Association of attentional network function with exon 5 variations of the **CHRNA4** gene

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Mutational analyses in xenopus oocyte and mice models indicate that the positive effect of nicotine on attention may be modulated by genetic variations within exon 5 of the alpha4 subunit of the nicotinergic acetylcholine receptor gene **CHRNA4**. The potential relevance of exon 5 is further emphasized by two recent family-based association studies of nicotine dependence because subgroups of nicotine-dependent subjects are thought to ‘self-medicate’ attentional deficits with nicotine. We investigated a synonymous single nucleotide polymorphisms (SNP): rs1044396, which has recently been associated with nicotine-dependence, plus two adjacent synonymous SNPs rs1044394 and rs1044393 in exon 5 of \(n = 47\) unrelated healthy Caucasian subjects (age: 22.7 ± 1.7 years; sex: \(n = 23\) males; regular smokers: \(n = 19\)). Attentional network function was assessed in supplementary motor area/anterior cingulate (SMA/ACC) and parietal cortex with functional magnetic resonance imaging during an attention-requiring visual oddball task. SNP rs1044396 showed genotype effects on attentional network function both in the SMA/ACC and parietal cortex in the absence of overt behavioral effects. In the parietal cortex, a gene-dosage effect was seen. Comparable genotype effects were also found for the other two SNPs. This investigation provides first evidence that attentional network function may be modulated by genetic variations within **CHRNA4** exon 5. If confirmed, future studies need to address what ‘functional’ polymorphisms are causative for the observed effects.

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**INTRODUCTION**

Numerous behavioral studies in both animals and humans have consistently demonstrated a positive acute effect of nicotine on attentional performance (1–15). In line with this observation, functional imaging experiments have shown that nicotine optimizes attentional network function throughout the brain including the frontal and parietal cortices as well as the thalamus (16–23). Notably, it is also known that nicotine dependence is highly prevalent in patients who suffer from neuropsychiatric disorders that are associated with poor attentional capacity. For instance, patients suffering from attention deficit/hyperactivity disorder (ADHD) are exceptionally frequently nicotine-dependent and there is evidence that nicotine might be consumed by some people for self-medication of (sub-clinical) attentional deficits which predate the onset of nicotine consumption (24,25). Since attentional performance is partly genetically controlled (26), it is therefore conceivable that the genetic basis of attentional network function, which is still largely unknown so far, may involve the brain nicotinergic system.

The nicotinic acetylcholine (nACh) \(\alpha4\) subunit gene **CHRNA4** is a potential candidate gene for attentional network function. The gene is located on chromosome 20q13.3 and consists of six exons being distributed over approximately 17 kb of genomic DNA (27). The behavioral and physiological effects of nicotine are largely mediated via nACh receptors—in particular via the high affinity \(\alpha4\beta2\) subtype which is widespread throughout the brain comprising 90% of high-affinity nicotine binding sites in mammalian brain (28,29). Supported by investigations of a variety of different experimental nicotine agonists in behavioral rat...
models, the attentional effects of nicotine agonists are thought to result from stimulation of these high-affinity α4β2 receptors (30–32). Recombinant mice strains with an α4 nicotinic subunit containing a single point mutation in the pore-forming second transmembrane domain (M2) were found to render the α4 receptors hypersensitive to nicotine (34). There are also several reports of a possible association of cognitive deficits (‘mental retardation’) with a missense mutation in exon 5 that replaces a serine into phenylalanine in CHRNA4 which in turn affects the M2 domain of the receptor (35,36). Using patch clamp, it has been previously demonstrated in the *xenopus oocyte* model that this missense mutation in exon 5 induces distinct modifications of the receptor’s (ion channel) functional properties including a significant increase in acetylcholine sensitivity (37). It is therefore of particular interest that two large family-based studies of male Chinese (38) and European–American subjects (39) have associated measures of nicotine dependence with single nucleotide polymorphisms (SNPs) within exon 5 of CHRNA4. This is because it is conceivable that genetic variations in exon 5 may affect nicotine receptor sensitivity and—by extension—attentional network function which in turn may promote in a subgroup of smokers the initiation and maintenance of nicotine abuse for reasons of self-medication of attentional deficits.

It was the purpose of the present study to address the question whether genetic variation within exon 5 of CHRNA4 are associated with attentional network function as assessed with functional magnetic resonance imaging (fMRI).

RESULTS

Genotype distribution and subject/experimental parameters

Genotype and allele distribution of the main SNP of interest rs1044396 with a minor allele frequency (MAF) of 0.48 was similar to what has been reported previously (38,39) and genotype counts did not significantly deviate from those expected according to the Hardy–Weinberg equilibrium (40). The MAFs of the two other SNPs rs1044394, rs1044393 were low (0.08, 0.14, respectively) and therefore, the less frequent homozygous genotype was discarded for further group comparisons. Task and subject parameters across genotype rs1044396 are provided in Table 1. The only significant between-genotype group differences were found for sex and education. No corresponding significant group differences were found for the two other SNPs rs1044394, rs1044393 (data not shown).

Attentional network function, smoking status, years of education, reaction time

In Table 2, regions of maximum BOLD-response are shown. Peak maxima are seen in the left SMA, in the left middle cingulate of the ACC and left precentral gyrus. A strong BOLD-response maximum is also found in the postcentral gyrus (parietal cortex). These maxima, which are located within the selected volume of interests (VOIs) (see below), as well as several additional weaker maxima essentially conform with previous reports on the BOLD-response pattern during oddball task conditions (e.g. 40). Figure 1 depicts the overall BOLD-response in the entire group of subjects in the area of the SMA/ACC and parietal cortex. In agreement with our previous analysis of a subset of the present sample (41), the BOLD-response is stronger in the left when compared with the right hemisphere, particularly in the parietal region where activity is virtually absent on the right side. This was previously discussed by us to reflect the specific task conditions with right thumb button press in response to target stimuli (41).

In subsequently conducted VOI analyses in both regions, no statistically significant effects of smoking status (regular smoker versus non-smokers) were seen on BOLD-response (bihemispheric SMA/ACC: Z = 0.7; df = 1, 45; P = 0.419; left parietal cortex: F = 0.0; df = 1, 45; P = 0.962) when using smoking status as fixed factor and the BOLD-response (mean Z-value) as dependent variable. Likewise, no significant effects of smoking status were observed when instead using the number of ‘activated’ voxels (BOLD-response threshold: Z ≥ 2.3) as dependent variable: bihemispheric SMA/ACC: F = 0.2; df = 1, 45; P = 0.670; left parietal cortex: F = 0.3; df = 1, 45; P = 0.584. A significant effect of smoking status was only observed, as previously reported by Musso et al. (42), when exclusively comparing the bihemispheric SMA/ACC in a subset of subjects, i.e. never-smokers versus smokers who are smoking at least 10 cigarettes per day (F = 4.5; df = 1, 22; P = 0.045). Accordingly, smoking status was not considered further for the subsequent genotype analyses of the entire subject sample.

We also did not further take into consideration ‘years of education’ because no significant correlations were seen with VOI mean Z-value bihemispheric SMA/ACC BOLD-response (R = −0.03; P = 0.861) or with VOI mean Z-value left parietal BOLD-response (R = −0.21; P = 0.184). On the other hand, attentional network BOLD-response differences were found between males and females, the latter showing stronger BOLD-responses both in the bihemispheric SMA/ACC and left parietal cortex, although these differences just missed statistical significance. SMA/ACC (mean Z-value): males = 0.41 ± 0.60, females = 0.72 ± 0.65; t = −1.7; P = 0.100), SMA/ACC (n of voxels Z > 2.3): males = 18.3 ± 39.5, females = 43.8 ± 61.2; t = −1.7; P = 0.099), left parietal cortex (mean Z-value): males = 0.44 ± 0.85, females = 0.96 ± 1.04; t = −1.9; P = 0.068), left parietal cortex (n of voxels Z > 2.3): males = 18.5 ± 40.5, females = 52.1 ± 78.7; t = −1.8; P = 0.074). Therefore, we included sex as a covariate into the subsequently conducted statistical calculations.

Mean reaction time or mean intrasubject reaction time variability was not significantly correlated with the mean Z-value (BOLD-response) in the selected VOIs (data not depicted). However, non-significant trend correlations were observed for all three VOIs (left and right medial frontal, left parietal) with mean reaction time (P < 0.10). These correlations were negative indicating stronger attentional network activation in those subjects with shorter reaction time which is in line with previous reports from our group (41).
Table 1. Experimental and subject parameters by genotype group (rs1044396)

| Parameter                        | A/A (n = 13) | A/G (n = 23) | G/G (n = 11) | P  
|----------------------------------|--------------|--------------|--------------|----
| Age (years)                     | 22.5 ± 1.7   | 22.4 ± 1.7   | 23.6 ± 1.7   | 0.178  
| Sex (M/F)                       | 9/4          | 8/15         | 6/5          | 0.047  
| Smoking (yes/no)                | 5/8          | 8/15         | 6/5          | 0.825  
| Education (years)               |              |              |              |       
| Intelligence (IQ)               | 114.9 ± 11.6 | 118.9 ± 11.7 | 118.9 ± 7.2  | 0.689  
| Reaction time (ms)              | 526.6 ± 46.0 | 507.3 ± 59.3 | 538.1 ± 59.3 | 0.443  
| Reaction time variability (ms)  | 87.9 ± 16.9  | 97.5 ± 30.1  | 97.5 ± 30.1  | 0.574  
| Head movement (mm, absolute)    | 0.55 ± 0.35  | 0.35 ± 0.31  | 0.36 ± 0.50  | 0.403  
| Head movement (mm, relative)    | 0.13 ± 0.23  | 0.07 ± 0.05  | 0.07 ± 0.04  | 0.357  
| VOI-size (n of voxels)          | 578.9 ± 61.5 | 540.9 ± 60.8 | 553.6 ± 50.2 | 0.187  

Difference significant with genotype as factor was obtained with ANOVA except for sex and smoking status (χ²) comparing A/A versus A/G (1) and A/G versus G/G (2).

Table 2. Brain regions of maximum BOLD-responses during visual oddball task

<table>
<thead>
<tr>
<th>Talairach Atlas (Brodman area)</th>
<th>Cytoarchitectonic maps (maximum probability)</th>
<th>MNI (x,y,z)</th>
<th>Peak Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left medial frontal gyrus (BA6)</td>
<td>Left supplementary area (area 6)</td>
<td>−4, −20, 56</td>
<td>4.49</td>
</tr>
<tr>
<td>Left medial frontal gyrus (BA6)</td>
<td>Left supplementary area (area 6)</td>
<td>−2, −8, 52</td>
<td>4.41</td>
</tr>
<tr>
<td>Left cingulate gyrus (BA24)</td>
<td>Left middle cingulate (area 6)</td>
<td>−4, −10, 44</td>
<td>4.34</td>
</tr>
<tr>
<td>Left precentral gyrus (BA6)</td>
<td>Left precentral gyrus (area 6)</td>
<td>−28, −20, 70</td>
<td>4.10</td>
</tr>
<tr>
<td>Left postcentral gyrus (BA3)</td>
<td>Left postcentral gyrus (area 3a)</td>
<td>−32, −30, 46</td>
<td>4.61</td>
</tr>
<tr>
<td>Left postcentral gyrus (n.a.)</td>
<td>Left superior temporal gyrus (OP 1)</td>
<td>−54, −22, 14</td>
<td>3.70</td>
</tr>
<tr>
<td>Right postcentral gyrus (BA2)</td>
<td>Right supramarginal gyrus (area 2)</td>
<td>66, −26, 12</td>
<td>3.20</td>
</tr>
<tr>
<td>Left middle temporal gyrus (BA19)</td>
<td>Left middle temporal gyrus (n.a.)</td>
<td>−48, −64, 12</td>
<td>2.90</td>
</tr>
<tr>
<td>Left insula (n.a.)</td>
<td>Left superior temporal gyrus (TE 1.0)</td>
<td>−42, −26, 14</td>
<td>3.45</td>
</tr>
<tr>
<td>Left thalamus (n.a.)</td>
<td>Left thalamus (n.a.)</td>
<td>−14, −22, 2</td>
<td>3.90</td>
</tr>
<tr>
<td>Ventral posterior medial nucleus</td>
<td></td>
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</tr>
</tbody>
</table>

Brain regions of maximum BOLD-responses (unclustered) in the entire sample (n = 47). From left to right: regions in Talairach-Tournoux Atlas with Brodmann Areas, Cytoarchitectonic Probability Map Atlas with macro-labels and maximum probability areas. Corresponding Montreal Neurological Institute (MNI) coordinates of peak BOLD-response (Z-value).

Attentional network function and genotype

In Figure 2, separate voxel-by-voxel analyses are presented for each genotype group (rs1044396). Differences of BOLD-responses become apparent between genotype groups both in SMA/ACC and parietal cortex. The strongest BOLD-response was seen in A/A carriers. In comparison, A/G carriers showed a diminished BOLD-response, particularly in SMA/ACC. Virtually no activity was detected in G/G carriers. BOLD-response differences between genotype groups are confined by a voxel-wise group contrast between A/A and G/G carriers (Fig. 3). In the contrast analyses of homozygous carriers, the peak activation maximum genotype group differences were seen in the left ACC/SM in Z = 2.9 at −4, −16, 52 mm and in the left parietal cortex with Z = 3.7 at −54, −16, 12 mm.

A subsequently conducted VOI-analysis of the SMA/ACC brain region (Fig. 4) showed a significant main effect of genotype (rs1044396) on attentional network function in SMA/ACC with genotype as fixed factor, sex as covariate and SMA/ACC BOLD-response (mean VOI Z-value) as dependent variable (F = 3.4; df = 2, 43; P = 0.042). When instead using the number of ‘activated’ voxels (BOLD-response threshold: Z > 2.3) as dependent variable, no significant effect was seen (F = 0.9; df = 2, 43; P = 0.411). An additionally conducted separate mean Z-value VOI-analysis of the genotype effects in left and right SMA/ACC showed that the genotype effect on medial prefrontal BOLD-response was somewhat stronger in the right hemisphere (F = 3.8; df = 2, 43; P = 0.029) than in the left hemisphere (F = 1.9; df = 2, 43; P = 0.159).

Similar results were obtained for the left parietal cortex VOI (Fig. 5), although now a clear gene-dosage effect became apparent: a significant main effect of genotype (rs1044396) was seen on left parietal network function with genotype as fixed factor, sex as covariate and left parietal BOLD-response (mean VOI Z-value) as dependent variable (F = 3.3; df = 2, 43; P = 0.047). When instead using the number of ‘activated’ voxels (BOLD-response threshold: Z > 2.3) as dependent variable, statistical significance was missed (F = 2.9; df = 2, 43; P = 0.068).

A significant main effect was also observed for the adjacent SNP rs1044394 (F = 5.1; df = 1, 45; P = 0.024) on the mean Z-value BOLD-response in SMA/ACC VOI which was again stronger for the right hemispheric VOI (F = 9.7; df = 1, 45; P = 0.003) than for the left hemispheric VOI (F = 0.3;
df = 1, 45; \( P = 0.622 \)). On the other hand, a corresponding genotype effect was not seen for the left parietal region \( (F = 1.2; df = 1, 45; P = 0.277) \). However, the number of heterozygous carriers was small \( (n = 7) \) when compared with homozygous carriers \( (n = 40) \). Likewise, a significant main effect was seen for SNP rs1044393 on SMA/ACC BOLD-response \( (F = 4.6; df = 1, 45; P = 0.037) \). An additionally conducted separate analysis of the left and right VOI in SMA/ACC revealed that the genotype effect on BOLD-response was again stronger in the right hemisphere \( (F = 5.2; df = 1, 43; P = 0.027) \) than in the left hemisphere \( (F = 0.4; df = 1, 43; P = 0.522) \). Again, however, no comparable genotype effect was found for the left parietal region \( (F = 0.9; df = 1, 45; P = 0.355) \).

**DISCUSSION**

Our investigation provides first evidence that attentional network function in humans may be modulated by genetic variations within \( CHRNA4 \) exon 5. The results are intriguing because (i) mutational analyses in mouse and oocyte models have indicated that exon 5 may be critical for proper function of the high-affinity alpha4beta2 nicotine receptor (34,37);
(ii) nicotine is well known to impact on attentional network function (1–23); and (iii) genetic variations of CHRNA4 exon 5 have been implicated in nicotine dependence (38,39). Accordingly, our findings are in agreement with our original notion that genetic variations in CHRNA4 may affect nicotine receptor sensitivity and—by extension—attentional network function which in turn may promote in a subgroup of smokers the initiation and maintenance of nicotine abuse for reasons of self-medication of attentional deficits. From a clinical perspective, our findings are potentially meaningful with regard to several neuropsychiatric disorders that are characterized, among other features, by attentional deficits and nicotine abuse including ADHD, schizophrenia, epilepsy and nicotine dependence. On the other hand, it still needs to be addressed what SNP in CHRNA4 is directly responsible for altered function of the nACh receptor α4 subunit. Interpreting their findings on nicotine dependence, Feng et al. (38) argued by referring to a promoter analysis in transgenic mice (43) that the investigated synonymous exon 5 SNPs might be in linkage disequilibrium (LD) with functional SNPs either upstream of the transcription initiation site or in intron 1. Alternatively, it is also conceivable that the synonymous SNPs within exon 5 may affect pre-mRNA splicing (44,45) and thereby cause the observed changes of attentional network function.

There is further evidence from several sides that CHRNA4 exon 5 may indeed play a role in several neuropsychiatric disorders. Scheffer et al. (46) described an inherited form of an atrial epilepsy that they termed autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). So far, six different mutations have been identified in ADNFLE, four of them are located in CHRNA4 and two in CHRNB2 (36,37,47,48). These mutations do not seem to lead to any gross abnormalities; the patients have normal neurological examination, magnetic resonance scans mostly reveal no pathology and interictal EEGs are often normal. Picard et al. (49) described 71 patients from 19 families with three different types of autosomal dominant partial epilepsies and screened them for a history of psychiatric disturbances. They found that behavioral disorders were most common in the ADNFLE (43%), compared with familial temporal lobe epilepsy (13%) and partial epilepsy with variable foci (23%). As there has been a considerable interest in the association between epilepsy and schizophrenia/psychosis for many decades (50), the high frequency of psychiatric diagnoses among patients with ADNFLE may not be an artefact. Actually, in one recent study of an ADNFLE family, the affected members who carried an insertion of an additional leucine (776ins3) in exon 5 of the CHRNA4 gene were found to have an unusual high rate of serious psychiatric disorders with mostly psychosis or schizophrenia-like clinical features (51). In addition, there are several reports of a possible association of cognitive deficits (‘mental retardation’) and a missense mutation in exon 5 that replaces a serine into phenylalanine.
In conclusion, it is possible that nAChR mutations might be a rare cause of schizophrenia even in patients without epilepsy. A less spectacular but more frequent mechanism may involve a contribution of certain nAChR gene variants to schizophrenia susceptibility. In such a model common gene variants would subtly alter the function of the nAChR, conferring a susceptibility effect to the genetically complex predisposition of schizophrenia. Such genetic effects can manifest themselves in different phenotypic aspects and their detection therefore requires a careful clinical characterization of the patient samples (e.g. attentional capacity and network activity).
While the findings in ADNFLE families suggest a possible link between variations in the CHRNA4 (and CHRNA2) genes and schizophrenia (broadly defined), there are also reports that indicate a relationship with attention deficit disorder. For instance, a recent family-based association study (52), which systematically screened for sequence variations in the coding regions as well as intron/exon junctions of the α4 subunit, found a significant association for a 5′ intron 2 SNP and severe attentional problems. The location of the SNP was considered to be compatible by affecting pre-mRNA stability or splicing. Similarly, Comings et al. (53) also reported a, although marginally, significant association of attention deficit disorder and a dinucleotide polymorphism in the same gene.

In summary, we found an association of CHRNA4 exon 5 gene variations with attentional network function as revealed by an fMRI experiment. If our findings in this relatively small sample are confirmed independently, future studies have to address particularly two questions. First of all, we need to know what ‘functional’ polymorphisms are causative for the observed effects on attentional network function. Secondly, larger samples should be investigated which include well-defined clinical populations including those with clinically relevant attentional network deficits and strong nicotine dependence. Given that two very recent studies did not find an association of nicotine dependence with CHRNA4 exon 5 SNPs including rs1044396 (54,55), such clinical studies might be able to further clarify whether genetic variations in CHRNA4 exon 5 are associated with nicotine dependence or a subgroup of smokers with attentional network deficits.

MATERIALS AND METHODS

Subjects

An investigation of n = 47 healthy subjects (mean age: 22.7 ± 1.7, 23 males, 19 regular smokers) was carried out with fMRI during an attention-requiring visual oddball task. Subjects were recruited via local newspaper announcements. Only unrelated Caucasian subjects (local university students) were included in the study. All subjects were right-handed. Participants were only investigated if there was no evidence for any medical or neurological condition that could interfere with the purpose of the study, or if there was no history for any psychiatric DSM-IV axis I or axis II disorder including current or recent drug or alcohol abuse as assessed by a Structured Clinical Interview (56), a formal medical and neurological examination including urine toxicology for illegal drug abuse screening, routine blood tests and an urine investigation as well a clinical EEG session. Only subjects with an IQ > 80 were included in the study as assessed by the HAWIE-R (Hamburg-Wechsler Intelligenz test) Scale (57) which is largely equivalent with the full-scale Wechsler Adult Intelligence Scale-R (58). Smokers were smoking on average 12.2 ± 6.4 cigarettes/day with an average smoking history of 6.3 ± 2.3 years. Non-smokers were either never-smokers (n = 22) or former smokers (n = 6). Written informed consent was obtained from all study participants. The study was approved by the Ethics Committee of the Johannes Gutenberg-University in Mainz (Germany).

Behavioral task

Before the experiment, smokers were allowed to smoke ad libitum and imaging was conducted after approximately 1 h of nicotine abstinence. Thus, we expected acute nicotine effects to be minimal from prior smoking as well as no nicotine withdrawal effects during the imaging session (59).

Subjects were required to perform a visual oddball task. The task has been designed to measure the attentional network brain response (41), which has been previously found to be abnormal in smokers during this particular task conditions (42). We employed an ‘event-related’ fMRI design with presentation of 160 visual stimuli (40 targets, 120 non-targets, checkerboard reversal) by means of a back-projection system onto a translucent screen using the ‘Presentatio’ software package (Neurobehavioral Systems Inc.). Subjects were instructed to respond as quickly and accurate as possible to each stimulus by pressing either the left button (non-target) or the right button (target). Stimuli were presented with a duration of 500 ms in counter-balanced and pseudorandomized order at ‘jittered’ inter-stimulus intervals (ISIs) of 6000 ± 500 ms between stimulus onsets. The relatively short and pseudorandomized ISIs were chosen because a similar stochastic design was successfully used in a previous fMRI study (60). As behavioral outcome measure, motor responses (latency, intra-subject variability) were recorded through a fiber optic response box. The total duration of the task was 960s.

Magnetic resonance image scanning

Imaging was conducted with a 1.5 T Siemens Sonata® scanner using an 8-channel head coil. In order to avoid head movements, the head of each subject was tightly fixed with cushions during the scanning procedure. To facilitate localization and co-registration of functional data, structural scans were acquired using T1-weighted MRI sequences [Magnetization prepared rapid gradient echo (MP-RAGE): TR/TE = 2860/3.93 ms, flip angle = 15°, 176 slices, slice thickness = 1 mm, matrix: 176 × 256 × 256. While subjects performed the visual oddball task, event-related fMRI data [TR/TE = 3000/60 ms, flip angle = 90°, field of view (FOV) = 192 × 192 mm², matrix = 64 × 64, 25 axial slices, voxel dimensions 3.0 × 3.0 × 5.5 mm³] were collected. Stimulus presentation was triggered continuously by slice acquisition of functional images. The imaging experiment was previously validated in a subset of the present sample to measure attentional network function (41).

Image analysis

fMRI-analysis was performed with FSL (FMRIb’s Software Library, www.fmrib.ox.ac.uk/fsl). After motion artefact correction using the SIEMENS motion correction, the remaining motion artefacts were < 1 mm and therefore, no subject was excluded for excessive motion. The following pre-statistics processing was applied: Employing different modules of the FSL-software package, we conducted motion correction using MCFLIRT (61), non-brain removal using BET (62), spatial smoothing using a Gaussian kernel of FWHM = 6 mm,
mean-based intensity normalization of all volumes by the same factor and highpass temporal filtering (Gaussian-weighted LSF straight line fitting, with sigma = 9.0 s). General linear model (GLM) time-series statistical analysis of individual data sets was carried out using FILM (FMRIB’s Improved Linear Model) with local autocorrelation correction (63). Registration of functional images to high-resolution structural images was carried out using FLIRT (61,64). For further analysis of the functional data, we used one explanatory variable (i.e. visual target stimulus) convolved with a Double-Gamma hemodynamic response function. The Double-Gamma function is a mixture of two Gamma functions—a standard positive function and a small delayed, inverted Gamma to model the late undershoot. We calculated cluster-corrected \( Z > 2.3 \) voxel-by-voxel BOLD target responses for each individual followed by a group level mixed effect analysis which was conducted with FLAME (FMRIB’s Local Analysis of Mixed Effects) (65) with spatial normalization to Montreal Neurological Institute (MNI) space and applying a cluster significance thresholded of \( Z > 2.3 \) (64,66,67). For visual display of the spatially transformed group contrasts, Z-maps (voxelwise, uncorrected; \( Z > 1.6, P < 0.05 \)) of the functional data were imported to AFNI (Analysis of Functional Neuro Images software; http://afni.nimh.nih.gov/afni/afni) and cortical surface maps (inflated) were created with SUMA (Surface Mapping AFNI). The precise localization of maximum BOLD-responses was determined with AFNI using three common reference systems: Talairach-Tournoux Atlas, Cytoarchitectonic Probability Map Atlas and MNI.

Subsequently, we conducted VOI analyses for group comparisons as previously described (41). These analyses were based on region-of-interests which we have identified in previous electromagnetic source analyses and fMRI studies under similar task conditions (41,60,68–71). One VOI encompasses the (bihemispheric) medial frontal lobe (MFL) with maximum activation in the supplementary motor area (SMA) and includes the adjacent dorsal anterior cingulate cortex (ACC) (left: mean volume = 374.5 ± 53.1 voxels; right: mean volume = 163.5 ± 18.9 voxels). The other VOI includes the parietal cortex (left hemisphere only), an area which is activated in conjunction with the electromagnetic P300-wave (mean volume = 350.4 ± 33.6 voxels). VOIs were created on the basis of the spatially transformed group ‘activation’ masks of the entire group of \( n = 47 \) subjects (thresholded at \( Z > 2.3 \)) which were subsequently transformed back to individual brain size which resulted in individual VOI-sizes for each subject. Within the resulting VOIs of each individual subject, we calculated the mean \( Z \)-value as well as the number of ‘activated’ voxels above a statistical threshold of \( Z > 2.3 \).

Genotyping

DNA was extracted from anticoagulated venous blood using standard techniques. Three synonymous SNPs from exon 5 of CHNRA4 were selected (rs1044394, rs1044393, rs1044396). These SNPs span a region of 990 bp encompassing SNP rs1044396, which has been associated with nicotine dependence in the two studies of Feng et al. (38) and Li et al. (39). Genotyping of SNPs was carried out by a TaqMan nuclease assay (72) using Taqman MGB probes and primer provided by the Assays-on-Demand service (Applied Biosystems, Foster City, CA, USA). The samples were amplified with GeneAmp PCR System 9700 thermocyclers (Applied Biosystems). Allelic discrimination was performed by measuring the fluorescence intensity of reporters at the polymerase chain reaction (PCR) end-point using an ABI Prism 7900 HT system and the SDS software version 2.1 (Applied Biosystems).

Statistics

Statistical analyses were conducted with ANOVA, \( t \)-tests, \( \chi^2 \) tests or regression analyses (Spearman \( R \)) as appropriate.

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Conflict of Interest statement: None declared.

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