Genetic architecture of dystonia
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Citation for published version (APA):

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CERVICAL DYSTONIA AND GENETIC COMMON VARIATION IN THE DOPAMINE PATHWAY

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ABSTRACT

Cervical dystonia, a late onset focal dystonia, has a complex genetic background. Multiple lines of evidence point to a role for aberrant dopamine levels in dystonia. We assessed whether common variation within genes that regulate brain dopamine levels and in key genes of the dopamine metabolic pathway, modulate the risk for cervical dystonia. DNA was collected from 363 Dutch CD patients and a cohort of Dutch control individuals. Haplotype-tagging single nucleotide polymorphisms (SNPs) complemented with selected variants of functional importance in COMT, DAT, TH, MAO-A and -B, DDC and DBH were investigated. We tested the 143 markers in single-SNP, haplotype and epistasis analyses. We did not find an association with any of the selected 143 SNPs in these key dopamine genes. Our data shows that common variations in key genes of the dopamine pathway do not contribute to dystonia risk in the Dutch population. Possibly, risk alleles in this pathway may be rarer than detectable in this study, or might be located in downstream dopamine signalling pathway. Alternatively, found dopamine level changes are secondary to the dystonia disease processes.
INTRODUCTION

Cervical dystonia (CD), a primary adult-onset focal dystonia, is the most frequent form of dystonia in Caucasians (prevalence 1 in 4,500)\(^1\) with an assumed complex genetic background. Changes in dopamine (DA) signalling have been implicated in the pathogenesis of dystonia: mutations in GTP cyclohydrolase I and Tyrosine Hydroxylase, both essential in DA production, cause dystonia (DYT5, MIM128230); imaging studies suggest a hyperdopaminergic state in focal\(^2\) and familial dystonias\(^3\); post-mortem measurement of tissue in the striatum showed a significant increase in the striatal DOPAC/DA ratio in dystonia patients, suggestive of increased DA turnover\(^4\); DA changes are found in striatum of dystonia animal models (see for a review Tanabe and colleagues\(^5\)).

The presynaptic DA transporter (DAT, SLC6A3, OMIM: 126455) and catechol-O-methyltransferase (COMT, OMIM 116790) are crucial for the regulation of synaptic DA levels in the brain. DAT is the main regulator of dopamine signaling; it recycles DA back into the presynaptic terminal, thus terminating synaptic firing. In the 3′-UTR of DAT a 40-bp variable number of tandem repeat (VNTR) polymorphism is present, which acts as a modulator of gene transcription. The 9-repeat allele is associated with increased DAT availability in striatum, compared to the 10-repeat allele.\(^6\)

In COMT, four SNPs located in the promoter region (rs6269) and coding region His62His (rs4633), Leu136Leu (rs4818), and Val158Met (rs4680)) form three functional haplotypes. These variants modulate protein translation efficiency by altering the mRNA secondary structure, influencing enzymatic activity.\(^7\) DAT and COMT genes interact non-additively to modulate cortical function during executive processing.\(^8\) Other enzymes involved in production and degradation of DA, and thus control the synaptic DA availability, are the Tyrosine Hydroxylase (TH, OMIM191290), DOPA decarboxylase (DDC, OMIM07930), dopamine β-hydroxylase (DBH, OMIM09312), and monoamine oxidase A and B (MAOA, OMIM309850; MAOB, OMIM309860) genes. (Figure 1)

As disturbance of DA levels in the striatum has been observed in dystonia, genetic factors influencing the synaptic DA availability may be associated with CD. In this study we assessed whether common variation within genes that regulate DA levels and in key genes of the DA metabolic pathway, modulate the risk for CD. Three hundred sixty three Dutch CD patients and a large cohort of Dutch control individuals were studied for common variation in COMT, DAT, TH, MAO-A and -B, DDC and DBH by using a haplotype-tagging approach complemented with selected SNPs of functional importance.
Study populations

All studied patients and controls were Dutch Caucasians. All procedures were carried out with written consent of the subjects involved and with the ethical approval of the Academic Medical Center Amsterdam institutional review board (www.amc.nl).

CD patients – A group of 363 patients (66.9% female) with idiopathic non-familial CD were genotyped. All were diagnosed with primary dystonia based on the accepted criteria and included in a clinical database. Mean age at examination was 56.7 years (SD 13.0) and mean age at dystonia onset was 41.9 years (SD 13.6).

Three control groups were used (Table 1):

1. Controls for DAT and COMT: 720 sex-matched controls from the Dutch National Blood Bank;
2. Controls for DA metabolism: as genome-wide data from the Rotterdam study III (ERGO Young) was available, we used these as control data for the dopamine pathway. This included genome-wide genotyping data from 2082 control participants (55.6% female) genotyped with Human610K Beadchips from Illumina (http://www.illumina.com). The mean age was 53.75 years with a range of 45-95 years; and
3. Controls for MAO-A and MAO-B: because the X chromosome was not covered in this genome-wide array, we genotyped (SNPlex Genotyping system, Life Technology) the informative SNPs in MAO-A and MAO-B in 1308 controls of The Longitudinal Aging Study Amsterdam (LASA control cohort). LASA is an ongoing cohort study of people aged 55–85 years.

Figure 1. Components and enzymes in the DA metabolic pathway. TH = tyrosine hydroxylase, DDC = DOPA decarboxylase, DBH = dopamine β-hydroxylase, PNMT = phenylethanolamine N-methyltransferase, COMT = catecholamine-O-methyltransferase, MAO = monoamine oxidase, DOPAC = dihydroxyphenylacetic acid, MHPG = 3-methoxy-4-hydroxyphenylglycol.

(adapted from: http://www.genome.jp/kegg/pathway/map/map00350.html)
**SNP selection and Genotyping**

COMT and DAT: rs6269, rs4818 and rs4680 tag the common functional haplotype blocks in COMT.\(^{11}\) SNPs were genotyped with a TaqMan discrimination assay from Applied Biosystems (http://www.appliedbiosystems.com). The 40-bp VNTR in the DAT gene was amplified following a standard protocol with previously described primers.\(^6\) PCR products were separated by electrophoresis in an ethidium bromide-stained 3% agarose gel, effectively separating different VNTR numbers.

**DA Metabolic pathway SNP screen:** SNPs within Tyrosine Hydroxylase (\(TH, OMIM191290\)), DOPA decarboxylase (\(DDC, OMIM107930\)), dopamine\(\beta\)-hydroxylase (\(DBH, OMIM609312\)) monoamine oxidase A (\(MAOA, OMIM309850\)) and MAOB (\(OMIM309860\)) (Figure 1) were assessed using a haplotype-tagging approach, complemented with variants selected for possible functional importance. Haplotype-tagging SNPs were selected using the Haploview program with \(r^2\) threshold set on 0.8. We identified 1,918 SNPs in the six candidate genes (NCBI dbSNP database, build 125) including 5kb of flanking sequence on 5’- and 3’-side. From this, 139 SNPs were selected following our selection criteria: (a) SNPs with minor allele frequency (MAF) > 5% according to dbSNP, HapMap databases or re-sequencing in 47 Caucasian individuals; (b) SNPs in potentially functional regions: at a potential transcription factor (TF) binding site in the promoter region (up to 15 kb in front of the first two exons) according to P-Match or TFSEARCH programs, or located within the coding region, or located within the 3’-UTR, especially at a potential microRNA binding site according to the miRBase or miRanda programs, or in a splicing site according to the NetGene2 program, B5 at conserved region (75% consistent rate) according to the Vista program; (c) the independent SNP, or the minor allele of a SNP specifically represents one or several subset haplotype allele(s) in a linkage disequilibrium (LD) block -according to the HapMap data; (d) Including haplotype tagging SNPs of the HapMap data; (e) The maximum distance between SNPs was about 5 kb. In brief, SNPs were distributed with a maximum of 5kb distance, had a minimum MAF of 5%, were haplotype tagging SNPs (haplotypes with a frequency of 0.05 and higher) or were in potential functional regions.

**Re-sequencing** – To determine the allele frequency of SNPs in potentially functional region (promoter region, exons and 3’-UTR) of \(TH, DDC\) and \(MAOA\) and \(MAOB\) genes,
we re-sequenced in total 21 kb of fragments within these genes using 47 healthy Dutch individuals of European ancestry. We detected 132 SNPs and 31 (23%) of them are informative (MAF >5%) SNPs, and 21 informative SNPs were selected for our association studies according to our selection criteria.

The SNPs were genotyped with three assays of the SNPrlex Genotyping system 48-plex (Life Technology), following the standard protocol. Electrophoresis and data collection was performed with an ABI3730 DNA analyzer from Applied Biosystems. Genotypes were called with GeneMapper® Software v4.0 provided by ABI. The Human610K Beadchip from Illumina includes 620,901 SNPs, 30 of our selected “dopa” SNPs were included in this panel. Using Markov Chain based haplotyper (MACH; version 1.0.16) the remaining 71 “dopa” SNPs were imputed.

**Imputation of data** – Markov Chain based haplotyper (MACH; version 1.0.16) on every dataset to impute genotypes for all participants of European ancestry with haplotypes derived from initial low coverage sequencing of 112 European ancestry samples in the 1000 Genomes Project (as of August 2009, http://www.sph.umich.edu/csg/abecasis/MACH/download/1000G-Sanger-0908.html). For all datasets, data were imputed by a two-stage design. The first stage generated error and crossover maps as parameter estimates for imputation on a random subset of 200 samples per study over 100 iterations of the initial statistical model. We used these parameter estimates to generate maximum likelihood estimates of allele numbers per SNP on the basis of reference haplotypes for the datasets during the second stage of the imputation. SNPs with RSQR quality estimates of less than 0.30 as indicated by MACH were excluded from analyses of the datasets, because imputed genotypes below this threshold are probably of poor quality. Mean MAF of 101 SNPs is 0.29 (0.017-0.5), mean Quality of imputation is 0.97 (0.81 – 1.00; SD 0.04), mean R² 0.94 (0.19 – 1.00; SD 0.11). Genome-wide genotyping data from 2082 control participants from the Rotterdam study III were used.

**Data Analysis**

Single variant association was performed using PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/), using additive, dominant and recessive genetic models and allele association. The cutoff p-value of Hardy-Weinberg equilibrium was 0.001. Haplotype construct in COMT, haplotype association and gene–gene interaction analysis was performed using Haplostats (http://cran.r-project.org/web/packages/haplo.stats/index.html). The rare haplotypes were pooled into a single category. The most frequent haplotype is the default and chosen as the base-line category for the design matrix. Logistic regression analysis using an additive mode of association and sex as covariate was performed. Power calculations were performed using the online available Genetic Power Calculator (http://pngu.mgh.harvard.edu/-purcell/gpc/).
RESULTS

Summary statistics of genotyping – Of 139 SNPs in the SNPlex, 2 probes failed for all CD samples, and 3 SNPs (2.1%) did not reach the 90% genotyping threshold in the CD group and were excluded from further analysis. Mean genotype failure rate was 2.9% (range 0.8% – 8.8%). For the TaqMan experiments, genotype quality was high (overall missing values: rs6269 = 0.5%; rs4818 = 2.2%; rs4680 = 0.5%) and PCR amplification failure for analysis of the DAT VNTR was 3.8%. All studied SNPs in the controls were in Hardy-Weinberg equilibrium. For this study (360 cases and 720 controls, the lowest number of controls used) a 85% power to detect association of SNPs with a MAF of 0.05 with an effect of OR 1.7 is calculated.

COMT and DAT – In DAT, the 10-VNTR and 9-VNTR alleles together accounted for 97% of detected variation (range 8-11 repeats). No significant differences in 10/9-allele frequencies (p = 0.41) and genotype frequencies (p = 0.13 (2df); dominant model p = 0.15 (1df)) were detected between cases and controls. In COMT no statistical significant differences in allele frequencies for rs6269 (p=0.5896), rs4818 (p=0.6818) and rs4680 (p=0.8596) were observed between CD cases and controls (total genotyping rate of 98%). Haplotype analyses revealed six haplotypes tagged by rs6269-rs4818-rs4680. The three most frequent haplotypes, accounting for over 99% of all detected haplotypes, are studied for folding effects and enzymatic activity by Nackley et al.11 The frequencies of the haplotypes coding for the High enzyme activity variant (rs6269-rs4818-rs4680: G-G-G) and Low enzyme activity haplotype (A-C-G) were similar in cases and controls. (see Table 2).

Table 2. Association study with functional haplotype constructs (rs6269|rs4818|rs4680) of the COMT gene.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Activity</th>
<th>MAF CD</th>
<th>MAF Control</th>
<th>t-test</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-G-G</td>
<td>high</td>
<td>0.38</td>
<td>0.36</td>
<td>0.22</td>
<td>0.64</td>
</tr>
<tr>
<td>A-C-G</td>
<td>low</td>
<td>0.08</td>
<td>0.09</td>
<td>0.36</td>
<td>0.55</td>
</tr>
<tr>
<td>A-C-A</td>
<td>average</td>
<td>0.54</td>
<td>0.54</td>
<td>0.01</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Previous studies showed additive and multiplicative interactions for the effects of the DAT VNTR and COMT haplotypes on brain activity.12 We did not find a significant interaction of effects for VNTR genotype (9 repeats as minor allele) with low or high COMT activity haplotype, resulting in an OR of 1.24 (95%CI 0.43 – 3.56; p= 0.68) and OR 1.06 (95%CI 0.60 – 1.87; p= 0.83), respectively after sex correction. To look if the extreme combinations (low activity COMT – DAT 10-VNTR (high DA levels) and high activity COMT – DAT9-VNTR (low DA levels) were overrepresented in the CD group, we assessed the frequencies of these combinations, using a autosomal dominant model for 9R-VNTR DAT. Low-activity COMT – DAT 10R had a frequency of 24.4% in cases and 22.5% in controls (p=.679); high activity COMT – DAT9R was seen in 17.8% of cases and 16.8% of controls (p=.817).
Variants in DA metabolizing proteins – The 139 genotyped SNPs in the DA pathway were located within TH (13 SNPs), COMT (12), MAO (36), DDC (51) and DBH (27). Most SNPs were located in intronic regions (110 SNPs), others in coding regions (10 SNPs) or UTRs (19 SNPs). (data available on request and online: http://www.prd-journal.com/article/S1353-8020(12)00342-2/addOns) We examined allele and additive model association between 363 CD cases and imputed data from 2082 Rotterdam Study controls. After Bonferroni multiple testing correction, 2 SNPs remained significant in allelic association: rs4074905 in TH (p = 0.04418) and the coding variant rs6271 in DBH (p = 1.402 x 10^{-23}). Both SNPs were tested with TaqMan assays in 720 additional healthy controls (Control Group 1: Blood Bank samples). Between this second control group and CD patients no association was found for either SNP: rs4074905 p = 0.28 (Pearson) OR = 0.86 (95% CI: 0.66 – 1.12) and rs6271 p= 0.59 with OR = 1.165 (0.66 –2.02).

DISCUSSION

Here, we performed the first pathway-based association study in CD. In this population, common variation in DA producing and metabolizing genes does not increase the risk for cervical dystonia. Single variant and haplotype association in COMT and DAT, as well as global analysis for interaction, did not show a significant difference between cases and controls. Inefficient cortical function has been related to both unusually high as unusually low dopamine activity. Maximal and minimal dopamine levels are produced by specific combinations of low activity COMT – DAT 10-VNTR and high activity COMT – DAT 9-VNTR, respectively. The frequencies of these extreme combinations were not significantly different in cases and controls in our study.

Next to COMT and DAT, we studied common genetic variation in other key proteins of dopamine production and metabolism (TH, MAO-A, MAO-B, DBC, DDH). From the first SNP screen, comparing CD patients with ERGO Young population, two significant associations were found (rs4074905 and rs6271). However, replication failed in a second control group (Dutch National Blood bank). Found frequencies were also similar to MAF in European samples in dbSNP 132. Therefore, we conclude that in our population common variation in key proteins in the dopamine pathway is not associated with CD.
We choose for a pathway-based approach, as this is less random than single SNP association studies, and requires only a fraction of the samples needed for genome-wide approaches to have sufficient power. Our study had 85% power to detect allelic effects of variants with a MAF of 0.05 with an odds ratio of 1.7. It should be realized that smaller OR might be expected and will be missed with this study size. Negative findings could also reflect type II errors due to the stringent multiple testing correction (Bonferroni). Also, it is possible that in our population no such association exists, however we cannot exclude associations of dopamine variants in other populations or in different types of primary dystonia. Also, the use of variable control series and use of imputation could lead to a lack of association. Risk alleles with a frequency of 5% or higher were studied. In genetic complex diseases like CD also risk alleles with lower frequencies (rare and low-frequency variants) could well be involved.

In summary, although previous imaging and functional studies in primary dystonia pointed to aberrant basal ganglia dopamine handling, our data shows that common variations in key genes of the dopamine pathway do not contribute to dystonia risk in the Dutch population. Possibly, risk alleles in this pathway may be more rare than detectable in this study, or might be located in downstream DA signalling pathway (e.g. dopamine receptors) or, alternatively, dopamine level changes are secondary to the dystonia disease processes.

ACKNOWLEDGMENTS

We are grateful to all participants. We thank Dr. Michael Tanck for his statistical expertise. This study was supported by the Prinses Beatrix Fund.
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