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Molecular and behavioral analysis of the intron 2 repeat polymorphism in the canine dopamine D4 receptor gene

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Genetic polymorphisms in the human dopamine D4 receptor (*DRD4*) gene, especially the exon 3 variable number of tandem repeats (VNTR), have been related to several psychiatric disorders and personality traits. A homologous exon 3 VNTR has been described in dogs, and we previously showed an association between the *DRD4* exon 3 polymorphism and activity/impulsivity trait in German shepherds. In this study, we present a detailed analysis of the intron 2 VNTR of the *DRD4* gene. A short and a long form of the intronic variation were identified in 678 unrelated dogs from five breeds and in 22 wolves. For molecular analysis, the intron 2 region was cloned into a promoterless luciferase reporter vector that led to an elevation in transcriptional activity. Moreover, an allelic difference in promoter activity was detected, and a repressive effect of the long allele was observed. Behavioral analysis of 96 unrelated German shepherds showed a significant association between the social impulsivity endophenotype of the Greeting Test and both the exonic ($P = 0.002$) and the intronic ($P = 0.003$) VNTRs of the *DRD4* gene. Moreover, an additive effect of the two polymorphisms was also shown (Spearman's rho = 0.356, $P = 0.0004$). In conclusion, these results give further support to our previous findings that the *DRD4* gene is associated with dog behavior. We also present molecular evidence for the functional role of the intron 2 VNTR in the canine *DRD4* gene.

Keywords: Canine, dopamine D4 receptor, endophenotype, exon 3 VNTR, German shepherd, intron 2 VNTR, social impulsivity

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Genetic analysis of dog behavior has great potential given that the canine social system and complex social cognition are known to be closely related to those in humans in several aspects, unlike the widely used rodent and primate models (Hare & Tomasello 2005; Miklósi *et al.* 2004). Accordingly, recent studies have suggested the consideration of canine populations as a model system for the investigation of the genetic background of human behavior (Bjornerfeldt *et al.* 2008; Miklósi 2008; Overall 2000; Saetre *et al.* 2006; Spady & Ostrander 2008).

Association studies between candidate gene polymorphisms and personality traits in humans were initiated by Ebstein *et al.* (1996), who showed an association between the dopamine D4 receptor (*DRD4*) gene exon 3 variable number of tandem repeats (VNTR) polymorphism and novelty seeking personality trait. This was followed by numerous studies (Ebstein 2006, review), and a meta-analysis of case–control and family-based studies showed an association between this VNTR and attention deficit hyperactivity disorder (ADHD), indicating the crucial role of the *DRD4* and its polymorphisms not only in behavior but also in psychiatric conditions (Faraone & Khan 2006). Another functionally active length polymorphism was also described in the 5′ noncoding region of the human *DRD4* gene (D'Souza *et al.* 2004; Kereszturi *et al.* 2007) and found to be associated with ADHD (Kereszturi *et al.* 2007; Kustanovich *et al.* 2004).

The exon 3 VNTR of the *DRD4* gene has also been identified in other mammalian species such as horses (Hasegawa *et al.* 2002), nonhuman primates (Livak *et al.* 1995) and dogs (Niimi *et al.* 1999), and it was analyzed in association with personality traits in horses (Momozawa *et al.* 2005) and nonhuman primates (Bailey *et al.* 2007). The canine exon 3 VNTR was found to be related to excitability, aggression (Niimi *et al.* 1999) and reactivity (Ito *et al.* 2004). Furthermore, we previously described an association between the *DRD4* exon 3 VNTR and the activity/impulsivity endophenotype in German shepherd police dogs (Hejjas *et al.* 2007). Besides the exon 3 VNTR, a novel 17-bp insertion/deletion length polymorphism was identified in intron 2 of the dog *DRD4* gene by Nara *et al.* (2005). The putative functional effect of the intron 2 polymorphism on gene expression level has not been tested. Here, we aimed to study the functional role of the intronic VNTR in the canine *DRD4* gene. Moreover, we present a thorough association analysis between *DRD4* gene polymorphisms and social impulsivity.

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Materials and methods

Animals

A total of 687 unrelated animals of five dog breeds (Belgian Tervueren, $n = 101$; Belgian Groenendael, $n = 105$; Belgian Malinois, $n = 50$; German shepherd, $n = 323$; Siberian husky, $n = 99$) and 22 European gray wolves (*Canis lupus*) were used in this study. Genetic analyses and behavioral testing of the animals were approved by the owners.

Phenotyping

The applied Greeting Test was based on the Social Contact Test described by Svartberg (2005) as a part of the dog mentality assessment test used in numerous studies (Ruefenacht *et al.* 2002). The test was performed on 96 unrelated German shepherds (mean age: 3.8 ± 2.8, 54% male and 46% female) as follows. The experimenter (a stranger to the dog, E) approached the leashed dog, speaking continuously to the animal. When the dog acted 'friendly' (i.e. moving toward the experimenter and tail wagging) or showed neutral behavior, E stepped toward the dog and patted its head, back and shoulder successively. E then stepped 1 m sideways within reach of the leash. If the dog followed her, she patted the dog again. Otherwise, E waited 2–3 seconds and then called the dog. If the dog still did not approach, E crouched and called it again. If there was still no response, E went to the dog and patted it, then left. If the dog avoided E or showed aggressive behavior (i.e. barking or growling), E crouched (out of reach) and tried to call the dog. If the dog showed an approaching tendency and was not aggressive, E stepped next to the dog and patted it (see above). If the dog did not change its behavior, the trial was terminated after 30 seconds.

Social impulsivity was coded as follows: 0 points if the dog did not act friendly, 1 point if the dog showed friendly behavior but did not follow the experimenter when stepping away and 2 points if the dog followed the experimenter when stepping away. Higher scores in this variable indicate increased interest in novel social companions, which has been described as social impulsivity by Fairbanks (2001).

Genotyping

A buccal smear was collected, and DNA was isolated by the Gentra purification kit (Valencia, CA, USA). The intron 2 VNTR was amplified by the Qiagen HotStarTaq DNA-polymerase kit (Qiagen, Valencia, CA, USA). The forward primer (5′-GCC ATC AGC GTG GAC AGG T-3′) was obtained from the study of Nara *et al.* (2005), and the reverse primer (5′-GCC CTG GCG GTT GTA ACT CA-3′) was designed using the OLIGO 5.0 software. The following thermocycle was applied: 95°C for 15 min, then 35 cycles of 94°C for 1 min, 65°C for 30 seconds and 72°C for 1 min. The final extension was 72°C for 10 min. Polymerase chain reaction products were 193 and 210 bp, corresponding to the *P* and *Q* alleles, respectively. Raw genotype data of the exon 3 VNTR were obtained from our previous study (Hejjas *et al.* 2007).

Plasmid constructs

The pGL3-Basic (pGL3-B) and pGL3-Control (pGL3-C) luciferase reporter vectors (Promega, Madison, WI, USA) were used to clone the +28 722 348 to +28 722 523 region of canine *DRD4* intron 2 (according to ENSCAFG00000006562 in Ensembl database). Primers containing *Xho*I and *Hin*dIII or *Mlu*I and *Xho*I recognition sites were used for pGL3-B and pGL3-C constructs, respectively. For pGL3-B, sense 5′-AAC **CTC GAG** GCC ATC AGC GTG GAC AG-3′ and antisense 5′-GCC **AAG CTT** GCC CTG GCG GTT GTA ACT CA-3′ primers were used; for pGL3-C, sense 5′-AAC **ACG CGT** GCC ATC AGC GTG GAC AG-3′ and antisense 5′-GCC **CTC GAG** GCC CTG GCG GTT GTA ACT CA-3′ primers were used. (Bold letters indicate the recognition sites of the above-mentioned restriction endonucleases.) The templates were German shepherd genomic DNA samples with known genotypes.

Cell culture

The SK-N-FI (neuroblastoma) cell line was grown in Dulbecco's modified Eagle's medium, high glucose (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% nonessential amino acids. HeLa cells were cultured in minimal essential Eagle medium (Gibco) supplemented with 10% fetal bovine serum, 1% nonessential amino acids and 1% Na pyruvate. Both cell lines were grown at 37°C with 5% CO_2.

Transient transfections

Cell lines were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). A mixture of 0.3 μg reporter construct and 0.3 μg pCMV-β-gal and 6 μl Lipofectamine 2000 was used to transfect 1.2×10^6 SK-N-FI or 0.5×10^6 HeLa cells plated 24 h before transfection in six-well plates.

Luciferase and β-galactosidase assay

Cells were extracted by three consecutive freeze–thaw cycles and subsequent centrifugation. Supernatants were used for enzyme activity determination. Luciferase and β-galactosidase activities were detected using the Luciferase Assay System kit (Promega) and the *O*-nitrophenyl-β-D-galactopyranoside cleavage rate, respectively. Luminescence was measured using a Mithras LB940 multilabel reader (Berthold Technologies, Bad Wildbad, Germany). Luciferase promoter activity was normalized to β-galactosidase activity. Three parallel trials were used in all transfections and all experiments were performed in triplicate.

Statistical analysis

Lewontin's *D′* value was calculated using the GOLD 1.0 software package (Abecasis *et al.* 2000) to assess linkage disequilibrium between the *DRD4* intron 2 and exon 3 VNTRs. Statistical analysis for transcriptional data was performed using one-way ANOVA followed by the Tukey–Kramer multiple comparison test (GRAPHPAD INSTAT 3.05). The raw scores of social impulsivity were used as dependent variables, and *DRD4* exon 3 and intron 2 VNTR genotypes were tested as the grouping factor with sex and age as covariates for univariate analysis of variance (ANOVA) using SPSS for Windows 13.0. Pairwise multiple comparisons of genotype group means were calculated with *post hoc* tests (Tukey Honestly Significant Differences). To assess joint effects of the two VNTR genotypes, an ordinal variable was computed, and nonparametric correlations (Spearman's rho) were used to test the association between the behavioral trait and the double genotype groups.

Results

Allele and genotype distribution of the *DRD4* intron 2 VNTR in dogs and wolves

The intron 2 VNTR in the dog *DRD4* gene was described by Nara *et al.* (2005) to be an insertion/deletion of a 17-bp-long region; however, the detailed structure of the polymorphism was not discussed. Based on a thorough sequence analysis, we found that the 17-bp region was present in triplicate in the longer (*Q*) allele, while the middle module was deleted in the shorter (*P*) variant (Fig. 1). The three 17-bp modules were separated by 13- and 12-nucleotide-long spacer sequences, respectively that were found to be remarkably homologous. Thus, we refer to this polymorphism as the *DRD4* intron 2 VNTR.

The allele and genotype frequencies of the intron 2 VNTR were determined in 101 Belgian Tervuerens, 105 Belgian Groenendaels, 50 Belgian Malinois, 323 German shepherds,

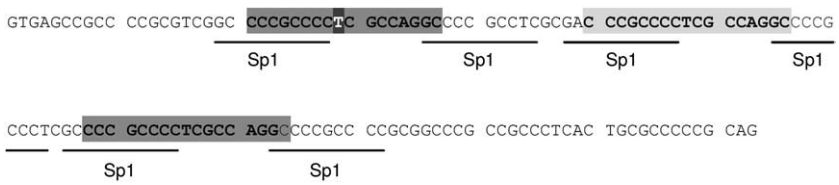

Figure 1: Structure of the *DRD4* intron 2 VNTR. This figure shows the entire sequence of the canine DRD4 intron 2 long (*Q*) allele (133 bp). Dark boxes depict the repetitive sequences present in the short (*P*) form. The light box indicates the extra 17-bp repeat element of the long variant. The white T nucleotide in the first module is the position of the CT SNP that is in complete linkage with the VNTR. The long allele carries a T (as shown in the figure), whereas the short form contains a C at this site. Putative Sp1 transcription factor-binding sites are also shown based on *in silico* sequence analysis using the TFSEARCH (Searching Transcription Factor Binding Sites v. 1.3) web-based search engine.

99 Siberian huskies and 22 European gray wolves (*C. lupus*) (see Table 1). Both long (*Q*) and short (*P*) alleles of intron 2 VNTR were detected in all five dog breeds. Interestingly, in the 22 European gray wolves tested, no *Q* allele was found. No significant deviation from Hardy–Weinberg equilibrium was detected in any of the dog breeds (Belgian Tervueren: $P = 0.982$, Belgian Groenendael: $P = 0.692$, Belgian Malinois: $P = 0.926$, German shepherd: $P = 0.217$, Siberian husky: $P = 0.915$).

Analysis of linkage disequilibrium between the DRD4 intron 2 and exon 3 VNTRs

Lewontin's *D′* value was calculated to evaluate linkage disequilibrium of the intron 2 and exon 3 VNTRs in the *DRD4* gene (Table 1). In the European gray wolf, linkage disequilibrium could not be assessed because these animals were not polymorphic at the studied intron 2 site. Complete linkage disequilibrium ($D' = 1$) was observed in the Belgian Tervueren and Groenendael populations, while *D′* values for the other dog breeds varied between 0.760 and 0.886.

Molecular analysis of the dog DRD4 intron 2

To investigate the putative promoter characteristics of the intron 2 region, the entire intronic sequence was cloned into the pGL3-B promoterless vector upstream of the luciferase reporter gene. pGL3-C constructs were also generated to test the potential repressor function of intron 2, with the analyzed region ligated upstream of the simian virus 40 (SV40) constitutively expressing promoter and the luciferase gene. Both the *P* and *Q* polymorphic variants were cloned into both constructs. Although, a single nucleotide polymorphism (SNP) was also detected at position 28 722 399 (according to ENSCAFG00000006562) in the first module of the intron 2 VNTR, it is known that this SNP is in complete linkage disequilibrium with the VNTR (Nara *et al.* 2005; Fig. 1). Accordingly, our *P* allele constructs contained the C variant, while *Q* allele vectors carried the T form of the SNP. Transient transfections were carried out using SK-N-FI human neuroblastoma and HeLa human epithelial carcinoma cell lines in triplicate.

Intron 2 alleles cloned into the pGL3-B vector showed elevated transcriptional activity compared with the pGL3-B vector in both SK-N-FI and HeLa cells ($P < 0.001$; Fig. 2a). The *P* allele caused a sevenfold elevation, while the *Q* allele showed a fivefold increase in relative luciferase activity compared with the promoterless vector in the neuroblastoma cell line (Fig. 2a). Similarly, a moderate but still significant (3.5- to 4-fold) increase in transcriptional activity was measured for constructs containing either the *P* or *Q* allelic variant in non-neuronal HeLa cells. The pGL3-B P and Q vectors only showed different transcriptional activity patterns (Fig. 2a) in the neuronal SK-N-FI cell line: the shorter intronic variant showed significantly higher transcriptional activity compared with longer form ($P < 0.01$). Interestingly, in non-neuronal HeLa cells, this allelic difference was undetectable. These results raise the possibility that the dog *DRD4* intron 2 region may function as an alternative promoter for the gene.

In the pGL3-C experimental setup, vectors with the short (*P*) and long (*Q*) alleles were cloned upstream of the SV40 promoter. The intron 2 VNTR region with the short (*P*) form showed slightly lower transcriptional activity than the pGL3-C vector in the SK-N-FI cell line; however, this difference was not significant (Fig. 2b). On the other hand, the construct containing the *Q* allele showed significantly ($P < 0.001$) lower relative luciferase activity than the *P* variant. In contrast, two- to threefold transcriptional activity ($P < 0.001$) elevation was detected in HeLa cells using either the pGL3C-P or pGL3C-Q vectors (Fig. 2b); however, no allelic differences could be observed. These results suggest that the intron 2 VNTR region might regulate gene expression, however, in a cell type-specific manner.

Table 1: Genotype distribution of the *DRD4* intron 2 VNTR in five dog breeds and European gray wolves

	Genotype frequencies of *DRD4* intron 2 VNTR (%)				
Dog breeds	*P/P*	*P/Q*	*Q/Q*	*n*	*D′*
Belgian Tervueren	14.9	48.5	36.6	101	1.000
Belgian Groenendeal	29.5	45.7	24.8	105	1.000
Belgian Malinois	30.0	52.0	18.0	50	0.760
German shepherd	48.3	39.3	12.4	323	0.886
Siberian husky	3.0	5.1	91.1	99	0.781
European gray wolf	100.0	0.0	0.0	22	1.000

n, number of dogs investigated in each breed; *D′*, Lewontin's *D′* value for linkage disequilibrium between the *DRD4* intron 2 and exon 3 VNTRs.

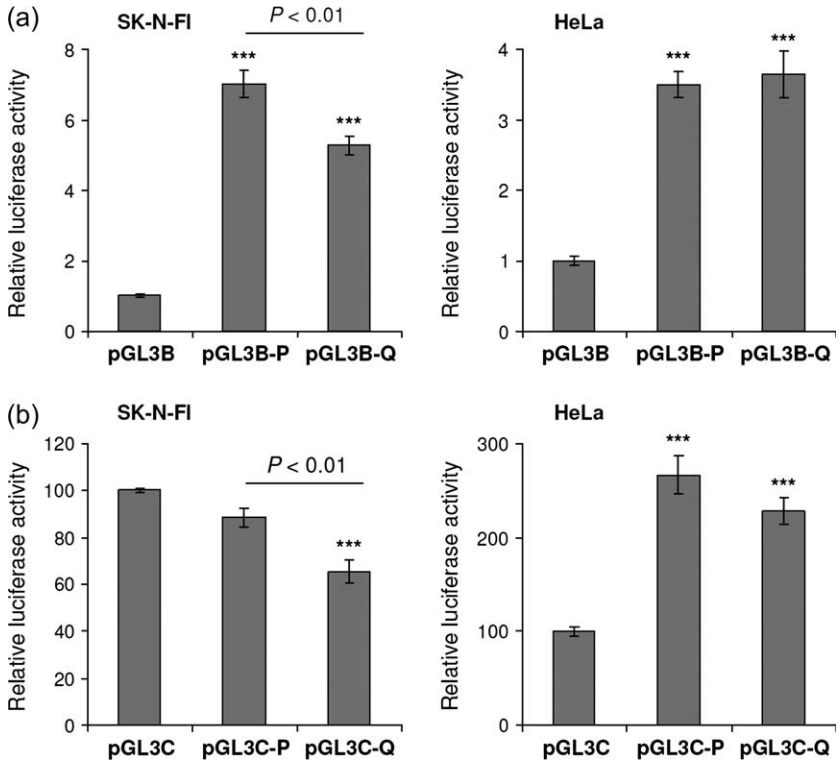

Figure 2: Functional characterization of the *DRD4* intron 2 VNTR. The entire *DRD4* intron 2 region (28 722 348–28 722 523 according to Ensembl ENSCAFG00000006562) carrying *P* or *Q* alleles of *DRD4* intron 2 VNTR were subcloned into the pGL3-B (a) and pGL3-C (b) luciferase reporter vectors. Transient transfections were performed in SK-N-FI and HeLa cells. Luciferase activity was normalized to β-galactosidase activity. Data are presented relative to the pGL3-B (a) or pGL3-C (b) activity and shown as mean ± SD. Results of a representative experiment are shown as measured in triplicate (***$P < 0.001$). Similar data were obtained from three independent experiments.

Association between social impulsivity and the intron 2 and exon 3 VNTRs of the DRD4 gene in German shepherds

The association between social impulsivity and the assessed *DRD4* polymorphisms was tested by ANOVA. Both the exon 3 and intron 2 VNTRs had a significant effect on the measured behavioral parameter by univariate analyses (exon 3: $F_{2,91} = 5.66$, $P = 0.005$, power = 0.851 and intron 2: $F_{2,89} = 5.34$, $P = 0.006$, power = 0.828; Table 2). *Post hoc* analyses of the exon 3 genotype group means showed significant differences between the 2/3 and 3/3 genotypes ($P = 0.001$) and the 2/2 and 3/3 groups ($P = 0.009$), while the mean values of the 2/2 and 2/3 categories did not differ significantly, suggesting a dominant effect of the short (2) allele. Therefore, the 2/2 homozygotes and the 2/3 heterozygotes were combined, and two groups (short allele present: 2/2 + 2/3 vs. short allele absent: 3/3) were tested in the subsequent analyses. It was observed that mean score for social impulsivity was significantly different ($F_{1,92} = 10.45$, $P = 0.002$, power = 0.892) between dogs with 3/3 genotype (1.36) and the other group of animals (2/2 + 2/3) that possessed at least one short allele (0.87; Table 2).

Post hoc analyses for intron 2 showed similar findings. Significant differences were found between the social impulsivity mean values of *P*/*Q* and *Q*/*Q* animals ($P = 0.014$) and those of *P*/*P* and *Q*/*Q* animals ($P = 0.042$). Mean values of the *P*/*P* and *P*/*Q* genotype groups did not differ significantly; thus, they were grouped for further analyses, and again two

Table 2: Significant association of the *DRD4* exon 3 and intron 2 VNTR genotypes and mean raw scores of social impulsivity

Polymorphic sites	Exon 3 VNTR			Intron 2 VNTR		
Genotype	2/2	2/3	3/3	*P*/*P*	*P*/*Q*	*Q*/*Q*
n	44	30	22	50	32	14
Social Impulsivity	0.93 ± 0.59	0.80 ± 0.61	1.36 ± 0.58	0.98 ± 0.60	0.84 ± 0.64	1.43 ± 0.51
F		$F_{2,91} = 5.66$			$F_{2,89} = 5.34$	
P (ANOVA)		0.005			0.006	
Genotype combinations	2/2 or 2/3		3/3	*P*/*P* or *P*/*Q*		*Q*/*Q*
n	74		22	82		14
Social Impulsivity	0.87 ± 0.60		1.36 ± 0.58	0.93 ± 0.61		1.43 ± 0.51
F	$F_{1,92} = 10.45$			$F_{1,90} = 9.06$		
P (ANOVA)	0.002			0.003		

categories (short allele present: *P*/*P* + *P*/*Q vs.* short allele absent: *Q*/*Q*) were defined. Similar to the above results, a significantly distinct ($F_{1,90} = 9.06$, $P = 0.003$, power = 0.846) mean score (1.43) was observed in the *Q*/*Q* genotype group compared with animals with at least one short allele at this site (*P*/*P* + *P*/*Q*, 0.93; Table 2).

Taking these results together with the observation discussed above that the two polymorphisms were in linkage disequilibrium, it was important to decide if the association was present with both polymorphisms only because they were linked or because they both had a separate influence on the phenotype. To address this question, the joint effect of the *DRD4* exon 3 and intron 2 categories was investigated. Based on the *post hoc* analyses presented above, four groups were formed: (1) short allele present at both sites (exon 3: 2/2 + 2/3 and intron 2: *P*/*P* + *P*/*Q*), (2) short allele present at the exon 3 VNTR only (exon 3: 2/2 + 2/3 and intron 2: *Q*/*Q*), (3) short allele present at the intron 2 VNTR only (exon 3: 3/3 and intron 2: *P*/*P* + *P*/*Q*) and (4) no short allele at either VNTR (exon 3: 3/3 and intron 2: *Q*/*Q*). Univariate analysis of the four double genotype categories resulted in a significant effect on the measured behavioral assay ($F_{3,88} = 4.63$, $P = 0.005$, power = 0.879). Figure 3 depicts that dogs lacking short allele at both VNTRs ($n = 10$, category 4) had a mean social impulsivity value of 1.6. It is notable that this value was higher than that of the other three categories (short allele present at both sites: 0.87, $n = 70$; short allele present at the exon 3 VNTR only: 1.0, $n = 4$; short allele present at the intron 2 VNTR only: 1.25, $n = 12$), and more importantly, it was also higher than the mean scores of the exon 3 3/3 or the intron 2 *Q*/*Q* groups (Table 2) when the two polymorphisms were tested separately. *Post hoc* analyses of the mean scores of the double genotype groups showed a significant difference between categories 1 (short allele present at both sites) and 4 (no short allele at either VNTR) ($P = 0.003$). Nonparametric correlation between a constructed ordinal variable for the above double genotype categories and the 3-point scale used for scoring social impulsivity showed a significant positive association (Spearman's rho = 0.356, $P = 0.0004$), supporting an additive effect of the *DRD4* exon 3 and intron 2 polymorphisms. This was more pronounced in the absence of short alleles.

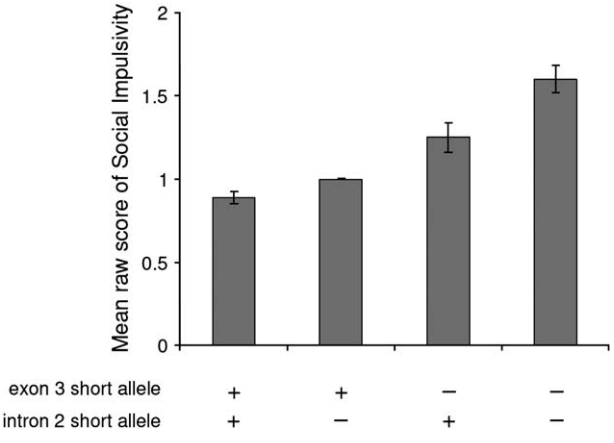

Figure 3: Effect of the *DRD4* exon 3 and intron 2 VNTRs on social impulsivity. The average of mean raw scores is plotted in the double genotype categories. Groups were defined based on the presence (+) or the absence (−) of the short allele at the intron 2 and exon 3 VNTRs, respectively, as described in detail in the *Results* section.

Discussion

It is known that numerous behavioral characteristics have a strong genetic background in several species. Moreover, a study investigating large dog populations has shown that personality traits are considerably inherited in German shepherds (Saetre *et al.* 2006). In addition, a number of studies have shown that the *DRD4* gene plays a role in the development of human and animal behavior. In our study, we investigated two polymorphisms (intron 2 and exon 3 VNTRs) of the canine *DRD4* gene at three different levels, genetic, functional and behavioral.

Allele and genotype frequencies of the intron 2 VNTR among five dog breeds (Belgian Tervuerens, Belgian Groenendaels, Belgian Malinois, German shepherds and Siberian Huskies) and European gray wolves have been described. To the best of our knowledge, three of the five dog breeds (Belgian Tervuerens, Groenendaels and Malinois) have not yet been tested for this polymorphism. The allele frequencies of German shepherds and Siberian huskies were published by Nara *et al.* (2005); however, the population they examined was relatively small compared with that of our study (19 Siberian huskies and 15 German shepherds, vs. 99 and 323 animals in our work, respectively; see Table 1).

In the present study, we also carried out a molecular analysis of the canine *DRD4* intron 2 because the functional role of intronic polymorphisms is more in question than that of exonic variants. The investigated region showed remarkable transcriptional activity in neuroblastoma and HeLa cell lines using an *in vitro* luciferase reporter system (Fig. 2). These results suggest that the dog *DRD4* intron 2 region may function as an alternative promoter for the gene. The existence of a secondary promoter-like sequence in intronic regions is known in human genes involved in neurotransmission. It was suggested that the human *DRD4* promoter might be within an intronic region (Todd & O'Malley 2001). This theory was based on previously reported discrepancies in the size of the *DRD4* messenger RNA (mRNA), which was originally reported to be 5.3 kb in a human neuroblastoma cell line as well as in the striatum, medulla, frontal cortex and limbic area of the rat and monkey brain (Van Tol *et al.* 1991). In contrast, the same mRNA was later found to be much shorter (1.5 kb) and expressed at higher levels in the corpus callosum, medulla and subthalamic nucleus (Matsumoto *et al.* 1996). These observed differences suggest that the human *DRD4* gene might have alternative intronic transcriptional start sites. This was also observed for the catechol-*O*-methyltransferase (*COMT*) gene, resulting in the formation of two distinct transcripts with different 5′ ends (Tenhunen *et al.* 1994).

An allelic difference was also described in the cell type-specific silencer function of the canine *DRD4* intron 2 VNTR (Fig. 2). Furthermore, our *in silico* investigation of the dog

DRD4 intron 2 sequence showed that the long variant possessed more (six) Sp1 consensus sequences, while the deletion of a 17-bp module resulted in the loss of two of these transcription factor-binding sites (Fig. 1). This could be responsible for allelic differences. Sp1-like factors are generally known to be activators; however, a negative regulatory transcriptional element in this family was identified in the murine brain [dopamine receptor regulating factor (DRRF)] (Hwang *et al.* 2001). Moreover, a highly overlapping expression profile was also shown for dopamine receptors and DRRF in mouse brain (D'Souza *et al.* 2002). The existence of such transcriptional machinery expressed only in neuronal cell lines might explain the different results obtained with the neuronal SK-N-FI and epithelial HeLa cell lines. It could be hypothesized that a canine homologue of DRRF might influence expression of the dog *DRD4* gene through the intron 2 VNTR; however, other factors could also be important.

An association between the *DRD4* gene and dog behavioral traits has scarcely been tested to date. Ito *et al.* (2004) divided 1535 animals from 23 different dog breeds into two groups based on allele frequencies of the *DRD4* exon 3 VNTR: group A contained dogs with the short allele and group B contained animals with longer variant of this polymorphism. The dog breeds in group B were characterized by a higher average value of 'aggressiveness' and lower 'reactivity' scores compared with animals in group A. On the other hand, combined analysis of dog breeds with various allele frequencies may result in spurious associations because of the possible effect of population stratification (Hamer & Sirota 2000). To avoid false-positive results, we focused on a single breed, the German shepherd, in our previous study of the genetic analysis of behavior in which an association was found between the *DRD4* exon 3 VNTR and the activity/impulsivity dimension (Hejjas *et al.* 2007) of the recently validated dog-ADHD rating scale filled out by the owners (Vas *et al.* 2007). In this study, we used a more objective ethological test, which was based on direct behavioral observation of individual animals. In the widely applied Greeting Test, we measured the variable of social impulsivity among German shepherd dogs encountering a friendly stranger. We found evidence that both the exon 3 and intron 2 VNTRs contribute to the tendency of German shepherds to approach a stranger in a friendly way. This is an important aspect of dog's personality, especially if it is expected to adjust its behavior to the complex social interactions in a human society, where such contacts with strangers frequently occur.

In summary, the current study presents a molecular and behavioral analysis of two polymorphisms of the *DRD4* gene in dogs. We describe evidence for a functional role of the intron 2 VNTR and show an association between both variations and the behavior of German shepherds. These data broaden our knowledge of the *DRD4*, one of the main players in personality.

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