Joint analysis of the NACP-REP1 marker within the alpha synuclein gene concludes association with alcohol dependence

D. Bönsch¹, T. Lederer², U. Reulbach¹, T. Hothorn³, J. Kornhuber¹ and S. Bleich¹,*

¹Department of Psychiatry and Psychotherapy, ²Institute of Forensic Medicine, and ³Department of Medical Informatics, Biometry and Epidemiology, Friedrich-Alexander-University of Erlangen-Nuremberg, 91054 Erlangen, Germany

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Various studies have linked alcohol dependence phenotypes to chromosome 4. One candidate gene is NACP (non-amyloid component of plaques), coding for alpha synuclein. Recently, it has been shown that alpha synuclein mRNA is increased in alcohol-dependent patients within withdrawal state. This increase is significantly associated with craving, especially obsessive craving. On the basis of these observations, the present study analysed two polymorphic repeats within the NACP gene. We found highly significant longer alleles of NACP-REP1 in alcohol-dependent patients compared with healthy controls (Kruskal–Wallis test, $\chi^2 = 99.5; df = 3, P < 0.001$). In addition, these lengths significantly correlate with levels of expressed alpha synuclein mRNA ($\chi^2 = 8.83; df = 2, P = 0.012$). The present results point to a novel approach for a genetic determination of craving, a key factor in the genesis and maintenance not only of alcoholism but also of addiction in general.

INTRODUCTION

In mission Indians, an alcoholism severity phenotype has been mapped to chromosome 4 to a region containing a cluster of genes, coding for alcohol dehydrogenase peptide and alpha synuclein (1). Results from this paper hint towards a possible involvement of a gene apart from the two neighboured alcohol dehydrogenase genes ADH1B and ADH1C. In addition several other linkage studies point to the same region (2–4). Furthermore, alpha synuclein maps to a quantitative trait locus for alcohol preference, and expression of alpha synuclein in different brain areas is increased in rats whose alcohol preference is inbred (5). Most recently, evidence has been presented that alpha synuclein levels are elevated in midbrain dopamine neurons of chronic cocaine abusers (6). Furthermore, an increased expression of alpha synuclein mRNA in patients with alcoholism has been observed, which correlates to craving in addicted patients with alcoholism (7).

Alpha synuclein is involved in dopaminergic neurotransmission (8). It regulates synaptic dopamine homeostasis (9) and influences the expression of dopamine synthesis genes such as GTP cyclohydrolase, TH and aromatic acid decarboxylase (10). Dopaminergic transmission has been suggested to be a main mechanism mediating reinforcement, withdrawal and craving associated with alcohol addiction (11). The dopamine-containing projection from the ventral tegmental area of the midbrain to the nucleus accumbens is critically involved in the mediation of seeking behaviour in addiction disorders (12).

Alpha synuclein plays a major part in Parkinson’s disease (8,13–15). Mounting evidence suggests an inverse association among cigarette smoking, alcohol drinking, coffee consumption and Parkinson’s disease (16–18). Apart from the possibility of environmental risk factors, genetic susceptibility is discussed as a major factor. This may lead to speculation, which genes involved in Parkinson’s disease might influence behaviour concerning substance abuse.

Several polymorphisms within NACP have been extensively analysed in patients suffering from Parkinson’s disease. Especially for NACP-REP1, which is located 5’ of the alpha synuclein promoter, various studies have shown importance for alpha synuclein expression (19–21). Its significance for Parkinson’s disease is yet unclear with controversial findings in several studies (22). However, the role of the repeat within intron 4 is not well understood.

*To whom correspondence should be addressed. Tel: +49 91318536362; Fax: +49 91318534105; Email: stefan.bleich@psych.imed.uni-erlangen.de

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Therefore, the aim of this study was to investigate whether genetic polymorphisms, influencing the expression of alpha synuclein differ between patients with alcoholism compared with healthy controls.

RESULTS

A TC-rich repeat downstream of the exon 4/intron 4 boundary was described by Xia et al. (23). Four different alleles ranging from 265 to 362 bp were identified in a Caucasian population whereby in different diseases such as Alzheimer’s disease, Lewy-body disease or Parkinson’s disease no correlation of repeat length and disease status was observed. In the present study, 70 patients and 89 controls (140 and 178 alleles, respectively) were analysed for the repeat in intron 4. As shown in Figure 1A, no significant differences between alcohol-dependent patients and controls were observed ($\chi^2 = 7.0; \text{df} = 3, P > 0.05$). We also did not find a significant trend in this group so that a further increase in case/sample numbers did not seem to deliver any additional informations. Both groups showed allele frequencies comparable to the frequencies reported in control populations in previous studies (23).

*NACP*-Rep1 is a polymorphic complex repeat site located ~10 kb upstream of the translational start of *NACP*. The repeat is highly interesting because several association studies have demonstrated a link between certain alleles and an increased risk of sporadic Parkinson’s disease in different populations. The significance of these observations is underlined by findings describing an increased expression of alpha synuclein depending on the allele length (20). Figure 1B illustrates the distribution of alleles within *NACP*-REP1 in our sample: in total, 135 patients and 101 controls were investigated (270 and 202 alleles, respectively). Astonishingly, alcoholic patients show completely different frequencies compared with healthy controls with highly significant expanded alleles ($\chi^2 = 99.5; \text{df} = 3, P < 0.001$). Controls show a maximum at allele 269 (~60%), whereas patients with alcoholism show a maximum at allele 271 (50%). The most significant difference in allele distribution is found for the two longest alleles 271 and 273 as well as the shortest allele 267.

It was further analysed whether these findings on the genomic level also have an effect on alpha synuclein expression. Alpha synuclein mRNA was quantified and mRNA levels were correlated with the allele lengths: the allele lengths of *NACP*-REP1 correlate with levels of alpha synuclein mRNA whereby the repeat lengths are significantly associated with higher alpha synuclein mRNA expression (Kruskal–Wallis test, $\chi^2 = 8.83; \text{df} = 2, P = 0.012$) (Fig. 2).

The estimated differences and simultaneous confidence intervals of levels of alpha synuclein indicate that the global difference stems from a large effect between long and short allele lengths (estimated difference 1.189 with 90% confidence interval of 0.126–2.251), whereas no effect between intermediate and short (0.434; 0.349 to 1.218) and long and intermediate allele lengths (0.755; 0.181 to 1.690) can be established.

This indicates that the regulative role of *NACP*-REP1 is not only restricted to cell culture systems but also can be demonstrated in human blood cells.

DISCUSSION

To our knowledge, this is the first study evaluating repeats within the *NACP* gene in patients with alcoholism. Allele frequencies between controls and patients did not reveal any
suggested to be a main mechanism mediating reinforcement, seeking behaviour (5). Dopaminergic transmission has been neuronal dopamine release, to cocaine (6) and alcohol (7). Most recently, it has been shown that SNCA triplication is associated with alcohol craving, an important factor for development, maintenance and relapse of alcoholism. Further studies will help to elucidate the possible role of polymorphisms within NACP as well as the function of alpha synuclein in the pathophysiology of addictive disorders.

**MATERIALS AND METHODS**

**Subjects**

The present prospective case–control study was approved by the Institutional Review Board of the University of Erlangen-Nuremberg. All southern (franconian) German subjects had been informed and had signed a consent form before participating in any part of the study. Caucasian controls (mean age 44.3, SD 11.2; range 20–72) were matched to Caucasian patients by age and smoking. Only males were included into both groups because of the small sample size of females. The male healthy controls had no disorder meeting Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria, and alcohol consumption did not exceed 30 g ethanol intake per week. Two experienced psychiatrists (D.B. and U.R.) independently examined all healthy subjects who were recruited from the Department of Ophthalmology (University of Erlangen-Nuremberg). Study characteristics of both patients and controls, such as sociodemographic data, period of alcohol consumption, last alcohol intake, daily intake of alcohol, estimation of lifetime drinking, smoking behaviour or medical history, were taken using a modified semi-structured interview (7,27) (data not shown). All male patients (mean age 43.8, SD 9.2; range 24–65) from the Franconian Alcoholism Research Studies (FARS) (28) were active drinkers or medical history were not enrolled.

**Genetic and laboratory analyses**

Fasting blood samples for DNA extraction were drawn on admission in ethylenediaminetetraacetic (EDTA) acid-containing tubes and were stored at −80 °C immediately after collection. A Y-STR core set of DYS 19, DYS 389 I, DYS 389 II, DYS 390, DYS 391, DYS 392, DYS 393, DYS

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**Figure 2.** Additive repeat length and alpha synuclein mRNA expression. Short, 0–4: 267/267; 267/269; 267/271; 269/267; 269/269; intermediate, 6–8: 267/273; 269/271; 269/273; 271/271 and long, 10–12: 271/273; 273/273. *NACP*-REP1 lengths significantly correlate with levels of expressed alpha synuclein mRNA (Kruskal–Wallis test, \( \chi^2 = 8.83; df = 2, P = 0.012 \)).

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**Table 1.** Additive repeat length and alpha synuclein mRNA expression. Short, 0–4: 267/267; 267/269; 267/271; 269/267; 269/269; intermediate, 6–8: 267/273; 269/271; 269/273; 271/271 and long, 10–12: 271/273; 273/273. *NACP*-REP1 lengths significantly correlate with levels of expressed alpha synuclein mRNA (Kruskal–Wallis test, \( \chi^2 = 8.83; df = 2, P = 0.012 \)).

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**Delta CT of alpha synuclein in alcoholics versus controls.** Short, intermediate and long *NACP*-REP1 lead to a higher expression of alpha synuclein mRNA as compared with healthy controls. The atte length of *NACP*-REP1 influences the expression of alpha synuclein. It has been shown that there is a considerable variability in alleles of the same length (24) which might confound conventional genotype association analysis. Therefore, further investigations must clarify the influence of variability within alleles of the same length with respect to alpha synuclein expression levels. However, this latter effect was clearly shown in alleles with different lengths (19–21). Moreover, it has been shown that the expression of alpha synuclein is elevated in patients with alcoholism whereby higher mRNA alpha synuclein levels were significantly linked to alcohol craving (7). Most recently, it has been shown that SNCA triplication results in a doubling in the amount of alpha synuclein protein in blood which possibly demonstrates a correlation between peripheral and central expressions (25). This is further in line with our preliminary results showing that protein concentrations in blood correlate to alpha synuclein mRNA levels in patients with alcoholism (data not shown). The results of the present study are in accordance with previous studies linking alpha synuclein levels, which promote neuronal dopamine release, to cocaine (6) and alcohol seeking behaviour (5). Dopaminergic transmission has been suggested to be a main mechanism mediating reinforcement, withdrawal and craving associated with alcohol addiction (11). In addition, the present findings are consistent with the observation of an altered mRNA expression of alpha synuclein in frontor and motor cortices of human alcoholics (26). The present study provides first evidence for a linkage of higher levels of synuclein and genetic distribution of alleles within *NACP*-REP1 in patients with alcoholism. This hints towards a novel pathophysiologic mechanism in alcoholism. Longer alleles of *NACP*-REP1 lead to a higher expression of alpha synuclein which is associated with alcohol craving, an important factor for development, maintenance and relapse of alcoholism. Further studies will help to elucidate the possible role of polymorphisms within *NACP* as well as the function of alpha synuclein in the pathophysiology of addictive disorders.
438 and DYS 439 was used to analyse relatedness. In total, 46 distinct alleles were observed in our sample whereby no identical haplotype was observed within the 135 patients. Furthermore, no significant differences were observed compared to an European population sample of 16 779 haplotypes in a set of 120 populations (DYS 19, DYS 389 I, DYS 389 II, DYS 390, DYS 391, DYS 392, DYS 393) and an European population sample of 3054 haplotypes in a set of 18 populations (DYS 483, DYS 439), respectively ($P = 0.42–0.91$).

The regions of the NACP gene containing the repeat polymorphisms were amplified by PCR using the following primers: REP1 (CCT GGC ATA TTT GAT TGC AA) and REP2 (GAC TGG CCC AAG ATT AAC CA) according to Xia et al. (29); Intron4-F (CCT CTG GTA TTC CCA GTC TC) and Intron4-R (CAC ATT ATG TAT ATT TAA AGG TG) (23) with both reverse primers being 5'-fluorescently labelled with TAMRA. PCR was performed according standard procedures and products were analysed on an ABI-sequencer (ABI 320) together with a ROX-labelled size standard (Amersham) and scored by GeneScan (version 2.0).

Total RNA was extracted from whole frozen EDTA-blood using a modified Qiaqen-protocol: a phenol-extraction in Qiagen (Qiagen) was followed by column-purification with RNaEasy Mini Kit (Qiagen) including DNase digestion. Reverse transcription was performed by using poly-dT primers and AMV Reverse Transcriptase from the Roche cDNA Synthesis System. Quantitative PCR was performed using SYBR Green I Master Mix buffer (Applied Biosystems), and reactions were run on an iCycler (BioRad) using a three-step standard protocol with an annealing temperature of 66°C. GAPDH was used as internal standard, and ΔCT values were calculated from differences between GAPDH and alpha synuclein. All experiments were repeated three times. We used the following primer pairs: GAPDH-F (CACCAGGCGCTGTTTAACTCTGGTA) and GAPDH-R (CTTGACGGTGCCATGGAATTGGC); A-Syn-F (GCCAAGGAGGGAGTTGTGGCTTAACTCTGGTA) and A-Syn-R (CTGTTGCCACCCCATGCACCCTCC).

### Statistical analyses

Distributions of all utilized variables were tested using Kolmogorov–Smirnov. As none of the variables was normally distributed, statistical analysis was done by non-parametric methods such as correlation analysis by Spearman, Chi-squared statistic and Kruskal–Wallis test. Sum scores were built from statistical and product values were calculated from differences between GAPDH and alpha synuclein. All experiments were repeated three times. We used the following primer pairs: GAPDH-F (CACCAGGCGCTGTTTAACTCTGGTA) and GAPDH-R (CTTGACGGTGCCATGGAATTGGC); A-Syn-F (GCCAAGGAGGGAGTTGTGGCTTAACTCTGGTA) and A-Syn-R (CTGTTGCCACCCCATGCACCCTCC).

Windows 12 (SPSS Inc., Chicago, IL, USA), confidence intervals were computed using the multcomp add-on package (30) within the R system (31) for statistical computing (version 2.0.1).

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