sea Region of Turkey, several members of which have manifested or are at risk for developing HD.

3.1.1. A Large Turkish Kindred from Tokat Affected with HD

In this kindred, peripheral blood samples were collected from six individuals with typical HD symptoms, 10 asymptomatic family members at 50 or 25 per cent risk for HD, and one female patient with Parkinson Disease-like symptoms.

Informed written consent for participation was obtained from each individual, studied in this thesis (Appendix A). For all patients, HD clinical diagnosis was firmly

established. The age of onset of HD was estimated as the age, at which motor or cognitive symptoms first occurred.

3.2. DNA Samples

Nine DNA samples with previously identified TAA genotypes (GRIK2 gene), were kindly provided by Dr. Silke Metzger, University of Tübingen, Germany.

3.3. Oligonucleotide Primers

Sequences of the primers, used in the amplification of the HD gene CAG repeat region, GRIK2 gene rs10548788 polymorphism (Paschen et al., 1994), NR2A gene rs1969060 polymorphism (Arning et al., 2004) and NR2B gene rs1806201, rs890 and rs1042339 polymorphisms (Arning et al., 2004), are given in Table 3.1.; these primers are purchased from Iontek, Istanbul.

Table 3.1. Oligonucleotide primer pairs used in PCR

Primer Name	**Primer Sequence**
P3F	Forward: 5'-TCTGCTTTTACCTGCGGCC-3'
HD3	Reverse: 5'-GGCGGTGGCGGCTGTTGCTGCTGC-3'
GRIK2F	Forward: 5'-CAACACCTTTTCTCTAACCCC-3'
GRIK2R	Reverse: 5'-CTCGGCCAGTTTTTACAACTTG-3'
NR2A060F	Forward: 5'-GGTTTTAAGATTTGTGCCAGG-3'
NR2A060R	Reverse: 5'-CTTAGACCGAGTTGGCAACA-3'
NR2B201F	Forward: 5'-GGTCATTTCTAGCCTCTCTGGA-3'
NR2B201R	Reverse: 5'-ATACTATGGGGCCGGTGGT-3'
NR2B890F	Forward: 5'-GCTGTCAGCCATTCCTGTT-3'
NR2B890R	Reverse: 5'-CATGAATTTAGCCAGAGCCTC-3'
NR2B339F	Forward: 5'-GACCACAAGCGCTACTTCAG-3'
NR2B339R	Reverse: 5'-TGTCATACAGGTTGCCTGCT-3'

3.4. Enzymes

Ex Taq DNA Polymerase	:	5U/µl, Takara, Japan
Go Taq DNA Polymerase	:	5U/µl, Promega, USA
AspI	:	10U/µl, Roche, Germany
DdeI	:	10U/µl, Roche, Germany
PsuI (XhoII)	:	5U/µl, Fermentas, Lithuania
TspRI (TscAI)	:	1U/µl, Fermentas, Lithuania

3.5. Chemicals

All solid and liquid chemicals used in this study, were purchased from Merck (Germany), Sigma (Germany) and Amresco (USA), unless stated otherwise in the text.

3.6. Buffers and Solutions

3.6.1. DNA Extraction

Lysis Buffer	:	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclease Buffer	:	10 mM Tris-HCl (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulfate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)

Proteinase K	:	20 mg/ml in dH_2O, Promega, USA
Sodium Chloride (NaCl)	:	2.5 M saturated stock solution
Absolute Ethanol (EtOH)	:	Riedel-de Haën, Germany
TE Buffer	:	20 mM Tris-HCl (pH8.0) 1 mM Na_2EDTA (pH8.0)

3.6.2. Polymerase Chain Reaction (PCR)

5X Go Taq Flexi Buffer	:	Proprietary formulation ($MgCl_2$ free) Promega,USA
Magnesium Chloride ($MgCl_2$)	:	25 mM, Promega, USA
Deoxyribonucleotides (dNTPs)	:	100 mM of each dNTP Promega, USA
Q Solution (5X)	:	Proprietary formulation Qiagen, Germany

3.6.3. Restriction Enzyme Analysis

Buffer B (10X)	:	10 mM Tris-HCl (pH 7.5) 10 M $MgCl_2$ 0.1 mg/ml BSA Fermentas, Lithuania

Buffer B (10X)	:	100 mM Tris-HCl 1 M NaCl 50 mM $MgCl_2$ 10 mM 2-mercaptoethanol (pH 8.0) Roche, Germany
FastDigest Buffer (10X)	:	Proprietary formulation Fermentas, Lithuania
Buffer H (10X)	:	500 mM Tris-HCl 1 M NaCl 100 mM $MgCl_2$ 10 mM Dithioerythritol (pH 7.5) Roche, Germany

3.6.4. Agarose Gel Electrophoresis

10X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric Acid 20 mM Na_2EDTA (pH 8.3)
Ethidium Bromide (EtBr)	:	10 mg/ml
Agarose	:	Agarose Basica Le, Prona, EU
6X Loading Dye	:	10 mM Tris-HCl (pH 7.6) 0.03 per cent Bromophenol Blue 0.03 per cent Xylene Cyanol FF 60 per cent glycerol 60 mM EDTA Fermentas, Lithuania

DNA Ladder	:	100 base pair (bp) Fermentas, Lithuania

3.6.5. Polyacrylamide Gel Electrophoresis

40 per cent Acrylamide Stock (19:1)	:	38 per cent Acrylamide 2 per cent N, N'-Methylene Bisacrylamide
8 per cent Instagel Solution	:	8 per cent Acrylamide Solution (19/1) 8 M Urea 1X TBE buffer (pH 8.3)
Ammonium Persulfate	:	10 per cent APS (w/v) in dH_2O
TEMED	:	N,N,N,N-tetramethylethylenediamine
PAGE Loading Dye	:	95 per cent formamide 20 mM EDTA 0.05 per cent xylene cyanol 0.05 per cent bromophenol blue

3.6.6. Silver Staining

Staining Buffer (Buffer B)	:	0.1 per cent $AgNO_3$ (w/v) in dH_2O
Developing Buffer (Buffer C)	:	1.5 per cent NaOH 0.01 per cent $NaBH_4$ 0.015 per cent formaldehyde in dH_2O

3.7. Kits

Nucleic Acid Isolation Kit	:	Roche, Germany
Gel Extraction Kit	:	Qiagen, Germany

3.8. Equipment

Autoclaves	:	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	:	440-47N, Kern, France
Centrifuges	:	5415, Eppendorf, Germany Universal 16R, Hettich, Germany Allegra X22-R, Beckman Coulter, USA
Deep Freezers	:	2021D (-20^{0}C), Arçelik, Turkey Sanyo (-70^{0}C), Sanyo, Japan
DNA Isolation	:	MagNA Pure Compact Instrument Version 1.0 Roche, Germany
Documentation System	:	GelDoc Documentation System BIO-RAD, USA
Electrophoretic Equipments	:	i-MyRun-N, CosmoBio Co., Ltd, Japan Minicell Primo E320, Thermo, USA Mini Sub Cell GT, BIO-RAD, USA Wide Sub Cell GT BIO-RAD, USA Sequi-Gen Cell, BIO-RAD, USA

Heat Block	:	Thermostat Heater 5320 Eppendorf, Germany
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer HS31, UK
Ovens	:	MD 554, Microwave Oven Arçelik, Turkey EN 400 (37°C), Nuve, Turkey BD53 (56°C), Binder, Germany
Water bath	:	1083 Shaking Water Bath, GFL, Germany
Power Suppliers	:	EC 135-90 Thermo, USA Model 200, BRL, USA
Refrigerators	:	4250T, Arçelik, Turkey
Spectrophotometer	:	NanoDrop ND-1000, Thermo, USA
Thermocyclers	:	TC 312, Techne, UK Techgene, Progene, UK Techne, Progene, UK Touchgene Gradient, Progene, UK
Vortex	:	Fisons WhirliMixer, UK Reax Top, Heidolph, Germany
Water Purification	:	WaTech Water Technologies, Turkey

4. METHODS

4.1. DNA Extraction from White Blood Cells

4.1.1. Sodium Chloride Extraction

Ten mililiters of peripheral blood sample was collected from each individual into sterile vacutainer tubes containing K_2EDTA as anticoagulant, and stored at 4^0C until extraction.

The blood samples were transferred into 50 ml sterile falcon tubes. Three volumes of ice-cold red blood cell (RBC) lysis buffer was added and the cells were allowed to lyse at 4^0C for 15 minutes. After a 15-minute incubation period, the samples were centrifuged at 5000 rpm for 10 minutes at 4^0C to precipitate the nuclei of the lysed cells. The supernatant was discarded, and the pellet was resuspended with 10 ml lysis buffer by vortexing. The samples were centrifuged again at 5000 rpm for 10 minutes at 4^0C, and after centrifugation the supernatant was discarded. If the precipitate was dark reddish, indicating contamination with red blood cell debris, the last resuspension and the centrifugation steps were repeated. The supernatant was discarded, the nuclear pellet was resuspended in three ml of ice-cold nuclei lysis buffer and vortexed until clumps were dissolved. The samples were incubated with 30 µl Proteinase K (20 mg/ml) and 50 µl 10 per cent SDS at 56^0C for three hours or at 37^0C overnight.

After incubation, 10 ml 2.5 M NaCl was added to the samples, and the tubes were shaken well before centrifugation. They were then centrifuged at 5000 rpm for 30 minutes at room temperature. The supernatant, containing the DNA, was precipitated with two volumes of absolute ethanol by gently inverting the tubes, until the DNA thread became visible. The DNA was fished out and transferred into an Eppendorf tube, and kept at room temperature until ethanol evaporated. The DNA was then dissolved in 100-1000 µl TE buffer, left at room temperature for overnight, and then stored at 4^0C.

If there was no visible DNA precipitation after the addition of ethanol, the tubes were kept at -70^0C for at least two hours. Then, they were centrifuged at 14000 rpm for 30 minutes at 18^0C. After centrifugation, the supernatant was discarded, and the DNA pellet was dissolved in 80-100 µl TE buffer.

4.1.2. DNA Extraction by MagNA Pure Compact

MagNA Pure Compact is a fully automated nucleic acid extraction system, that uses the specifically designed MagNA Pure compact nucleic acid isolation kit. The extraction principle of this system is based on the affinity of magnetic beads for the DNA. During extraction, DNA binds to the magnetic glass particles, and in the elution step, it is removed from the beads. From 1 ml blood sample, 200 µl DNA in buffer solution is obtained, which can be further diluted according to the concentration required.

4.2. Analysis of the Extracted DNA Samples

4.2.1. Agarose Gel Electrophoresis

The quality and quantity of the DNA was evaluated by running the samples on a one per cent agarose gel. The gel was prepared by dissolving one gram of agarose in 100 ml 0.5X TBE buffer and by boiling it. After cooling to 50^0C, ethidium bromide, which is a DNA-intercalating agent, that enables to visualize the DNA under UV light, was put into the mixture in a final concentration of 0.5 mg/ml, the solution was poured onto a gel plate, and the combs were inserted. When the gel solidified, the combs were removed and the plate was placed into an electrophoresis tank, containing 0.5X TBE buffer. Each DNA sample was mixed with 6X loading dye to a final concentration of 1X, and they were loaded into the slots of the gel with a micropipette. The gel was run at 150 V for 10-15 minutes. The DNA bands were visualized under UV light and recorded; the quality and quantity of the DNA sample was determined by comparing its intensity with known DNA samples.

4.2.2. Spectrophotometric Measurement of DNA

The exact amount and concentration of the DNA sample was determined by spectrophotometric measurement using a NanoDrop Spectrophotometer. In the NanoDrop Spectrophotometer, a pulsed xenon flash lamp provides the light source and the spectrometer is used to analyze the light after passing through the sample. The instrument is controlled by PC-based software, and the data is logged in an archive file on the PC.

1 µl of DNA was pipetted into the end of the receiving fiber optic cable of the NanoDrop Spectrophotometer, and the source fiber optic cable was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. Optical density of the DNA was measured at 260 nm (OD_{260}).

4.3. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a method to amplify specific regions of the target DNA. Fifty to hundred ng of genomic DNA was used as template in all PCR assays. After addition of DNA and all PCR components, the volume of each PCR was completed to 25 µl with distilled water, and amplification was initiated in a thermal cycler. After completion of reaction, the samples were run in a two per cent agarose gel to test the quality and quantity of the amplified sample. For this purpose, five µl of each PCR product was mixed with one µl of 6X loading dye and run at 175 V for 15-20 minutes. The 100 bp ladder was used as the DNA size marker on the gel. The gel was visualized and recorded under UV light as described in 4.2.1.

4.3.1. PCR Protocol for HD Gene CAG Repeat Region

To amplify the CAG repeat region in the first exon of the HD gene, the following PCR conditions were applied with the PCR components shown in Table 4.1.

Initial Denaturation	:	95^0C	5 minutes	
Denaturation	:	95^0C	1 minute	} 30 cycles
Annealing	:	60^0C	1 minute	
Extension	:	72^0C	1 minute	

Table 4.1. CAG Repeat Region PCR components

Components	**Volume (µl)**	**[Stock]**	**[End]**
$MgCl_2$	2,2	25 mM	2,2 mM
dNTP	0.8	25 mM	0.8 mM
P3F	0.5	12,5 µM	0.25 µM
HD3	0.5	12,5 µM	0.25 µM
Q Solution	5.0	5X	1X
Buffer	2.5	10X	1X
Ex Taq DNA Polymerase	0.2	5 units/µl	0.04 units/µl

4.3.2. PCR Protocols for Polymorphisms of Glutamate Receptor Genes

To amplify the polymorphisms on the glutamate receptor genes, the following PCR conditions were applied with the primer annealing temperatures shown in Table 4.2.

Initial Denaturation	:	94^0C	5 minutes	
Denaturation	:	94^0C	45 seconds - 1 minute	} 28 - 30 cycles
Annealing	:	30 seconds		
Extension	:	72^0C	1 minute	
Final Extension	:	72^0C	5 minutes	

4.2. Annealing temperatures of the PCR assays

Polymorphism	Annealing Temperature
GRIK2 rs10548788	62.1^0C
NR2A rs1969060	58.8^0C
NR2B rs1806201	60.5^0C
NR2B rs890	58.8^0C
NR2B rs1042339	59^0C

PCR components are shown in Table 4.3.

Table 4.3. Glutamate receptor genes polymorphisms: PCR components

		VOLUMES (µl)					
Components	**[Stock]**	**GRIK2 rs10548788**	**NR2A rs1969060**	**NR2B rs1806201**	**NR2B rs890**	**NR2B rs1042339**	**[End]**
$MgCl_2$	25 mM	2	3	1	1	1	1 – 3 mM
dNTP	25 mM	0.8	0.8	0.8	0.8	0.8	0.8 mM
Forward Primer	12,5 µM	1	1	1	1	1	0.5 µM
Reverse Primer	12,5 µM	1	1	1	1	1	0.5 µM
Buffer	5X	5	5	5	5	5	1X
DNA Polymerase	5 units/µl	0.2	0.2	0.2	0.2	0.2	0.04 units/ µl

4.4. Restriction Enzyme Analysis

Restriction enzyme (RE) analysis was performed for detection of single nucleotide polymorphisms (SNPs) (Table 4.4).

Table 4.4. Restriction enzymes, their recognition sites and the expected results of the enzyme digestion (N: any nucleotide; S: C or G; R: A or G, Y: C or T, ↓: cutting site sense strand, ’: cutting site antisense strand)

SNP	Gene	RE	Recognition Site	SNP Type on the Gene	Result
rs1969060 C / T	NR2A	DdeI	5’-C↓TNA’G-3’	TGCC↓TCA’G	digested
				TGCTTCAG	undigested
rs1806201 T / C	NR2B	TspRI	5'-‘NNCASTGNN↓-3'	‘CCCACTGCA↓AC	digested
				CCCACCGCAAC	undigested
rs890 G / T	NR2B	PsuI	5'- R↓GATC’Y-3'	G↓GATC’T	digested
				TGATCT	undigested
rs1042339 G / A	NR2B	AspI	5'-GACN↓N’NGTC-3'	GACT↓C’CGTC	digested
				GACTCCATC	undigested

Restriction enzyme reaction mixture components and incubation temperatures are given in Table 4.5.

Table 4.5. Restriction enzyme analysis: Components

	DNA	RE		Buffer		dH_2O	Incubation Temperature
NR2A rs1969060	3 µl	DdeI	0.5 µl	Buffer H	1 µl	5.5 µl	37^0C
NR2B rs1806201	3 µl	TspRI	0.7 µl	FastDigest Buffer	1 µl	5.3 µl	65^0C
NR2B rs890	3 µl	PsuI	0.5 µl	Buffer B	1 µl	5.5 µl	37^0C
NR2B rs1042339	3 µl	AspI	0.2 µl	Buffer B	1 µl	5.8 µl	37^0C

After incubation, the digested PCR products were directly mixed with two µl of 6X loading dye and electrophoresed on a two per cent agarose gel together with the 100 bp

size marker at 175 V for 15-20 minutes. The gel was then visualized under UV light and the bands were recorded. The expected agarose gel band patterns are shown in Figure 4.1.

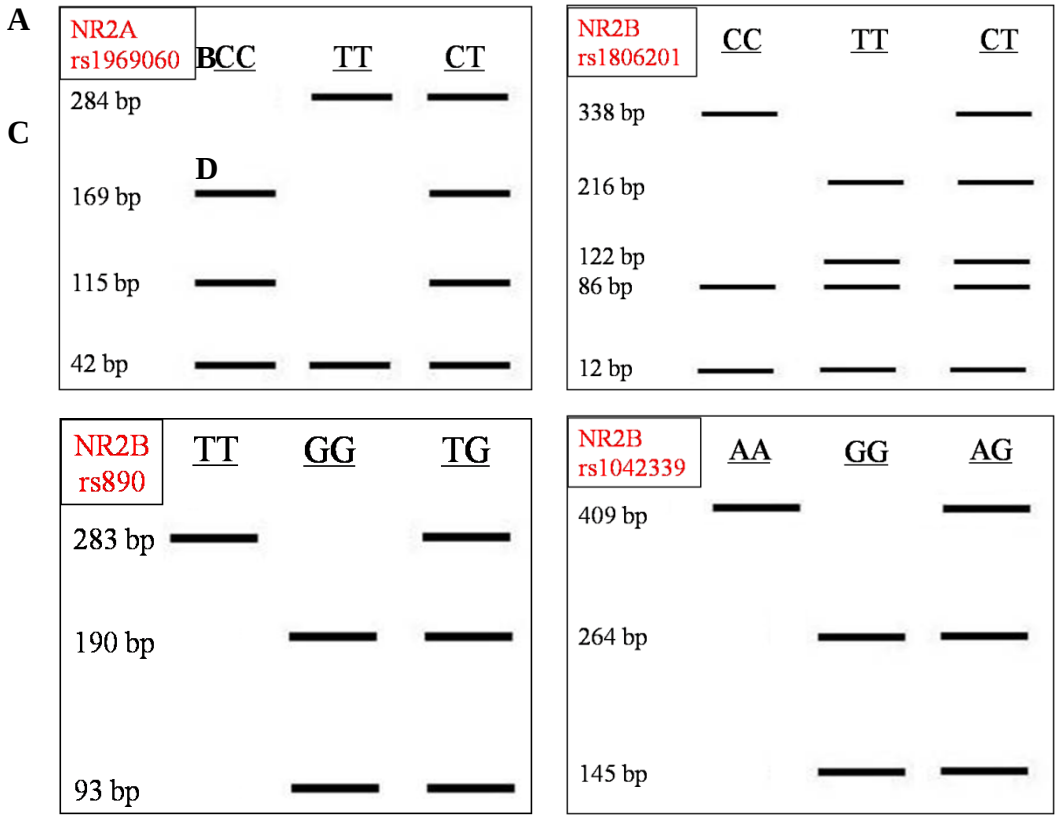


Figure 4.1. Illustration of DdeI (A), TspRI (B), PsuI (C) and AspI (D) digestions on agarose gels

4.5. Polyacrylamide Gel Electrophoresis

To identify the TAA repeat genotypes on the GRIK2 gene, the samples were run on an eight per cent polyacrylamide gel with known controls.

4.5.1. Preparation of Denaturing Polyacrylamide Gels

The 38 x 50 cm sequencing apparatus was assembled using 0.75 mm spacers. Fortyfive ml of eight per cent Instagel was mixed with 350 µl of 10 per cent APS and 35 µl of TEMED, and immediately poured between two glass plates. The sharks-tooth comb was

inserted in inverted position. The gel was allowed to polymerize for at least 45 minutes before the electrophoretic run.

4.5.2. Denaturing Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out in 1X TBE buffer pre-heated for five minutes in a microwave oven to 55-60^{0}C. The gel was prerun in the hot buffer for 15 minutes at a constant power of 70 W to allow the gel temperature to rise to 50-55^{0}C. The DNA samples were mixed with PAGE loading dye in 1:1 ratio, denatured at 95^{0}C in the incubator for five minutes, and immediately chilled on ice. The comb was reoriented into the correct position to form wells, and the upper part of the gel was cleaned from urea and gel particles by rinsing the slots with electrophoresis buffer. For each sample 2.5 µl was loaded into wells, and the gel was run at a constant power of 65 W. The duration of the electrophoresis was approximately two and a half hours; this was adjusted according to allele sizes and predicted distances between the alleles. After electrophoresis, the gel was silver-stained to visualize the alleles (see Section 4.7.3).

4.5.3. Silver Staining

After electrophoresis, the glass plates were separated. The gel usually remained attached to one of them, and was removed with the help of a filter paper. The gel was transferred to a container, Buffer B was added carefully and shaken for 10 minutes. Then, the gel was washed twice with dH_2O to remove excess silver. It was incubated in Buffer C until bands appeared. If the bands were weakly stained and difficult to analyse, the staining procedure was repeated. Finally, the gel was washed with dH_2O at least for five times, and was sealed in a transparent folder.

4.6. Determination of Fragment Sizes by GeneScan Analysis

To identify TAA repeat genotypes in the GRIK2 gene, all samples were subjected to GeneScan Analysis using ABI PRISM® 310 Genetic Analyzer (Iontek Ltd., İstanbul). GeneScan system is a method of capillary electrophoresis that is used to determine fragment sizes. GeneScan software compares the length of PCR fragments with its size

marker, makes a standard curve, and calculates the fragment sizes according to this standard curve.

To determine PCR fragment sizes by GeneScan Analysis, PCR products should be labeled by a fluorescent dye. For this purpose, a 5'FAM-labeled forward primer was used to amplify the TAA repeat region in the GRIK2 gene. First, samples with previously identified TAA genotypes were amplified and analysed by GeneScan system. The correlation was established between TAA repeat numbers and their corresponding fragment sizes. Using the formula, which represents the slope of the line in the correlation of TAA repeats and fragment sizes, GRIK2 gene TAA repeat genotypes of patients were calculated.

4.7. DNA Sequencing

DNA sequencing is the method of choice for the identification of CAG repeat numbers in HD gene. For this purpose, the samples were sent to Iontek Ltd., Istanbul.

4.7.1. Preparation of PCR Samples for DNA Sequencing

Samples, showing a positive band pattern in agarose gel electrophoresis, were analysed by DNA sequencing to identify CAG repeat numbers, on both normal and expanded alleles. Since the normal and expanded PCR fragments need to be sequenced one by one, PCR products were run on two per cent agarose gels until the bands separated. Then, they were cut from the gel using a scalpel, and extracted from agarose gel with the QIAquick Gel Extraction Kit by following the instructions of the manufacturer. Finally, the extracted PCR products were run in a two per cent agarose gel to visualize the quality and quantity of the purification.

4.8. Statistical Analysis

Frequencies of the variations of the glutamate receptor-coding genes, the mean value of expanded and normal CAG repeat numbers and the mean value of age of onset of the study group were determined.

Since the relationship between the age of onset in Huntington's Disease and the expanded CAG repeat length is curvilinear, the variability in the age of onset that is attributable to the expanded CAG repeat number was calculated by linear regression analysis. The relationship between the age of onset to the expanded CAG repeat number is best represented by logarithmic transformation of the age of onset. Regression analysis was performed, using the logarithmically transformed age at onset as the dependent variable, and the size of expanded CAG repeat as the independent variable.

Using the framework of generalized linear models in analysis of variance, modifier roles of glutamate receptor variations and also the modifier role of normal CAG repeat number on the age of onset were determined.

The general linear model procedure provides regression analysis and analysis of variance for one dependent variable by one or more factors and/or variables. The factor variables divide the population into groups. For regression analysis, the independent variables are specified as covariates.

In this analysis, the logarithmically transformed age at onset was set as the dependent variable, since the expanded and normal CAG repeat numbers and the GRIK2 gene TAA repeat numbers are quantitative variables, they were set as covariates; since NR2A and NR2B genotypes are categorical variables, they were set as fixed factors. Sex-specific addition of genotype variations to the effect of expanded CAG repeat number was also analysed. All statistical analyses were carried out under consideration of the effect of the expanded CAG repeat numbers. SPSS version 11.5 for Windows was used for all statistical analyses.

5. RESULTS

5.1. Analysis of the CAG Repeat Expansion

5.1.1. PCR Amplification and DNA Sequencing

The CAG repeat region in the first exon of the HD gene was amplified by PCR (Figure 5.1). For sequencing, the normal and the expanded bands were extracted from agarose gel, separately (Figure 5.2).

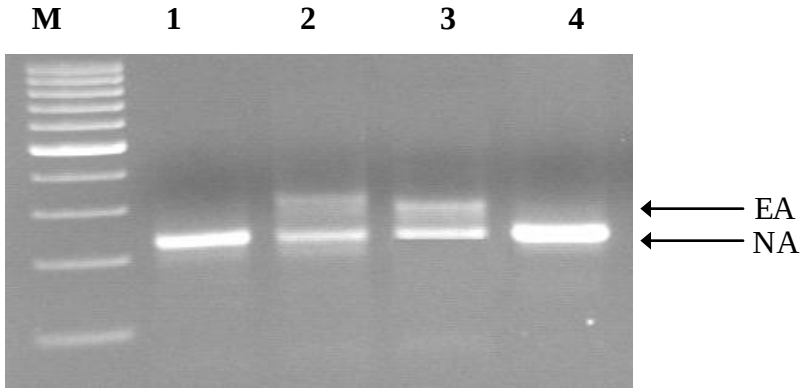


Figure 5.1. PCR amplification of the HD gene CAG repeat region
M: 100 bp ladder, lane 1: negative control, lane 2: positive control, lanes 3 and 4: HD samples, NA: normal allele, EA: expanded allele

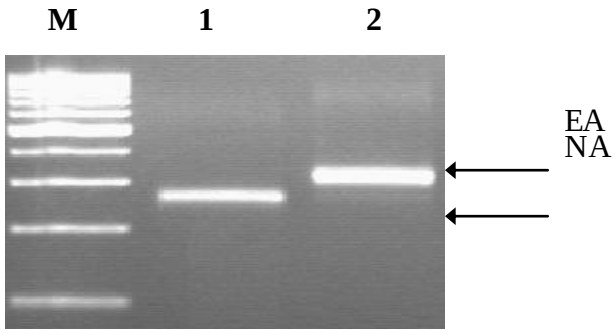


Figure 5.2. PCR products after extraction from agarose gel
M: 100 bp ladder, lane 1: normal allele, lane 2: expanded allele

Products (expanded and normal alleles) were sequenced to identify the CAG repeat numbers (Figures 5.3 and 5.4).

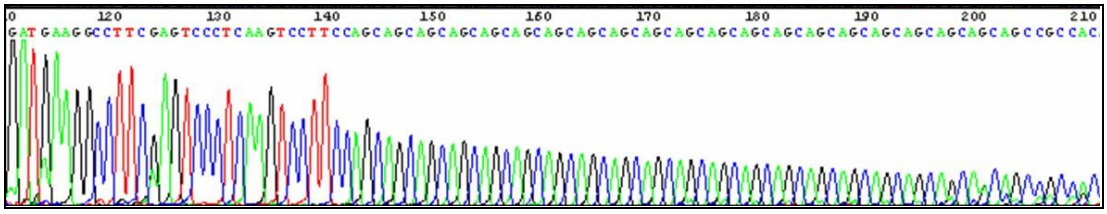


Figure 5.3. Chromatogram: DNA sequencing of the normal allele

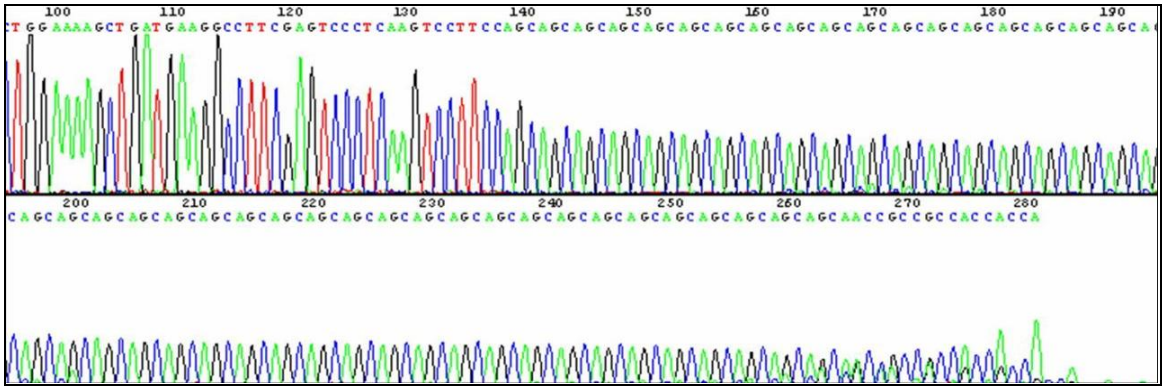


Figure 5.4. Chromatogram: DNA sequencing of the expanded allele

5.1.2. Distribution of the CAG Repeat Numbers in the Study Population

The study population, consisting of 108 Turkish HD patients, was classified according to the CAG repeat numbers (Table 5.1).

Table 5.1. Distribution of the CAG repeats within the study population

Variable	Minimum	Maximum	Most frequent allele	Mean ± Standard Deviation
Expanded CAG repeat number	39	101	44 (15.7 per cent)	46.6 ± 7.9
Normal CAG repeat number	8	29	17 (23.1 per cent)	19.2 ± 3.4

5.2. A Large Turkish Kindred from Tokat Affected with HD

A large Turkish kindred, with many individuals affected with HD or at risk for developing HD, was investigated in the framework of this thesis. Table 5.2. compiles the ages of onset, molecular diagnosis, CAG repeat numbers and inheritance of the mutant HD allele for each individual of this family. Pedigree of the family, spanning five generations, is shown in Figure 5.5.

Table 5.2. Features of the family members studied

Case Number	Age of Onset	Molecular Diagnosis	Normal CAG Length	Expanded CAG Length	Inheritance
III. 1	PD-like symp.	Negative	---	---	---
IV. 1	20	Positive	22	44	Maternal
IV. 2	40	Positive	18	44	Maternal
IV. 3	35	Positive	18	45	Maternal
IV. 4	40	Positive	17	44	Maternal
IV. 5	44	Positive	28	45	Maternal/Paternal
IV. 6	37	Positive	29	45	Maternal/Paternal
IV. 7	Presymptomatic	Negative	---	---	---
IV. 8	Presymptomatic	Negative	---	---	---
IV. 9	Presymptomatic	Negative	---	---	---
V. 1	Presymptomatic	Negative	---	---	---
V. 2	Presymptomatic	Positive	20	46	Maternal
V. 3	Presymptomatic	Positive	20	47	Maternal
V. 4	Presymptomatic	Negative	---	---	---
V. 5	Presymptomatic	Positive	19	46	Paternal
V. 6	Presymptomatic	Negative	---	---	---
V. 7	Presymptomatic	Negative	---	---	---

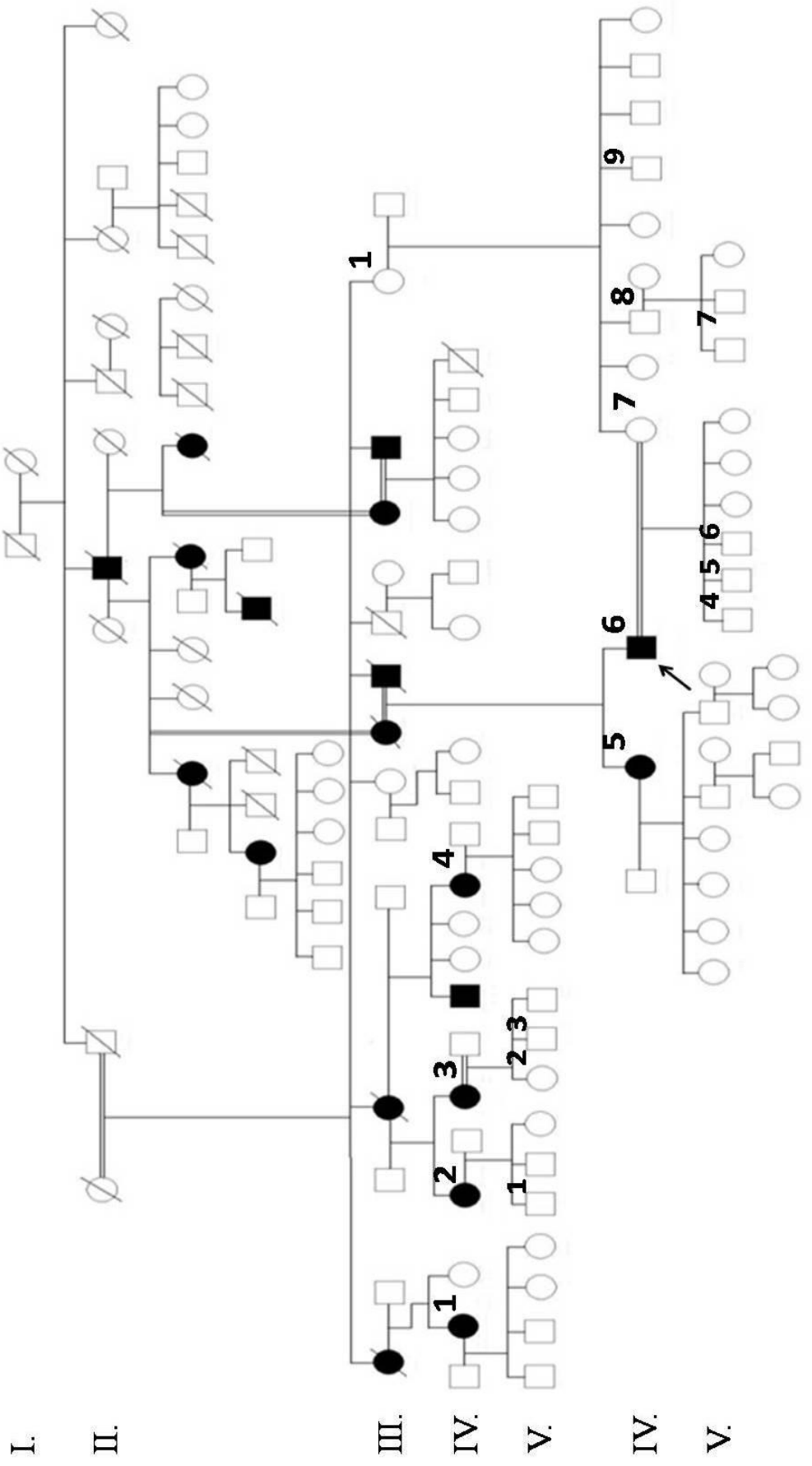


Figure 5.5. Pedigree of the family from Tokat

The kindred originates from Tokat, which is a medium-sized city in the Middle Black Sea region of Turkey, with a population of 620.000. Symptomatic patients and asymptomatic at-risk individuals of the family were investigated in the field. Consanguineous marriages were frequent in the family. Among the individuals studied, cases homozygous for the mutant HD allele were not found. Patient IV.1 could not be seen, since she was living in a small village far away from Tokat. All symptomatic patients had motor symptoms. Motor symptoms of patient IV.4 had just started with only, but apparent, involuntary movements of both thumbs.

All affected and several presymptomatic members of this family had a prominent skin lesion (Figure 5.6).

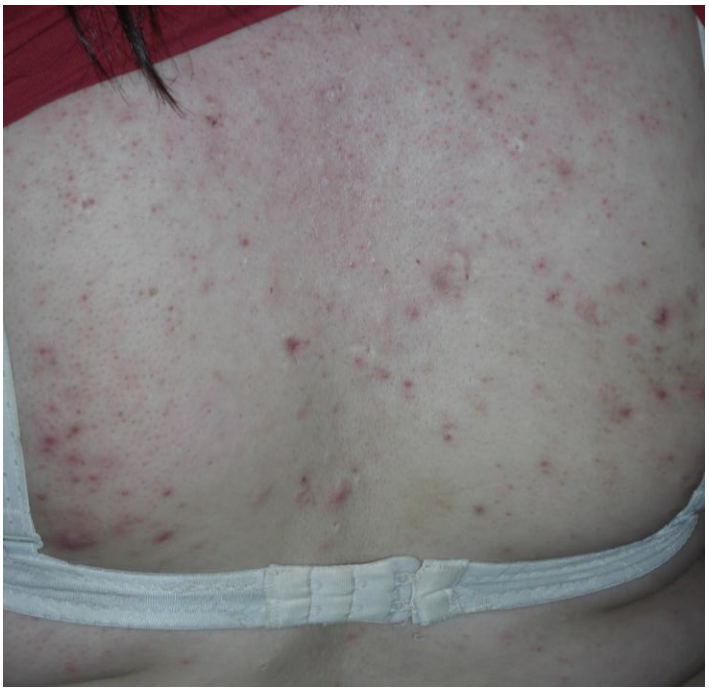


Figure 5.6. Skin lesions of the patient V.2

The possible correlation between these skin lesions and HD were investigated, to understand whether these lesions were exclusively seen in mutant HD gene carriers, as the clinicians of the family suspected. Our results indicated that all presymptomatic individuals, carrying the mutant HD allele, had skin lesions; still there were four individuals with skin lesions who did not carry the mutant allele (Table 5.3).

Table 5.3. Presence of skin lesions among presymptomatic individuals

Case Number	HD Diagnosis	Skin Lesions
IV. 7	Negative	Positive
IV. 8	Negative	Negative
IV. 9	Negative	Positive
V. 1	Negative	Positive
V. 2	Positive	Positive
V. 3	Positive	Positive
V. 4	Negative	Positive
V. 5	Positive	Positive
V. 6	Negative	Negative
V. 7	Negative	Negative

5.3. Analysis of the GRIK2 Gene rs10548788 Polymorphism

5.3.1. PCR Amplification, GeneScan Analysis and PAGE

The GRIK2 gene rs10548788 polymorphism (polymorphic TAA repeat region) was amplified by PCR using 5'-FAM-labeled primers (Figure 5.7). PCR products were subjected to GeneScan Analysis to determine the fragment sizes of both alleles (Figure 5.8).

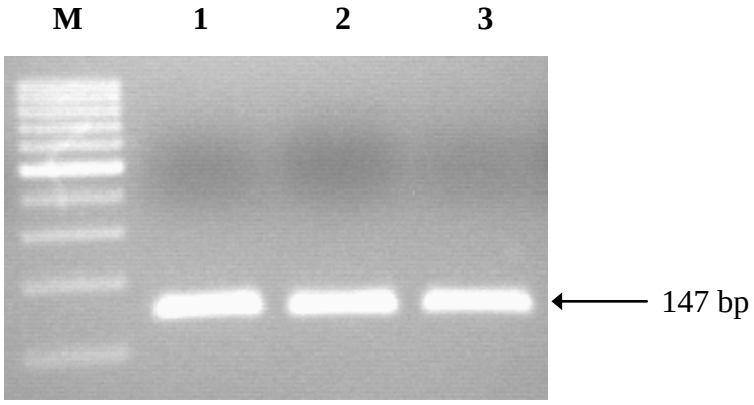


Figure 5.7. PCR results of the GRIK2 gene rs10548788 polymorphism
M: 100 bp ladder; lanes 1, 2, 3: amplification products

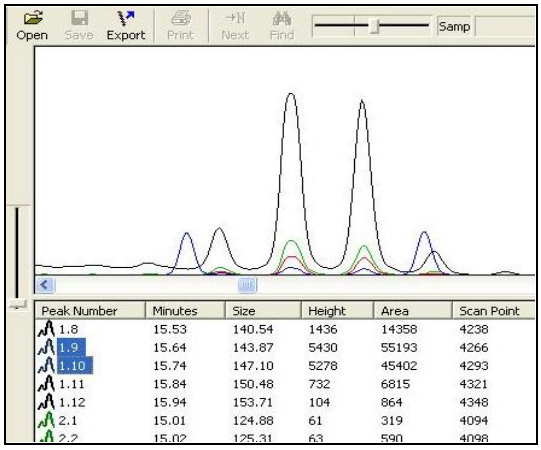


Figure 5.8. Electropherogram: GeneScan Analysis of the GRIK2 gene rs10548788 polymorphism

57 samples were simultaneously analyzed by polyacrylamide gel electrophoresis (PAGE). PCR products of the GRIK2 gene rs10548788 polymorphism were run on eight per cent polyacrylamide gels together with controls (Figure 5.9).

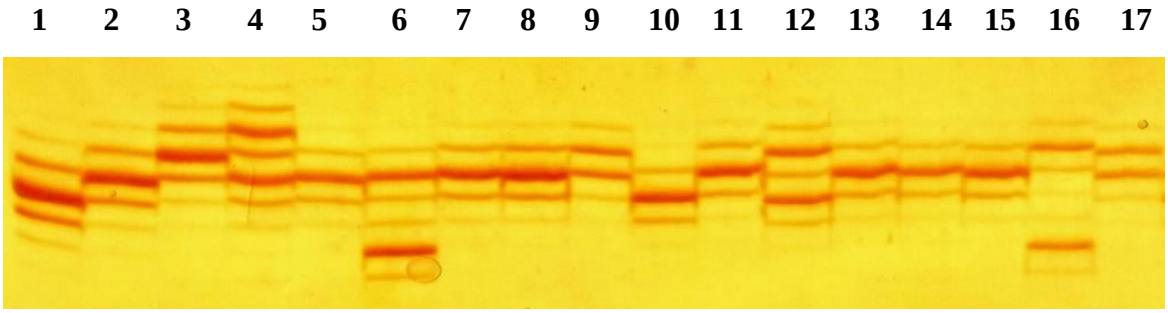


Figure 5.9. PAGE analysis of the GRIK2 gene rs10548788 polymorphism
Lane 1: control 1 (13/13 TAA repeats), lane 2: control 2 (14/14 TAA repeats), lane 3: control 3 (15/15 TAA repeats), lane 4: control 4 (16/14 TAA repeats), lanes 5-17: study group samples

5.3.2. Determination of TAA Repeat Genotypes from GeneScan Fragment Sizes

To determine the TAA repeat numbers of the study group, samples with known TAA numbers were subjected to GeneScan analysis. The correlation between TAA repeat numbers and fragment sizes was established according to the known samples (Table 5.4).

Table 5.4. TAA genotypes and fragment sizes of known samples

Controls	TAA Genotypes	Fragment Sizes in GeneScan Analysis
sHD445	9 / 14	128.6 / 144.1
sHD516	10 / 14	131.5 / 143.9
sHD515	11 / 14	134.3 / 143.9
CEPH 135001	12 / 12	137.3
CEPH 1701	13 / 13	140.5
CEPH 133402	14 / 14	143.7
CEPH 134001	15 / 15	147.0
CEPH 140801	16 / 14	150.0 / 143.4
CEPH 134502	17 / 14	153.3 / 143.5

The graph, showing the TAA repeat numbers, plotted against the corresponding fragment sizes, is shown in Figure 5.10.

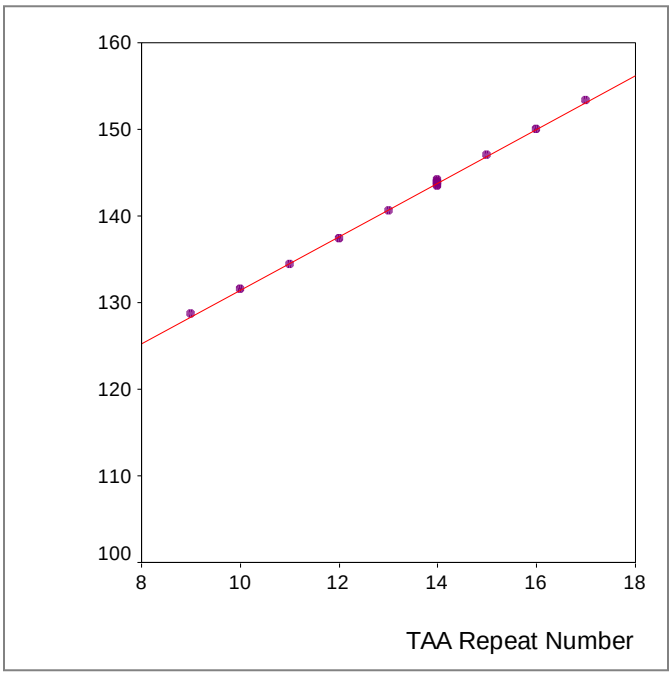


Figure 5.10. TAA genotypes of samples with known TAA lengths, plotted against fragment sizes

As the R^2 value indicates, there is a strong correlation between TAA repeat numbers and their corresponding fragment sizes, as expected. Using the formula below, which represents the slope of the line in the correlation of TAA repeat number and fragment size, the GRIK2 gene TAA genotypes of patients were calculated.

$$\text{TAA Repeat Number} = \frac{\text{Fragment Size} - 100.475}{3.097}$$

Allele frequencies of the GRIK2 gene rs10548788 polymorphism are shown as percentages by a pie chart in Figure 5.11.

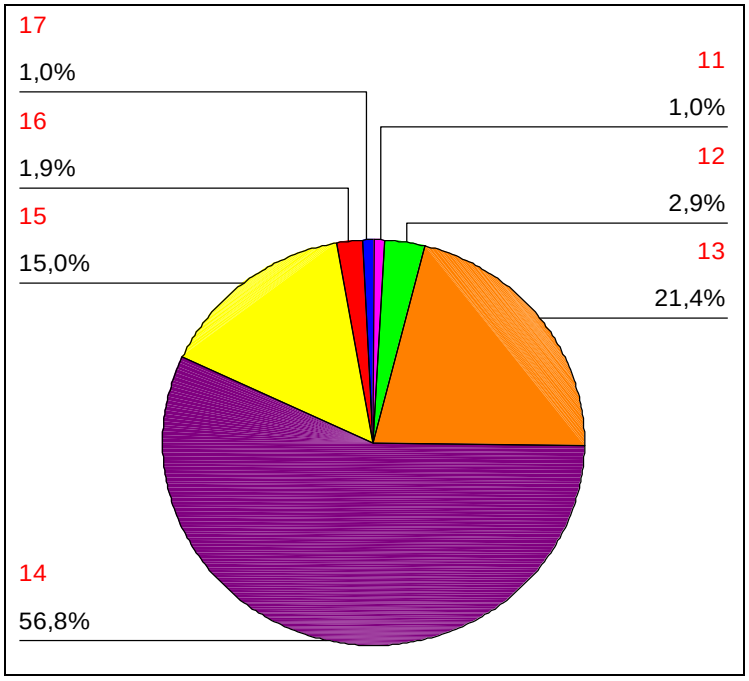


Figure 5.11. Allele frequencies of the GRIK2 gene rs10548788 polymorphism

5.4. Analysis of the NR2A Gene rs1969060 Polymorphism

The NR2A gene rs1969060 polymorphism was analyzed with PCR (Figure 5.12), followed by restriction enzyme digestion (Figure 5.13).

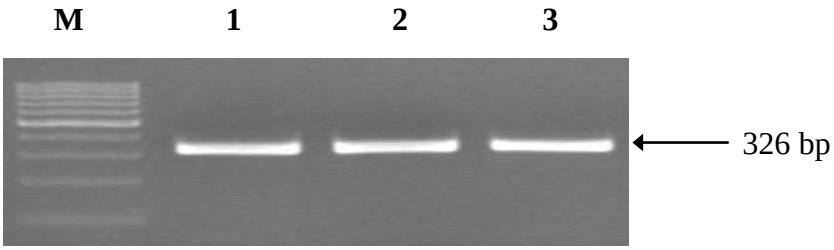


Figure 5.12. PCR results of the NR2A rs1969060 polymorphism
M: 100 bp ladder, lanes 1, 2, 3: amplification products

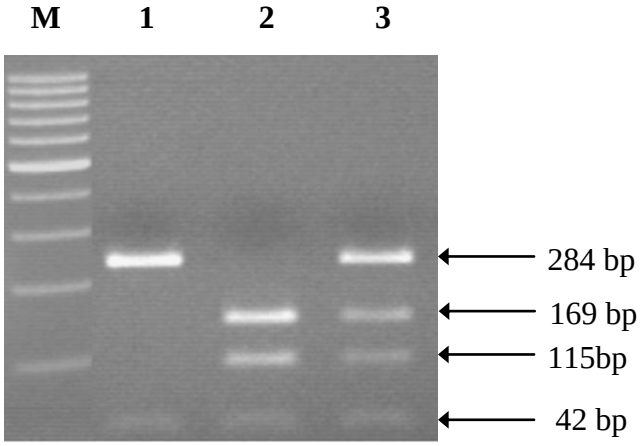


Figure 5.13. Digestion of the NR2A rs1969060 polymorphism with DdeI
M: 100 bp ladder, lane 1: homozygous product with TT genotype, lane 2: homozygous product with CC genotype, lane 3: heterozygous product with CT genotype

The genotype and allele distribution of the NR2A rs1969060 polymorphism are given in Table 5.5.

Table 5.5. NR2A rs1969060 genotypes and frequency of alleles

NR2A rs1969060		**Study Group (n=103)**
Genotypes	**TT**	63 (61.2 per cent)
	CT	38 (36.8 per cent)
	CC	2 (2 per cent)
Alleles	**T**	164 (79,7 per cent)
	C	42 (20.3 per cent)

5.5. Analysis of the NR2B Gene rs1806201 Polymorphism

The NR2B gene rs1806201 polymorphic region was amplified by PCR (Figure 5.14), genotypes were further determined by restriction enzyme analysis (Figure 5.15).

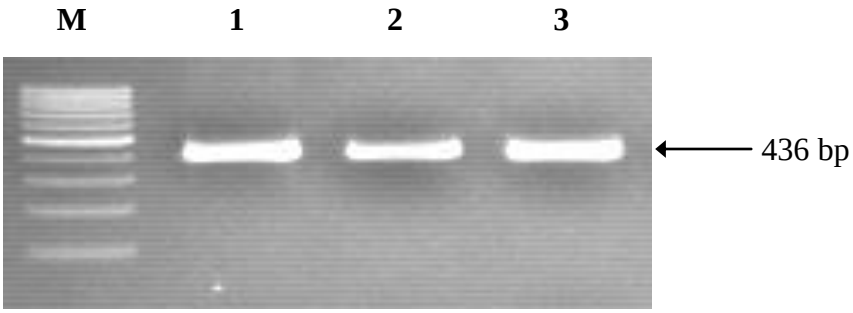


Figure 5.14. PCR results of the NR2B gene rs1806201 polymorphism
M: 100 bp ladder, lanes 1, 2, 3: amplification products

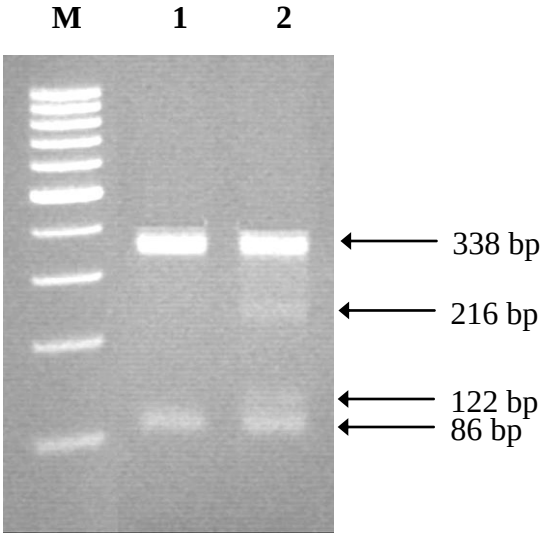


Figure 5.15. Digestion of the NR2B rs1806201 polymorphism with TspRI
M: 100 bp ladder, lane 1: homozygous product with CC genotype, lane 2: heterozygous product with CT genotype

The genotype and allele distribution of the NR2B rs1806201 polymorphism are shown in Table 5.6. In none of 108 patients, the TT genotype was observed.

Table 5.6. NR2B rs1806201 genotypes and frequency of alleles

NR2B rs1806201		Study Group (n=103)
Genotypes	CC	61 (59.2 per cent)
	CT	42 (40.8 per cent)
	TT	0 (0 per cent)
Alleles	C	164 (79,7 per cent)
	T	42 (20.3 per cent)

5.6. Analysis of the NR2B Gene rs890 Polymorphism

The NR2B gene rs890 polymorphic region was amplified by PCR (Figure 5.16).

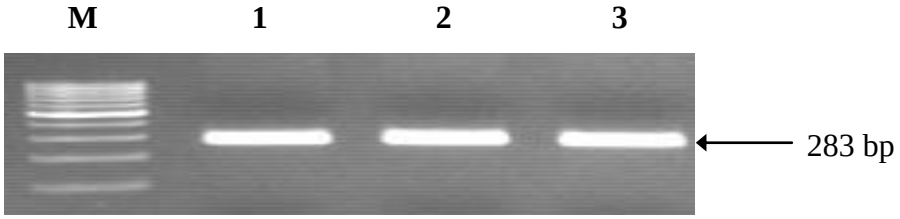


Figure 5.16. PCR results of the NR2B gene rs890 polymorphism
M: 100 bp ladder, lanes 1, 2, 3: amplification products

To identify the NR2B rs890 genotypes, PCR products were digested by PsuI restriction enzyme (Figure 5.17).

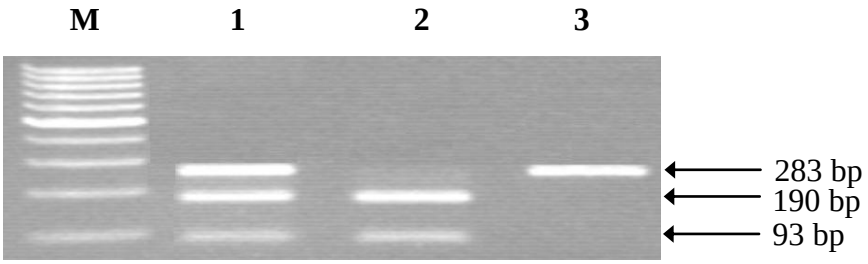


Figure 5.17. Digestion of the NR2B rs890 polymorphism with PsuI
M: 100 bp ladder, lane 1: heterozygous product with TG genotype, lane 2: homozygous product with GG genotype, lane 3: undigested product with TT genotype

The genotype and allele distribution of the NR2B rs890 polymorphism are shown in Table 5.7.

Table 5.7. NR2B rs890 genotypes and frequency of alleles

NR2B rs890		Study Group (n=103)
Genotypes	GG	25 (24.3 per cent)
	GT	54 (52.4 per cent)
	TT	24 (23.3 per cent)
Alleles	G	104 (50,5 per cent)
	T	102 (49.5 per cent)

5.7. Analysis of the NR2B Gene rs1042339 Polymorphism

To study the NR2B gene rs1042339 polymorphism, the region was amplified by PCR (Figure 5.18), the PCR products were digested by AspI restriction enzyme (Figure 5.19).

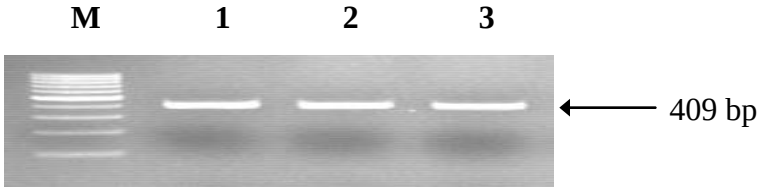


Figure 5.18. PCR results of the NR2B gene rs1042339 polymorphism
M: 100 bp ladder, lanes 1, 2, 3: amplification products

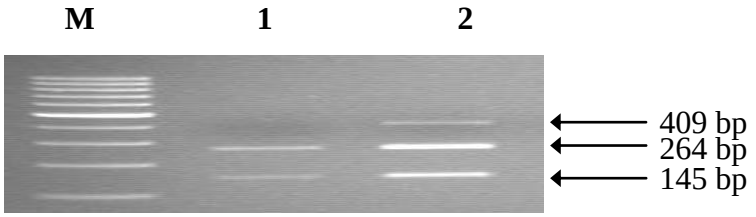


Figure 5.19. Digestion of the NR2B rs1042339 polymorphism with AspI
M: 100 bp ladder, lane 1: homozygous product with GG genotype, lane 2: heterozygous product with AG genotype

The genotype and allele distribution of the NR2B rs1042339 polymorphism are shown in Table 5.8.

Table 5.8. NR2B rs1042339 genotypes and frequency of alleles

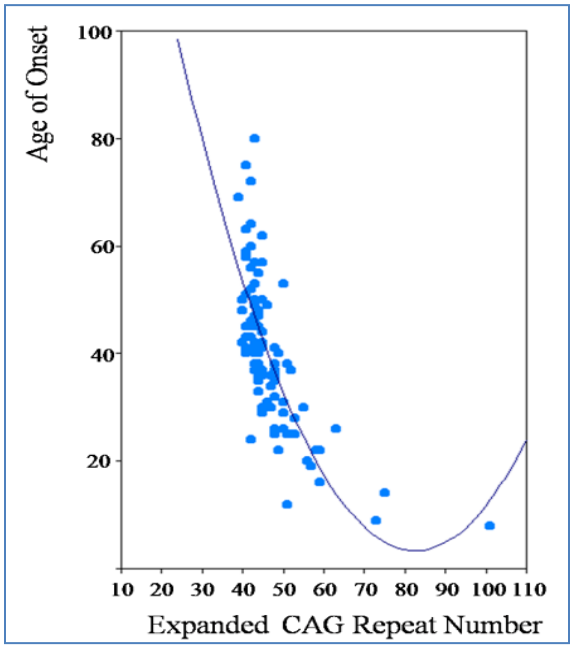
NR2B rs1042339		**Study Group (n=103)**
Genotypes	**GG**	98 (95.1 per cent)
	AG	5 (4.9 per cent)
	AA	0 (0 per cent)
Alleles	**G**	201 (97.5 per cent)
	A	5 (2.5 per cent)

5.8. Statistical Analysis

In the framework of this thesis, 103 unrelated Turkish HD patients and five affected family members (Chapter 5.2) were studied. Within the study population, the mean value of the age of onset, ranging from 8 to 80, was calculated to be 40.8 ± 13.8 (AO ± standard deviation).

5.8.1. Effects of Glutamate Receptor Genes on Age of Onset in HD Patients

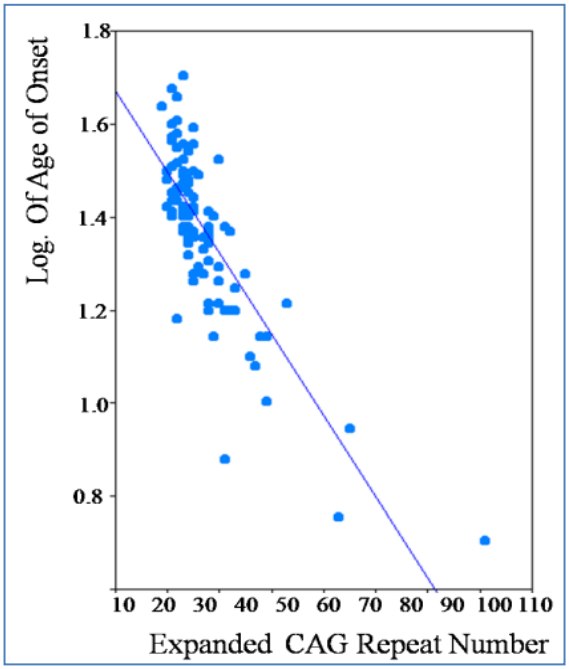
To determine the effect of expanded CAG repeat on the age of onset, linear regression analysis was applied (Figure 5.20). According to this analysis, 56.6 per cent of the variance in the age of onset was found to be determined by the expanded CAG repeat number, representing a curvilinear relationship.



$R^2 = 0.56$

Figure 5.20. Age of onset plotted against number of expanded CAG repeats

Since the relationship between repeat length and age of onset is curvilinear, age of onset was logarithmically transformed; a better fit was obtained, explaining 64.6 per cent of the variance [log (Age) = α + β (expanded CAG repeat number)] (Figure 5.21).



$R^2 = 0.64$

Figure 5.21. Log. of age of onset plotted against the expanded CAG repeat numbers

To determine the modifier effects of variations in the glutamate receptor genes, the method of generalized linear models in analysis of variance was used. The effect of expanded CAG repeats were considered in each calculation. Neither the polymorphisms in the glutamate receptor genes, nor the normal CAG repeat numbers, resulted in a statistically significant increase in the R^2 value (Table 5.9).

Table 5.9. Analysis of polymorphisms in candidate genes that may modify the age of onset in addition to the contribution of the expanded CAG repeat

Gene (polymorphism)	**R^2**	**ΔR^2**	**Additional explained variance (per cent)**	***P* value**
HD CAG	0.646	-	-	<0.0005
HD CAG + Normal CAG	0.650	0.004	0.61	0.302
HD CAG + GRIK2 TAA1	0.647	0.001	0.15	0.621
HD CAG + GRIK2 TAA2	0.650	0.004	0.61	0.331
HD CAG + NR2A (rs1969060)	0.647	0.001	0.15	0.629
HD CAG + NR2B (rs1806201)	0.648	0.002	0.30	0.434
HD CAG + NR2B (rs890)	0.663	0.017	2.5	0.096
HD CAG + NR2B (rs1042339)	0.647	0.001	0.15	0.629

5.8.2. Effects of Glutamate Receptor Genes on Age of Onset in Female and Male HD Patients

Out of 103 patients studied, 48 were female and 55 were male. The possible effect of gender difference on the age of onset was studied under consideration of the expanded CAG repeat numbers. No effect of gender difference on age of onset was found in the above population (Table 5.10).

Table 5.10. Investigation of the effect of gender difference on the age of onset in HD

Factors	**R^2**	**ΔR^2**	**Additional explained variance (per cent)**	***P* value**
HD CAG	0.646	-	-	<0.0005
HD CAG + Gender	0.648	0.002	0.30	0.448

5.8.3. Effects of Glutamate Receptor Genes on Age of Onset in a Selected Patient Group with 40-50 CAG repeats

Modifier effects of the glutamate receptor gene variations and the normal CAG repeat number were investigated in a subgroup of patients, consisting of 86 patients, whose expanded CAG repeat numbers were between 40 and 50 (Table 5.11). No significant effect of candidate genes on age of onset was found in this subgroup of patients.

Table 5.11. Analysis of polymorphisms in candidate genes that may modify the age of onset in patients with CAG repeats in the range of 40-50

Gene (polymorphism)	**R^2**	**ΔR^2**	**Additional explained variance (per cent)**	***P* value**
HD CAG	0.366	-	-	<0.0005
HD CAG + Normal CAG	0.372	0.006	1.61	0.398
HD CAG + GRIK2 TAA1	0.370	0.004	1.08	0.478
HD CAG + GRIK2 TAA2	0.371	0.005	1.34	0.427
HD CAG + NR2A (rs1969060)	0.373	0.007	1.87	0.653
HD CAG + NR2B (rs1806201)	0.368	0.002	0.54	0.628
HD CAG + NR2B (rs890)	0.405	0.039	9.62	0.077
HD CAG + NR2B (rs1042339)	0.372	0.006	1.61	0.388

5.8.4. Effects of Glutamate Receptor Genes on Age of Onset in Six Consanguineous Patients Belonging to the Large Kindred in Tokat

To determine the effect of expanded CAG repeat number on age of onset in this family, linear regression analysis was applied to six affected individuals. The CAG repeat number was not found to be significantly correlated with the age of onset (Figure 5.22).

$R^2 = 0.12$

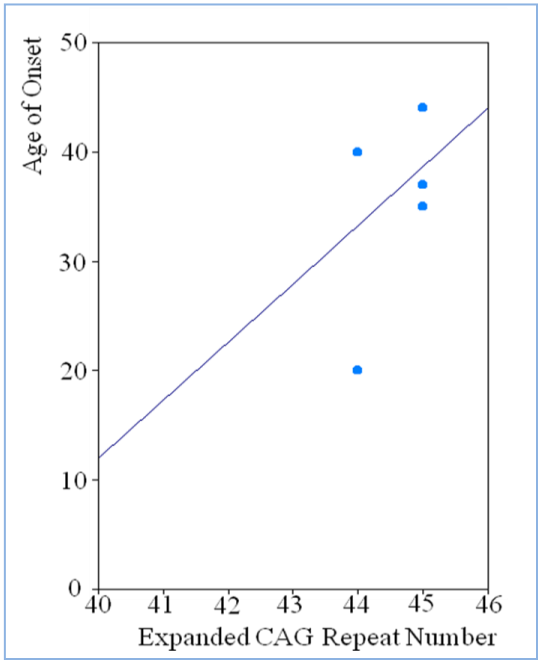


Figure 5.22. Age of onset plotted against number of expanded CAG repeats in the family from Tokat

Variations in the glutamate receptor genes were also determined for these six individuals (Table 5.12).

Table 5.12. Genotypes of the candidate modifier genes in the symptomatic members of the family from Tokat

ID	GRIK2 TAA1	GRIK2 TAA2	NR2A rs1969060	NR2B 1042339	NR2B rs1806201	NR2B rs890
IV. 1	14	14	TT	GG	CC	GG
IV. 2	13	14	TT	GG	CT	GG
IV. 3	14	14	CT	GG	CT	TG
IV. 4	14	15	TT	GG	CC	GG
IV. 5	14	14	TT	GG	CT	GG
IV. 6	14	14	CT	GG	CC	GG

To identify the modifier effects of the normal CAG repeat number and the variations of the glutamate receptor genes, the method of general linear models in analysis of variance was applied. None of these variations were found to be correlated with the age of onset in these six individuals (Table 5.13).

Table 5.13. Analysis of variations in glutamate receptor genes that may modify the age of onset in the symptomatic members of the family from Tokat

Gene (polymorphism)	R^2	ΔR^2	Additional explained variance (per cent)	*P* value
HD CAG	0.121	-	-	0.500
HD CAG + Normal CAG	0.141	0.020	-	0.808
HD CAG + GRIK2 TAA1	0.309	0.188	-	0.433
HD CAG + GRIK2 TAA2	0.309	0.188	-	0.433
HD CAG + NR2A (rs1969060)	0.241	0.120	-	0.540
HD CAG + NR2B (rs1806201)	0.268	0.147	-	0.494
HD CAG + NR2B (rs890)	0.177	0.056	-	0.679
HD CAG + NR2B (rs1042339)	0.121	0.000	-	0.500

6. DISCUSSION

Huntington's Disease is a devastating neurodegenerative disorder that is inherited in an autosomal dominant manner. Disease symptoms, which usually appear between 35 and 50 years of age, are cognitive decline, psychiatric disturbances and motor dysfunction (Margolis and Ross, 2003). In 1993, the causative mutation for HD was identified as the expansion of polymorphic CAG repeats in the first exon of the HD gene which resides on chromosome 4 (The Huntington's Disease Collaborative Research Group, 1993). The expanded CAG repeats are translated into a polyglutamine tract in the N-terminal of the huntingtin protein (Borrell-Pages et al., 2006).

Until today, a huge amount of studies were focused to elucidate the process of selective neuronal degeneration seen in HD. Several mechanisms were proposed to have roles in neurodegeneration process, however the exact way of pathogenesis have not been understood yet. Toxic gain of function of the mutant huntingtin and also loss of normal huntingtin function were suggested to explain HD pathogenesis. Studies with HD mice showed evidences of mutant protein aggregation, mitochondrial dysfunction, transcriptional dysregulation, apoptosis, ubiquitin-proteasome system abnormalities and excitotoxic cell death. It is most likely, that in neurons, different pathological mechanisms contribute to the effects of each others, and subsequently, lead to neuronal neurodegeneration. Clear understanding of the HD pathogenesis, will help to develop treatments for this incurable disorder (Gil and Rego, 2008).

To date, a few studies on Huntington's Disease, have been published in Turkey. A previous study from our center, reported a large number of HD families, originating from all over Turkey, in whom the disease was firmly diagnosed. The diagnosis was later confirmed by molecular analysis (Ersoy, 2005). According to this study, Huntington's Disease is homogeneously distributed in Turkey. Although previous studies are useful sources for the characterization of Huntington's Disease in the Turkish population, it is still of primary importance to extend our previous knowledge to gain a deeper understanding.

6.1. Patient Population

In the framework of this thesis, 108 Turkish HD patients, including 102 unrelated individuals and six affected members of a large kindred, were investigated. Besides the patients, 10 asymptomatic family members at 50 or 25 per cent risk for HD, and one female patient with Parkinson Disease-like symptoms were analysed.

For all patients, the age of onset was estimated as the age, at which motor or cognitive symptoms were first noticed. In the study population, the age of onset ranged from 8 to 80 years, and the mean age of onset was 40.8±13.8, which is relatively low, when compared to European populations (Arning et al., 2005; Saft et al., 2004). This relatively low value may be the result of our cohort's expanded CAG repeat numbers, but may also be due to the genetic background of the Turkish population. Out of 108 HD patients, seven were regarded as juvenile HD patients, whose ages of onset were below 20 years.

In our study population, expanded CAG repeat numbers varied from 39 to 101. The most frequent allele was the one with 43 CAG repeats. CAG repeats on the normal allele ranged from 8 to 29, 17 being the most frequent number. These findings are in accordance with the data, that is previously published on Turkish HD patients.

In the framework of this study, a large Turkish HD family, comprising several members in five generations, was investigated. The index case, was admitted to the Neurology Department of Gaziosmanpaşa University Medical School in Tokat, with clinical symptoms of involuntary movements of body parts, balance problems and walking disability. He also had cognitive decline, apparent with defective memory functions. After he was clinically diagnosed with Huntington's Disease, family history was investigated, and other affected members of the family and at risk individuals were identified. All affected members of the family had involuntary choreic movements. Investigation of the family pedigree revealed that consanguineous marriages were frequent in the family. However, there were not any homozygous cases for the mutant HD gene among the members studied. This family represents a good example of Turkish mating habits,

common in Anatolia, with a high incidence of consanguineous marriages, especially between first cousins.

In this family, all affected and several presymptomatic members had prominent skin lesions. The possible correlation between these skin lesions and HD were investigated to understand, if these lesions were exclusive to mutant HD gene carriers. No correlation between skin lesions and Huntington's Disease could be established. The high frequency of consanguineous marriages between close relatives may result in recessive disorders. Thus, the skin lesions in this family may be a consequence of a recessive trait that is, however, not linked to the mutant HD gene.

The phenomenon of anticipation is well established in Huntington's Disease: The age of onset decreases in successive generations. Large expansions of CAG repeats happen particularly in spermatogenesis; as a result, patients with juvenile onset mostly inherit the disease from their fathers. In the family from Tokat, the expanded CAG repeat numbers of the patients investigated were either 44 or 45. Among six HD patients, four had inherited the disease from their mothers. In the other two symptomatic siblings, the inheritance pattern could not be defined, since their parents were consanguineous and both were affected with HD. The normal CAG repeat numbers of these two siblings were in the range that shows meiotic instability. Since the parents of all HD patients in the family were not available, the expansion size of CAG repeats during transmission could not be studied.

Among 10 at risk individuals studied, three patients were found to carry the expanded HD allele. Two patients had inherited the disease from their mothers, and the CAG repeats were expanded for one or two CAGs during transmission. One patient had inherited the disease from his father, and also his CAG repeat number had increased for one CAG during transmission. There are not any juvenile onset patients in the family, so far. In this family, the expanded CAG repeat numbers of patients were found to slightly increase during transmisson to the next generation. Since most of the patients and the presymptomatic individuals inherited the disease from their mothers, there were no huge expansions, which is common for paternal transmissions in Huntington's Disease.

This large kindred from Tokat is a good representative of Huntington's Disease in Turkey. Clinical and genetic investigation of large HD families would help to increase our current knowledge on the genotype-phenotype correlations and the phenomenon of anticipation in Huntington's Disease. Follow up studies of such families would turn out with significant new results, when studying Huntington's Disease in the Turkish population.

6.2. Investigation of Genetic Modifiers of Age of Onset

In the last 10 years, investigation of genetic modifiers with association studies attracted much interest in the area of genetics, since the effects of genetic modifiers could be established in some diseases, like penetrance, age of onset or clinical features. To date, identification of genetic modifiers, illustrates the importance of dissecting the complex networks of gene and protein interactions that underlie variations between individuals.

Currently, it is well-established that the age of onset in HD is inversely correlated with the expanded CAG repeat number. This correlation was found to account for 42-73 per cent of the variation in the age of onset. Although expanded CAG repeat number is the strongest determinant of the age of onset, several lines of evidence suggest, that other genetic factors beyond the HD gene, modify the age of onset in HD. Identification of possible modifiers, and understanding the molecular and cellular basis, by which modifier genes exert their influence in HD, will provide insights into neuropathological mechanism of HD, and will shed light into therapeutic interventions (Metzger et al., 2006).

In our cohort, the expanded CAG repeat length explains 56.6 per cent of the variance in the age of onset. However, since the relationship between the CAG repeat length and the age of onset is curvilinear, the age of onset was logarithmically transformed. By logarithmic transformation of the age of onset, the best fit of our data was obtained, explaining 64.6 per cent of the variance which is in accordance with the previous findings.

In this study, polymorphisms of glutamate receptor genes, which were previously found to have modifier effects on the age of onset were investigated in Turkish HD patients. Among all glutamate receptor genes, GRIK2 gene, encoding kainate receptor

subtype GluR6, was extensively studied in the literature, as a modifier of age of onset. Previous studies reported that the TAA repeat polymorphism in the 3' untranslated region of the GRIK2 gene explains 2 to 13 per cent of the variance in the age of onset. According to earlier results, individuals with 16 TAA repeats in the GRIK2 gene were shown to have significantly earlier age of onset than expected (Chattopadhyay et al., 2003; MacDonald et al., 1999). However, the same correlation could not be observed in a larger study, which consisted of 980 European HD patients (Metzger et al., 2006).

In our study group, the three most frequently detected alleles of the GRIK2 gene were those with 14, 15 and 13 TAA repeats, which is in accordance with the previous reports. Modifier effect of the GRIK2 gene TAA repeat polymorphism (rs10548788) was investigated in our study population, under consideration of the expanded CAG repeat numbers. Our results did not demonstrate any significant association between the GRIK2 genotype and the age of onset. Although the R^2 value increased from 0.646 to 0.650, the difference did not reach statistical significance. This result may be a consequence of the low frequency of the critical 16 TAA repeat allele in our study population.

It is well established that NMDA receptors are the primary mediators of excitotoxicy. NMDA receptors are multimeric complexes, composed of two NR1 subunits together with two region-specific NR2 subunits. In the cortex and striatum, NR2A and NR2B subtypes are predominant, and NR2C and NR2D subtypes are expressed in small amounts. In the literature, genes encoding the NR2A and NR2B receptor subtypes were proposed as modifiers of the age of onset in HD. A study with 167 HD patients attributed 12.3 per cent additional variance to NR2B genotype variations and 4.5 per cent to NR2A genotype variations (Arning et al., 2005). Replication of this study with 443 HD patients from a Venezuelan kindred, showed significant association with NR2A genotype variations, but not with NR2B genotype variations (Andresen et al., 2007).

In our study, four different polymorphisms in the NR2A and NR2B genes, which were previously shown to have modifier effects, were investigated. Allele frequencies of all these polymorphisms in the Turkish population were found to share close similarity to that of European populations. Modifier effects of these polymorphisms were investigated in our cohort when the effect of the expanded CAG repeat number was taken into account.

However, our results did not show any significant correlation of these polymorphisms with the age of onset. Among the polymorphisms investigated, the modifier effect of the rs890 polymorphism in the NR2B gene may be regarded as a trend, which might explain 2.5 per cent additional variance in the age of onset; but it is still not significant.

Previous studies suggested the normal CAG repeat number as one of the modifiers of age of onset (Djousse et al., 2003, Kehoe et al., 1999). In our study, the modifier effect of the normal, unexpanded CAG repeat number was also investigated. However, no significant effect on the age of onset was observed.

As the precise magnitude of the effect of any genetic modifier on the age of onset is dependent on many independent variables, it is very important to replicate the same observations with different studies to establish a confirmed association of a genetic modifier with the age of onset. However, the lack of replication of these association tests in the Turkish population does not imply that the original association results of other studies are inaccurate. It is well known that interpreting genetic association tests are complex. Also, a significant association in a particular population may not replicate in a new population, as the allele frequencies of the polymorphisms in the investigated genes may be different. The patients of the previous studies were from German, English, European, Eastern American and Eastern Indian descent; our study population was composed of Turkish HD patients only.

Although, the allele frequencies of all investigated polymorphisms, in the study, were found to be very similar to that of European populations, the lack of reproduction of previous results may also be a consequence of the relatively small size of our study population. To increase the power of our study, more HD patients with well-defined clinical and familial history are needed.

Previous studies suggested that, the effects of modifier genes on age of onset, differantiate between female and male patients. As an example, NR2B gene rs1806201 polymorphism was shown to have significant correlation with the age of onset only in female patients, suggesting that poor-defined sex-specific mechanisms may be influencing

the disease onset. One of the proposed mechanisms of the gender effect is the possible effect of estrogen on synaptic distributions of NMDA receptors (Arning et al., 2007).

In our study group, there were 48 female and 55 male patients, the number of patients in these two groups were close enough to be feasibly compared. Polymorphisms that we have investigated did not show significant difference between the ages of onset of female and male patients.

Statistical analysis of candidate modifier genes was applied to a subgroup of patients, consisting of 86 patients with 40 to 50 CAG repeats on the expanded allele. In this range, it becomes more difficult to predict the age of onset using expanded CAG repeats. In line with this knowledge, in this subgroup of patients, the expanded CAG repeat number explained 36.6 per cent of the variance in the age of onset (Table 5.11). However, none of the polymorphisms investigated in the glutamate receptor coding genes, resulted in significant increase in the R^2 values. Only the effect of the NR2B rs890 polymorphism increased, explaining 9.6 per cent of the variance (in addition to the effect of expanded CAG repeats), but this was statistically not significant (p=0.07).

It is of particular importance to elucidate modifiers of the age of onset in patients deriving from the same family, because members of the same family are expected to carry similar gene backgrounds. In the kindred from Tokat, there were huge differences between the onset ages in the patients with the same expanded CAG repeat numbers. As an example, patients IV.1, IV.2 and IV.4 have all 44 CAG repeats in the mutant HD allele, but the age of onset of patient IV.1 was 20 which was 20 years earlier than the patients IV.2 and IV.4 (Figure 5.5). When the modifier effects of glutamate receptor genes and the normal CAG repeat number on the age of onset in patients of the Tokat kindred were investigated, an insignificant correlation was found, this lack of correlation may be interpreted as a result of the low number of patient samples. Only six affected members of this kindred, were analysed in this thesis. However, the family includes a large number of presymptomatic members, and prospective studies with more members of this kindred would result in valuable information.

To the best of our knowledge, only a few studies on Huntington's Disease have been published in Turkey. This study provides valuable data for the understanding of the genetic and clinical features of Huntington's Disease in the Turkish population. We were not able to demonstrate any significant association between glutamate receptor gene variations and the age of onset. This may be a consequence of the genetic background of the Turkish population, but it may also be a result of the relatively small study group. Studying these polymorphisms in a larger study group, or investigation of other candidate modifier genes in the Turkish population may confirm these findings.

7. CONCLUSION

Huntington's Disease is a devastating disorder with no known cure. To develop effective therapies, a better understanding of the disease pathogenesis is essential. Revealing mechanisms of neurodegeneration in HD is like assembling a puzzle in which every single piece has a critical importance. Disease symptoms in HD usually strikes in the middle age and age of onset is known to be modified by genetic factors beyond the HD gene. To detect genetic polymorphisms associated with age of onset in HD, is a difficult problem, because the effects of these polymorphisms can be small. To reach significant levels, study populations need to be large.

The results of our study did not demonstrate any significant association between glutamate receptor-coding genes and age of onset, but this lack of replication does not rule out the roles of these genes in HD. In the literature, there are lots of contradictory results for each polymorphism studied in this thesis. Analysis of such polymorphisms in a larger study population is needed, but this would have extended the scope of this thesis. We believe that the present study is a first step towards this direction.

APPENDIX A: INFORMED WRITTEN CONSENT AND INFORMATION SHEET

KATILIMCILAR İÇİN BİLGİ ve OLUR FORMU

Proje yürütücüsü: Prof. Dr. A. Nazlı Başak
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34342, Bebek İstanbul.

Telefon (iş): (212) 359 66 79

Proje konusu: Huntington Hastalığı (HD), otozomal dominant kalıtım gösteren, geç-başlangıçlı bir sinir hücresi hastalığıdır. Bireyin beyin dokusunda, selektif sinir hücresi kaybı sonucunda hastalık belirtileri ortaya çıkar. Hastalığın klinik özellikleri, başlıca üç temel grup altında toplanmıştır: Hareket bozuklukları, psikiyatrik bozukluklar ve zihinsel bozukluklar. Hareket bozukluklarından en sık karşılaşılanı, "kore" olarak da adlandırılan ve hastalığın farkedilmesini sağlayan en keskin bulgu olan istemsiz hareketlerdir. Hastalığın sık karşılaşılan diğer semptomları ise; depresyon, kişilik değişiklikleri, anlama ve algılama bozukluklarıdır.

Huntington Hastalığı, 4. Kromozomun kısa kolu üzerinde bulunan Huntington genindeki CAG üçlü nükleotitlerinin tekrar artışı sonucunda oluşur. Huntington geninin 1. eksonundaki CAG tekrar sayısı normal bireylerde 6-34 arasında iken, 40 ve üzeri tekrar sayısı taşıyan bireyler HD hastası olurlar. Artmış sayıda CAG tekrarı içeren genin ürünü olan mutant huntingtin proteini, hücre içerisinde normal fonksiyonunu gösterememekle beraber, spesifik enzimler aracılığı ile parçalara ayrılır ve bu parçalar hücre içerisinde birikerek hücrenin normal işleyişini bozarlar. Bu durum, beyindeki sinir hücrelerinin ölümü ile sonuçlanır, ve hastalık kliniği ortaya çıkar.

Öngörülen proje çerçevesinde Huntington hastası bulunan ailelerde DNA analizi yapılacaktır. Hastalık, otozomal dominant olarak kalıtıldığı için, hasta olan bir bireyin çocuğunun HD hastası olma riski %50'dir.

Çalışmaya katılmanız tamamen isteğinize bağlıdır. Araştırmaya katılmayı kabul ettiğiniz takdirde sizden on mililitre kan örneği alınacaktır. Yapacağımız araştırmanın size bir risk getirmesi beklenmemektedir. Kan aldırmanın genelde hiçbir zararı olmamasına karşın, nadiren çok az kanama ve morarmaya yol açabilir.

İstediğiniz zaman çalışmaya katılmaktan vazgeçebilirsiniz. Bu durumda sizden almış olduğumuz örnek, eğer arzu ederseniz imha edilecektir; yoksa isminiz her zaman gizli tutulmak kaydı ile, başka araştırmalar için de kullanılabilir. İlişikteki nörolog hekim tarafından doldurulan bilgi formundaki bilgiler araştırma ekibi tarafında görülecektir. Bu bilgilerin laboratuvardaki araştırmacılarla paylaşılmasını kabul etmeniz gerekmektedir.

Araştırmanın sonucunda aranan bilgi elde edildiği takdirde, ailenizin bireylerine genetik danışma hizmeti verilebilecektir. Toplumumuzda genetik altyapının belirlenmesi, hastalığın moleküler temelinin aydınlatılmasına katkıda bulunacaktır. Bunun sizin ailenize yararlı olup olmayacağını şimdiden söylemek mümkün değildir ve size bu konuda söz veremeyiz. Araştırmanın ilerde başka bireylere yarar sağlaması olasılığı yüksektir. Çalışmaya kan verilerek gösterilecek gerekli toplumsal duyarlılık çalışma açısından büyük önem taşımaktadır.

Bu formu imzalamadan önce, çalışma ile ilgili sorularınız varsa lütfen sorun. Daha sonra sorunuz olursa Prof. Dr. A. Nazlı Başak'a (Telefon: 212 359 66 79 / 359 72 98) ve ilgili nörolog hekiminize (adı ve telefon numarası formda belirtilmiştir) sorabilirsiniz. Araştırma ile ilgili haklarınız konusunda Boğaziçi Üniversitesi İnsan Araştırmaları Etik Kurulu'na danışabilirsiniz.

Adres ve telefon numaranız değişirse, bize haber vermenizi rica ederiz.

Bana anlatılanları ve yukarıda yazılanları anladım. Bu formun bir kopyasını aldım. Çalışmaya katılmayı kabul ediyorum.

Katılımcı veya vasisinin ismi ve imzası: **Tarih:**

Hastaya yakınlığı:

Şahit ismi ve imzası:

Klinisyen ismi:

Adresi:

Telefonu:

İmzası:

HD HASTA BİLGİLERİ

Adı / Soyadı :

Cinsiyeti :

Doğum yılı / yeri :

Hastalık başlangıç yaşı / yılı:

Klinik bulgular:

Motor Bozukluklar

İstemsiz hareketler	**□ var**	**□ yok**
Disartri	**□ var**	**□ yok**
Denge Kaybı	**□ var**	**□ yok**
İstemsiz kas seğirmeleri (distoni)	**□ var**	**□ yok**
Sakarlık	**□ var**	**□ yok**

Psikiyatrik Bozukluklar

Depresyon	**□ var**	**□ yok**
Sinirlilik (İritabilite)	**□ evet**	**□ hayır**

Zihinsel Bozukluklar

Hafıza	**□ var**	**□ yok**
Konsantrasyon güçlüğü	**□ evet**	**□ hayır**

Diğer semptomlar (varsa belirtiniz) ……..

Aile öyküsü (aile ağacı) :

Akraba evliliği:

Özgeçmiş (hastalık, travma, toksinler vs) :

Adres / telefon :

Gönderen Doktor / Klinik:

Telefon / Fax:

E-mail:

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