Molecular genetic aspects of Huntington's Disease

Ph.D. Thesis

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List of abbreviations

AD	Alzheimer Disease
A2M	α2-macroglobulin
AP2	adaptor protein 2
ApoE	apolipoprotein E
ATP	Adenosine-triphosphate
BDNF	Brain-derived neurotrophic factor
βAM	β amiloid
CAG	citosin, adenin, guanin
ChAT	cholin-acetyl-transferase
CNS	central nervous system
DRPLA	Dentatorubral-pallidoluysian atrophy
FDG PET	Fluoro-deoxy-glucose Positron Emission Tomography
GAPDH	gliceraldehyd-3-phosphate-dehydrogenase
HD	Huntington's Disease
HIP-1	Huntingtin-interacting protein 1
HIP-2	Huntingtin-associated protein 2
IT	interesting transcript
mAb	monoclonal antibody
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NFT	neuronal fibrillary tangles
NII	neuronal intranuclear inclusion
NMDA	N-methyl-D-aspartate
3-NP	3-nitropropionic acid
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
SBMA	Spinobulbar muscular atrophy
SCA	Spinocerebellar ataxia
SDH	succinate dehydrogenase

Original Papers related to the Ph.D. thesis

I. **Jakab K**, Endreffy E, László A, Vécsei L, Raskó I: Molekuláris genetikai diagnózis Huntington-kóros betegeinknél. Clin Neurosci/Ideggy Szle 1995, 48: 292–295.

II. **Jakab K**, Gárdián G, Endreffy E, Kalmár T, Bachrati Cs, Vécsei L, Raskó I: Analysis of CAG repeat expansion in Huntington's disease gene (IT-15) in a Hungarian population. Eur Neurol 1999, 41: 107–110.

III. Kálmán J, Juhász A, Majtényi K, Rimanóczy Á, **Jakab K,** Gárdián G, Raskó I, Janka Z: Apolipoprotein E polymorphism in Pick's disease and in Huntington's disease. Neurobiol of Aging 2000, 21: 555–558.

IV. **Jakab K,** Gárdián G, Kalmár T, Endreffy E, Vécsei L, Raskó I: Huntington-kóros betegek molekuláris genetikai vizsgálata hazánkban. Gyermekgyógyászat 2001, 52: 43–47.

V. **Jakab K,** Novák Z, Engelhardt J, Kemény L, Kálmán J, Vécsei L, Raskó I: UVB irradiation-induced apoptosis increased in lymphocytes of Huntington's disease patients. NeuroReport 2001, 12: 1653–1656.

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1. Janka Z, Juhász A, Rimanóczy Á, Boda K, Márky-Zay J, Palotás M, Kuk I, Zöllei M, **Jakab K,** Kálmán J: α2-macroglobulin exon 24 (val-1000-IIe) polymorphism is not associated with late-onset sporadic Alzheimer's dementia in the Hungarian population. Psychiatric Genetics, 2002 (in press).

2. Beniczky S, Kéri Sz, Antal A, **Jakab K,** Nagy H, Benedek Gy, Janka Z and Vécsei L: Somatosensory evoked potentials correlate with genetics in Huntington's disease. NeuroReport 2002; 13. (in press).

Chapters and abstracts related to the thesis

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2. **Jakab K**: Huntington-kór és egyéb choreával járó mozgászavarok In: Parkinson kór és egyéb mozgászavarok. Ed: Takáts A. Melania, Budapest. 2001.

3. **Jakab K**. Molekuláris genetikai diagnózis Huntington-kóros betegeknél. In: Neurológia a harmadik évezred hajnalán. Ed: Vécsei László, Tajti János, Gárdián Gabriella, Springer, 2001.

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6. **Jakab K:** Trinucleotid repeat expansio okozta neurológiai kórképek. I. Neurogenetikai Tudományos Ülés, Szombathely, Clin Neurosci/Ideggy Szle 1997, 50: 407.

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9. **Jakab K**, Novák Z, Engelhardt J, Kemény L, Vécsei L, Raskó I: UVB sugárzással indukált apoptosis vizsgálata Huntington-kóros betegek lymphocytáiban. Magyar Klinikai Neurogenetikai Társaság Tudományos Ülése, Szombathely, 2001, absztrakt könyv.

Summary

The spectrum of inherited neurological diseases characterized by an unusual type of mutation – trinucleotide repeat expansion – is continuously increasing, HD is just one of these neurodegenerative diseases. Unstable expanded trinucleotide repeats were an entirely novel and unprecedented disesase mechanism when first discovered in 1991.

In that type of trinucleotide repeat expansion disorder, named polyglutamin diseases, modest expansions of CAG repeats within the coding sequences cause toxic gain-of-function mutation. The CAG repeats encode polyglutamine tracts in the gene product, that cause it to aggregate within the cells, and/or the polyglutamine expansion results in altered protein-protein interactions, that manifest as a toxic gain of function activity. Eight neurodegenerative diseases belong to polyglutamin disorders: HD, Kennedy disease (SBMA), spinocerebellar ataxias (SCA1, 2, 3, 6, 7), dentatorubral-pallidoluysian atrophy (DRPLA).

Huntington's disease is a neurodegenerative disorder with autosomal dominant inheritance. The main symptoms are choreiform, involuntary movements, personality changes and dementia associated with progressive striatal and cortical atrophy.

Striatal cells are not equally affected by the degenerative process. The neuronal loss is selective for GABAergic medium-size spiny neurones which sends efferents to the globus pallidus and substantia nigra.

The genetic mutation responsible for HD has been identified as an expansion of the CAG trinucleotid repeat length present in IT 15 (Interesting Transcript) gene which is mapped to chromosome 4p16.3. An inverse correlation was found between the age of onset of the disease and the number of CAG repeats. HD shows anticipation, in which subsequent generations display earlier disease onsets due to intergenerational repeat expansion.

The normal huntingtin is distributed in peripheral organs as well as in the central nervous system. The function of the normal huntingtin is not fully understood yet. Wild-type huntingtin up-regulates transcription of brain-derived neurotrophic factor (BDNF), a pro-survival factor produced by cortical neurons that is necessary for survival of striatal neurons. Normal huntingtin has an anti-apoptotic effect in neurons in vitro. Several findings are consistent that huntingtin is involved in regulating synaptic plasticity. The N-terminal fragments of the mu-

tant huntingtin form aggregates catalyzed by the tissue transglutaminase enzyme. The aggregates form intranuclear inclusions in specific areas of the CNS.

Several proteins have been identified which interact with huntingtin. The expanded polyglutamine tract in the mutated huntingtin may modify the normal interaction of huntingtin with other proteins or may result in new interactions, leading in both cases to neuronal death.

Molecular studies on mutated huntingtin showed that neurodegeneration in HD involve apoptosis, because huntingtin is a substrate for apopain, a key enzyme in apoptosis.

Our first goal was to introduce and offer a PCR-based molecular genetic diagnostic test to characterize HD patients and asymptomatic carriers in Hungary.

The most important part of our work is to detect the asymptomatic carriers and give them a possibility to prevent the progressive neuronal destruction and/or rescue the striatal neurons. We identified 25 asymptomatic carriers long before the manifestation of the clinical symptoms. Early detection and late onset of the disease render these asymptomatic carriers well suited for effective therapeutic intervention, if this will be developed. Prenatal genetic test for HD is also available, if needed.

The available data proved that the distribution of repeats in HD is population dependent, it seemed appropriate to determine and characterize the pattern of CAG repeat distribution in HD patients and healthy control individuals in our country.

The CAG repeat expansion of IT 15 gene in Hungarian HD patients was similar to that reported from other countries in Western Europe. The prevalence of HD and also the average length of the CAG repeat size in normal individuals might indicate that the population of Hungary is mixed, resembling those of Europe.

The same tendency has been observed in the case of the apolipoprotein E (apoE) polymorphism. The polymorphism of apoE gene has been implicated as a risk factor in the pathomechanism of several forms of dementia, such as Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson disease and Creutzfeld-Jakob disease. The aim of our investigation was to determine the apoE genotype in two other dementing disorder, HD and Pick's disease and normal controls. Our study revealed that the apoE 4 allel frequency is higher in Pick's disease, than in HD and normal controls. In Pick's disease the apoE protein is also present in the intraneuronal Pick body, and the apoE4 allel could play a role in the pathomechanism of this tau-protein related form of dementia. Contrary to this observation the apoE allele fre-

quencies in HD were similar to those in the control population suggesting that the inheritance of the different apoE allels is not equally important for the pathogenesis of all neurodegenerative disorders with dementia.

Since the inheritance pattern of the triplet expansion in HD showed parent-dependent characteristics, therefore in two relatively big Hungarian families the role of parental origin of the repeat expansion on the stability of CAG repeats was analyzed. As the CAG repeat instability is much more enhanced in one of the examined families than in the other, this suggests a still unknown factor influencing the dynamic mutation. It could be related to the status of the mismatch repair system.

One of the key issues in understanding the clinical features of HD was to establish the role of the huntingtin protein. Neuronal cell death is likely to be caused mainly by genetic gain-of-function mechanisms, involving abnormalities of protein folding, chaperone interactions, alterations in gene transcription, loss of neurotrophic support and involvement of signal transduction pathways and apoptosis. Although the exact details are unclear, the cellular mechanisms by which mutant huntingin kills neurons are beginning to be revealed. Recently investigations have focused on the role of apoptosis in neurodegeneration. The aim of our experiments was to provide a further support of the huntingtin-mediated cell toxicity, which induces apoptosis. We wanted to know whether the increased sensitivity to apoptosis is also present in non-neural tissue of the patients. Huntingtin is widely expressed in the CNS as well as in non-neural tissues, so functional abnormalities can be expected also outside of the brain. The susceptibility of lymphocytes from HD patients, asymptomatic carriers to UVB irradiation-induced apoptosis was examined and compared to normal controls. Our study proves that in HD not only the cells of the central nervous system are prone to apoptosis, but also the peripheral lymphocytes. We established that increased susceptibility of HD cells to apoptosis is not restricted to neurons. Mutant huntingtin may promote apoptosis in all cell types, but only a restricted neuronal subpopulation is the most sensitive to this process causing neurodegeneration.

Another mechanism possibly involved in HD pathogenesis are impairment in energy metabolism, mitochondrial dysfunction in striatal neurons. The presence of apoptotic features in HD does not exclude the possibility of glutamate-mediated excitotoxicity, decreased energy metabolism, and the defect in mitochondrial complex II-III. An interesting hypothesis can be

proposed establishing a connection between huntingtin, apoptosis and mitochondrial dysfunction. Huntingtin is a substrate of caspase-3, a key enzyme of apoptosis, in addition, huntingtin is also known to interact with GAPDH, a protein involved both in energy metabolism (glycolysis) and in apoptotic pathways. Abnormal interaction between mutated huntingtin and GAPDH could cause decreased glucose metabolism in the basal ganglia in HD and might also stimulate apoptosis. Interaction of GAPDH with the mutant huntingtin may decrease the glycolytic activity of this enzyme, progressively leading to energy deficit. To support this theory we examined the glycolytic function of GAPDH in HD fibroblasts. HD brain is known to exhibit metabolic abnormalities, therefore we considered the possibility that metabolic alterations might also be manifested in non-neural HD cells, such as fibroblasts. Cultured skin fibroblasts from four HD patients and an age-matched control were used to measure the activity of GAPDH. All HD patients' fibroblast extract exhibited decreased GAPDH activity compared to the normal control. These preliminary data are consistent with the hypothesis that GAPDH enzyme has functional role in the pathomechanism of HD, and these metabolic changes could be detected systemically, not restricted to neurons.

Our PET study using 18F-FDG as a tracer provided another piece of evidence for an alteration of glucose metabolism and energy impairment in HD patients. Cerebral glucose metabolism was severely reduced in the caudate nucleus of HD patients even at an early stage of the disease. Our results demonstrate that genetically identified HD gene carriers can show normal neurological function for many years before the disease becomes clinically manifest, but subtle metabolic changes could be detected by 18F-FDG PET. The measurement of striatal glucose hypometabolism could be used as a marker of the disease progression and it could be useful for testing the effect of possible future treatment.

There are currently no cures or even effective treatments for HD. However, recent advances in understanding the basic mechanisms of CAG expansion and polyglutamine toxicity have renewed the hopes that a therapeutic strategy might someday be possible. The next challenge is developing a therapy to decrease the toxicity of mutant huntingtin. Despite an incomplete understanding of toxicity, it is generally accepted that aggregation of mutant huntingtin fragments is the causative factor. Antibodies and number of small molecules, peptides inhibitors are being developed to block aggregation, and improve cell survival.

Metabolic abnormalities including mitochondrial deficit, decreased glucose metabolism, ATP depletion also play a role in HD neurodegeneration. Clinical trials are underway to evaluate the efficacy of free-radical scavengers, creatine and co-enzyme Q. These agents are protective, and improve the energy production in mitochondria.

The enhanced apoptotic process observed in HD may serve as a therapeutic target for the treatment. If caspase activation occurs early enough in the disease progression then disease onset could be blocked by the use of caspase inhibitors, which might provide protection by blocking a general cell death pathway. Although effective therapy is not possible yet, rapid advances in the understanding of the basic mechanisms of the neurodegeneration in HD are leading to expanded approaches towards therapeutic strategies and a hope for cure.

Contents

I. Introduction

Huntington's disease is a neurodegenerative disorder with autosomal dominant inheritance. The main symptoms are choreiform, involuntary movements, personality changes and dementia associated with progressive striatal and cortical atrophy.

The word "chorea" is derived from the Latin choreus and the Greek choros having to do with dancing. Thomas Sydenham (1624–1689), an English physician who is now considered the "father of chorea", elaborated the first clinical descriptions of choreic movements. George Huntington was the first, who presented a paper entitled 'On Chorea' in 1872, whereby he discussed the symptoms of chorea, described key features of HD that set it apart from other forms of chorea, and established HD as a separate disease entity. Quickly thereafter, reports from all over the world were published describing hereditary chorea.

I.1. Clinical features

There is a geographic variation in the prevalence of HD. It is close to 1:10 000 in most Western European countries and countries populated by European immigration (Squitieri et al., 1994).

Clinical symptoms develop very rapidly after onset, and compose a three-part picture with motor symptoms, psychiatric disturbances and cognitive impairment (Adams and Victor's Principles of Neurology, McGraw-Hill, NY, 2001). HD generally has an adult onset at an average age of 40-45 and the progression of the disease inevitably leads to death within 10-15 years. Occasionally it manifests in younger age. This juvenile type of the disease is paternally inherited and more severe.

Motor disabilities progress over that period from a hyperkinetic form to an akineto-rigid syndrome. The earliest motor signes are eye movement abnormalities, followed by appearance of orofacial dyskinesias, other dyskinesias involving the head, neck, trunk, arms and finally chorea. Chorea is "a state of excessive, spontaneous movements, irregularly timed, ran-

domly distributed and abrupt. Severity may vary from restlessness with mild intermittent exaggeration of gesture and expression, fidgeting movements of the hands, unstable, dance-like gait, to a continuous flow of disabling, violent movements" (defined by the World Federation of Neurology) (*Barbeau et al.*, 1981). Resulting from chorea, a general clumsiness in tasks requiring the use of hands and arms is observed. The remarkable intensity and amplitude of these abnormal movements gradually impair the patient's ability to work and reduce their social life. The gait of the patients is slow, stiff, and unsteady. As the disease progresses choreiform movements reduced, the hyperkinetic syndrome being replaced by bradykinesia, rigidity and dystonia.

Cognitive decline has been observed in early stage of the disease. Neurophysiological testing of HD patients pointed to HD as a model of subcortical dementia (*Beatty et al.*, 1988). The progressive lesion of the striatum disconnects the prefrontal cortex from the basal ganglia circuitry, interrupting the flow of information arising from the cerebral cortex. This is thought to result in a frontal-type syndrome, despite the absence of any primary lesion of the frontal cortex. Cardinal features of HD dementia are difficulties in retrieving memories, slowed information processing, cognitive inflexibility (*Cummings*, 1993).

Patients' personality changes dramatically, they may be suspicious, irritable, impulsive, eccentric, untidy, or excessively religious. Poor self-control may be reflected in outbursts of temper, fits of despodency, alcoholism. Disturbances of mood, particularly depression is common, and may constitute the most prominent symptoms early in the disease. These emotional disturbances and changes in personality can reach such proportions as to lead a virtual psychosis with delusions or hallucinations .

I.2. Neuropathology

Atrophy of the caudate nucleus and putamen bilaterally is the characteristic abnormality, usually accompanied by cortical atrophy. The caudatal atrophy alters the configuration of the frontal horns of the lateral ventricles, in addition, the ventricles are diffusely enlarged. The increasing atrophy can make the overall size of the HD brain smaller, than normal.

The striatal degeneration begins in the medial part of the caudate nucleus and spreads, tending to spare the nucleus accumbens. Vonsattel established a standard grading system for the degree of striatal neurodegeneration (*Vonsattel et al.*, 1985). The degree of atrophy of the striatum has been shown to correlate well with the severity of motor and psychiatric symptoms (*Myers et al.*, 1988).

Striatal cells are not equally affected by the degenerative process. The neuronal loss is selective for GABAergic medium-size spiny neurones which sends efferents to the globus pallidus and substantia nigra.GABAergic projection neurons contain the modulatory substances enkephalin and substance P. (*Reiner et al.*, 1988; *Beal et al.*, 1987). In addition, a large majority of these neurons also contain the calcium binding protein calbindin (*DiFiglia et al.*, 1989). Striatal interneurons are largely unaffected, at least in the early course of the disease (*Kowall et al.*, 1987). Cholinergic interneurons are large and aspiny, GABAergic interneurons are medium aspiny neurons, with subpopulations that contain either NADPH-diaphorase, nitric oxide synthase (NOS), or parvalbumin or calretinin (*Cicchetti et al.*, 1996). Analysis of post mortem HD striatum reveals a dramatic loss of striatal GABAergic medium spiny projection neurons, with a relative sparing of cholin-acethyl-transferase (ChAT) interneurons and astrocytosis (*Ferrante et al.*, 1987). All major striatal afferents such as dopaminergic and serotoninergic afferents are left relatively unaltered by the degenerative process. Striatal levels of dopamine or dopaminergic markers are not decreased in HD striatum (*Beal et al.*, 1990).

Other areas of intense cell loss and astrogliosis include the two segments of the globus pallidus, the substantia nigra pars reticulata, the subthalamic nucleus and several thalamic nucleus. The common feature to all these areas is that they belong to the basal ganglia circuitry and connected to the striatum.

Neurodegeneration in the cerebral cortex is generally observed in HD mainly in layers III, V, and VI. (*Hedreen et al.*, 1991).

I.3. Genetics

The genetic mutation responsible for HD has been identified as an expansion of the CAG trinucleotid repeat length present in IT 15 (Interesting Transcript) gene which is mapped to chromosome 4p16.3. (*HD Collab Res Group*, 1993). The HD gene is 210 kb, containing 67 exons encoding a protein named huntingtin. The CAG repeat expansion is located in the coding region of the gene at the 5' end of the first exon. The CAG repeat number is polymorph and unstable. In healthy individuals the CAG repeat ranges from 9-36 (median:19), while patients with HD it ranges between 36-121 (median:44) (*Kremer et al.*, 1994). There is a border zone between normal and abnormal CAG repeat lengths (36-39) because of the incomplete penetrance of the disease phenotype (*Rubinstein et al.*, 1996).

An inverse correlation was found between the age of onset of the disease and the number of CAG repeats.Longer CAG expansion is associated wit shorter disease duration, faster rate of deterioration and greater neuronal loss in the striatum (*Furtado et al.*, 1996).

The repeat number can grow in each successive transmission. HD shows anticipation, in which subsequent generations display earlier disease onsets due to intergenerational repeat expansion (*Trottier et al.*, 1994). In each individual somatic and gonadal mosaicism can be observed. The CAG repeat number is the highest in the striatal and cortical neurons (*Telenius et al.*, 1994).

I.4. Molecular mechanisms

Huntingtin is a 350 kD protein, which consists of 3144 amino acids. Each CAG triplet codes for the amino acid glutamine, resulting in a polyglutamin stretch in the huntingtin protein. As the CAG repeat number grows in the gene, the growing polyglutamine tract produces a mutant huntingtin with increasingly aberrant properties (*HD Collab Res Groups*, 1993).

The normal huntingtin is distributed in peripheral organs as well as in the central nervous system (*Sharp et al.*, 1995). In the brain, neurons are enriched in huntingtin as compared to glial cells (*Li et al.*, 1993). The huntingtin immunoreactivity is mostly localized in synaptic vesicles (*DiFiglia et al.*, 1995), clathrin-coated membranes along dendrites (*Velier et al.*,

1998), associated with Golgi and the mitochondria (*Gutekunst et al.*, 1998). The level of expression and the regional distribution of the mutated huntingtin in the brain as well as in the peripheral tissues were rather similar to those of the normal protein (*Aronin et al.*, 1995). At the cellular level differences have been found in HD brains, in particular an abnormal nuclear accumulation of the N-terminal fragments of huntingtin, described as neuronal intranuclear inclusion (NII) (*DiFiglia et al.*, 1997).

The function of the normal huntingtin is not fully understood yet. It is essential during development for gastrulation and neurogenesis (*Nasir et al.*, 1995), and it is important for neuronal survival in adults. Wild-type huntingtin up-regulates transcription of brain-derived neurotrophic factor (BDNF), a pro-survival factor produced by cortical neurons that is necessary for survival of striatal neurons in the brain (*Zuccato et al.*, 2001). Normal huntingtin has an anti-apoptotic effect in neurons in vitro (*D. Rigamonti*, 2000). Huntingtin is also involved in vesicle trafficking in the secretory and endocytotic pathways (*Kegel*, 2000). Several findings are consistent in with huntingtin being involved in regulating synaptic plasticity.

Polyglutamin region of huntingtin self-associate to form polar zippers (strands that assemble into sheets or barrels by hydrogen bonding), promoting aggregation. (*Perutz MF*, 1999). The N-terminal fragments of the mutant huntingtin form aggregates catalyzed by tissue transglutaminase enzyme and produce intranuclear inclusions.

Several proteins have been identified to interact with huntingtin. The expanded polyglutamine tract present in the mutated huntingtin may modify the normal interaction of huntingtin with other proteins or may result in new interactions, leading in both cases to neuronal death. The CAG repeat expansion affects the quaternary structure of the protein, so that many domains of the mutated huntingtin could be involved in new protein-protein interactions, even though the N-terminal domain of the protein is the most likely to be involved.

Huntingtin-interacting protein 1 (HIP-1) plays a role in cytoskeleton regulation and it is also associated with chlatrin-coated vesicles containing a site for direct binding to the chlatrin heavy chain. As the huntingtin polyglutamine tract becomes expanded, huntingtin has significally less affinity for HIP-1 (*Kalchmann et al.*, 1997).

Another protein, Huntingtin-associated protein 1 (HAP-1) is also known to interact with huntingtin. The partial co-localization of HAP-1 with NOS, has suggested that an abnormal

interaction between huntingtin and HAP-1 could result in NO-mediated neuronal death (*Li et al.*, 1996).

The N-terminal of huntingtin has been shown to bind to a glycolytic enzyme GAPDH. Interaction of GAPDH with the mutant huntingtin may impair the glycolytic activity of this enzyme leading to energy deficit in the cell (*Kuida et al.*, 1996).

Molecular studies on mutated huntingtin showed that neurodegeneration in HD may involve apoptosis, because huntingtin is a substrate for apopain (*Goldberg*, 1996). Apopain also named caspase 3, a cystein protease, is a key enzyme in apoptosis. Caspase 3 cleaves huntingtin to toxic N-terminal fragments. The proteolytic activity of caspase 3 is increased in the presence of mutated huntingtin. Apoptotic neurons were detected by TUNEL staining in the striatum of HD patients in post mortem brain samples. (*Dragunow et al.*, 1995; *Thomas et al.*, 1995). Activated caspase 8 was also identified in neuronal intranuclear inclusions in HD brains (*Sanchez et al.*, Neuron 22 1999).

I.5. Animal models of HD

Animal models of HD were constructed by generating transgenic mice, or excitotoxic striatal lesioning, or by mitochondrial toxins inducing energy impairment.

In the **excitotoxic striatal lesion models** histological, biochemical and behavioral similarities to HD are observed, provided strong support of excitotoxic hypothesis for HD. Activation of glutamate receptors by a relatively high concentrations of glutamate produces neurotoxic events through the same excitotoxic cascade: induces a massive intracellular influx of Na^{+} and Ca^{++} cations which can activate a number of biochemical pathways (activation of phospholipases, proteases, kinases) leading to irreversible alterations and finally cell death. Intrastriatal injection of kainate induced striatal neurodegeneration in rats (*Beal et al.*, 1986), but do not perfectly reproduced the histopathology of HD. Both projection and NADPH containing interneurons were killed by the excitotoxin, as opposed to the relative sparing of striatal interneurons in HD. In contrast, intrastriatal injections of quinolinic acid, a NMDA-selective glutamate agonist, induced a prefential degeneration of GABAergic neurons and a relative sparing of NADPH-diaphorase interneurons, and ChAT interneurons (*Beal et al.*,

1989). Excitotoxic striatal lesions can also replicate some of behavioural aspects of HD, including motor symptoms and frontal-type cognitive deficits in rats. These findings suggested the involvement of NMDA receptors in the etiology of HD.

Direct injection of great variety of **mitochondrial toxins** (malonate, rotenone, 3-acetylpyridine, 3 nitropropionic acid) could also lead to pattern of striatal atrophy similar to HD. These studies show that impairment in energy metabolism, not accompanied by changes in extracellular glutamate concentrations, can result in a secondary excitotoxic insult (*Novelli et al.*, 1988). 3-NP inhibits succinate dehydrogenase (SDH) which is active in complex II of electron chain transport, causing a decrease in ATP synthesis (*Alexi et al.*, 1998). This leads to a loss of function of Na^+/K^+ -ATPase, causing cells to depolarize (*Riepe et al.*, 1994). The depolarization has been shown to cause the revers operation of the Na^+/Ca^{++} exchange pump leading to an increase in Ca^{++} influx. In addition, depolarization also releases the Mg^+ block of the NMDA receptor, causing a higher intracellular Ca^{++} influx. 3-NP-induced striatal lesioning involves both necrotic and apoptotic cell death (*Alexi et al.*, 1998). 3-NP also induces activation of caspase 3 (*Keller et al.*, 1998). Lesioning with 3-NP provides an animal model of HD which closely resembles the human disease, in both pathology and symptomatology. Locomotor dysfunction, chorea, dystonia, dyskinesia are observed in these primate animal models (*Brouillet et al.*, 1995). Animals treated chronically with 3-NP show a loss of GABAergic projection neurons with a relative sparing of NADPH diaphorase neurons accompanied by astrogliosis (*Beal et al.*, 1993). 3-NP administered over a long period of time to selectively lesion the striatum, it allows for the first time the creation of an animal model of HD which reproduces the slow progressive nature of the disease.

Knock-out mouse model. It was found that, homozygous knock-out of HD gene in mice was embryonic lethal, indicating that huntingtin has an essential role in early embryonic development (*Duyao et al.*, 1995; *Nasir et al.*, 1995; *White et al.*, 1997).

In **transgenic mouse models** the mutant HD gene or part of it, is inserted randomly into the mouse genome, leading to expression of a mutant protein in addition to the endogenous, normal huntingtin. The first successful mouse model of HD was generated by overexpressing exon 1 of the human gene IT15 encoding huntingtin with long (145-157) CAG repeats termed R6 mice (*Mangiarini et al.*, 1996). Several features of the human HD were obvious in these

animals. Motor deficits can be measured in these mice as early as 5-6 weeks of age (*Carter et al.*, 1999). Behavioral abnormalities appeared at age of 8 weeks, followed by an early death at 10-13 weeks of age. The mice have severe phenotype with low weight, diabetes, tremor and convulsions (*Mangiarini et al.*, 1996). At autopsy brain weight is reduced, but neuronal death is minimal compared with the behavioral symptoms. Immunostaining with antibody against the N-terminal portion of huntingtin detected inclusions in the nucleus of most neurons. Nuclear inclusions were preceded by an abnormal location of huntingtin in the nucleus (*Davies et al.*, 1997). NII were found only in those transgenic animals that developed motor symptoms, however, no neuronal loss was seen in these mice.

Knock-in transgenic mouse models were generated by insertion of HD mutation into the mouse Hdh gene (which encodes the mouse huntingtin), leading to homozygocity or heterozygocity for the mutation. The mutant gene was expressed under its natural promoter and in the appropriate genomic context of the mouse Hdh gene (*Barnes et al.*, 1993). Theoretically knock-in models should be optimal to reproduce human pathology, because they are the most faithful reproduction of the disease genotype. In practice, however these models were disappointing initially, because the mice showed no motor symptoms. Generation of additional models were produced and much delayed motor anomalies were reported in a model with 150 CAG repeats (*Lin et al.*, 2001). A consistent feature of the different models of knock-in mice is the presence of microaggregates of huntingtin in the brains of mice at the age of 2-6 months (*Wheeler et al.*, 2000; *Menalled et al.*, 2000; *Li et al.*, 2000). Further crucial information obtained with these models is the evidence for an age-related instability of expanded CAG repeats in neurons, despite the fact that neurons do not divide (*Kennedy et al.*, 2000). This instability is region specific, larger increases in CAG repeat number was found in the striatum and cortex.

I.6. The aim of the present study

1. Since at the beginning of our study results concerning the characteristics of the interesting mutation in Huntington's disease just started to emerge, our first goal was to introduce and offer a PCR-based molecular genetic diagnostic test to characterize HD patients and asymptomatic carriers in Hungary.

2. The available data proved that the distribution of repeats in HD is population dependent, it seemed appropriate to determine and characterize the pattern of CAG repeat distribution in HD patients and healthy control individuals in our country.

3. The inheritance pattern of the triplet expansion in HD showed parent-dependent characteristics. Therefore in two relatively big Hungarian families the role of parental origin of the repeat expansion on the stability of CAG repeats was analyzed.

4. The apolipoprotein allele distribution could be a susceptibility factor in certain neurodegenerative disorders. Our aim was to determine the apolipoprotein E genotype in HD with other two neurodegenerative diseases, such as AD and Pick's disease.

5. Alteration of glucose metabolism in HD plays a major role in neurodegeneration. Therefore PET scanning of HD brains was performed and the activity of one of the key enzymes in glucose metabolism (GAPDH) was determined.

6. One of the key issues in understanding the clinical features of HD was to establish the role of the huntingtin protein. It was not clear whether it has pathological role only in the brain or in other tissues also. That was the reason why peripheral lymphocytes from HD patients were characterized related to their apoptotic ability.

II. Materials and methods

II.1. Determination of CAG repeat expansion in HD patients and healthy controls

II.1.1. Selection of patients and controls for molecular genetic studies

Fifty-two HD patients' and 25 asymptomatic carriers' DNA samples were collected from 55 unrelated families. In 17 families 2-13 individuals were tested. In 3 of 17 families the affected parents died years before the development of the method of genetic screening. The diagnosis of HD was established by neurologists and confirmed by geneticist. Clinical details with documented neurological examination including CT, MRI and PET were available for 48 patients. In addition, the number of CAG repeats was assessed in 70 normal controls. All of the patients, their relatives and controls voluntary participated in the study. We followed the guidelines for the molecular genetics predictive test in HD (*Broholm et al.*, 1994).

II.1.2. Isolation of genomic DNA from peripheral blood

Blood samples were collected from the patients, family members and healthy controls on the basis of informed consent. DNA was prepared from leucocyte nuclei according to a standard method (*Maniatis et al.*, 1989). Briefly, 4 ml peripheral blood was anticoagulated with 1 ml of 5% EDTA and haemolysed by freeze thawing. White blood cells were lysed with the addition of 45 ml lysis buffer (0.32 M sucrose, 5 mM $MgCl_2$, 10 mM TRIS-HCl pH:7.4, 1% Triton-X 100). Nuclei were pelleted by centrifugation, washed in PBS twice and resuspended in 1 ml solution of 75 mM NaCl, 50 mM EDTA. Nuclei were lysed and digested with the addition of 100 µl 10% SDS and 22 µl 25 mg/ ml Proteinase K (Merck) overnight at 55°C. DNA was phenol extracted, precipitated with ethanol and dissolved in TE.

II.1.3. Determination of CAG repeats

The CAG repeat number has been determined after PCR amplification of the appropriate DNA region as described by Watanabe et al., 1996. The oligonucleotid primers used for PCR amplification were the following:

HD-1 5' – ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC – 3'

HD-3 5' - GGCGGTGGCGGCTGTTGCTGCTGCTGCTGC – 3'

This primers encompassed only the CAG repeat excluding the polymorph CCG region residing at the 3' end of the CAG repeats. The amplification was carried out in 50 µl total volumes, containing 500 ng genomic DNA, 20 pmol of each primers, 250 µM Σ dNTP, 1.5 U Pfu polymerase enzyme in Pfu buffer (Stratagene). The amplification was carried out in the following cycles: 95°C 5 min 1x, 95°C 1 min, 65°C 2 min, 72°C 1.5 min 35x, 72°C 8.5 min 1x, 4°C. 10 µl PCR product was mixed with gel loading buffer, and separated on 8% polyacrylamid gel, electrophoresed together with pBR322 molecular weight marker. The size of CAG repeats was calculated by an UVP gel documentation system (Gel Base, UVP, England).

II.2. Apolipoprotein E genotyping in HD

II.2.1. Patients

The genomic DNA of 28 HD patients was extracted from peripheral blood leucocytes according to the standard method. The CAG repeat number was determined after PCR reaction, as described earlier. HD patients were 49.4±8.7 years old. The CAG repeat number ranged between 37-49. In addition 79 healthy controls and 36 patients with Pick's disease were examined. Informed consent was obtained from all individuals and the protocol was approved by the ethical committee of the Univerity of Szeged.

II.2.2. Determination of ApoE alleles

ApoE alleles were determined by a polymerase chain reaction-based strategy. PCR reaction was performed in a PTC 100 Thermal Controller MJ Res. Inc. thermal cycler. The final volume of PCR solution was 25 µl, containing 20 µM of each primer (5'-TCCAAGGAGCTGCAGGCGGCGCA-3' and 5'-ACAGAATTCGCCCCGGCCTGGTACACTGCCA-3'), 300 ng DNA, 1.25 µl of dNTPs (20 mM), consisting a mix of 5 mM of each, 1.5 µl (25 mM) MgCl2, 2.5 µl (5%) di-methyl-sulfoxide, 0.5 U Taq DNA polymerase (Promega) in 67 mM TRIS-HCL buffer (pH 8.8). The initial denaturation was 5 min at 95 °C, followed by 30 cycles of 30s at 94 °C de-naturation, 22 s at 63 °C annealing and extension for 30 s at 72 °C. A final extension for 3 min at 72 °C completed the amplification procedure. The amplified DNA was digested with 5 U CfoI (Promega) overnight at 37 °C and the DNA fragments were separated on 8% nonde-naturing acrylamide gel. The gel was stained with 0.5 µg/ml ethidium bromide and apoE genotype was determined by the pattern of DNA fragments present.

Statistical analysis: the apoE allele frequencies were compared with the Pearson $\chi 2$ test. The Monte Carlo significance (2-sided) level was set at the 95% interval. The program SPSS 1995 was used for all statistical analysis.

II.3. [(18)F]FDG PET examination of HD patients and asymptomatic carriers

Two symptomatic HD patients in early stage of the disease were studied. They were 31 and 54 years old with 59 and 41 CAG repeat number. Six asymptomatic carriers were examined, mean age 34±SD. Their CAG repeat number varied between 38 and 45. Each individual under-went a general neurological examination before PET scanning. HD patients showed mainly be-havioral signs and mild choreic movements without severe cognitive decline. None of the asymptomatic carriers had any neurological symptoms. PET scans with the tracers [18F]fluoro-deoxyglucose ([18F]FDG) were performed to study the glucose metabolism in the caudate nu-cleus and putamen. The scanning was carried out in PET Centrum in the University of Debre-cen. The study was approved by the ethical committee of the Human Investigation Review Board of University of Szeged. Informed consent was obtained from each individual.

II.4. Examination of UVB irradiation-induced apoptosis in HD lymphocytes.

II.4.1. Patients and samples

Peripheral blood mononuclear cells (PBMC) were prepared from venous blood of eight HD patients, two asymptomatic carriers and ten healthy controls by FICOLL sedimentation. HD patients were 49,6±15.7 (mean±S.D.) years old, asymptomatic carriers were 37 and 45 years old, control individuals were 45±10.8 years old. The number of CAG repeat ranged from 44 to 70 in HD patients. The asymptomatic carriers had 43 and 45 CAG repeats. All of the patients, asymptomatic carriers and controls voluntary participated in the study. The guidelines for the molecular genetics predictive testing in HD was followed.

II.4.2. UVB irradiation.

For induction of apoptosis PBMC were irradiated by XeCl 308 nm UVB laser (Lambda Physic LPX 105E) in uncovered tissue culture plates in PBS. Laser radiation was delivered at 20 Hz, and radiation doses were 70-200-300 mJ. After irradiation the PBMC were washed once and suspended in RPMI 1640 (GIBCO, Scotland) supplemented with 10% AB+ heat inactivated human serum, 2 mM L-glutamine and antibiotics. The cells were cultured at 37 °C in 5% CO2 for 20 h.

II.4.3. Immunofluorescence assay for flow cytometry

PBMC were fixed in 2% paraformaldehyde for 30 min. washed in PBS and permeabilised in 0.1% saponin in PBS supplemented with 1% bovine serum and 0.02% NaN3 for 15 min on ice. The cells were washed in PBS containing 1% BSA, the supernate was discarded and each sample was stained with 20 µl APO2.7-PE mAb (Immunotech, Marseille, France) and 5 µl anti-human CD3-FITC mAb (Dako, Glostrup, Denmark) in 75 µl PBS for 25 min at room temperature. The APO2.7-PE mAb reacts to a 38 kDa mitochondrial membrane protein exposed on cell undergoing apoptosis. To exclude false positive reactions control samples were stained with isotype-matched anti-mouse mAb. After washing cells were resuspended in 0.5 ml PBS.

II.4.4. Flow cytometry

Sample data were aquired on FACStar and FACSCalibur (Beckton Dickinson, USA) flow cytometer. Samples were analyzed using CellQuest software (Beckton Dickinson, USA). The percentage of APO2.7-PE positive CD3+ lymphocytes were determined.

II.4.5. Statistical analysis

One-way ANOVA was followed by the Fisher's LPSD test to determine significant differences between groups. $p<0.05$ was considered statistically significant.

II.5. Determination of GAPDH activity in fibroblast cultures

II.5.1. Skin biopsy and production of primary human fibroblast cultures

Skin biopsies were taken from 4 HD patients, and 1 normal control by punch techniques in local anaesthesia. HD patients were 67, 52, 46, 27 years old, the control was 40 years old. The number of CAG repeat were 44, 45, 48, 59 in HD patients. All individuals voluntary participated in the study. Informed consent was obtained from the individuals, and the protocol was approved by the local ethical committee.

Skin biopsies were minced into small pieces, than one-two pieces were placed under sterile cover slips and attached to the bottom of a 60 mm Petri dish by the means of sterile vaseline. Dulbecco's MEM with 10% fetal calf serum was added and the dishes were incubated at 37 °C in 5% CO_2. The outgrowth of fibroblasts could be observed after several days of incubation. The cover slips were removed and the dishes were incubated further with several changes of medium till the fibroblasts reached confluency. The transfer of cell to other dishes was achived after trypsin treatment.

II.5.2. GAPDH enzyme assay in HD and control fibroblast extracts

The cultured fibroblasts were separated from the substratum by trypsinisation and centrifuged for 20 min at 2000 g, 4 C°. The supernatants were removed and the pellet were resuspended in 1 ml buffer (20 mM TRIS-HCl pH: 7.0 , 1 mM DTE, 1 mM EDTA, 1 mM PMSF).

The cells were sonicated 5 times, for 5 sec, with 30 sec intervalls. The suspension was repelleted by centrifugation (25 min, 50 000 g, 4 °C). The supernatants contained the fibroblast extract, and the membrane-pellet was removed. The cell-free suspension contains huntingtin which was identified with immunoblot techniques.

An aliquot of this suspension was used to determine the GAPDH activity. The assay mixture contained 2 mM glyceraldehyd-3-phosphate (GAP), 4.3 mM NAD+, 4.3 mM Na_2HAsO_4, 50mM TRIS-HCl buffer pH:8 and the homogenate. The rate of appearance of NADH was monitored at 340 nm (absorption coefficient: ε 340=6220 M-1) by spectrophotometric measurements at 37 °C. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of the product per minute (U/mg).

III. Results

III.1. CAG repeats in HD patients, asymptomatic carriers and controls

All of the 52 HD patients and 25 asymptomatic carriers had CAG repeat expansion in the HD gene and all of them were heterozygotes. The expanded CAG repeats ranged from 36 to 70 (median: 45). The most frequent HD allels contained 40-48 CAG repeats. There were four juvenile HD patients associated with high CAG repeat number (58, 59, 67, 70). We found three HD patients and seven carriers who belonged to the border zone, where the CAG repeat length was between 36-39. In the control group, one individual had also 36 CAG repeats as an intermediate allel, but this person's family history was negative for HD.

The CAG repeat numbers in 70 healthy controls were assessed as 10-36 (median: 18) (*Fig. 1.*; *Fig.2.*).

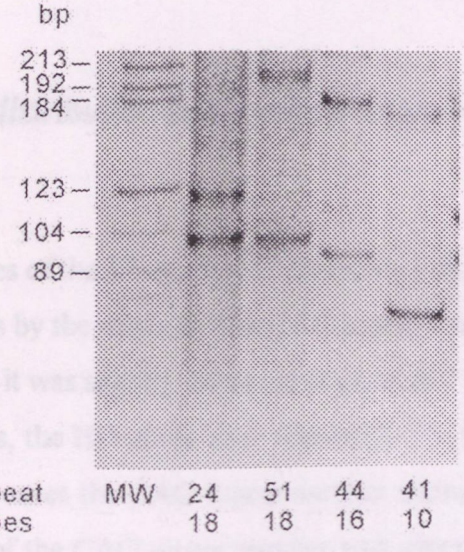

Fig. 1. PCR amplification of the DNA region carrying CAG repeat expansion. Electrophoretic picture of amplified DNA from 3 patients with HD (lanes 3–5) and from 1 control (lane 2). Molecular weight marker is in lane 1. The corresponding CAG repeat haplotypes: 24/18, 51/18, 44/16, 41/10.

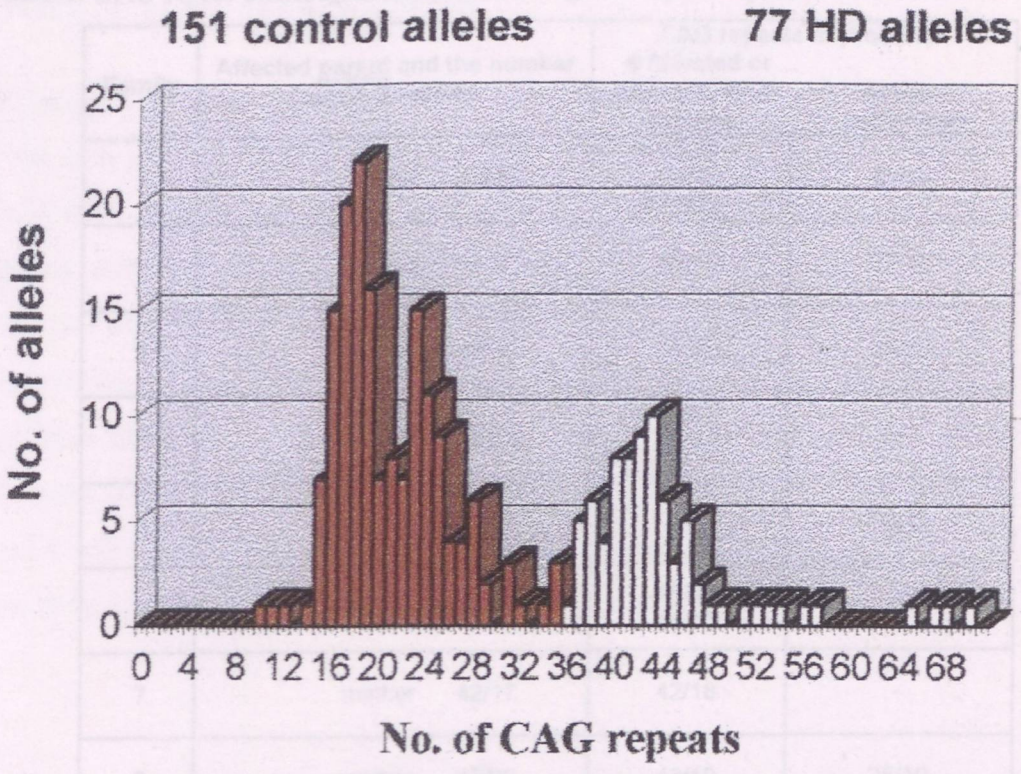

Fig. 2. Distribution of CAG repeats on the chromosomes of controls and HD patients. The most frequent CAG repeat number on the control chromosomes was 18, while that on the chromosomes from HD patients was 45.

III.2. Repeat distribution and parental origin of CAG repeat expansion in 17 HD families

In 12 families of the 17 examined HD families the expanded CAG repeats were transmitted to the 22 probands by the mothers. The CAG repeat instability was mildly affected in the maternally inherited cases, it was slightly increased or equal in 7 probands, and was decreased in 10 cases.

In 5 families, the HD allels were transmitted to the 9 probands by the fathers. In the paternally inherited cases the CAG repeat number showed higher instability, with marked increasing. Decrease of the CAG repeat number was observed only in one proband. In 2 families the parents with HD had healthy children, they did not transmit the disease allel.

Six symptomatic HD patients, 18 asymptomatic carriers and 10 healthy individuals were identified by PCR analysis in the 17 examined HD families.

Table 1. CAG repeat distribution and parental origin of repeat expansion in 17 families with HD

Family	Affected parent and the number of CAG repeats	CAG repeats in probands	
		■ Affected or asymptomatic carriers	healthy individuals
1.	mother 45/18	■ 45/20 42/19 ■ 46/22	21/16
2.	mother 37/22	45/19	22/16
3.	mother 43/15	46/22	29/19
4.	mother 46/22	46/13 45/13	–
5.	mother 41/16	–	24/17
6.	father 43/17	46/18 45/13	–
7.	mother 42/17	42/18	–
8.	mother 43/25	43/19	25/19
9.	father 45/18	■ 46/20	–
10.	mother 44/26	43/18	–
11.	father † unknown	■ 70/17	26/11
12.	father 39/15	37/16 39/16 39/17 40/17	16/13 16/12
13.	mother 44/24	39/20	24/16
14.	father 48/20	–	27/18
15.	mother † unknown	■ 58/10 42/17	–
16.	mother † unknown	■ 59/15	–
17.	mother † unknown	36/17 38/19	–

III.3. Analysis of two large HD families

HD family I.

In the first generation, the HD allel was transmitted by the father to his four offsprings. Three of them (two sisters and one brother) inherited the HD gene causing severe disease symptoms, and they all died at the age of 45-55, without genetic testing. In the third generation, HD symptoms were developed in three young persons at age of 12-22-25 years old, indicating a higher CAG repeat number (70, 58, 55). The progression of the disease was rapid in all cases and the symptoms of HD were very severe. One HD patient with 55 CAG repeat number committed suicide at age of 30 years old. One asymptomatic carrier with 42 CAG repeat number and one healthy individual were also found in the third generation *(Fig.3.)*.

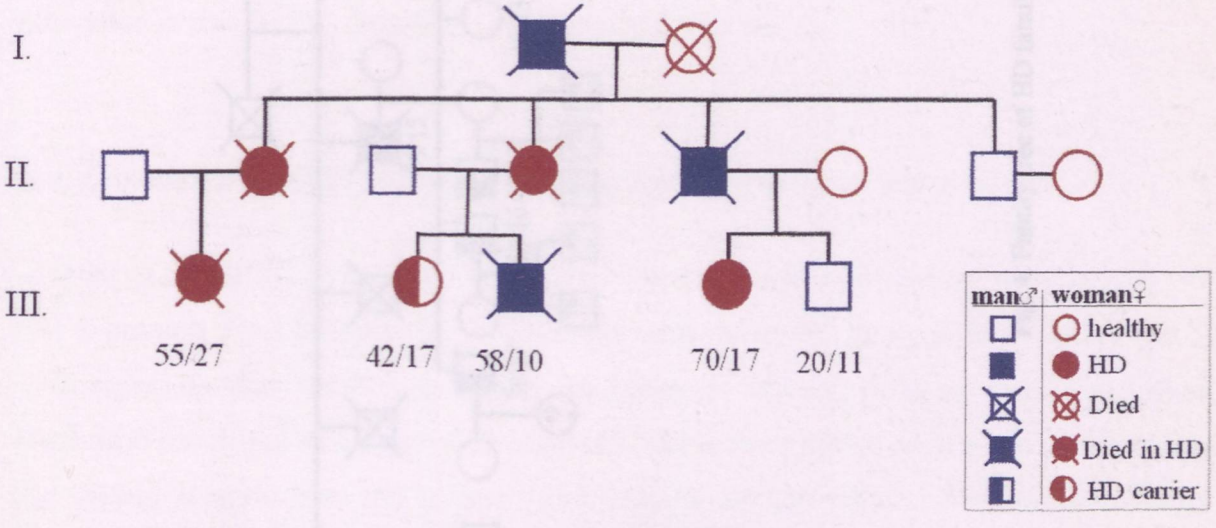

Fig 3. Family tree of HD family I.

HD family II.

The mother with HD of the first generation had six children, three of them inherited the Huntington disease with a late adult onset at an average age of 55-60 years old. One patient of the second generation was available for genetic testing. He had 39 CAG repeat number, the symptoms of the disease were mild motor abnormalities and cognitiv decline with slow pro-

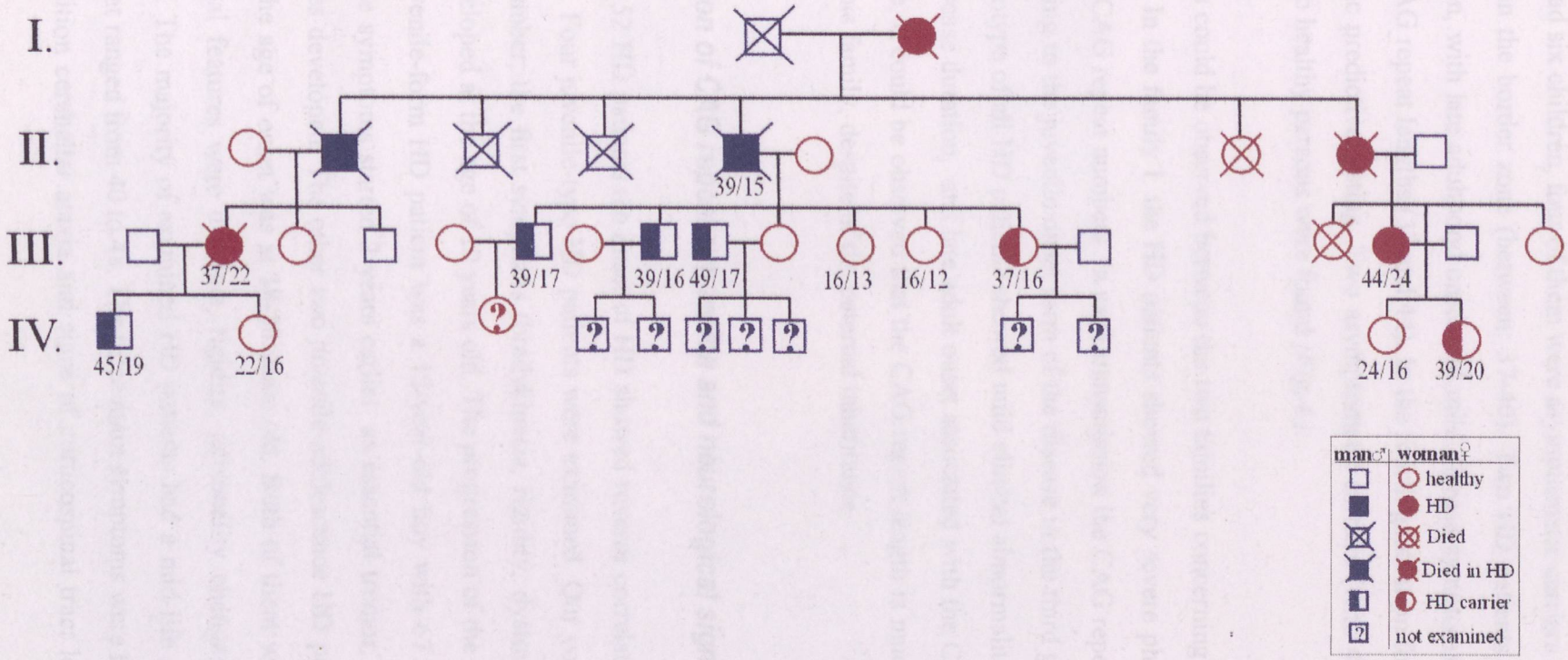

Fig. 4. Family tree of HD family II.

gression. He had six children, four of them were asymptomatic carriers with slightly increased CAG repeats in the border zone (between: 37-40). Two HD patients were identified in the third generation, with late adulthood onset and mild disease symptoms, associated with relatively short CAG repeat lengths (37 and 44). In the fourth generation four persons were available for genetic predictive testing. Two asymptomatic carriers (with 45 and 39 CAG repeat length) and two healthy persons were found *(Fig.4.)*.

Differencies could be observed between the two families concerning the CAG repeat number instability. In the family I. the HD patients showed very severe phenotype in all generation with high CAG repeat numbers. In each transmission the CAG repeat instability has been increased, leading to the juvenile onset form of the disease in the third generation. In the family II. the phenotype of all HD patients showed mild clinical abnormalities with slow progression, longer disease duration, and late adult onset associated with the CAG repeat numbers in the border zone. It could be observed that the CAG repeat length is much more stable in each generation in this family, despite of the paternal inheritance.

III.4. Correlation of CAG repeat expansion and neurological signs of HD

In all of the 52 HD patients the onset of HD showed reverse correlation with the CAG repeat expansion. Four juvenile-type HD patients were examined. Our youngest patient had 70 CAG repeat number, the first symptoms (bradykinesia, rigidity, dystonia, cognitive decline, irritability) developed at the age of 10 years old. The progression of the disease was very fast. The second juvenile-form HD patient was a 12-year-old boy with 67 CAG repeat number. Atypical disease symptoms started 2 years earlier, as essential tremor, later anxiety and behavioral changes developed. The other two juvenile-adolescence HD patients had 58 and 59 CAG repeats, the age of onset was at 18-20 years old. Both of them were early stage of the disease. Cardinal features were dystonia, rigidity, personality changes leading isolation in their social life. The majority of examined HD patients had a mid-life onset at age of 40-50 with CAG repeat ranged from 40 to 48. The three main symptoms were found in all of the HD patients, in addition cerebellar ataxia and signs of corticospinal tract lesion were also com-

mon. The smallest CAG repeat number was 37 with the onset of symptoms at age 55. One HD patient with 39 CAG repeat showed the first signs of the disease at age 60. Seven asymptomatic carriers had the CAG repeat expansion in the border region between 36 – 39. It is difficult to predict wether the disease symptoms will develop or not in these cases.

In all HD patients who were subjected to MRI or/and CT analysis atrophy of the caudate nucleus and later whole brain atrophy were seen *(Fig.5.)*

[(18)F]FDG-PET has also been performed in a few HD patients, showing reduced glucose metabolism in the region of the basal ganglia *(Fig. 6.)*.

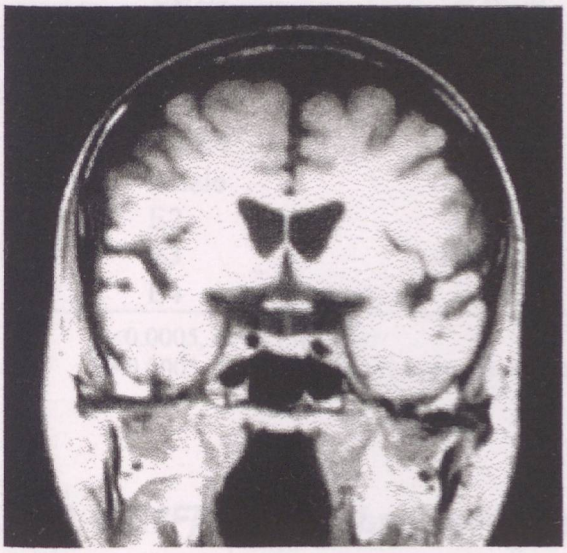

Fig. 5. MRI picture showes nucleus caudatus atrophy and whole brain atrophy in a HD patient.

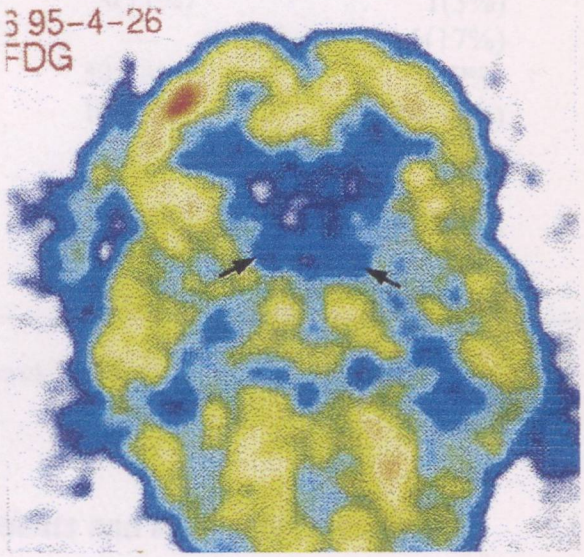

Fig. 6. [(18)F]FDG-PET detects reduced glucose metabolism in the basal ganglia of a HD patient

III.5. Apolipoprotein E genotype and allel frequencies in HD

Apolipoprotein E 2, 3, 4 allel frequencies were determined in 28 HD patients, compared to 79 healthy controls and 36 Pick's disease patients with frontal-type dementia. The majoritiy of HD patients (68%) were apoE 3/3 homozygotes, the rest were heterozygotes, 18% of HD patients showed apoE 2/3 allels and in 14 % of the HD patients occurred the apoE 3/4 allel.

There were no apoE 2/2 or 4/4, or 2/4 allels in the HD group. The distribution of the apoE genotypes among the HD, Pick's disease and controls subjects are showen in *Table 2.*

HD patients did not differ significantly from the control individuals in the distribution of apoE genotypes.

Table 2. Apolipoprotein E genotype and allele frequencies in different groups

Genotype	Huntington's disease n=28	Controls n=79	Pick disease n=36 no. of persons (%)
E2/E2	—	—	—
E2/E3	5(18%)	9(11%)	1(3%)
E2/E4	—	—	6(17%)
E3/E3	19(68%)	59(75%)	7(19%)
E3/E4	4(14%)	10(13%)	20(55%)
E4/E4	—	1(1%)	2(%)
Allele			
E2	5(9%)	9(6%)	7(%)
E3	46(82%)	137(87%)	35(48%)[a]
E4	5(9%)	12(7%)	30(42%)[b]

[a]$p<0.0005$, df= 1 (38.068);
[b]$p<0.0005$, df= 1 (38.468) vs. controls; Pearson x^2 test with 2-sided Monte Carlo significance

III. 6. [18F]FDG PET abnormalities in HD patients and asymptomatic carriers

In both early stage HD patients' PET scans showed typically reduced glucose metabolism in the caudate nucleus.

A 29-year-old asymptomatic carrier with 45 CAG repeat number, and a 28-year-old asymptomatic carrier with 38 CAG repeat number had normal glucose uptake in the basal ganglia. In the other 4 asymptomatic carriers (27-30-44-47 years old, with CAG repeat number: 45, 43, 45, 43) reduced [(18)F]FDG uptake was detected asymmetrically in the striatum, in addition decreased [(18)F]FDG uptake was observed globally in the cortex. *(Fig. 7.)*.

The preliminary data showed that the metabolic deficit in the basal ganglia is much more intense in patients with developed HD than in asymptomatic carriers. The PET data seemed not to be in correlation with the length of CAG repeats. It rather seemed that the glucose metabolism

depends on the age of the asymptomatic carriers. There must be other factors which also influence the glucose metabolism, supported by the findings that two asymptomatic carriers had normal PET, and two other asymptomatic carriers in the same age showed abnormal PET.

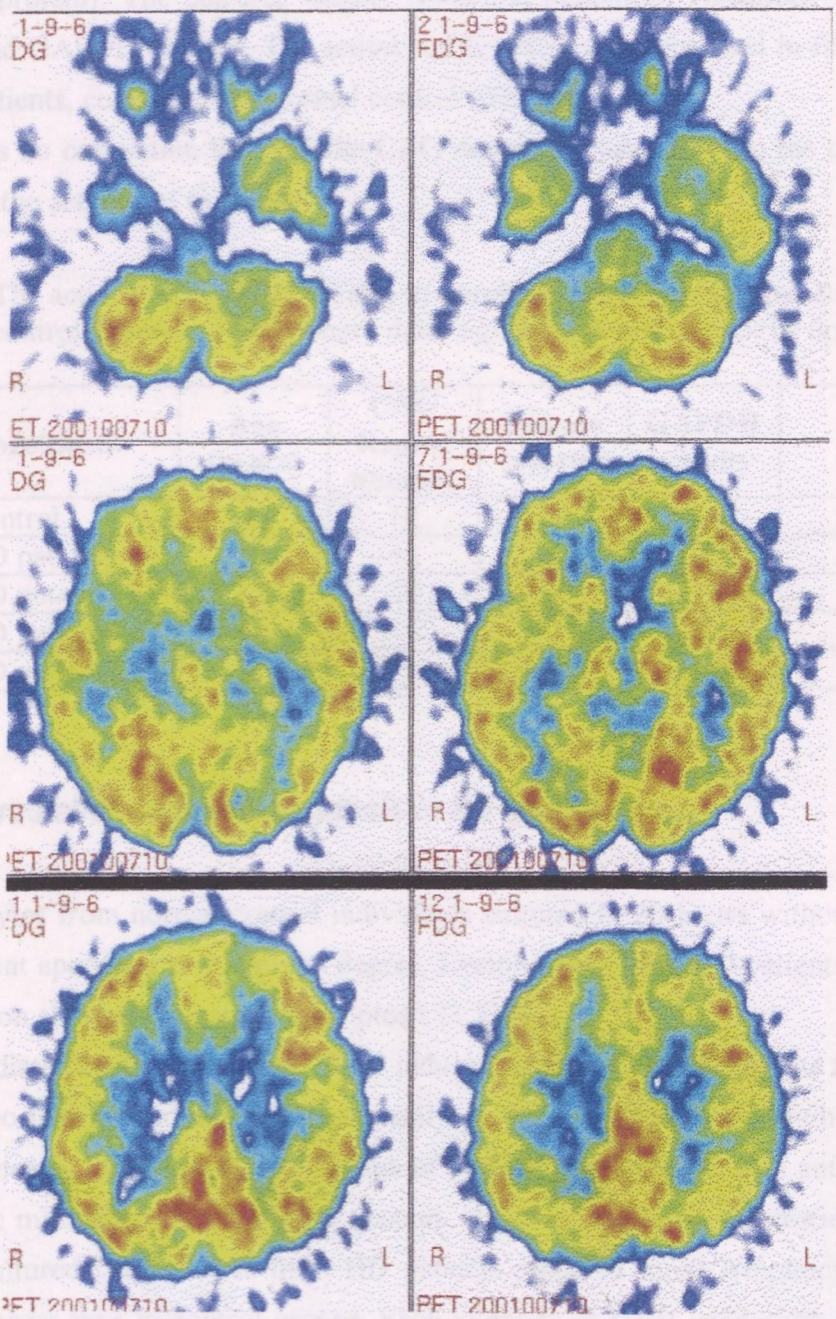

Fig. 7. [(18)F]FDG–PET picture of the 44 years old asymptomatic carrier with 45 CAG repeats showed reduced [(18)F]FDG uptake asymmetrically in the striatum

III.7. Impairment of GAPDH glycolytic function in HD patients' fibroblast extracts

Cultured skin fibroblasts from 4 HD patients and age-matched control were prepared to study the glycolytic activities of GAPDH enzyme (a key enzyme in glycolysis and a huntingtin binding protein). The cell-free extract contained huntingtin visualized by immunoblott technique and GAPDH enzyme. The activity of GAPDH was decreased in fibroblast extracts of all HD patients, compared to a normal control. *(Table 3.)*

There was no correlation between the CAG repeat, the age of onset, the disease duration, severity and the activity of GAPDH.

Table. 3. The activity of GAPDH (U/mg) in fibroblast extracts of HD patients and normal control (These are preliminary data, significance test can not be done.)

Fibroblasts	Age (years)	CAG Repeat number	Disease severity	GAPDH activity	%
Control	40	18	–	0,392	100
HD patients 1.	67	45	++	0,278	71
HD patients 2.	52	48	++	0,300	77
HD patients 3.	46	44	++	0,228	58
HD patients 4.	27	59	+++	0,286	73

III.8. UVB irradiation-induced apoptosis in HD lymphocytes

Lymphocytes from normal control individuals cultured in 20 hours without UVB irradiation underwent apoptosis in a minimal degree. Lymphocytes from HD patients cultured in the same condition showed no increased apoptosis (3.43%).

UVB irradiation with intensity of 70 mJ induced apoptotic changes in one fifth of lymphocytes from normal individuals cultured exactly in the same condition. Higher intensities of irradiation linearly increased the percentage of lymphocytes labelled with antibody recognizing apoptotic mitochondrial membrane protein. However, the same intensities of UVB irradiation of cultured lymphocytes from HD patients rendered more lymphocytes to undergo apoptotic process than in control groups. Each intensity of UVB irradiation applied induced apoptosis in significantly higher percentage of lymphocytes from HD patients than from normal controls ($p< 0.0171$, $p< 0.0099$, $p< 0.0126$) *(Fig. 8.)*.

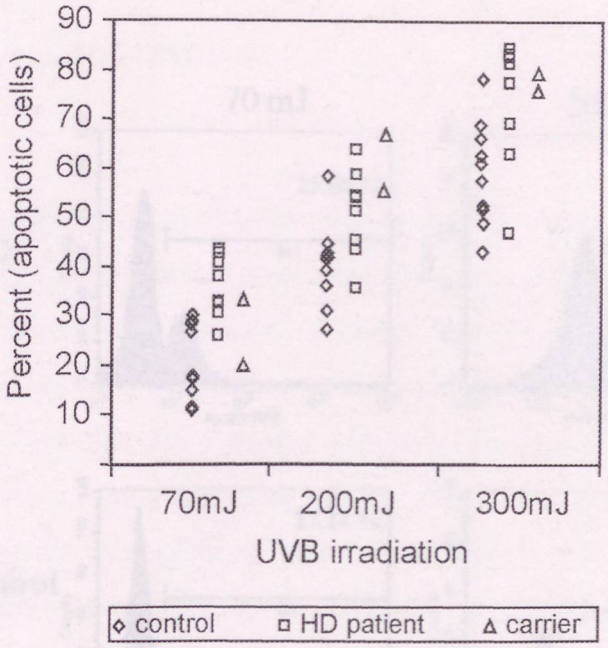

Fig. 8. 70, 200 and 300 mJ UVB irradiation induced apoptotic cell death is significantly higher in eight HD patients and two asymptomatic carriers compared to ten normal controls. $p < 0.0171$ (70 mJ), $p < 0.0099$ (200 mJ), $p < 0.0126$ (300 mJ). The points represent the percentage of apoptotic cells from each individual.

The reactivity seems to be uniform. All of the samples from HD patients exposed to irradiation showed higher proportion of apoptotic cells than any of the control ones *(Fig 9.)*. Furthermore the same rate of induced apoptosis was noted in the lymphocytes of two asymptomatic carriers of HD gene. The enchancement of the apoptotic reaction of lymphocytes did not correlate with the rate of HD progression, or with the stage of the disease. Because of the relatively short ranges of CAG repeat expansion (median:45) in HD patients the rate of apoptotic change in lymphocytes could not be correlated reliably with the length of CAG repeat expansion. *(Fig 10.)*

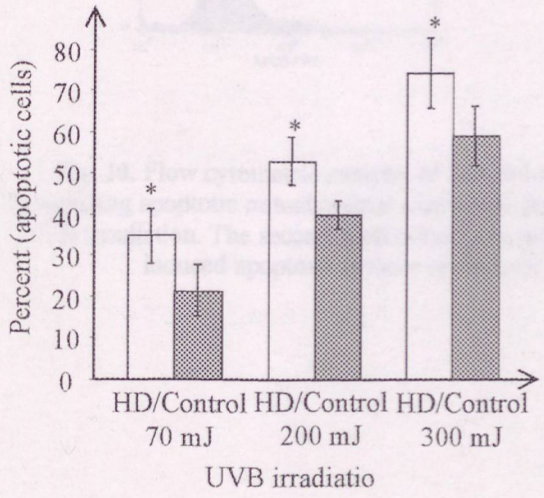

Fig. 9. Each intensity of UVB irradiation applied induced apoptosis in significantly higher percentage of lymphocytes from HD patients than from normal controls.

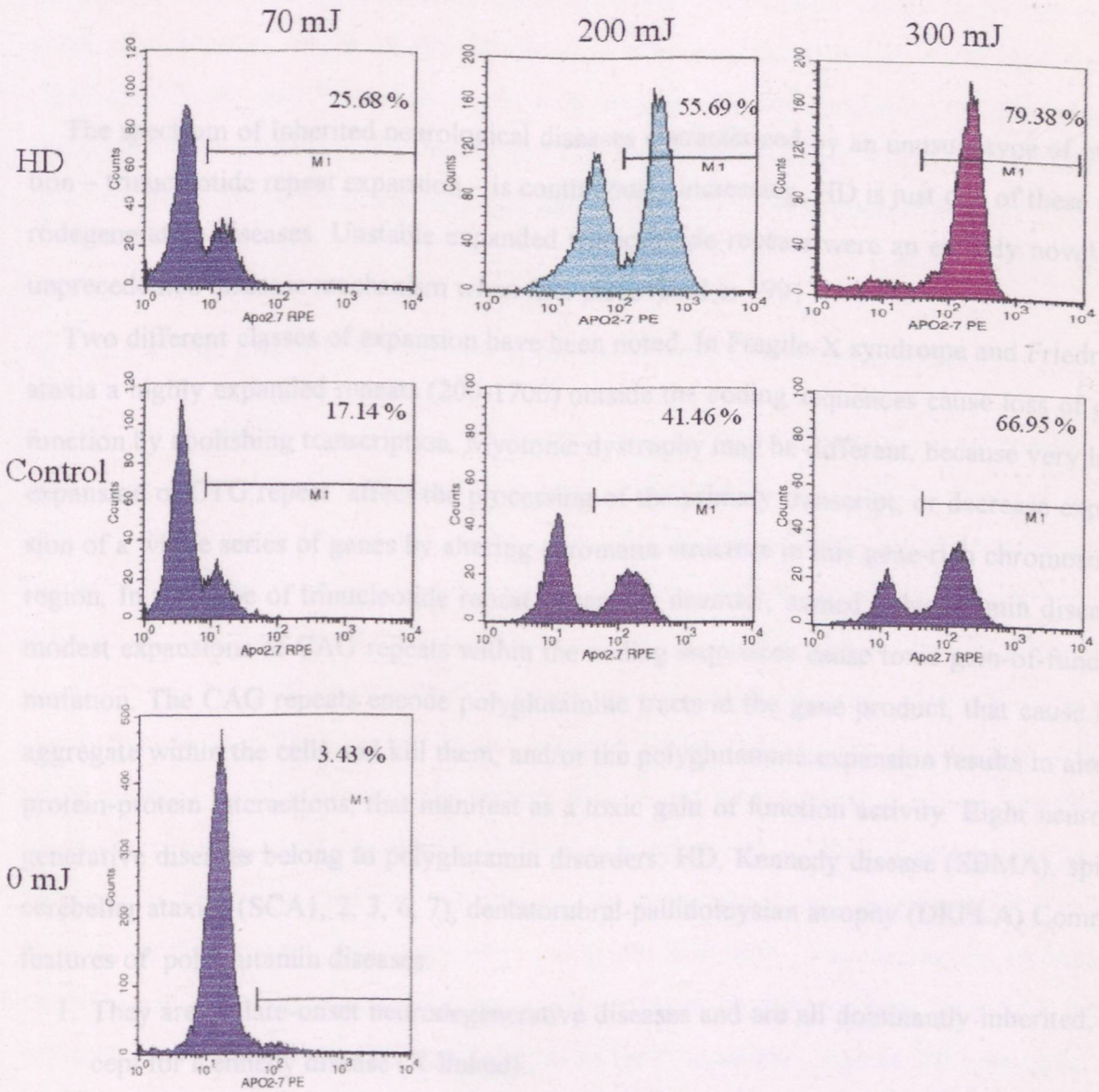

Fig. 10. Flow cytometric analysis of HD and control lymphocytes labelling with Apo2.7-PE antibody recognizing apoptotic mitochondrial membrane protein. Apoptosis was induced by 70 mJ, 200 mJ and 300 mJ UVB irradiation. The second profile indicates proportion of apoptotic cells within the MI region. The UVB induced apoptosis is more enchanced in HD patients in each experimental paradigm.

IV. Discussion

The spectrum of inherited neurological diseases characterized by an unusual type of mutation – trinucleotide repeat expansion – is continuously increasing, HD is just one of these neurodegenerative diseases. Unstable expanded trinucleotide repeats were an entirely novel and unprecedented disesase mechanism when first discovered in 1991.

Two different classes of expansion have been noted. In Fragile-X syndrome and Friedreich ataxia a highly expanded repeats (200-1700) outside the coding sequences cause loss of gene function by abolishing transcription. Myotonic dystrophy may be different, because very large expansion of CTG repeat affect the processing of the primary transcript, or decrease expression of a whole series of genes by altering chromatin structure in this gene-rich chromosomal region. In that type of trinucleotide repeat expansion disorder, named polyglutamin diseases, modest expansions of CAG repeats within the coding sequences cause toxic gain-of-function mutation. The CAG repeats encode polyglutamine tracts in the gene product, that cause it to aggregate within the cells and kill them, and/or the polyglutamine expansion results in altered protein-protein interactions, that manifest as a toxic gain of function activity. Eight neurodegenerative diseases belong to polyglutamin disorders: HD, Kennedy disease (SBMA), spinocerebellar ataxias (SCA1, 2, 3, 6, 7), dentatorubral-pallidoluysian atrophy (DRPLA).Common features of polyglutamin diseases:

1. They are all late-onset neurodegenerative diseases and are all dominantly inherited, except for Kennedy disease (X-linked).
2. The expanded allel is transcribed and translated, the trinucleotide repeat encodes a polyglutamine tract in the protein.
3. There is a threshold repeat size, below which the repeat is non-pathogenic and above which it causes disease.
4. The larger the repeat above the threshold, the earlier is the age of onset.

When the polyglutamin tract exceeds the threshold length, the protein aggregates, forming an inclusion body that apparently kills the cell. Neuronal cell death caused by protein aggregates is a common thread in the pathology of polyglutamin diseases, Alzheimer disease, Parkinson disease, and Prion diseases. In each disorder different protein aggregates cause selective neurondegeneration. The mechanisms of selective neurodegeneration remain to be discovered. HD is one of the best known and most intensively studied disease of them.

IV.1. Instability and the pathogenic potential of CAG repeat sequences

The human genome has a very high proportion of DNA sequences that are repeated. Tandem repeats in coding DNA include very short nucleotide repeats, moderately sized repeats and very large repeats that include whole genes. Tandem trinucleotide repeats are frequent in the human genome. Although there are 64 possible trinucleotide sequences, there are only 10 different trinucleotide repeats. Most of these are known as polymorphic microsatellite markers, but in addition, certain repeats show anomalous behavior which can cause abnormal gene expression. In each case, repeats below a certain length are stable in mitosis and meiosis, while above a threshold length, the repeats become extremely unstable. These unstable repeats are virtually never transmitted unchanged from parent to child. Both expansion and contractions can occur, but there is bias toward expansion. Long, disease-causing repeats show dynamic mutation, that means extreme meiotic instability. The mechanism of dynamic mutation and the molecular genetic basis of repeat instability has not been identified. Arrays of triplet repeats may be able to form alternative DNA structures, such as DNA hairpins, triplex or quadruplex DNA structures which might contribute to their instability (*Sinden et al.*, 1999.) However, when sequences capable of forming such structures are put into transgenic mice, they do not show dynamic mutation, suggesting that effect other than DNA structure, such as repair efficiency and/or genomic location may influence the instability of repeats. Recent findings have confirmed that CAG expansion occurs during the repair of strand breaks and implicates the mismatch repair system in the mutation process. (*Kovtun et al.*, 2001). It had been speculated that expansion occurs by polymerase slippage during cell proliferation or meiotic recombination. In germ cells, expansion occurs late in sperm development, when cells

are haploid and are postmitotic and postmeiotic. Therefore, CAG expansion cannot depend on mitotic replication or meiotic recombination and must arise from the repair of strand breaks. After a strand break, gaps can form by folding CAG repeats into hairpins. Hairpins that form from CAG repeats contain an A-A mismatch base every third position between two C-G pairs. Msh2, the member of mismatch repair system, might stabilize this loop structures preventing the reannealing of the complementary strands at the break site, increases the lifetime of the gap, and forcing its repair. Repair of the gaps traps DNA loops, which are the precursors for expansion. Repair can occur through a simple fill in reaction, resulting CAG repeat expansion. The surprising discovery was that, the mismatch repair system, which normal function is to remove mispaired bases, plays an important role in the dynamic mutation process. The loss of Msh2 can attenuate expansion in animals provided the first evidence that expansion can be stopped in vivo. This is an unexpected result, because Msh2 loss was originally identified as predisposing to colon and other cancers, enhancing dinucleotid repeat insability. (*Manley et al.*, 1999; *De Wind N et al.*, 1995.)

IV.2. CAG repeat distribution, parental origin and instability of CAG repeats in Hungarian patients

The aim of our work was to introduce the PCR-based methodology to determine the CAG expansion in HD gene. It is important to establish the correct genetic diagnosis of HD. Tóth T. et al. have already examined CAG repeats using silver staining in HD patients (*Tóth et al.*, 1997), but the CAG haplotype of IT 15 gene has not been analyzed yet in Hungarian population. Generally, when the disease has been observed in its fully developed form, its recognition requires no great clinical effort. The main difficulty arises with patients who lack a family history, but they have progressive chorea, psychopathological changes and dementia. This difficulty has been overcame since the mutation was identified. We modified the PCR method described by Watanabe to measure the CAG repeat length in HD gene. The sensitivity and specificity of this diagnostic test for HD is 99.9-100%. We confirmed the diagnosis of HD in 52 patients. The most frequent HD allels contained 40-48 CAG repeats indicating adult onset of the disease with typical neurological signs. The four juvenile-type HD patients had more

severe akineto-rigid symptoms in accord to higher CAG repeat number. An invers correlation was found between the age of onset of the disease and the number of CAG repeat and linear correlation was established between the CAG repeat length and the severity of clinical symptoms. In the border zone (CAG repeat number: 36-39) it is difficult to predict the age of onset and the rate of the disease progression because of the incomplete penetrance of the HD gene. Introducing of this genetic diagnostic test in Hungary it is now possible to confirm or exclude the presence of the HD allel in the relatives of HD patients. The most important part of our work is to detect the asymptomatic carriers and give them a possibility to prevent the progressive neuronal destruction and/or rescue the striatal neurons. We identified genetically 25 asymptomatic carriers long before clinical symptoms develop. Early detection and late onset of disease render these asymptomatic carriers well suited for effective therapeutic intervention, if this will be developed. We followed the guidelines for molecular genetics predictive test in HD, and psychologist supervised the patients thereafter. Prenatal genetic test for HD is also available, if needed.

We examined repeat distribution, parental origin, and instability of CAG repeat in 17 HD families. Both CAG repeat expansion and contraction was observed. The disease was maternally transmitted in 10 families, the CAG repeat instability was mildly affected. In the other 5 families where the HD gene was inherited paternally shown much greater CAG repeat instability was seen toward expansion resulted from the mechanism during spermatogenesis. Only two parents with HD did not transmit the HD gene to their children, so the inheritance of the disease was interrupted. The CAG repeat distribution was analyzed in two large HD families. As the CAG repeat instability is much more enhanced in one of the examined families than in the other one, it suggests a still unknown factor influencing the dynamic mutation. It could be related to the status of the mismatch repair system.

IV.3. The role of huntingtin

In spite of the fact however that the genetic defect is defined in HD however, the role of the gene product (huntingtin) and the pathomechanism of the selective neurodegeneration is still largely unknown. Neuronal cell death is likely to be caused mainly by genetic gain-of-function mechanisms, involving abnormalities of protein folding, chaperone interactions, al-

terations in gene transcription, loss of neurotrophic support and involvement of signal transduction pathways and apoptosis. Although the exact details are unclear, the cellular mechanism by which mutant huntingin kills neurons are beginning to be revealed. The molecular link between polyglutamine expansion in huntingtin and the consequent death of the striatal neurons has been elusive. The expanded polyglutamine tract must be the key factor in the cellular toxicity. Several of these polyglutamine proteins such as huntingtin are known as transcriptional coactivators. Huntingtin may normally interact with proteins in transcription complexes, and the interaction could be aberrant, when the polyglutamine is expanded. Polyglutamine toxicity could cause abnormalities in the regulation of gene transcription. On the other hand, the N-terminal polyglutamine region of huntingtin can form aggregates and inclusion bodies. Aggregations of huntingtin fragments are ubiquitinated and associate with the proteosome apparatus, the multimeric enzyme complex that degrades abnormally folded proteins. Toxicity could emerge as the misfolded proteins accumulate and associate with proteosomes but are resistant to degradation. The depletion of proteosomal activity might lead to cellular toxicity. This mechanism could be relevant for other neurodegenerative diseases that involve abnormally folded proteins, such as Parkinson's disease and ALS. (*Bence et al.*, 2001) Although a gain-of-function theory provide the best model of HD and polyglutamine diseases, it is possible that a loss of the normal function of the protein with an expanded polyglutamine also contributes to disease pathogenesis. Polyglutamine expansion might reduce the normal capacity of huntingtin to stimulate brain-derived neurotrophic factor (BDNF). Loss of BDNF and the presence of huntingtin-aggregates in the striatal neurons together contribute to neuronal death.

Recently investigations have focused on the role of apoptosis in neurodegeneration. The aim of our experiments was to provide a further support of the huntingtin-mediated cell toxicity, which induces apoptosis. We wanted to know whether non-neural tissue shows increased sensitivity to apoptosis. Huntingtin is widely expressed throughout the CNS as well as in non-neural tissues, so functional abnormalities can be expected also outside of the brain. The susceptibility of lymphocytes from HD patients, asymptomatic carriers to UVB irradiation-induced apoptosis was examined and compared to normal controls. Our study proves that in HD not only the cells of the central nervous system are prone to apoptosis, but also the pe-

ripheral lymphocytes. (*Sawa et al.*, 2000). We established that increased susceptibility of HD cells to apoptosis is not restricted to neurons. UVB irradiation was used to induce apoptosis in lymphocytes and an immunohistochemical detection of Apo2.7 expression in irradiated cells was utilized to monitor the effect. Sawa et al. applied different methods for induction of apoptosis in cultured HD lymphoblasts. In their study the number of cells displaying apoptosis markers was doubled in HD lymphoblasts cultures. In our work the same tendency of susceptibility of CD3+ lymphocytes to differently induced apoptosis was noted. Both sets of experiments however substantiated that under different stress conditions higher number of HD lymphocytes respond with apoptosis. Even having mutant huntingtin the HD lymphocytes function normally, because they have much shorter lifetime than neurons. Mutant huntingtin may promote apoptosis in all cell types, but only a restricted neuronal subpopulation is the most sensitive to this process causing neurodegeneration. Targeted disruption of HD gene in mice resulted in embryonic lethality and increased apoptosis in the embryonic ectodermal cells (*Nasir et al.*, 1995). These data suggest that normal huntingtin has an anti-apoptotic effect. The lower incidence of cancer in HD patients, but not in their healthy relatives support the theory, that mutant huntingtin can protect against cancer inducing apoptosis in preneoplastic cells (*Sorensen et al.*, 1999).

There is increasing evidence that apoptosis may play an important role in neurodegeneration. Apoptosis is carried out by cascades of protein-cleaving enzymes called caspases. In particular caspase-3 and caspase-8 have been implicated in neuronal death in HD. Huntingtin has been involved in apoptotic pathways, both the normal and the mutant huntingtin being cleaved by caspase-3. Once cleaved, the N-terminal part of huntingtin triggers apoptosis by acting in the nucleus, independently of the presence of intranuclear inclusions (*Sadou et al.*, 1998). Huntingtin may be seen as a pro-apoptotic protein whose mutation results in increasing apoptotic properties. Previous studies had found that mutant huntingtin leads to the activation of caspase-8. (*Sanchez et al.*, 1999). Other investigators have shown that normal huntingtin interacts with a protein known as HIP1. But the details of the molecular mechanism were unclear. The current hypothesis of the cell death in HD was established by Gervais and colleagues. Their studies of cultured cells show a possibly new apoptotic pathway, in connection with huntingtin-mediated toxicity. Huntingtin and HIP-1 were shown to colocalize to chlatrin-

coated vesicles. HIP-1 contains binding sites to the chlatrin heavy chain and the adaptor protein-2 (AP2). Chlatrin and AP2 are needed for endocytosis and chlatrin-rich endocytotic zones are present in presynaptic nerve terminals. Huntingtin and HIP-1 are involved in regulating synaptic plasticity, including vesicle fusion with the plasma membrane and endocytosis.

As the huntingtin polyglutamine tract becomes expanded, huntingtin has less affinity for HIP-1. Unbound HIP-1 would be free to interact with a newly identified protein, Hip-1 protein interactor (Hippi) that appears to be essential for forming a death-effector complex involving caspase-8. Activation of caspase-8 can trigger alterations in mitochondria, which releases the cytochrom c protein, forming a protein complex, apoptosome. Apoptosome activates proteolytic enzymes, such as caspase-3, which cleaves huntingtin, producing toxic protein fragments, that can self-aggregate and form inclusions in the cytoplasm and nucleus. Inclusions enhanced cellular toxicity through its modulation of transcription. HIP-1 – Hippi – caspase 8 complex could act as a cytoplasmic trigger of apoptotic cascade that eventually leads cell death. It is not clear whether these inclusions are required for neuronal cell death, because caspase-3 can kill neurons independently. (*Gervais et al.*, 2002) Gervais results suggest that mutation in huntingtin reducing its interaction with HIP-1, might alter endocytotic

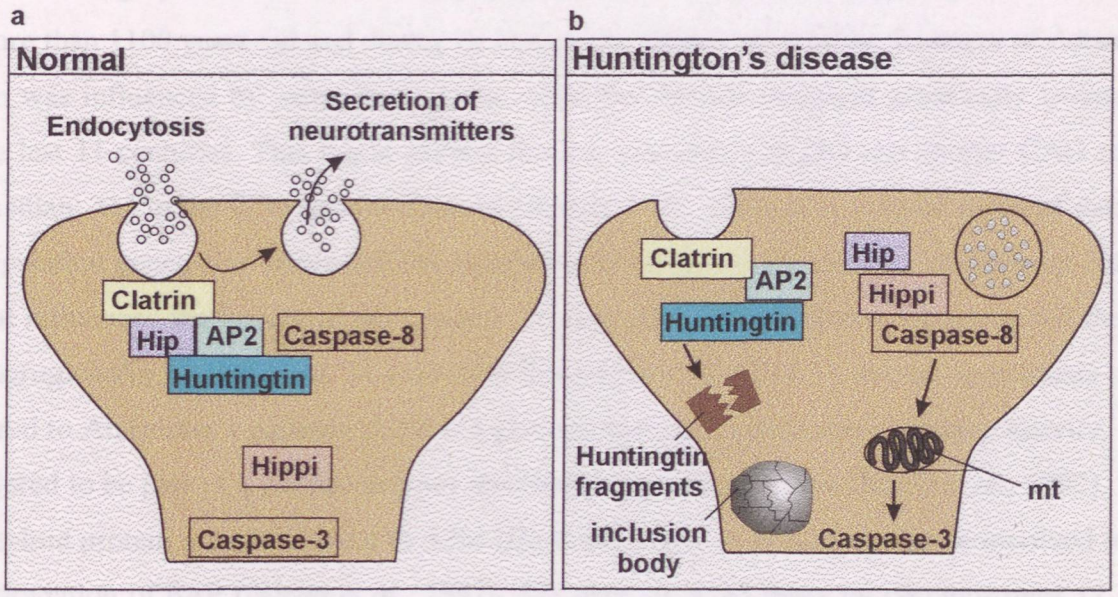

Fig. 12. Current explanation of neuronal cell death in HD
(Mattson MP, Nature 2002; 415: 377. – modified by Jakab K.)

processes leading to synaptic dysfunction in neurons and the activation of caspase cascades specifically at synapses. But further work will be required to establish whether this occurs in HD patients. We need to know in what cells Hippi is expressed normally in the human brain, and whether its expression or subcellular localization is changed in HD patients, what the relationship is between Hippi expression and the selective vulnerability of striatal neurons.

IV.4. ApoE polymorphisms in neurodegenerative diseases of Hungarian patients

The CAG repeat expansion of IT 15 gene in Hungarian HD patients was similar to that reported from other countries. In addition we determined the CAG repeat size in 70 healthy controls. The median of normal CAG repeat number was 18, the same as reported from Western European countries. There is a geographic variation in the prevalence of HD, associated with the higher CAG repeat size on normal chromosomes (*Kremer et al.*, 1994). In Western Europe and North America it is 5-10:100 000, while this figure in Asia and Africa is only 0.2-0.4:100 000, in Finland 0.5:100 000 (*Squitieri et al.*, 1994). The prevalence of HD and also the average length of CAG repeat size on normal individuals might indicate that the population of Hungary is mixed, resembling populations in Western Europe. However, the country is more than 1100 years old and during its history the genetic pool of the founders of Asian origin was influenced by many immigrants, while the Finnish remained genetically isolated in the last 11 centuries. This might explain the low prevalence of HD and shorter CAG repeat (median: 16) in the IT15 gene in Finland (*Ikonen et al.*, 1992) It is a useful piece of data for population genetics to prove that the population of Hungary is a melange of different nations that influenced the history of our country. To support this presumption, we examined the α2-macroglobulin (A2M) exon 24 (val-1000-Ile) polymorphism in 51 normal individuals compared to Alzheimer's patients. A2M is a glycoprotein with proteinase inhibitory activity, considered to be part of a general defence mechanism against proteases (*Borth et al.*, 1992). Beta amyloid protein is tightly bound to A2M (*Du et al.*, 1997). It is supposed to be involved in the degradation of βAP (*Narita et al.*, 1997). The gene of A2M maps on chromosomes 12. The exon 24 (Val-1000-Ile) GG genotype was reported to be associated with AD, as a genetic risk factor (*Blacker et al.*, 1998). Until now there were no data available about the A2M polymor-

phism in the normal Hungarian population. Our results indicated that the distribution of A2M genotype and allel frequencies were different in the normal Hungarian population from that in the Finnish. The Hungarians have less G allel (28%) than the Finnish (43%) and show more similarities to the other reported Caucasian ethnic groups such German, Italian and American (28-32%) (*Bullido et al.*, 2000).

The same tendency has been observed in the case of the apolipoprotein E (apoE) polymorphism. The polymorphism of apoE gene has been implicated as a risk factor in the pathomechanism of several forms of dementia, such as Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson disease and Creutzfeld-Jakob disease (*Saunders et al.*, 1993). Nevertheless, no explanation is available as concerns the role of the apoE molecule in the pathomechanism of neurodegenerative disorders. This lipoprotein interacts with the components of both senile plaques and neurofibrillary tangles in AD. ApoE 3 isoform prevents tau-protein hyperphosphorylation in vitro (*Strittmater et al.*, 1993), allowing it to function normally in the stabilization of microtubular structure and function. ApoE 4 allel could be a susceptibility factor for AD causing tau hyperphosphorylation. The aim of our investigation was to determine the apoE genotype in two other dementing disorder, HD and Pick's disease and normal controls. Our study revealed that apoE 4 allel frequency is higher in Pick's disease, than in HD and normal controls. In Pick's disease apoE protein is also present in the intraneuronal Pick body, and apoE4 allel could play a role in pathomechanism of this tau-protein related form of dementia. As oppose to this the apoE allele frequencies in HD were similar to those in the control population suggesting that the inheritance of the different apoE allels is not equally important in all neurodegenerative disorders with dementia. The lack of association of the apoE4 allel with HD could be interpreted that, although HD is a dementing disorder, different brain structures are affected than in AD and Pick's disease. HD has no tau-protein related pathology and the mutant huntingtin may not interact with apoE molecule. Pick bodies, like the NFT are also tau-protein immunoreactive, and the apoE molecule might interfere with the development of Pick bodies and the process of neurodegeneration.

IV.5. The role of impairment energy metabolism in neurodegeneration

Another mechanism possibly involved in HD pathogenesis are impairment in energy metabolism, mitochondrial dysfunction in striatal neurons. The presence of apoptotic features in HD does not exclude the possibility of glutamate-mediated excitotoxicity, decreased energy metabolism, and the defect in mitochondrial complex II-III. An interesting hypothesis can be proposed establishing a connection between huntingtin, apoptosis and mitochondrial dysfunction. Huntingtin is a substrate of caspase-3, a key enzyme of apoptosis, in addition, huntingtin is also known to interact with GAPDH, a protein involved both in energy metabolism (glycolysis) and in apoptotic pathways. Abnormal interaction between mutated huntingtin and GAPDH could cause decreased glucose metabolism in the basal ganglia in HD and might also stimulate apoptosis.The mitochondria has a central role in apoptosis, controlling early stages of the apoptotic cascade through the release of protein such as cytochrom C or apoptosis inducing factor (AIF) (*Susin et al.*, 1996). There is a consistent defect in mitochondrial complex II-III in the striatum of HD patients, leading to respiratory chain failure (*Beal et al.*, 1995). Therefore it is conceivable that mitochondrial abnormalities could increase the possibility of chronic energy impairment and apoptotic cell death in HD striatum. This process can be potentiated by the mutant huntingtin at various steps (mitochondrial defect, GAPDH and caspase-3 activities).

Interaction of GAPDH with the mutant huntingtin may decrease the glycolytic activity of this enzyme, progressively leading to energy deficit (*Burke et al.*, 1996). To support this theory we examined the glycolytic function of GAPDH in HD fibroblasts. HD brain is known to exhibit metabolic abnormalities, therefore we considered the possibility that metabolic alterations might also be manifested in non-neural HD cells, such as fibroblasts. Cultured skin fibroblasts from four HD patients and an age-matched control were used to measure the activity of GAPDH. All HD patients' fibroblast extract exhibited decreased GAPDH activity compared to the normal control. These preliminary data are consistent with the hypothesis that GAPDH enzyme has functional role in the pathomechanism of HD, and these metabolic changes could be detected systemically, not restricted to neurons (*Cocper et al.*, 1998).

Our PET study using 18F-FDG as a tracer provided another piece of evidence for an alteration of glucose metabolism and energy impairment in HD patients. Cerebral glucose me-

tabolism was severely reduced in the caudate nucleus of HD patients even at an early stage of the disease. Two young asymptomatic carriers had normal glucose uptake, while two other asymptomatic carriers showed abnormal PET in the same age and no correlation was found between the PET abnormality and the CAG repeat number. Decreased glucose metabolism was also observed in two mid-life asymptomatic carriers. These data suggest that there is an impairment of glucose metabolism in the basal ganglia of HD patients, contrary to asymptomatic carriers. Our results demonstrate that genetically identified HD gene carriers can show normal neurological function for many years before the disease becomes clinically manifest, but subtle metabolic changes could be detected by 18F-FDG PET. The measurement of striatal glucose hypometabolism could be used as a marker of the disease progression and it could be useful for testing the effect of possible future treatment.The cause of the reduction of striatal glucose metabolism observed by 18F-FDG PET in HD patients is unclear (*Antonini et al.*, 1996).

IV.6. Therapeutic interventions and strategies in HD

There are currently no cures or even effective treatments for HD. Therapeutic strategies have remained elusive because little is known about either the mechanisms of CAG expansion or the mechanism of polyglutamine-mediated neuronal cell death. However, recent advances in understanding the basic mechanisms of CAG expansion and polyglutamine toxicity have renewed hope that a therapeutic strategy might someday be possible. These strategies range from methods that counteract the causes of cell death to strategies that bypass the mechanisms of cell death to cell replacement therapy. Combinatorial approaches provide considerable improvements in rescuing and protecting neurons from degeneration.

The strong dependence of the character of the disease on the CAG repeat length has raised the possibility that stopping CAG expansion at the DNA level might prevent disease progression and diminish disease severity. The finding that loss of Msh2 attenuates expansion in animals provided the first evidence that expansion can be stopped in vivo, and has given the hope that a therapeutic intervention of a repair complex could be used to attenuate, or delay the onset of disease.

The next challenge is developing a therapy to decrease the toxicity of mutant huntingtin. Despite an incomplete understanding of toxicity, it is generally accepted that aggregation of mutant huntingtin fragments is the causative factor. Antibodies and number of small molecules, peptides inhibitors are being developed to block aggregation, and improve cell survival. It has recently been reported that huntingtin can regulate transcription of brain-derived neurotrophic growth factor (BDNF), which is important for the growth of striatal neurons. Loss of BDNF could contribute to neuronal cell death, so treatment with BDNF might improve neuronal survival. A number of other neurotrophic factors have been found to rescue striatal neurons in animal models of HD (*Zuccato et al.*, 2001).

Metabolic abnormalities including mitochondrial deficit, decreased glucose metabolism, ATP depletion also play a role in HD neurodegeneration. Replenishing impaired energy metabolism might offset toxicity by restoring ATP levels. Clinical trials are underway to evaluate the efficacy of free-radical scavengers, creatine and co-enzyme Q. These agents are protective, and improve the energy production in mitochondria (*Tarnopolsky et al.*, 2001).

Anti-excitotoxin therapy is also a promising approach for neuroprotection in HD. In HD animal models these drugs have been very effective in protecting against excitotoxins. Clinical trials for lamotrigine have already been completed. Lamotrigine blocks voltage-gated sodium channels, thus inhibiting glutamate release. Lamotrigine was succesful in reducing chorea in HD patients, but it failed to affect the disease progression (*Kremer et al.*, 1999). Currently, trials are underway to test the efficacy of remacemide, another blocker of excitatory pathway (*The Huntington Study Group*, 2001).

The enhanced apoptotic process observed in HD may serve as a therapeutic target for the treatment. If caspase activation occurs early enough in the disease progression then disease onset could be blocked by the use of caspase inhibitors, which might provide protection by blocking a general cell death pathway. Minocycline is a derivate of the antibiotic tetracycline that crosses the blood-brain barrier and inhibits caspase 1 and 3. Clinical trials are planned to test the efficacy of minocycline, that inhibits the cell death pathway, and improves neuronal survival in animal models (*Chen et al.*, 200).

To offset the severe phenotype at later stages of disease progression surgical strategies have been developed in which transplantation of embryonic stem cells replaces lost neurons in

the striatum. Embryonic grafts placed in quinolinic-acid-treated animals improved motor functions. This approach is highly invasive and will be useful at the advanced stages of HD (*Watts et al.*, 2000).

Although effective therapy is not possible yet, rapid advances in the understanding of the basic mechanisms of the neurodegeneration in HD are leading to expanded approaches towards therapeutic strategies and a hope for cure. Early genetic detection of asymptomatic HD gene carriers and late onset of the disease render this patients particularly well suited for effective therapeutic intervention.

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