doi:10.1111/j.1365-2052.2007.01657.x

Association of polymorphisms in the *dopamine D4 receptor* gene and the activity-impulsivity endophenotype in dogs

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Summary

A variable number of tandem repeats (VNTR) polymorphism in exon 3 of the human *dopamine D4 receptor* gene (*DRD4*) has been associated with attention deficit hyperactivity disorder (ADHD). Rodents possess no analogous repeat sequence, whereas a similar tandem repeat polymorphism of the *DRD4* gene was identified in dogs, horses and chimpanzees. Here, we present a genetic association study of the *DRD4* VNTR and the activity-impulsivity dimension of the recently validated dog-ADHD Rating Scale. To avoid false positives arising from population stratification, a single breed of dogs (German shepherd) was studied. Two *DRD4* alleles (referred to as *2* and *3a*) were detected in this breed, and genotype frequencies were in Hardy–Weinberg equilibrium. For modelling distinct environmental conditions, 'pet' and 'police' German shepherds were characterized. Police German shepherds possessing at least one *3a* allele showed significantly higher scores in the activity-impulsivity dimension of the dog-ADHD Rating Scale than dogs without this allele ($P = 0.0180$). This difference was not significant in pet German shepherds. To the best of our knowledge, this is the first report of an association between a candidate gene and a behaviour trait in dogs, and it reinforces the functional role of *DRD4* exon 3 polymorphism.

Keywords activity-impulsivity, dog, *dopamine D4 receptor* gene, genetic association, polymorphism.

Introduction

Domestic dogs (*Canis familiaris*) have the potential to model various aspects of human social behaviour (Hare & Tomasello 2005) including attachment (Topál *et al.* 2005), communicative abilities (Miklosi & Soproni 2006) and temperament traits (Draper 1995). Dogs also offer an opportunity for understanding the genetic basis of behaviour regulation (Ostrander & Comstock 2004) and provide new insights into human genetic diseases (Ostrander *et al.* 2000). The investigation of dogs provides additional advantages compared with humans, such as reduced genetic heterogeneity within breeds and long extent of linkage disequilibrium. Moreover, the dog's unique evolutionary history in the human ecological niche (Schleidt & Shalter 2003) and its naturalistic socialization to humans provide further applicability of a comparative approach (Gomez 2005).

The variable number of tandem repeats (VNTR) in exon 3 of the *dopamine D4 receptor* gene (*DRD4*) is one of the most thoroughly investigated candidate genes in human studies, which has been related to the personality trait 'novelty seeking' (Ebstein *et al.* 1996) and to a frequent child psychiatric problem, attention deficit hyperactivity disorder (ADHD) (Faraone *et al.* 2005). Genetics plays a crucial role in the development of these traits, with heritability (h^2) estimates of ADHD as high as 80% (Thapar *et al.* 2000). A similar repeat polymorphism of the *DRD4* gene has been identified in other mammalian species, such as horses (Momozawa *et al.* 2005), non-human primates (Livak *et al.* 1995) and dogs (Niimi *et al.* 1999), although not in rodents (e.g. O'Malley *et al.* 1992). The dog *DRD4* VNTR consists of 27-, 39-, and 12-bp units, resulting in seven alleles, as

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Accepted for publication 2 August 2007

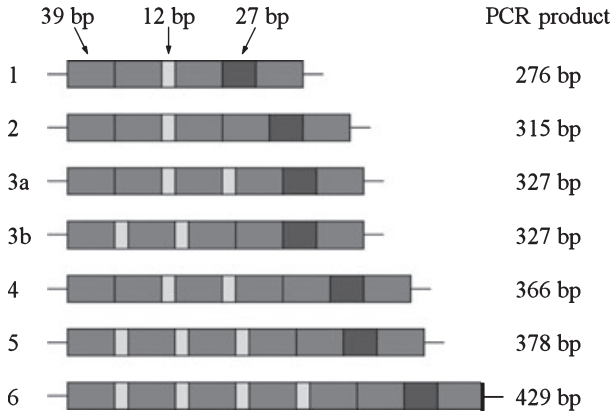

Figure 1 Diagram of the repeat region in exon 3 of the *dopamine D4 receptor* (*DRD4*) gene. Seven alleles were identified by Niimi *et al.* (1999) consisting of 27- (dark block), 39- (medium coloured) and 12-bp (light block) units. The name of the alleles and the size of the corresponding PCR product are also shown.

shown in Fig. 1 (Niimi *et al.* 1999). These *DRD4* gene variations may be related to behavioural traits such as excitability and aggression (Niimi *et al.* 1999). The *DRD4* VNTR allele frequencies of 23 dog breeds have been determined and correlated with behavioural differences by Ito *et al.* (2004). The 23 breeds were divided into two main groups based on the allele frequencies of this polymorphism. Dogs belonging to group A possessed a higher frequency of alleles *2* and *3a*, whereas the *3b*, 5 and *6* alleles were more frequent among the animals of group B. Phenotypes of the dogs were analysed using a questionnaire to owners, and it was observed that animals of group B obtained a higher average score of 'aggressiveness' and a lower value of 'reactivity' compared with individuals in group A. A slightly different approach was employed in our association study of the *DRD4* VNTR. To avoid false positives arising from population stratification of sexually isolated, closed breeding populations (Hamer & Sirota 2000), we analysed the behavioural effect of the exon 3 polymorphism of the *DRD4* gene in a single breed – the German shepherd – rather than comparing various breeds.

The investigation of endophenotypes (i.e. a well-characterized component of a complex trait that can be precisely, preferably numerically measured, and is believed to be genetically determined) is an emerging approach in genetic association studies, as it holds promise for discovering the complex genetic background of multifactorial phenotypes, such as psychiatric disorders. Parental questionnaires are frequently used for quantitative behavioural studies of human infants, as they can be applied for the high-throughput measurement of several endophenotypes. Using a similar approach, questionnaires for dog owners about behaviour traits of their dogs are now used (Hsu & Serpell 2003). The human ADHD Rating Scale (ADHD RS) Parent Version questionnaire (DuPaul 1998) was adapted and validated for dog owners (Vas *et al.* 2007), and used in our study to characterize activity-impulsivity and attention of German shepherds raised in two environmental conditions (pet dogs and police dogs).

Materials and methods

Genotyping

Buccal epithelial cells were obtained from 241 unrelated German shepherds by the Department of Ethology (Budapest, Hungary) and by the Hungarian National Police Training School for Police Dog Handlers (Dunakeszi, Hungary). DNA was extracted using the Gentra DNA purification kit. The first PCR amplification of *DRD4* VNTR was performed in a 10-μl reaction mixture containing 0.25 U DNA-polymerase, 1x Q-solution and 1x buffer (final $MgCl_2$ concentration 1.5 mM) from the Qiagen HotStarTaq DNA polymerase kit; 1 μM of both the forward (D1c: 5'-CGC GCG TCG GGC CAA GCT G-3') and the reverse (D2c: 5'-GCG GGG GGC AGG GGG CG-3') primers; 5 ng DNA template and 200 μM of each dNTP. PCR primers were designed by the OLIGO 5.0 software based on the published gene sequence (Niimi *et al.* 1999). PCR products were separated by 1.5% agarose–2% Metaphor composite gel electrophoresis and visualized by ethidium bromide. Another independent PCR reaction was performed to separate the *3a* and *3b* alleles according to Niimi *et al.* (1999), using the forward primer in combination with an allele specific reverse primer D4dogBR.

Phenotyping

Pet dog owners and the guides of the police dogs filled out the recently validated dog-ADHD RS Owner Version questionnaire (Vas *et al.* 2007; Appendix S1), containing 13 items (seven items from the activity-impulsivity scale, for example, 'It leaves from its place when it should stay'; and six items from the inattention scale, for example,'It's difficult for it to concentrate on a task or play'). Scale scores were calculated for each dog as the sum of the scores given by the owner (range: 0–3). A total of 189 German shepherds (138 males, 51 females, age mean ± SD: 4.69 ± 2.88 years) were characterized. Based on the training and housing conditions, dogs were classified as either pets (55 males, 47 females, 2.86 ± 2.08 years) or police dogs (83 males, four females, 6.70 ± 2.22 years). SPSS for Windows was used for all statistical analyses. Distribution of genotype frequencies in the two subgroups were assessed by chi-square tests performed on a 2 × 3 contingency table. Association of the ADHD questionnaire scores with the *DRD4*-VNTR polymorphism was assessed by independent samples *t*-tests.

Results

Two alleles (the 315- and 327-bp variants) were detected in our sample and referred to as alleles *2* and *3a* respec-

Table 1 *DRD4* VNTR allele and genotype frequencies in the studied German shepherd population.

		Genotype frequencies (%)			Allele frequencies (%)	
Group	*n*	*2/2*	*2/3a*	*3a/3a*	*2*	*3a*
Police dog	95	49.5	42.1	8.4	70.5	29.5
Pet dog	146	38.3	45.2	16.4	61.0	39.0
Total group	241	42.7	44.0	13.3	64.73	35.3

DRD4, *dopamine D4 receptor*; VNTR, variable number of tandem repeats.

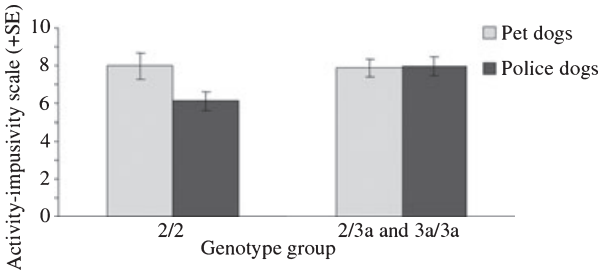

Figure 2 Activity-impulsivity scores of pet and police German shepherds as a function of *DRD4* variable number of tandem repeat (VNTR) genotypes. No difference was demonstrated in the activity-impulsivity scores between pet dogs with the *2/2* genotype vs. the *2/3a* and *3a/3a* combined genotype ($t_{(100)} = -0.134$, $P = 0.8951$). However, police dogs with the *2/2* genotype showed significantly lower activity-impulsivity scores compared with police dogs with the *2/3a* or *3a/3a* genotype ($t_{(85)} = 2.412$, $P = 0.0180$).

tively (see Table 1 and Fig. 1). The measured genotype frequencies (Table 1) fit well to the frequencies predicted by the Hardy–Weinberg equilibrium ($P = 0.8528$). Distribution of the genotypes in the two subgroups (pet dogs vs. police dogs) did not show any significant differences ($P = 0.1015$).

One hundred and eighty-nine of the 241 genotyped dogs were characterized by the dog-ADHD RS questionnaire (Appendix S1). No significant differences were found in average scores of activity-impulsivity ($t_{(187)} = -1.224$, $P = 0.223$) or in inattention scores ($t_{(187)} = 0.441$, $P = 0.660$) between the pet dogs and police dogs. Effect of sex and age were also analysed, as the majority of police dogs but not the pets were males, and police dogs were older on average. We did not find any significant effect of sex (two-samples *t*-test $t_{(187)} = -0.762$, $P = 0.447$ for activity-impulsivity and $t_{(187)} = -0.057$, $P = 0.955$ for inattention). Moreover, age was not correlated with the inattention questionnaire scores (Spearman correlation, $\rho = 0.044$, $P = 0.553$), and the correlation between age and activity-impulsivity questionnaire scores was weak ($\rho = -0.206$, $P = 0.005$).

Raw scores of the dog-ADHD RS scales according to the *DRD4* VNTR genotypes are shown in Table 2. For statistical analysis, the rare *3a/3a* genotype category was combined with *2/3a* heterozygotes. There was no difference in the activity-impulsivity scores between dogs with *2/2* genotype vs. the *2/3a* and *3a/3a* combined genotype group either in the total sample ($t_{(187)} = 1.228$, $P = 0.2209$) or in pet dog group ($t_{(100)} = -0.134$, $P = 0.8951$). In contrast, police dogs with *2/2* genotype showed significantly lower activity-impulsivity scores compared with police dogs with *2/3a* or *3a/3a* genotype ($t_{(85)} = 2.412$, $P = 0.0180$, see also Fig. 2). Similar comparisons did not show any significant differences for the inattention scores (total sample: $t_{(187)} = -0.120$, $P = 0.9074$; pet dogs: $t_{(100)} = 0.298$, $P = 0.7667$; police dogs: $t_{(85)} = -0.529$, $P = 0.5985$).

Discussion

In this study, the VNTR polymorphism of the *DRD4* gene was investigated in more than 200 Hungarian German shepherd dogs. The measured genotype frequencies were in Hardy–Weinberg equilibrium, suggesting that major stratification of the investigated sample was not observed. Our results are in a good agreement with the results of the Ito *et al.* (2004) study reporting the same two alleles (labelled as *2* and *3a* respectively) as the most frequent variants among 25 German shepherds. They reported two additional alleles in that breed (labelled as *3b* and 5 respectively) with low allele frequencies, whereas our sample did not contain those variants. The differences in the Hungarian and Japanese samples may reflect geographical alterations caused by a popular sire or a founder effect.

Phenotypic characterization of our dog sample was performed using a previously validated dog-ADHD RS questionnaire (Vas *et al.* 2007). Questionnaires as indirect

Table 2 Sum scores of dog-ADHD RS Owner Version questionnaires as a function of *DRD4* VNTR genotypes.

	Activity-impulsivity score[1]		Attention deficit score[1]	
Group	*2/2*	*2/3a* and *3a/3a*	*2/2*	*2/3a* and *3a/3a*
Pet	8.0 ± 0.7 (49)	7.9 ± 0.5 (53)	4.6 ± 0.4 (49)	4.7 ± 0.4 (53)
Police	6.1 ± 0.5 (34)	8.0 ± 0.5 (53)	5.0 ± 0.6 (34)	4.7 ± 0.4 (53)
Total	7.2 ± 0.5 (83)	7.9 ± 0.4 (106)	4.7 ± 0.3 (83)	4.7 ± 0.3 (106)

ADHD RS, attention deficit hyperactivity disorder rating scale; *DRD4*, *dopamine D4 receptor*; VNTR, variable number of tandem repeats.

[1] Mean ± standard error values are shown; number of animals is in parentheses.

behaviour evaluation methods have been validated for human subjects, as well as for dogs (Hsu & Serpell 2003). Our attempt to adapt the ADHD RS questionnaire for characterizing dog behaviour is based on the assumption that the human social environment provides a natural niche for dogs. Moreover, there are some similarities between the experiences of dogs and children in their respective social environment. Therefore, dogs might serve as an appropriate model of several human traits, such as child (hyper)activity. The data presented here demonstrated that the activity-impulsivity scale of the dog-ADHD RS may be a useful tool to assess the 'activity-impulsivity' endophenotype among dogs.

This study presents the first genetic association study of the *DRD4* gene polymorphism and the activity-impulsivity endophenotype in a dog model using a single breed population. Single breed samples assure homogenous genetic background for an association analysis of the phenotypic effect with a candidate gene. Our results show a significant association between the *DRD4* VNTR polymorphism and the activity-impulsivity score of police dogs. However, the same association was not significant among pet dogs. This may be because police dogs can be regarded as an environmentally homogeneous group (they all went through the same special training and they are kept in similar environmental conditions and under similar stress levels). The same genotype–phenotype association could not be demonstrated in pet dogs, where the various environmental effects, such as the attitude of the owners, the quality of training, etc. presumably surpass the subtle genetic effect of the *DRD4* polymorphism. It is also notable that the percentage of female dogs differed greatly in the police and pet dog samples. However, neither sex nor age of the dogs influenced the obtained questionnaire scores. These findings also point to a gene-environment interaction, an important element of behavioural genetic studies as shown in human studies (Caspi & Moffitt 2006). Our sample, however, was probably not large enough to show a statistically significant gene-environment interaction. Therefore, we intend to broaden our studies for more detailed characterization of various endophenotypes in dogs and their relation to environmental factors.

Acknowledgements

This research has been supported by the European Union (NEST 012787) and the OTKA (T029705).

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Supplementary material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2007.01657.x

Appendix S1 Dog-ADHD RS Owner Version questionnaire.

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