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NR1 Receptor Gene Variation is a Modifier of Age at Onset in Turkish Huntington's Disease Patients

Aysun Açar Hazer and Nagehan Ersoy Tunali

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Abstract

The length of the CAG repeat tract is the major determinant of age of onset (AO) of Huntington's Disease (HD). However, there remains a significant variance in AO when the expanded repeat size is ruled out. The search for genetic modifiers has revealed various candidate loci; however, many reports have been contradictory. The N-methyl-D-aspartate receptors (NMDAR) have been proposed as an important putative modifier. We aimed to determine whether polymorphisms in NMDAR-coding genes have an effect on the AO. We analyzed the association between GRIN1 (rs6293), GRIN2A (rs1969060), and GRIN2B (rs1806201, rs890) polymorphisms and AO of Turkish HD patients. According to our findings, expanded CAG repeat size explains 41.8% of the variance in AO. Upon classification of genotypes into CAG repeat length intervals, rs6293 can be considered as an AO modifier for Turkish HD patients with 50 or higher CAG repeats. In addition to that, we found a significant association of this polymorphism to HD, with the GG genotype constituting a risk factor. Candidate genetic modifiers should be tested in different populations since their effects may exist only in groups of specific ethnic origins. Defining such modifiers will help in complete understanding of HD pathogenesis and in designing therapeutic targets.

Keywords: Huntington's disease, polyglutamine repeats, age of onset, NMDA receptors, GRIN

1. Introduction

Huntington's disease (HD) is a progressive neurodegenerative disorder of the central nervous system characterized by involuntary movements, cognitive impairment, and emotional

disturbances [1]. The prevalence of the disease is about 1/10,000 among individuals of European descent [2]. The disease follows an autosomal dominant pattern of inheritance and is associated with an expanded block of CAG repeats in exon 1 of the IT15 gene [3]. The CAG repeats are translated into a polyglutamine (polyQ) tract near the N-terminus of the huntingtin (htt) protein, which acquires a deleterious gain of function in the mutant protein. This ubiquitously expressed protein is shown to be essential for development [4–6]. Analysis of htt-interactors revealed that htt may function in intracellular trafficking, neurotransmission, retrograde transport, cytoskeletal function, and transcriptional regulation [7–9].

Four CAG repeat size intervals associated with varying disease risks were defined. The alleles with up to 35 repeats are considered normal. However, repeats in the range of 29–35 have been shown to exhibit meiotic instability. Rare alleles with 36–39 repeats are in the reduced penetrance range, since some people with repeats in this range develop HD and others do not. On the other hand, alleles with 40 or more CAG repeats definitely cause HD in a normal life span [10].

HD usually strikes in the third to fourth decade of life and gradually worsens over a course of 10–20 years until death. The patients may differ in the type and severity of their symptoms, age of onset (AO), and disease duration. The number of CAG repeats in the IT-15 gene is the most important factor determining the AO. There is a significant inverse correlation between the AO and the CAG repeat length [11, 12]. However, the number of the CAG repeats does not allow an accurate prediction of AO, only 30–70% of the variance in AO can be explained by the repeat size alone [13–20]. The AO varies significantly among individuals with the same CAG size, and even monozygotic HD twins may show phenotypic discordance for the disease [21, 22]. This has led to a search for genetic modifiers and environmental factors that influence the AO. Diverse modifier candidate loci have been suggested to be associated with AO in HD such as GRIN, TP53, hCAD, UCHL1, BDNF, ASK1, and MTHFR [14, 17, 23–32]. However, many reports from different research groups have been contradictory.

The NMDA glutamate receptors (GRIN: glutamate receptor, ionotropic, N-methyl-D-aspartate) have been proposed as an important putative modifier, since glutamate (Glu) mediates fast excitatory neurotransmission in the brain. Neurodegeneration in HD is highly selective for striatal GABA-ergic medium-sized spiny neurons (MSNs) that project to the substantia nigra (SN) and globus pallidus [33]. The underlying mechanisms are poorly understood; nevertheless, models of neurodegeneration suggested excitotoxicity, oxidative stress, and impaired energy metabolism as relevant to selective pathogenesis in HD [34]. Excitotoxicity may play a major role in the pathogenesis of HD, because Glu is the principal excitatory neurotransmitter in the mammalian central nervous system (CNS) and its excessive extracellular levels for prolonged periods can lead to excitotoxicity [35]. Glutamate activates two classes of receptors in neurons: metabotropic glutamate receptors (mGluRs) coupled to G-proteins and ionotropic glutamate receptors (iGluRs) controlling ion channels. The most intensely studied subclass related to excitotoxicity is the NMDARs, since intrastriatal injections of an

NMDA receptor agonist, quinolinic acid, produce lesions similar to the HD neuropathology, selectively affecting the striatal projection neurons [36–38]. Upon activation, the NMDAR channel becomes permeable to Ca^{2+} and Na^+ , and NMDAR overactivity results in excessive Ca^{2+} loads. As homeostatic Ca^{2+} mechanisms are overwhelmed, a number of intracellular mechanisms are induced, leading to cellular damage by degradative enzymes [39].

Individual NMDARs are multimeric assemblies of subunits and the combination of NR1 with different NR2 subunits alters the characteristics of the NMDAR ion channel. NR2A-containing receptors are generally expressed at synapses and they are associated with developmental regulation and synaptic maturation; NR2B-containing receptors seem to be predominant at extrasynaptic sites. Therefore, the expression patterns of NR2A- and NR2B-containing NMDARs may have differential roles in mediating excitotoxic neuronal death [40]. The striatum, the most vulnerable region of the brain in HD, appears to express higher levels of NR2B subunit relative to other NR2 subunits when compared to other brain regions [41]. Studies with postmortem HD brains showing losses of striatal NMDAR-binding sites indicate that MSNs with higher levels of NMDAR expression are at particular risk [42]. A number of studies have demonstrated enhancement of the NMDAR currents in several transgenic HD mouse models [42–46]. Furthermore, an NR2B-selective hypothesis of mutant htt-mediated enhancement has been suggested [47]. Mutant protein caused an increase in the responses of NMDARs composed of NR1/NR2B, whereas NR1/NR2A NMDARs were not differentially affected by normal or mutant htt [48]. YAC72 MSNs were shown to be more susceptible to NMDA-induced toxicity than wild-type MSNs, and additionally, the enhancement of apoptosis by mutant htt in YAC46 and YAC72 MSNs was proportional to the length of the polyQ repeat, arguing a relationship of altered NMDAR signaling and mutant htt polyQ length [49]. These findings strongly support the hypothesis of excitatory amino acid-mediated and particularly NMDAR-induced cell death in HD. Therefore, NMDAR activity and/or subunit composition may have effects not only in the downstream effector pathways but also on the AO in HD patients.

Recent analysis by Arning and coworkers [17] indicated that variations of the NR2A and NR2B genes could explain the variance in AO especially in HD patients with CAG repeat lengths in the high 30s to the low 40s. In a replication study with Venezuelan kindreds, no evidence was obtained for the association of NR2B single nucleotide polymorphisms (SNPs); however, they found evidence for association of an NR2A SNP [24]. In an expanded HD cohort, Arning and coworkers confirmed the results of the previous study [14]. In addition to that, gender stratification of patients revealed differences in the variability in AO attributable to the CAG repeat number and highly significant differences in the AO association with the NR2A and NR2B variations [14].

In this study, we aimed to investigate the modifier effects of GRIN1, GRIN2A, and GRIN2B SNPs (rs6293, rs1969060, rs1806201, rs1042339, and rs890) on AO in Turkish HD patients. Defining genetic modifiers of AO in different ethnic populations is of great importance, since they may provide further clues to explain disease pathology and to construct neuroprotective strategies.

2. Materials and methods

2.1. Subjects and patient diagnosis

DNA samples of 102 unrelated HD patients (46 men and 56 women) were included in the study. Clinical examination and determination of motor AO was performed by experienced neurologists. The CAG repeat sizes of the patients were ascertained by polymerase chain reaction (PCR) using primers 5'FAM-HD3: 5'-GGCGGTGGCGGCTGTTGCTGCTGCTGCTGC-3' and P3F: 5'-TCTGCTTTTACCTGCGGCC-3', followed by capillary electrophoresis and fragment analysis using PeakScanner v.1.0 software (Iontek, Turkey). One hundred and two healthy age- and sex-matched controls were also genotyped for comparison. The control subjects and their families were free of neurological disorders. All subjects provided written informed consent for genotyping. The experiments performed comply with the current laws of Turkey, and the study was approved by the Halic University Human Researches Ethics Committee.

2.2. SNP genotyping

All studied SNPs were checked from the single nucleotide polymorphism database (dbSNP). Genotyping of GRIN1, GRIN2A, and GRIN2B polymorphisms was performed by PCR-RFLP analysis [17]. PCR was carried out in a final volume of 25 μ l with 50 ng of DNA, 2.0 mM Mg^{2+} , 0.8mM dNTP, 10 pmol of each primer, and 1 U Taq polymerase. The cycling conditions for each polymorphism included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48–60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (**Table 1**). PCR products were digested with 2 U of the appropriate restriction enzymes under optimal reaction conditions (**Table 1**) for 2 h and visualized on 2% agarose gels stained with ethidium bromide. Genotypes were confirmed with Sanger sequencing (Iontek, Istanbul) sequence analysis of 25 randomly selected samples.

2.3. Statistical analysis

Hardy-Weinberg (HW) test statistics was computed for each SNP. In order to compare allele frequencies between cases and controls, a standard case-control association analysis was performed using PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>). The dependence of AO on the expanded CAG repeat size was assessed by linear regression. The effects of the GRIN1, GRIN2A, and GRIN2B genotypes on AO were analyzed by multiple linear regression approach. The CAG repeat number and the respective genotypes were used as independent variables, and AO was used as the dependent variable. The CAG repeat size was considered as numerical, modifying genotypes were considered as nominal variables and they were encoded as "0, 1, 2." The odds ratios (OR) and independent-samples *T*-tests for genotypes were computed for the association of genotypes with AO. All the statistical analyses were performed with Statistical Package for Social Sciences (SPSS) version 17.0 for Windows.

Gene/polymorphism	Primer sequences 5' → 3'	Annealing temperature	Restriction enzyme	Allele sizes (bp)
GRIN1/rs6293	NR1F: CGTTCTTGCCGTTGATGA NR1R: GTAAGAGCCAGCAACGGAG	60.3°C	MspI	G allele: 138 + 113 + 59 + 114 A allele: 251 + 173
GRIN2A/rs19690600	2Ars1969060F: GGTTTTAAGATTTGTGCCAGG 2Ars1969060R: CTTAGACCGAGTTGGCAACA	60.3°C	DdeI	T allele: 280 + 45 C allele: 166 + 114 + 45
GRIN2B/rs1806201	2Brs1806201F: AGACTATTCGCTTCATGC 2Brs1806201R: GTGTGTTGTTTCATGGCTG	48.3°C	PstI	C allele: 210 T allele: 194 + 16
GRIN2B/rs890	2Brs890F: GCTGTCAGCCATTCCTGTT 2Brs890R: CATGAATTTAGCCAGAGCCTC	57.1°C	PsuI	G allele: 283 T allele: 194 + 89
GRIN2B/rs1042339	2Brs1042339F: GACCACAAGCGCTACTTCAG 2Brs1042339R: TGCATACAGGTTGCCTGCT	53.7°C	PsyI	G allele: 263 + 146 A allele: 409

Table 1. PCR and RFLP conditions of the studied polymorphisms.

3. Results

The patients were given the clinical diagnosis of HD upon neurological examination and thereafter clinical diagnosis was ascertained with molecular test results. The clinical symptoms of the patients included choreic movements, intellectual and cognitive impairment, dementia, and depression.

In the studied patient cohort, 22 different mutant alleles carrying 39–75 CAGs were determined; the most frequent range was between 42 and 47 repeats. The disease AO ranges between 16 and 80 years, the mean AO (\pm SD) being 40.75 (\pm 12.97) years (**Table 2**). There are wide variations in ages of onset for a given CAG repeat length in our patient population, which is a strong indicative of other genetic or environmental modifiers.

CAG repeat length	AO range (years)	AO mean (years)
39	69	69
40	42–73	55.25
41	20–75	43.71
42	35–60	50.27
43	40–80	52.25
44	28–48	41.58
45	24–57	38.88
46	37–52	42
47	30–62	40.23
48	26–36	32.8
49	28–45	37.6
50	27	27
51	25–39	30
52	25–36	31.17
53	25–32	28.5
54	23–35	29
55	23	23
56	19–23	21
58	22	22
59	16	16
67	22	22
75	16	16

Table 2. CAG repeat sizes of the patients and corresponding AO ranges and means.

Allele and genotype frequencies were deduced in HD cases and controls according to HW principles and evaluated with chi-squared goodness-of-fit test. In patients and controls, allele and genotype frequencies were found in HW equilibrium. All genotypes except the 3501A variant (rs1042339) in the GRIN2B gene were observed in the patient and control groups; therefore, this polymorphism was excluded from the analyses. Genotype frequencies of studied SNPs among HD patients and controls are summarized in **Table 3**.

Linear regression analysis has shown that 41.8% of the variance in AO could be attributed to the expanded CAG repeat size in the studied population ($R^2 = 0.418$, $p < 0.0001$). In order to evaluate the contributions of the studied SNPs to the AO, R^2 -values were determined for each SNP in conjunction with the effect of the expanded CAG repeat size. The R^2 -values of the SNP genotypes and the CAG repeats alone were compared to determine the change in R^2 (ΔR^2) (**Table 4**). In our HD patient cohort, the studied genotype variations at GRIN1, GRIN2A, and GRIN2B did not show any significant increase in the R^2 -values; therefore, they do not contribute to the variation in AO. Gender stratification of patients did not reveal any differences in the variability in AO attributable to the CAG repeat number and putative modifier polymorphisms. Upon classification of the CAG repeat sizes into four groups (40–45, >45, 40–50, and >50 CAGs) to expand the findings, a significant regression model was obtained only for GRIN1 rs6293 ($p = 0.016$) in the >50 CAGs group. The other groups did not reveal any significant regression models. The power of the analysis reached 99.9%, and at least 42

Gene polymorphism		GRIN1 rs6293	GRIN2A rs1969060	GRIN2B rs1806201	GRIN2B rs890	GRIN2B rs1042339
Genotypes		AA	CC	CC	GG	AA
		AG	CT	CT	GT	AG
		GG	TT	TT	TT	GG
Patients	%	42.16	1.96	44.12	26.47	0
		48.04	30.39	48.04	46.08	0
		9.8	67.65	7.84	27.45	100
	<i>n</i>	43	2	45	27	0
		49	31	49	47	0
		10	69	8	28	102
Controls	%	52.94	6.86	41.18	24.51	0
		43.14	27.45	49.02	54.90	0
		3.92	65.69	9.80	20.59	100
	<i>n</i>	54	7	42	25	0
		44	28	50	56	0
		4	67	10	21	102

Table 3. Genotype frequencies among patients and control subjects.

patients were essential to detect a significant effect with GRIN2A rs1969060, analysis of the other SNPs required 44 patients. With a sample population of 102 patients, we are beyond the minimum number of required patients.

Although there is no significant finding, the multiple linear regression data for GRIN2A rs1969060 could be regarded as a trend ($p = 0.132$) (Table 4). Moreover, HD patients with genotype CC had higher mean AO (50 years) than that of remaining HD patients (40 and 57 years) (Figure 1). The risk estimation analysis have shown that, compared to combined genotypes CT and TT, genotype CC can be regarded as protective (OR = 0.271). However, the observed differences in AO did not prove to be significant according to the results of independent samples t -test ($p = 0.31$).

	R^2	ΔR^2	% additional variance	p -value
HD CAG	0.418	–	–	0.000
HD CAG + GRIN1 (rs6293)	0.418	0.000	0	0.917
HD CAG + GRIN2A (rs1969060)	0.431	0.013	2.22	0.132
HD CAG + GRIN2B (rs1806201)	0.422	0.004	0.007	0.406
HD CAG + GRIN2B (rs890)	0.419	0.001	0.002	0.695

Table 4. Multiple linear regression analysis of the SNPs.

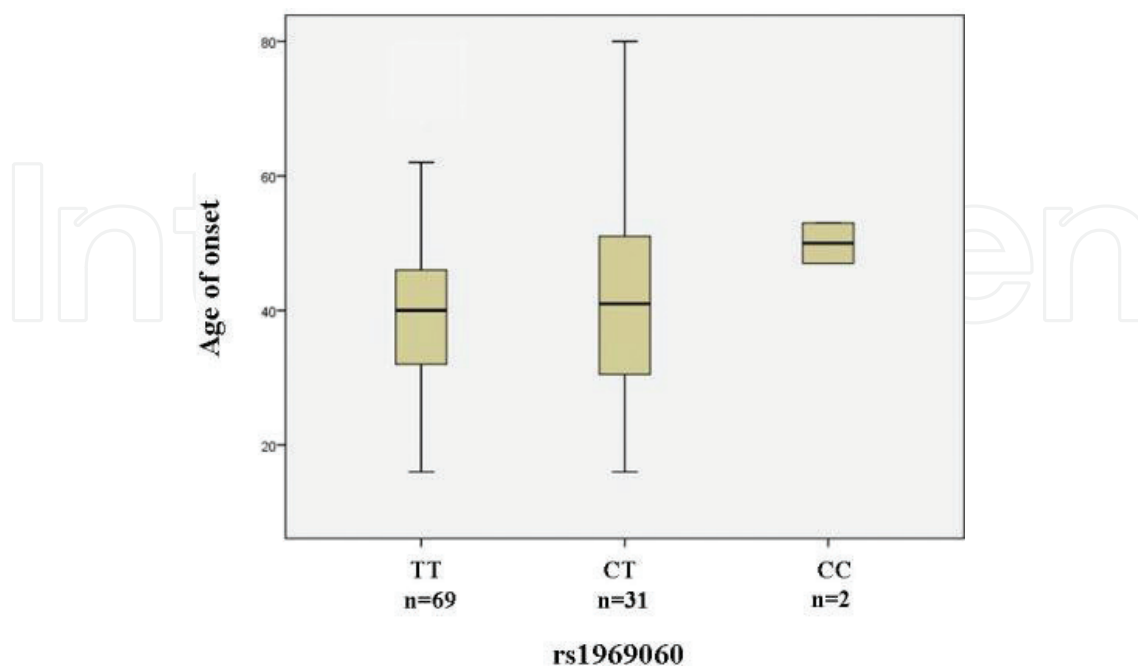


Figure 1. The AO distribution among rs1969060 genotypes is represented as box plots. For each genotype, the median AO is represented as a black bar, the quartile is shown as a solid box, and the range is indicated by margins.

In addition to that, although GRIN2B rs180620 polymorphism did not reveal an important regression model, HD patients with genotype TT had lower mean AO (35 years) than those having the remaining genotypes (41.18 years). However, upon independent samples *t*-test, this difference was not found to be significant ($p = 0.23$), either.

The result of simple associations test (**Table 5**) has shown that the distribution of GRIN1 rs6293 GG genotype was significantly different in patient and control groups ($p = 0.01478$). Upon this finding, risk analysis was performed and the OR value was calculated to be 2663, indicating that the GG genotype could be a risk factor in HD.

SNP	A1	F_A	F_U	A2	CHISQ	P	OR	L95	U95
rs6293	G	0.348	0.2321	A	5.942	0.01478	1.766	1.115	2.796
rs1806201	T	0.3137	0.3333	C	0.171	0.6792	0.9143	0.5979	1.398
rs890	T	0.5049	0.4804	G	0.2452	0.6205	1.103	0.7481	1.626
rs1969060	C	0.1716	0.2059	T	0.7844	0.3758	0.7988	0.4856	1.314

Table 5. Association analysis of SNPs using PLINK (A1: minor allele, A2: major allele, F_A: Frequency of minor allele in patients (F_U: frequency of minor allele in controls, CHISQ: chi-squared test, L95 and U95: lower and upper bounds of 95% confidence interval).

4. Discussion

It has been well established that the AO is inversely correlated with the number of CAG repeats in the mutant HD allele. On the other hand, the negative correlation between AO and repeat size is stronger for higher repeat numbers (earlier AO) and weaker for lower repeat numbers (later AO). This indicates that although the CAG size is the major determinant of onset age particularly in juvenile patients, factors other than the repeat size should contribute to onset in later ages [50]. Previous studies have shown that the CAG repeat number alone explained 30–70% of the variance in AO [13–15, 17–20]. In various studies, many gene polymorphisms were defined as possible candidates for explaining the remaining variance. The genes encoding htt-interacting proteins or proteins that are found to be involved in disease pathology were regarded as the major candidate modifiers. In this study, we aimed to evaluate the hypothesis that polymorphisms in GRIN1, GRIN2A, and GRIN2B genes that code for NMDAR subunits may contribute to explain the variability in AO in Turkish HD patients.

According to our findings, expanded CAG repeat size explains 41.8% of the variance in AO, which is in accordance with the results from other populations. However, when the entire observed CAG repeat sizes are considered, the remaining variance could not be attributed to any of the studied SNPs. Classification of SNP genotypes into four CAG repeat length intervals did not reveal any important findings either, except GRIN1 rs6293 in the >50 CAG repeats group ($p = 0.016$). As a result, rs6293 SNP can be considered as an AO modifier for Turkish HD patients with 50 or higher CAG repeats in their IT15 gene. Also, the results of the association test indicated a significant association of this SNP to HD ($p = 0.015$) and further risk analysis revealed that the GG genotype constitutes a risk factor (OR = 2.663).

Prior to this study, the effects of the SNPs in NMDAR-coding genes in determining AO in HD were investigated in the German population [14, 23] and a replication study was performed with Venezuelan kindreds [24]. In the Arning's work, German HD patients having 41–45 CAG repeats in their mutant HD alleles were intentionally selected. In this cohort, the contribution of the expanded CAG repeat size to the AO was calculated to be 30.8%, while variations in the GRIN2A (rs1969060) and GRIN2B (rs1806201) genes accounted for 4.5 and 12.3% of the remaining variance, respectively. In addition to that, GRIN2B rs1806201 (C2664T) variation resulted in a difference of 2.8 years between the genotypes and patients having TT genotype that had earlier AO than those having other genotypes [17]. In our sample population, although GRIN2B rs180620 polymorphism did not reveal an important regression model, TT genotype did show lower mean AO (35 years) than the remaining genotypes (41.18 years). Arning and coworkers expanded their findings with 8 additional SNPs and 83 additional HD patients with repeat sizes varying between 39 and 61 [14]. In that study, when GRIN2A and GRIN2B genotype variations were considered together in the multiple regression analysis, 7.2% of the additional variance could be explained. Furthermore, the classification of patients according to sex revealed a significant difference and 5.6% additional variance was attributed to C2664T genotype variations in women. Female HD patients with the CC genotype tended to have delayed onset compared to the other two genotypes. In our study, we could not deduce any significant results out of gender stratification. In the Venezuelan kindreds, GRIN2B 1806201 association could not be replicated; however, GRIN2A rs1969060 SNP was found to be associated with AO, with a very slight increase in R^2 ($\Delta R^2 = 0.003$). They found 3.9 year difference in the mean age of onset between the CT and TT genotype classes. They found that TT genotype was protective. On the contrary, in our population HD patients with CC genotype have about 10 years higher mean AO than that of the remaining patients, which may imply toward a protection effect. However, due to very few numbers of patients with the CC genotype, this result does not reach statistical significance. As mentioned by Andresen et al. [24], heterozygosity at this polymorphic locus varies greatly by geographical variation and the results should be evaluated carefully. The C allele frequency was established to be 17% in European, 52–56% in Asian and African, and 24% in Venezuelan populations. The data from our population are in accordance with that of the European population (17.2% in HD patients and 20.6% in control subjects).

Apart from our study, GRIN1 rs6293 was investigated only with the German HD patients with repeat numbers 41–45 [17], which demonstrated a trend for explaining the variance in AO ($p = 0.055$). On the contrary, we established GRIN1 rs6293 as an AO modifier for Turkish HD patients with 50 or higher CAG repeats in their IT15 gene.

In summary, the effects of the genetic variations in the GRIN1, GRIN2A, and GRIN2B genes have been investigated in a very few numbers of populations until now and the results could not be replicated. The major reason for this discrepancy could be that the effect of these polymorphisms on AO may exist only in groups of similar genetic backgrounds or of specific ethnic origin. Therefore, further investigations involving HD patients of various ethnic populations with a wide range of CAG repeat sizes are required to clarify the issue. However, these negative findings are far from ruling out NMDA receptors as an important biological effector in the pathophysiology of HD. Ample evidence suggests an important role for NMDARs in

selective vulnerability of MSNs and neuronal cell death in HD. While there is evidence that NMDARs play an important role in the pathogenesis of HD, their exact roles and the modifying effects of the polymorphism remain to be further investigated. It is clear that numerous supplemental studies are needed to establish a confirmed association of GRIN genes as genetic modifiers of AO in HD.

Conflict of interest

The authors declare that they have no conflict of interest.

Author details

Aysun Açar Hazer¹ and Nagehan Ersoy Tunali^{2*}

*Address all correspondence to: nagehan.ersoy@medeniyet.edu.tr

1 Department of Molecular Biology and Genetics, Halic University, Istanbul, Turkey

2 Department of Molecular Biology and Genetics, Istanbul Medeniyet University, Istanbul, Turkey

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