Doctoral (PhD) Thesis

Repeat polymorphisms of the dopaminergic neurotransmitter genes in dog

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I. INTRODUCTION

The molecular genetic background of psychological traits and psychiatric diseases has recently been in the focus of numerous studies. The genetic polymorphisms of the neurotransmitter systems could play an important role in the development of these phenotypes, Attention Deficit Hyperactivity Disorder (ADHD), novelty seeking, extraversion, neuroticism are some of the most thoroughly investigated traits. Psychogenetic studies demonstrated that heritability of most disorders is estimated to be between 0.4 and 0.6, whereas this value is as high as 0.7–0.8 for ADHD. Despite, it is known that a rather large number of genes as well as the environmental factors play a role together in the development of these complex traits, consequently it's important to take into consideration that the contribution of one or a few genes is usually very small, sometimes even hard to detect. Candidate gene association analysis is one of the most successful methods for the investigation of the genetic background of polygenic characteristics. Association analyses investigate the possible relationship between allelic variations of the selected gene(s) and the phenotypic traits of interest in a certain preferably large sample population. The candidate genes of psychological / psychiatric association analyses are most often selected from those playing a role in signal transduction or in the synthesis / degradation of neurotransmitters.

One of the most studied targets of the psychiatric genetic studies has been the variable number of tandem repeats (VNTR) polymorphism in exon 3 of the dopamine D4 receptor gene (DRD4) since an association was demonstrated between this polymorphism and novelty seeking personality trait more than a decade ago. Further studies revealed that attention deficit hyperactivity disorder shares several common characteristics with novelty seeking and demonstrated that this genetic variation can be considered as one of the risk factors of the disease.

Application of animal models is a general approach in behavioral genetic research, these studies employed traditionally rats and mice as model animals. On the other hand the application of dogs has recently become an emerging alternative, because their behavioral characteristics, e.g. social attachment, aggression and cooperation were demonstrated to be fairly similar to those of humans. Moreover, the relationship of dogs and their owners is also very similar to those between humans, consequently canines seem to be better models than rodents in several contexts. Their application in behavioral genetic studies is also supported by the fact that sequencing of the dog genome was completed in 2005, and since then more and more genes and their polymorphisms have been available in public databases. All these observations initiated the more thorough analysis of genetic variations in the genome of numerous canine breeds, moreover their association with different phenotypic traits is also getting into the focus of interest.

Although association studies clearly demonstrate the connection between candidate gene polymorphisms and the investigated trait, the causative relationship between the gene and the phenotype often remains uncertain. The biological consequence of genetic variations in exons is usually more comprehensible, as they often result in an amino acid change which can interfere the proper function of the protein in several ways (e.g. changing the three dimensional structure of the coded protein, destroying the active site of an enzyme / receptor etc.). On the other hand it is also getting clear that polymorphisms in non-coding regions (5' and 3' untranslated sequences, introns) are also of great importance because they may play a role in the regulation of gene expression. Several human studies described the *in vitro* functional analysis of such polymorphisms in promoter or intronic regions, while the number of similar investigations in dogs has been more limited so far.

II. OBJECTIVES

Genetic polymorphisms of the neurotransmission systems are intensively studied in human because of their putative influence on psychological traits and psychiatric disorders. Our **aim was to investigate the genetic variability of the DRD4 gene and the behavioral association in dogs**, as dogs are readily applicable as models for the analysis of human behavior.

1. **Association analysis of the DRD4 gene exon 3 VNTR and activity / impulsivity endophenotype of dogs.** Based on human studies to avoid false positives arising from population stratification we analyzed a single breed (German shepherds).
2. **Analysis of other polymorphisms of the DRD4 gene.** Population analysis of DRD4 exon 1 and intron 2 insertion/deletion polymorphisms in several dog breeds. Functional analysis of the intron 2 variation in luciferase reporter vector system.

Further repeat polymorphisms were searched in the canine neurotransmission system, these novel genetic variations can be important targets of subsequent association studies.

1. **Search for novel variable number of tandem repeats (VNTR) polymorphisms.** Identification of repeat sequences *in silico* in the dopaminergic system. Analysis of these regions with PCR-based methods in several dog breeds and wolves.
2. **Search for copy number variations (CNV).** Investigation of the dosage of some genes in the dopaminergic and serotonergic system of dog, as similar analyses haven't been done yet.

III. METHODS

Subjects

DNA samples of the investigated animals were collected by the Department of Ethology. Buccal epithelial cells were obtained from a number of dog breeds: 1) three subpopulations of the Belgian shepherd: groenendael (N=105), tervueren (N=101), and malinois (N=50); 2) 309 German shepherds, where we distinguished two environmentally different groups, such as pets (N=137) and police dogs (N=172); 3) 99 Siberian huskies and 4) 22 European gray wolves.

The collection of DNA was approved by the owners of the studied animals and by the Hungarian National Police.

Genotyping

The sequence of the investigated genes (dopamine D4 receptor, tyrosine hydroxylase, dopamine-β-hydroxylase, dopamine transporter) was obtained from the GenBank and Ensembl databases. The sequence of the exon 3 of the DRD4 gene was obtained from an earlier publication. The investigated genes were *in silico* searched for repetitive sequences applying the web-based Tandem Repeats Finder search tool.

The repeat number of the putative VNTR regions was investigated by polymerase chain reaction.

Copy number variation (CNV)

The gene dosage analysis was performed using qPCR (real-time PCR). Specific TaqMan probes and primers were designed for the investigated genes (*DRD4, TH, DBH, DAT, COMT, MAO-A, V1aR*). The 5' end of the probes was labeled with VIC or FAM reporter dye, while MGB (minor groove binding) quencher was employed at the 3' end of the TaqMan probes.

Transient transfection

The functional analysis was carried out in luciferase reporter system. The allelic variations of the intron 2 of the DRD4 gene were amplified by PCR using primers with restriction enzyme recognitions site and were cloned into pGL3-Basic and pGL3-Control reporter vectors. Transient transfection was performed in human neuroblastoma (SK-N-FI) and epithelial carcinoma (HeLa) cell lines. Vector containing β-galactosidase gene was applied as transfection control.

Phenotyping

Phenotypic characterization and the statistical evaluation were carried out in collaboration with the Department of Ethology supervised by Dr. Adam Miklosi. The previously validated dog-ADHD RS Owner Version questionnaire was used to investigate the activity-impulsivity endophenotype of dogs.

Statistical analysis

SPSS 10.0 statistical software package was used for the evaluation of the results. The rates of activity-impulsivity of of the pet and police German shepherds with different genotypes were assessed by independent samples t-tests. The analysis of the scale rates of the two groups of German shepherds was carried out by Mann-Whitney U-test, the possible effect of the age was analyzed by Spearman correlation.

Abbreviations:

DRD4: dopamin D4 receptor, TH: tyrosine hydroxylase, DBH: dopamine-β-hydroxylase, DAT: dopamine transporter, COMT: catechol-O-methyltransferase, MAO-A: monoamine oxidase A, V1aR: vasopressin 1a receptor, ADHD: attention deficit hyperactivity disorder, bp: basepair, VNTR: variable number of tandem repeats

IV. RESULTS

1. Two PCR-based methods were optimized for the analysis of the VNTR in exon 3 of the DRD4 gene in dogs. Two allelic variations (*2* and *3a*) were detected in the Germen shepherd population. The animals were separated into two different groups based on their living conditions: pet and police dogs. In our study we analyzed the putative relationship between the DRD4 gene exon 3 VNTR and the activity-impulsivity of dogs measured by the dog-ADHD questionnaire. Significant association could be demonstrated among police dogs: **animals with 2/2 genotype showed significantly lower activity-impulsivity scores compared to dogs with 2/3a or 3a/3a genotype (P = 0.018).**

2. The **DRD4 gene exon 3 VNTR was investigated in further dog breeds** (described in the Methods section in detail) **and a novel allelic variant** (allele "8") **was identified**, with a considerably high frequency in Siberian huskies (18.2%) and European gray wolves (29.6%).

3. The 24 bp insertion/deletion polymorphism in **exon 1 of the DRD4** gene described previously was analyzed in the above mentioned dog breeds and wolves. The genotype frequencies of the investigated populations showed a major difference, which was demonstrated to be statistically significant by the χ^2 test (**P < 0.0001**) regarding both VNTR polymorphisms of the DRD4 gene.

4. The 17 bp insertion/deletion in **intron 2 of DRD4** gene was identified earlier, our *in silico* analysis revealed however that this region can also be considered as a **VNTR** polymorphism. Functional characterization

of the region was carried out by *in vitro* system in two cell lines (SK-N-FI and HeLa), and it was shown that the intron 2 VNTR region possesses **promoter activity** and might **regulate gene expression**, however, in a cell type specific manner.

5. **Further repetitive sequences were found in the genes of the dog dopaminergic system with *in silico* search.** These regions were further analyzed in our dog populations, and it was found that the 36-bp-long sequence in intron 4 of the TH gene and the 38-bp-long sequence in intron 9 of the DAT gene was present either in a single copy or as a duplicated form. Furthermore, the 17-bp variation in intron 4 of the DBH gene was also polymorphic, either 1 or 3 repeats could be observed in our samples. The difference of the genotype frequencies among the breeds was statistically significant ($P < 0.0001$) regarding all polymorphisms, and the measured genotype frequencies didn't differ from the Hardy-Weinberg equilibrium.

6. A **real-time PCR based method was elaborated for** the search of the putative **copy number variations (CNVs)** of genes in the dopaminergic and serotonergic system. Sequence specific TaqMan probes were designed for 7 genes studied. Relative quantification (ΔC_T method) was used for the analysis of the gene dosages. According to our best knowledge, no analysis of CNVs in the dog genome has been published yet, consequently no specific endogenous control gene could be used, instead, data obtained for the investigated genes were compared to each other. Twenty animals were analyzed in each breed in our pilot study, however **we found neither deletion nor amplification in the investigated regions.** It concludes to the observation that no CNVs with higher than 5% occur in these regions of the genome in the investigated populations.

V. CONCLUSIONS

Application of animal models is an alternative approach in the field of the research of behavioral genetics. Dogs were used in our study because of the interesting fact, that canines possess a variable number of tandem repeats polymorphism in exon 3 of DRD4 gene similar to that of humans, whereas neither rats, nor mice have an analogue sequence variation in the appropriate gene segment.

One of the major aims of our work was the study of any putative association between the dog DRD4 exon 3 VNTR and some endophenotypes of the ADHD, because it was suggested that this gene is a candidate of the disease in human. Significant association was demonstrated between the activity-impulsivity scale of the dog ADHD rating scale and the 2/2 genotype of the DRD4 exon 3 VNTR in police German shepherds. A novel variant of the polymorphism was also identified and shown to be quite common among Siberian huskies and European gray wolves.

It was demonstrated that the 17 bp deletion in intron 2 of the DRD4 gene can also be considered as a VNTR. It was also shown, that the region possesses a promoter activity in *in vitro* reporter system, consequently might play a role in the regulation of gene expression.

Unknown polymorphisms were found *in silico* and *in vitro* in the the gene of the tyrosine hydroxylase, dopamine-β-hydroxylase and dopamine transporter. These novel polymorphisms can be important targets of further functional and association studies.

A real-time PCR based method was elaborated for the analysis of copy number of genes in the dopaminergic and serotonergic system. Although the experiments proved the reliability of the novel approaches, no CNVs were detected in the investigated regions.

VI. PUBLICATIONS

Publications related to the theses:

I. **Hejjas K**, Vas J, Topal J, Szantai E, Ronai Z, Szekely A, Kubinyi E, Horvath Z, Sasvari-Szekely M, Miklosi A. Association of polymorphisms in the dopamine D4 receptor gene and the activity-impulsivity endophenotype in dogs. Anim Genet. 2007 Dec;38(6):629-33. (IF.: 1.52)

II. **Hejjas K**, Vas J, Kubinyi E, Sasvari-Szekely M, Miklosi A, Ronai Z. Novel repeat polymorphisms of the dopaminergic neurotransmitter genes among dogs and wolves. Mamm Genome. 2007 Dec;18(12):871-9. (IF.: 2.279)

Further publication:

III. **Hejjas K**, Szekely A, Domotor E, Halmai Z, Balogh G, Schilling B, Sarosi A, Faludi G, Sasvari-Szekely M, Nemoda Z. Association between depression and the Gln460Arg polymorphism of P2RX7 Gene: A dimensional approach. Am J Med Genet B Neuropsychiatr Genet. 2008 Jun 9. *in press* (IF.: 4.463)

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