Association of a Functional NOS1 Promoter Repeat with Alzheimer’s Disease in the VITA Cohort

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Abstract. NO synthase, type I (NOS-I) has been suggested to play a role in the etiology of Alzheimer’s disease (AD). The gene encoding NOS-I harbors at least nine alternative first exons; in the promoter region of exon 1f, a polymorphic repeat (NOS1 ex1f-VNTR) has been described which influences gene expression and neuronal transcriptome. We have shown that short alleles of this repeat are associated with AD. Here, we sought to further explore this finding by investigating a longitudinal cohort sample from the Vienna-Transdanube-Aging (VITA) study consisting of 606 subjects enrolled at the age of 75 (of these, genotypes were available for 574 subjects) and followed up for 60 months. The ex1f-VNTR risk genotype was associated with AD in the total sample and at the second follow-up. Thus, either long alleles of NOS1 ex1f-VNTR are protective against disease or conversely, short alleles predispose to earlier onset of disease. As demonstrated, ex1f-VNTR interacted with the apolipoprotein E e4 risk allele (OR in the presence of both risk alleles 3.63; 95% CI: 1.45–9.12). These findings provide further evidence for an association of NOS1 with AD.

Keywords: Alzheimer’s disease, genetics, neuronal nitric oxide synthase (NOS1), polymorphism, risk factor, VITA study

INTRODUCTION

Alzheimer’s disease (AD) represents the most common form of neurodegenerative disorders. While there are familiar forms of early-onset Alzheimer’s demen-

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vant candidate genes have not yet been accomplished
[1]. Genome-wide association studies (GWAS) have
proven fruitful in the discovery of novel risk genes
in disorders such as diabetes, and thus have been also
applied in LOAD; however, also this approach conveys
several problems, e.g., that risk alleles might not be
tagged by the interrogated single nucleotide polymor-
phisms (SNPs). Not surprisingly though, recent GWAS
relicated the ApoE locus [2] (Harold et al., 2009),
but did not provide unequivocal evidence for novel risk
genes such as GAB2 [3] and GOLPH2 [4]. Thus, there
is still a need for candidate gene driven approaches to
consolidate and extend current knowledge [1]. Repli-
cation is essential in such studies, and while there are
numerous positive initial studies, most of the genes
fail to replicate, a phenomenon commonly referred to
as “the winner’s curse”.

Neuronal nitric oxide synthase (NOS1), which
is expressed at high levels throughout all brain areas
including hippocampus and frontal cortex [5], is a
highly relevant candidate gene for LOAD [6–8. It
also interacts with ApoE [9, 10], in that ApoE geno-
type appears to affect NO production. NOS1 displays
remarkable complex transcriptional regulation using
alternative first exons termed exon 1a to exon 1f,
resulting in different 5′-untranslated regions. Exons
1c and 1f are expressed most abundantly in the cen-
tral nervous system [11, 12]. In the promoter region
of exon 1f, we have described a variable number of
tandem repeats (VNTR) polymorphism [13–14]
term NOS1 Ex1f-VNTR. It is highly polymorphic
and consists of different numbers of dinucleotides
dichotomized in short and long alleles for associ-
ation studies. Most importantly, we could establish
that NOS1 Ex1f-VNTR is functional [14] as it results
in alterations of the neuronal transcriptome, such
as reduced expression of the NMDA receptor, and
reduced gene expression as evidenced by a reporter
gene assay. We have previously demonstrated that
this repeat is associated with LOAD in an Italian
case-control sample [15]. The risk variant, namely
being homozygous for short repeats, interacted with
the ApoE e4 risk allele thereby conveying a more
than 10-fold increase in the odds ratio to develop
LOAD. Here we sought to replicate our finding in an
independent cohort, the Vienna-Transdanube-Aging
(VITA) study [16]. This cohort was assessed at start-
ing age of 75 years and was followed every 30
month up to 60 months including detailed phenotyp-
ing for LOAD-relevant parameters. Importantly, the
longitudinal approach used in this study allowed test-
ing whether the association of NOS1 Ex1f-VNTR
short allele with AD affects age of onset and is sta-
ble over time.

MATERIALS AND METHODS

Subjects

Subjects of the present study were from the VITA
study, described previously in greater detail [16–18].
The VITA-Study investigates the residents of two
Viennese districts born between May 1925 and June
1926 (i.e., aged 75 years at inclusion). Out of 1505 con-
tacted individuals, 606 subjects consented in the study
at baseline and completed physical health check, ques-
tionnaires for education, psychosocial activities, and
neuropsychological examination. From those, NOS1
ex1f-VNTR genotype could be obtained in 574 indi-
viduals, whereas DNA extraction or genotyping failed
in 35 individuals. Follow-up at t = 60 mo was possible
in 394 subjects, 52 of which could only be assessed by
home visit, NOS1 ex1f-VNTR genotype was available
in 391 subjects.

Eighty one subjects out of the 606 participants at
baseline deceased between baseline and t = 60 mo, 92
subjects refused to take part again in the follow-up
investigation, 68 subjects were only willing to take
part in a telephone-interview providing only minimal
information (and thus were considered drop-outs),
and three subjects were lost due to follow-up. The
study flow chart is depicted in greater detail in Supple-
mental Figure S1 (available online: http://www.j-al
z.com/issues/23/vol23-2.html#supplementarydata02).

Individuals who did not take part in any follow-up
investigation performed poorer in memory, figure
copy, naming, set shifting abilities and in the Mini-
Mental Status Examination (MMSE) and showed a
greater severity of depression. Refusers who deceased
had a significant poorer performance in set shifting
abilities and nonverbal memory [19].

Subjects were diagnosed [20, 21] for depression
according to DSM-IV. A diagnosis of AD was estab-
lished applying the NINCDS-ADRDA criteria [22].
Vascular dementia (VD) was diagnosed according to
the NINDS-AIREN criteria [23]. Probable VD was
only diagnosed if focal neurological signs and signifi-
cant cerebral vascular lesions as evidenced by cranial
MRI were present. In the case the latter was lacking,
possible VD was diagnosed if dementia was present
along with focal neurological signs. Cerebral magnetic
resonance imaging (at baseline and at follow-up) was
available in nearly all cases, as were relevant serum
parameters such as calcium, vitamin B12, folic acid,
and thyroid hormones. Baseline demographic variables are given in Table 1 for the 574 subjects included in this analysis.

Diagnosis of AD includes pure AD subjects as well as mixed dementias (AD and vascular dementia, or AD and Lewy body dementia; present in 44 from 131 total AD cases). Subjects suffering from pure PD (n = 12) or other dementias such as FTD or LBD (n = 2 and 4, respectively) were included in the control group.

All participants passed through a consensus conference with regard to a diagnosis of possible or probable AD. The final diagnosis was made by an experienced geronto-psychiatrist. The number of AD cases given in the “total sample” (Table 2, Fig. 1) is the cumulative number of AD cases, i.e., the sum of all possible and probable cases in the whole cohort and differs from the number at t = 60 mo due to dropout of cases in the interval. The VITA study was carried out with the permission of the Ethics Committee of the City of Vienna, Austria and each participant gave a written informed consent.

Laboratory methods

DNA extraction

DNA was prepared from 2 ml EDTA-blood by the standard procedure of Proteinase K, aliquot and frozen at –70 °C.

STATISTICAL ANALYSIS

Overall, the frequency of the short-short NOS1 ex1f-VNTR genotype in the total sample was 21%, which is significantly different from a random distribution of 12.5% (p < 0.01, chi-square goodness of fit test). Chi-square tests were performed to test for group differences in allele and genotype frequencies. p-values <0.05 were considered significant. The odds ratio (OR) was calculated along with its 95% confidence interval (CI).

RESULTS

ApoE genotyping

The ApoE genotype was determined via PCR reaction with specific primers provided in the kit BNO-LiPA-ApoE (Immunogenetics, Gent, Belgium).

NOS1 genotyping

The Ex1f-VNTR polymorphism was determined by PCR and product size determination by means of fragment analysis on a CEQ8000 DNA-sequencer (Beckman-Coulter, Krefeld, Germany), as previously described [14]. Alleles have been dichotomized in short and long alleles as described, with up to 196 repeats being designated as short alleles.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows 12.0.1 (SPSS Inc., Chicago, IL, USA). Hardy Weinberg equilibrium was tested for using a goodness of fit test. Chi-square tests were performed to test for group differences in allele and genotype frequencies, p-values <0.05 were considered significant. The odds ratio (OR) was calculated along with its 95% confidence interval (CI).

Table 1

Demographic information on the investigated sample

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy</th>
<th>Poss. and prob. AD</th>
<th>Healthy</th>
<th>Poss. AD</th>
<th>Prob. AD</th>
<th>Healthy</th>
<th>Poss. AD</th>
<th>Prob. AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>355</td>
<td>19</td>
<td>440</td>
<td>50</td>
<td>25</td>
<td>285</td>
<td>73</td>
<td>33</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>229/326</td>
<td>9/14</td>
<td>14/226</td>
<td>30/40</td>
<td>7/18</td>
<td>102/183</td>
<td>40/53</td>
<td>7/26</td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.8±0.5</td>
<td>75.7±0.4</td>
<td>78.3±0.5</td>
<td>78.2±0.4</td>
<td>78.4±0.4</td>
<td>80.8±0.4</td>
<td>80.7±0.4</td>
<td>80.9±0.5</td>
</tr>
<tr>
<td>ApoE genotype (n positive)</td>
<td>116</td>
<td>4</td>
<td>72</td>
<td>18</td>
<td>9</td>
<td>55</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>ApoE-ε4 allele (mean ± SD)</td>
<td>27.9±1.5</td>
<td>23.7±2.7**</td>
<td>28.0±1.4</td>
<td>26.6±2.2**</td>
<td>21.3±4.5**</td>
<td>28.0±2.2</td>
<td>25.6±3.7**</td>
<td>16.2±8.4**</td>
</tr>
<tr>
<td>MMSE score (mean ± SD)</td>
<td>30.7±0.0</td>
<td>29.7±1.1**</td>
<td>30.4±0.0</td>
<td>29.5±1.0**</td>
<td>29.2±0.0</td>
<td>29.2±0.0</td>
<td>29.2±0.0</td>
<td>27.6±1.0</td>
</tr>
<tr>
<td>CDR Score (mean ± SD)</td>
<td>3.4±5.5</td>
<td>5.6±6.9</td>
<td>7.2±5.7</td>
<td>9.3±6.6*</td>
<td>9.3±6.3*</td>
<td>8.8±6.3</td>
<td>10.1±5.5</td>
<td>8.1±5.7</td>
</tr>
<tr>
<td>Hamilton score (mean ± SD)</td>
<td>2.4±2.4</td>
<td>5.0±3.4**</td>
<td>2.7±2.6</td>
<td>4.1±3.2**</td>
<td>4.5±2.8**</td>
<td>2.9±2.7</td>
<td>4.3±3.2**</td>
<td>3.7±2.5</td>
</tr>
<tr>
<td>SGDS (mean ± SD)</td>
<td>34.1±9.3</td>
<td>43.8±12.8**</td>
<td>35.4±9.4</td>
<td>38.3±9.4*</td>
<td>41.9±11.9*</td>
<td>36.5±10.8</td>
<td>37.9±10.2</td>
<td>39.6±10.1</td>
</tr>
<tr>
<td>STAI XI (mean ± SD)</td>
<td>35.5±9.6</td>
<td>42.0±11.3**</td>
<td>37.3±9.6</td>
<td>40.0±10.3**</td>
<td>37.1±10.2**</td>
<td>37.8±10.7</td>
<td>40.2±9.5</td>
<td>36.6±9.6</td>
</tr>
</tbody>
</table>

Poss. AD, possible Alzheimer’s dementia; Prob. AD, probable Alzheimer’s dementia according to NINCDS-ADRDA criteria. MMSE, Mini-Mental Status Exam; CDR, Clinical Dementia Rating; SGDS, Short Geriatric Depression Scale; STAI State-Trait Anxiety Inventory; *p < 0.05; **p < 0.01 from the respective “Healthy” group (Student’s t-test, two-sided).
is comparable to middle-aged German controls (22%, n = 3524; [14], and unpublished).

Table 2 gives the genotype frequencies of AD patients and controls; due to the relatively low number of LOAD patients, we did not perform an exploratory analysis to investigate for gender effects. Both AD and control populations were in Hardy-Weinberg equilibrium (not shown). In the total sample, there was a significant association of NOS1 ex1f-VNTR with LOAD when testing for genotypic associations. At the second follow up (n = 60 mo) however, there was a significant association of NOS1 ex1f-VNTR with LOAD (χ² = 6.8, df = 2, p = 0.03) not present at p = 60 mo and at baseline (p > 0.05). When considering the short-short risk genotype, delineated by our previous study [15], vs. all other genotypes (i.e., the long-long and short-long groups), results were significant for the total sample and the second, but not the first follow-up time point and baseline (total simple, p = 0.04; t = 60 mo, p = 0.06; t = 30 mo, p = 0.009). In that the short-short genotype was more prevalent in LOAD patients, corresponding to an OR of 1.66. The OR however was almost fourfold higher when comparing the frequency of LOAD in short-short vs. short-long and long-long genotype carriers, LOAD was significantly more often present in short-short genotype carriers as compared to the other groups at t = 60 mo as well as in the total sample (Fig. 1). Conversely, presence of depression was not associated with NOS1 ex1f-VNTR (all p > 0.05, not shown).

As expected, the frequency of the ApoE ε4-allele was significantly increased in AD as compared with controls (31 vs. 18%, χ² = 8.1, df = 1, p = 0.004; OR: 2.02, 95% CI: 1.30–3.17). To examine the combined influence of the NOS1 ex1f-VNTR short-short genotype and the ApoE ε4-allele on the risk to develop AD, we stratified our sample according to the presence of short-short genotype carriers as compared to the other groups. When comparing the frequency of LOAD in short-short vs. short-long and long-long genotype carriers, LOAD was significantly more often present at t = 60 mo as well as in the total sample (Fig. 1). Conversely, presence of depression was not associated with NOS1 ex1f-VNTR (all p > 0.05, not shown).

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DISCUSSION

We here attempted to replicate our previous finding [15] that short NOS1 ex1f-VNTR repeats are associated with LOAD in a G × G manner with ApoE4. To do so, a community-based sample was assessed at the age of 75 and followed up for 5 years allowing investigating whether the association remains stable and whether the risk allele affects age of onset, representing a novel approach in AD genetics. We could confirm that short NOS1 ex1f-VNTR repeats are associated with LOAD, and we replicated the interaction with ApoE4.

While investigating a longitudinal cohort design bears the disadvantage of a relatively low number of affected individuals (n = 106 at t = 80, corresponding to 27% of the sample) resulting in a possible lack of power to detect small effects, it conveys the benefit of proper matching and uniform distribution of a wide range of risk factors such as environmental toxins. Furthermore, the longitudinal design allows investigating factors predicting age of onset and disease course. Still, conclusions must be limited to LOAD, since our cohort starts at t=75 years. Furthermore, the relatively high drop-out rate must be considered a further limitation, which is however inherent to the longitudinal design in an elderly cohort.

As our findings in the VITA cohort closely resemble those of our previous case-control study, we consider NOS1 a replicated risk gene for LOAD. Lower significance levels might be attributed to decreased power due to the small number of patients as compared to an enriched case-control design. However, it is noteworthy that the association only became significant at the second, but not the first follow-up. The NOS1 risk genotype thus seems to predispose to a steeper slope in the risk to develop AD over the course of time, i.e., it seems to determine an earlier age at onset. On the other hand, carrying at least one long allele might be protective. Functional studies might reveal the precise underlying molecular mechanisms.

Carrying the short-allele genotype alone conveys an odds ratio between 1.47 (this study) and 2.37 [15] irrespective of its interaction with ApoE4. Thus, GWAS should be able to capture the risk variant, which was not the case as the repeat is not tagged by SNPs. Nevertheless, Liang and associates demonstrated linkage of LOAD with 12q22, the region harboring NOS1 [24]. It is intriguing that NOS1 ex1f-VNTR is functional on the molecular level [14], as it affects prefrontal functioning and expression of a reporter gene; this has been confirmed by an independent study also demonstrating an association of short repeats with Parkinson’s disease [25] as suggested previously [26]. This argues for the notion that NOS1 is a rather unspecific risk gene for neurodegenerative disorders. It has been suggested that increased NO production is involved in the pathogenesis of neurodegeneration by promoting neuronal damage via oxidative stress [6]. Thus, it appears to be counter-intuitive that the risk variant goes along with decreased gene expression. However, the consequences of reduced NOS1 exon 1f expression are unclear. As expression of exon 1c and exon 1f appears to be inversely correlated [27], decreased exon 1f expression might well go along with increased exon 1c expression and thus overall elevated NO production. Furthermore, NOS-1 is a "low-output" nitric oxide synthase as opposed to NOS-II, which is regarded a "high-output" NOS and more likely capable of causing oxidative damage. Rather, NO from NOS-I serves as a messenger molecule [28] which accomplishes hippocampal LTP. Reduced NO production in this brain region might contribute to defective LTP and further promote functional impairment in the case of neuronal damage. In AD, hippocampal NOS-I containing neurons appear to be highly susceptible to neurodegeneration [29], so that decreased NOS1 expression in concert with degeneration of NOS-I neurons likely results in impaired hippocampal nitricergic neurotransmission. Furthermore, dysregulation of NOS1 was shown to correlate with hippocampal nitrotyrosine formation [30, 31], pointing to an important mechanism, as several proteins involved in neurodegeneration (Drp1 [32], MAP1B [33], and XIAP [34]) are subject to regulatory nitrosylation. Of course, other molecular
mechanisms and neuroanatomical structures (such as the frontal cortex) might be implicated as well, which is subject to further studies.

Taken together, there is solid evidence that short NOS ex1-VNTR repeats, especially in interaction with ApoE4, convey genetic risk to develop LOAD. It thus seems worthwhile to further investigate the molecular pathways involved. Targeting the NO pathway is an innovative therapeutic principle which, despite still in its infancy, has been applied in rodent studies, where a NO donor compound proved beneficial in cognitive functioning [35] which led to human Phase I studies. Combined behavioral and genetic data thus argue for a contribution of dysregulated NOS functioning to AD and suggest that NOS is a worthwhile drug target opening new treatment avenues.

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