

DRD4 and *TH* gene polymorphisms are associated with activity, impulsivity and inattention in Siberian Husky dogs

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Summary

Both *dopamine receptor D4 (DRD4)* exon 3 and *tyrosine hydroxylase (TH)* intron 4 repeat polymorphisms have been linked to activity and impulsivity in German Shepherd dogs (GSDs). However, the results in GSDs may not be generalisable to other breeds, as allelic frequencies vary markedly among breeds. We selected the Siberian Husky for further study, because it is highly divergent from most dog breeds, including the GSD. The study sample consisted of 145 racing Siberian Huskies from Europe and North America. We found that this breed possesses seven *DRD4* length variants, two to five more variants than found in other breeds. Among them was the longest known allele, previously described only in wolves. Short alleles of the *DRD4* and *TH* repeat polymorphisms were associated with higher levels of activity, impulsivity and inattention. Siberian Huskies possessing at least one short allele of the *DRD4* polymorphism displayed greater activity in a behavioural test battery than did those with two long alleles. However, the behavioural test was brief and may not have registered variation in behaviour across time and situations. Owners also completed the Dog-Attention Deficit Hyperactivity Disorder Rating Scale (Dog-ADHD RS), a more general measure of activity and attention. Siberian Huskies from Europe with two short alleles of the *TH* polymorphism received higher ratings of inattention on the Dog-ADHD RS than did those with the long allele. Investigation of the joint effect of *DRD4* and *TH* showed that dogs possessing long alleles at both sites were scored as less active–impulsive than were others. Our results are aligned with previous studies showing that *DRD4* and *TH* polymorphisms are associated with activity–impulsivity related traits in dogs. However, the prevalence of variants of these genes differs across breeds, and the functional role of specific variants is unclear.

Keywords behaviour, canine, *dopamine receptor D4*, genetic association, sled dogs, *tyrosine hydroxylase*, VNTR polymorphism

Introduction

Dopaminergic genes, such as the *dopaminergic receptor D4 (DRD4)* gene and the *tyrosine hydroxylase (TH)* gene, have been the subject of recent behavioural genetics work in humans and other animals. *DRD4* encodes the D4 subtype of the dopaminergic receptor, whereas *TH* encodes the enzyme tyrosine hydroxylase, which is involved in the

synthesis of L-DOPA, dopamine's precursor. Dopamine itself is also the precursor of the catecholamines norepinephrine and epinephrine. Dopaminergic genes are suspected to influence behaviour, because dopamine is involved in the brain's reward system as well as in cognition, movement control and attention (Nieoullon 2002).

In particular, evidence suggests that polymorphisms of the *DRD4* gene play a role in activity–impulsivity related traits in humans. Much of the research in humans has focused on the variable number of tandem repeats (VNTR) polymorphism in exon 3 of *DRD4*. The five- and seven-repeat variants of this polymorphism have been associated with increased risk of attention deficit hyperactivity disorder (ADHD), whereas the more common four-repeat form

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appears to have a protective effect (Faraone *et al.* 2001; Maher *et al.* 2002; Li *et al.* 2006). The *DRD4* polymorphism also has been linked to impulsivity and financial risk-taking in humans (Dreber *et al.* 2009; Varga *et al.* 2012). In contrast, studies on the relationship between *TH* gene polymorphisms and activity–impulsivity are limited in number and have not yet demonstrated a clear association. For example, no association has been documented between *TH* gene polymorphisms and ADHD (Comings *et al.* 1995; Barr *et al.* 2000; Payton *et al.* 2001). However, this gene has been associated with neuroticism and other anxiety-related traits (Persson *et al.* 1997, 2000), and it has been suggested that neuroticism may be related to ADHD (Nigg *et al.* 2002).

Dopaminergic gene polymorphisms also have been linked to activity–impulsivity related traits in other species. Vervet monkeys possessing the five-repeat variant of the *DRD4* exon 3 polymorphism displayed greater novelty-seeking than did those with two copies of the six-repeat variant (Bailey *et al.* 2007). In passerine birds, a single nucleotide polymorphism on *DRD4* has been associated with differences in exploratory behaviour in both free-living individuals and lines selected for divergent levels of such behaviour (Fidler *et al.* 2007). Furthermore, variants of *DRD4* have been associated with feather-pecking behaviour, considered to be redirected exploratory behaviour, in laying hens (Flisikowski *et al.* 2009). In addition, reduced expression of the *TH* gene has been demonstrated in a rat model for ADHD (Leo *et al.* 2003).

In dogs, the *DRD4* and *TH* genes contain repeat polymorphisms similar to their human counterparts (Niimi *et al.* 2001; Hejjas *et al.* 2007a) and have been recently investigated for associations with activity–impulsivity related traits. In German Shepherd dogs (GSDs), both *DRD4* and *TH* gene polymorphisms were found to be linked to activity and impulsivity (Hejjas *et al.* 2007b, 2009; Kubinyi *et al.* 2012). GSDs working as police dogs and possessing at least one 327-bp long allele of the *DRD4* polymorphism showed significantly higher activity–impulsivity than did dogs lacking this allele (Hejjas *et al.* 2007b). In later studies, *DRD4* and *TH* gene polymorphisms also were found to be associated with activity–impulsivity and social impulsivity in pet GSDs (Hejjas *et al.* 2009; Kubinyi *et al.* 2012). Preliminary investigations into Belgian Tervueren dogs showed that *DRD4* polymorphisms might be linked with inattention as well (Hejjas *et al.* 2007a).

Although these findings have established a link between dopaminergic gene polymorphisms and activity–impulsivity in GSDs, they may not be generalisable to all breeds of dogs. Each breed with closed stud books is a genetically isolated population, and allelic frequencies of the *DRD4* and *TH* genes differ markedly among breeds (Ito *et al.* 2004; Hejjas *et al.* 2007a). Similarly, in humans, the frequencies of alleles and their associations with behaviour vary across populations. For example, in European populations, the

seven-repeat variant of the *DRD4* polymorphism is common and has been associated with ADHD (e.g. Holmes *et al.* 2000; Curran *et al.* 2001; Langley *et al.* 2009). In contrast, in East Asian populations, the seven-repeat variant is very rare (Chang *et al.* 1996), and the two-repeat variant has instead been linked with ADHD (Leung *et al.* 2005). Therefore, associations between dopaminergic gene polymorphisms and activity–impulsivity related traits need to be explored in a variety of breeds.

We selected the Siberian Husky for investigation for three reasons. First, a genome-wide survey revealed that Siberian Huskies have ancient origins, admixed with wolves, and are highly divergent from other dog breeds (Vonholdt *et al.* 2010). The Siberian Husky also displays high heterozygosity in microsatellite data compared with other breeds (Vonholdt *et al.* 2010), and this genetic diversity may enable us to reveal the genetic bases of dogs' behavioural variability in greater depth.

Second, the Siberian Husky is a popular sled dog, and racing individuals are often raised and kept in a more homogenous environment than are pet dogs. These homogeneous environments may facilitate the detection of gene–behaviour relationships. Third, genetic associations involving Siberian Huskies have been previously documented; this breed's genetic profile is associated with enhanced physical endurance (Huson *et al.* 2010). In this study, we tested racing Siberian Huskies for associations between *DRD4* and *TH* gene polymorphisms and activity–impulsivity related trait scores obtained from a questionnaire and a short behavioural test.

Materials and methods

Subjects

Owners attending sled dog races were recruited to participate in the study. A total of 169 purebred Siberian Husky sled dogs belonging to 69 owners were genotyped. Owners were asked to provide the kennel club registration numbers of their dogs as documentation of their purebred status. Forty-four owners reported their age; the owners' average age was 45.4 years ($SD = 11.2$). Sixty-five owners provided their sex, and 58.5% of them were female. Due to time constraints during the races, some owners did not complete the questionnaire or the behavioural tests or opted to participate in the study at other times. Unless otherwise noted, statistics presented are derived from a final sample of 145 dogs for which the following information was available: (i) age and sex of the dog; (ii) genotypes for *DRD4* exon 3 and/or *TH* intron 3 polymorphisms and (iii) questionnaire and/or behavioural test data. *DRD4* genotypes were available for all 145 dogs, and *TH* genotypes were available for 138 dogs. Questionnaire data were available for 140 dogs, and behavioural test data were available for 92 dogs.

To expand the sample size, dogs from both Europe and North America were tested. Ninety dogs were from Europe: 62 from Hungary; 13 from Austria; five from Romania; three each from Switzerland, Slovakia and the Netherlands; and one from the Czech Republic. Fifty-five dogs were from North America: 50 from the United States of America (USA) and five from Canada. The age of the dogs ranged from 1.0 to 13.5 years ($M = 4.70$ years, $SD = 2.89$). Seventy-nine dogs were males, and 66 dogs were females. Spay-neuter status was collected for 87 dogs, and of these, 57.5% were unaltered, whereas 42.5% were spayed or neutered (Table 1). Whenever possible, we excluded dogs that shared

more than one parent using the names of the sire and dam provided by the owners.

All procedures were approved by the Columbia University Institutional Review Board and the Institutional Animal Care and Use Committee. No special permission for the use of dogs in non-invasive studies is required in Hungary. Exemption from permission was granted by the University Institutional Animal Care and Use Committee at Eötvös Loránd University.

DNA sampling and genotyping

Buccal smears were collected, and DNA was isolated with the Genra purification kit. Repeat polymorphisms in *DRD4* intron 2 were not analysed as this region is not polymorphic in the Siberian Husky breed (Hejjas *et al.* 2009). The exon 3 polymorphism was analysed according to the procedure previously described in the study by Hejjas *et al.* (2007b). The first PCR amplification of *DRD4* VNTR was performed in a 10- μ l reaction mixture containing 0.25 U DNA polymerase, 1 \times Q-solution and 1 \times buffer (final MgCl₂ concentration 1.5 mM) from the Qiagen HotStarTaq DNA polymerase kit, 1 μ M 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 μ M of each dNTP. PCR primers were designed by 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 visualised by ethidium bromide. Another independent PCR 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. Repeat polymorphisms in *TH* intron 4 were analysed according to Hejjas *et al.* (2007a). In short, the PCR mixture contained 1 μ M of each primer, approximately 5 ng of DNA template 200 μ M dATP, dCTP, dTTP, 100 μ M of dGTP and dTTP, 0.025 U HotStarTaq DNA polymerase 1 \times buffer and 1 \times Q-solution supplied by the Qiagen HotStarTaq polymerase kit in a 10- μ l final volume. PCR cycle conditions and the separation of PCR products by gel electrophoreses were as described above.

Behavioural phenotyping

The behaviour of the dogs was characterised by two instruments: the Dog-ADHD Rating Scale (Vas *et al.* 2007) and a behavioural test battery.

Dog-ADHD rating scale

The Dog-ADHD RS (Appendix S1) was completed by the owners before the behavioural tests and in the presence of the experimenter. This questionnaire has been used and validated on both European and North American dogs as a general measure of activity and attention (Vas *et al.* 2007;

Table 1 Descriptive statistics of the studied Siberian Husky populations.

	EU	NA	ALL
Age, years, <i>M</i> (<i>SD</i>)	4.5 (2.8)	5.0 (3.0)	4.7 (2.9)
Sex, male, <i>n</i>	46	33	79
Neutered, <i>n</i> ¹	14	23	37
<i>DRD4</i> allele frequencies, %			
1	0.0	0.6	0.3
2	4.5	5.6	5.2
3	10.0	21.7	17.2
4	5.5	5.0	5.2
5	46.4	51.1	49.3
8	33.6	16.1	22.8
<i>DRD4</i> genotype frequencies, % (<i>n</i>)			
1/4	1.1	0.0	0.7 (1)
2/2	2.2	1.8	2.1 (3)
2/3	5.6	1.8	4.1 (6)
2/4	0.0	1.8	0.7 (1)
2/5	1.1	0.0	0.7 (1)
2/8	0.0	1.8	0.7 (1)
3/3	4.4	1.8	3.4 (5)
3/4	1.1	3.6	2.1 (3)
3/5	17.8	10.9	15.2 (22)
3/8	10.0	0.0	6.2 (9)
4/4	1.1	0.0	0.7 (1)
4/5	3.3	1.8	2.8 (4)
4/8	2.2	3.6	2.8 (4)
5/5	40.0	30.9	36.6 (53)
5/8	0.0	18.2	6.9 (10)
8/8	10.0	21.8	14.5 (21)
<i>H</i>	0.42	0.44	0.43
<i>n</i>	90	55	145
<i>TH</i> allele frequencies, %			
1	86.9	73.0	81.9
2	13.1	27.0	18.1
<i>TH</i> genotype frequencies, % (<i>n</i>)			
1/1	76.1	60.0	70.3 (97)
1/2	21.6	26.0	23.2 (32)
2/2	2.3	14.0	6.5 (9)
<i>n</i>	88	50	138

¹Neuter status was collected for a subsample of 87 dogs.

H = observed heterozygosity.

Wan *et al.* 2009; Lit *et al.* 2010). It is based on a widely used human parental ADHD survey (DuPaul *et al.* 1998) and consists of two subscales. The activity–impulsivity scale consists of seven items (for example, ‘Your dog fidgets all the time’), and the inattention scale consists of six items (for example, ‘Your dog’s attention can be easily distracted’). The scale score was calculated for each dog as the mean of the scores given by the owner on a four-point scale, from 0 (never) to 3 (very often).

Behavioural test

Dogs were observed in a test battery consisting of three subtests, which can be easily implemented by experimenters facing time constraints at dog shows, races or other competitive events. The test battery was conducted outdoors in a 15 × 15 m remote area of the sled dog race site. Some owners were unavailable to participate during the races and scheduled appointments to participate at or near their homes. Six Hungarian dogs were tested in public parks near their owners’ houses, and 14 dogs from the USA were tested on their owners’ properties. A video camera was placed on a tripod 4 m from the owner. A female experimenter conducted the tests (M.W. in North America, E.K. in Europe). The recordings were coded later by M.W. (Coder 1). To assess inter-observer reliability, another observer recoded all behavioural variables for 38 dogs (Coder 2).

Testing protocol

Spontaneous activity. The owner stands in place without paying attention to the dog while holding the dog on a leash (1.5–2 m). The dog is allowed to move freely within the range of the stretched leash and is not corrected or rewarded for any behaviour. The experimenter stays at a distance of at least 3 m from the dog without interacting with the dog. The test lasts for 1 min.

Coded variables:

- Head orientation: number of changes in head orientation
- Leg movement: number of seconds that the dog moves its legs
- Spontaneous activity level: subjective evaluation on a 1 (low) to 5 (high) scale

Greeting. The owner stands motionless next to the dog and holds the leash. He or she may speak during the test. The experimenter approaches the dog in a friendly manner (verbally greets the owner and the dog and smiles). She steps out of reach of the leash and waits for 3 s. If the dog does not display signs of aggression, she steps next to the dog and pets the dog on the head, back and sides from the withers to the croup area. The purpose of the handling was to simulate greetings with human strangers. After petting for up to 5 s, the experimenter steps 1 m away. She waits

for 3 s, handles the dog again as described and steps out of reach.

Coded variables:

- Approaches: (0) dog neither approaches the experimenter in the beginning of the test nor follows when she steps away, (1) dog either approaches or follows the experimenter and (2) dog both approaches and follows the experimenter
- Contact-seeking with the experimenter: (0) never, (1) occasionally and (2) often
- Tail-wagging: (0) never, (1) occasionally and (2) often
- Tucked or low tail: (0) never, (1) occasionally and (2) often
- Low posture: (0) never, (1) occasionally and (2) often
- Greeting activity level: subjective evaluation on a 1 (low) to 5 (high) scale

Collecting buccal sample. The experimenter asks the owner whether it is safe to handle the dog’s mouth. If so, the experimenter collects a buccal sample (cheek swab) using two plastic sticks with cotton swabs at one end. The experimenter carefully rotates the sticks while rubbing the inside of the mouth and puts the sticks inside a numbered and sealed plastic tube. If the dog does not stand motionless, then the owner can help by holding the dog. If the dog displays signs of aggression, the experimenter asks the owner to collect the sample. In the current study, the owner’s assistance was required in three cases. The procedure is repeated on the other side of the mouth, as well, for a total of four swabs. The swabs from each side of the mouth are placed in different tubes labelled as A and B. Coded variables:

- Movement during sample collection 1: while collecting sample A, (0) the experimenter is not able to collect the sample, and the owner does so; (1) the dog moves its head and body; (2) the dog moves its head; (3) the dog stands motionless
- Movement during sample collection 2: same as above, while collecting sample B

Statistical analysis

SPSS 17.0 for Windows was used for all statistical analyses. The chi-squared test for independence was used to assess Hardy–Weinberg equilibrium and linkage between genes. Cronbach’s alpha was calculated to assess the internal reliability of the questionnaire subscales, and the intraclass correlation coefficient (ICC) was used to assess inter-observer reliability in the coding of the behavioural test. To condense the items of the behavioural test battery, principal components analysis (PCA) was used with varimax rotation with eigenvalue >1 (Kline 1994). Because a large data set is recommended for PCA, we included all dogs for which behavioural data were available ($n = 113$). Principal component (PC) scores were calculated automat-

ically by SPSS software using the regression method. Cronbach's alpha was calculated to assess the internal reliability of the extracted PCs. Pearson's correlation coefficient was used to measure the relationship between questionnaire scale scores and PC scores.

Associations between *DRD4* or *TH* genotypes and questionnaire scores were tested in the full sample, as well as for the subsamples from each continent. Due to the smaller sample of dogs that participated in the behavioural test, associations for these measures are reported only for the full sample. Gene-behaviour associations were tested using linear mixed models with the dog's age, sex and genotype group ('short' or 'long'; see Results) as fixed effects. Random effects consisted of random intercepts for owner and continent of origin. Owner was included as a random effect to account for within-owner correlations in the data due to owners participating with multiple dogs. Continent was included in analyses of the full sample in order to account for population stratification effects that could contribute to false positive association. The inclusion of continent in the model also accounted for the effects of experimenter, as one experimenter was responsible for behavioural testing on each continent. The variance components matrix was used to model random effects. Separate models were performed for each gene on each of the questionnaire scales and behavioural PCs. Additional analyses of the full sample compared individuals with the various 'long *DRD4*' genotypes (5/5, 5/8 and 8/8). Similar comparisons could not be conducted in the 'short *DRD4*' group due to low sample sizes for individual genotypes. Finally, the association analyses were conducted with a combination of the *DRD4* and *TH* polymorphisms as the genotype variable; the results of these analyses are reported for the full sample due to the small sizes of the combined-genotype groups in the subsamples. Additional analyses of associations between the behavioural measures and other demographic variables (spay-neuter status and the number of dogs in the household) are included in Appendix S2. In addition, supplementary analyses comparing data from the current study with previously collected data on GSDs are available in Appendix S3, Table S1 and Fig. S1.

Results

Genotypes

We detected seven alleles of the *DRD4* exon 3 VNTR polymorphism in our Siberian Husky sample, ranging from one to eight repeats. The most frequent genotypes were 5/5 (36.6%) and 3/5 (15.2%) (Table 1). The 5/5, 3/5 and 3/3 genotypes were in Hardy-Weinberg equilibrium in the European and North American samples, $\chi^2 = 1.29$, $df = 1$, $P = 0.26$ and $\chi^2 = 0.24$, $df = 1$, $P = 0.62$

respectively. In *TH* intron 4, a 36-bp-long sequence was present either as a single copy (allele 1) or in a duplicate form (allele 2). The most frequent *TH* genotypes were 1/1 (70.3%) and 1/2 (23.2%) (Table 1). The *TH* genotypes were in Hardy-Weinberg equilibrium in the European, but not in the North American sample, $\chi^2 = 0.22$, $df = 1$, $P = 0.64$ and $\chi^2 = 5.70$, $df = 1$, $P = 0.02$ respectively. However, disequilibrium is common in domesticated species and in breeds with closed stud books. Because some of the *DRD4* genotypes and alleles were rare, we grouped individual genotypes according to the protocol generally applied for humans (e.g. Shao *et al.* 2006). The 'short *DRD4*' group included dogs possessing at least one copy of the 1, 2, 3a, 3b or 4 variants. The 'long *DRD4*' group consisted of individuals with the 5/5, 5/8 or 8/8 genotypes. The 'short *TH*' group included dogs with the 1/1 genotype, whereas the 'long *TH*' group consisted of dogs with at least one long (2) allele. Both *DRD4* and *TH* are localised on chromosome 18 (Hejjas *et al.* 2007a). However, *DRD4* exon 3 and *TH* intron 4 were not linked in our sample: dogs possessing short *DRD4* alleles randomly possessed short or long *TH* alleles ($\chi^2 = 0.40$, $df = 1$, $P = 0.53$).

Reliability of questionnaire subscales

Reliability was high for the two subscales of the Dog-ADHD RS. Cronbach's alpha for the seven items comprising the activity scale was 0.80, and Cronbach's alpha for the six items comprising the inattention scale was 0.85.

Inter-observer reliability in behavioural test

Inter-observer reliability was strong for the coded behavioural variables. ICCs between Coder 1 and Coder 2 ranged from 0.82 to 0.92.

Principal component extracting

Principal components analysis grouped the variables into three PCs that accounted for 61.8% of the total variance (Table 2). A scree plot supported a three-component solution. All items loaded higher than 0.5 on a total of three PCs. PC 1, titled 'Sociability', included the following items from the greeting test: approaches, contact-seeking, tail-wagging, low tail and low posture (Cronbach's alpha = 0.76). Positive loadings were obtained for the first three variables, whereas negative loadings were obtained for low tail and low posture. PC 2, labelled 'Activity', included the following items: head orientation, leg movement and both activity ratings from the spontaneous activity and greeting subtests (Cronbach's alpha = 0.44). PC 3, labelled 'Movement during Handling', consisted of the items measuring movement during both DNA sample collections (Cronbach's alpha = 0.62).

Table 2 Principal component structure, loadings of items, explained variance, Cronbach's alpha and eigenvalues of principal components.

Behavioural variables	Sociability	Activity	Movement during handling
Head orientation	-0.09	0.64	0.06
Duration of moving the legs	0.09	0.88	-0.07
Spontaneous activity level	0.05	0.93	-0.09
Approaches	0.73	-0.04	0.17
Contact-seeking with the experimenter	0.75	-0.04	0.02
Tail-wagging	0.64	0.17	-0.41
Low tail	-0.76	-0.24	0.13
Low posture	-0.62	-0.04	-0.05
Greeting activity level	0.34	0.62	-0.23
Movement during sample collection 1	-0.09	0.00	0.82
Movement during sample collection 2	0.14	-0.12	0.82
Explained variance (%)	23.91	23.02	14.82
Cronbach's alpha	0.76	0.44	0.62
Eigenvalue	3.24	2.12	1.44

Loadings greater than 0.60 are in bold.

Relationships between questionnaire and behavioural test scores

A marginally significant correlation was found between activity scores from the questionnaire and behavioural test, $r(85) = 0.21$, $P = 0.05$. None of the other correlations between questionnaire and behavioural test scores was significant, indicating divergence in the two types of measures.

Effects of DRD4 and TH genotypes on Siberian Husky behaviour

Significant results and trends from the genotype-behaviour analyses are reported below, and parameter estimates are presented in Tables S2 and S3. After accounting for the effects of sex, age, owner and continent of origin in the full sample, we found that Siberian Huskies possessing at least one short *DRD4* allele displayed greater activity in the behavioural tests than did those with two long alleles, $F(1, 81) = 5.53$, $P = 0.02$ (Fig. 1a). In addition, dogs with the short allele tended to receive higher ratings on the activity-impulsivity scale of the questionnaire in the full sample (Fig. 1b), as well as in the European sample, although the results were only marginally significant, $F(1, 139) = 3.22$, $P = 0.08$ and $F(1, 87) = 2.85$, $P = 0.10$ respectively. Individuals with the 'long' 5/5, 5/8 and 8/8 genotypes did not differ from each other on any of the measures.

In the full sample, Siberian Huskies with two short *TH* alleles received marginally higher activity-impulsivity and inattention ratings on the questionnaire and marginally lower activity scores in the behavioural test than did dogs with the long allele [activity-impulsivity: $F(1, 134) = 3.02$,

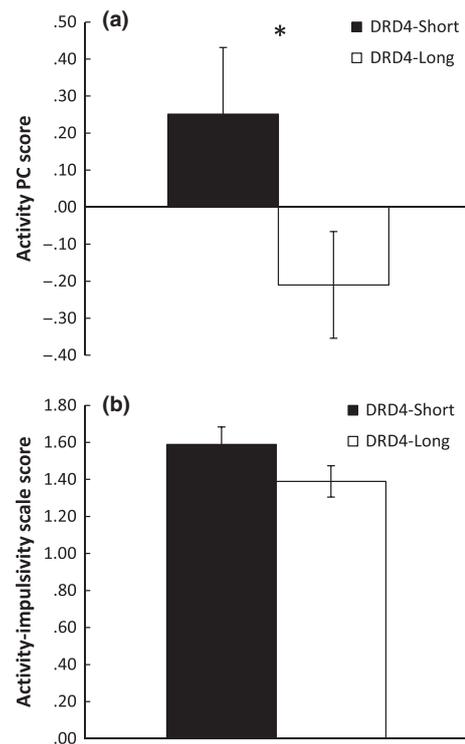


Figure 1 Associations between *DRD4* genotype and (a) activity principal component scores ($M \pm SE$) from the behavioural test and (b) activity-impulsivity scale scores ($M \pm SE$) from the questionnaire. Siberian Huskies with at least one short allele displayed greater activity ($P = 0.02$) and received marginally higher activity-impulsivity ratings ($P = 0.08$). Model-fitted values account for sex, age, continent of origin and owner identity. * P -value < 0.05.

$P = 0.08$; inattention: $F(1, 130) = 2.80$, $P = 0.10$; activity PC: $F(1, 70) = 3.22$, $P = 0.08$]. Neither sociability nor movement during handling in the behavioural test was associated with *DRD4* or *TH* polymorphisms. In the European sample, Siberian Huskies with two short *TH* alleles received significantly higher inattention ratings and marginally higher activity-impulsivity ratings on the questionnaire than did those possessing the long allele, $F(1, 83) = 4.27$, $P = 0.04$ and $F(1, 85) = 3.81$, $P = 0.05$ respectively (Fig. 2).

To investigate the joint effect of the *DRD4* and *TH* genotypes, four groups were formed in the full sample: (i) short allele present at both sites ($n = 41$); (ii) short *DRD4* and long *TH* alleles ($n = 16$); (iii) long *DRD4* and short *TH* alleles ($n = 56$); and (iv) long *DRD4* and long *TH* alleles ($n = 25$). The combined-genotype groups differed in activity-impulsivity ratings, $F(3, 133) = 4.35$, $P = 0.006$ (Fig. 3a). Dogs with long alleles for both genes were rated as less active-impulsive than were dogs with any other genotype combination. In addition, the combined-genotype groups varied in activity during the behavioural test, $F(3, 68) = 4.05$, $P = 0.01$ (Fig. 3b). Dogs with the combination of short *DRD4* and long *TH* alleles were the most active,

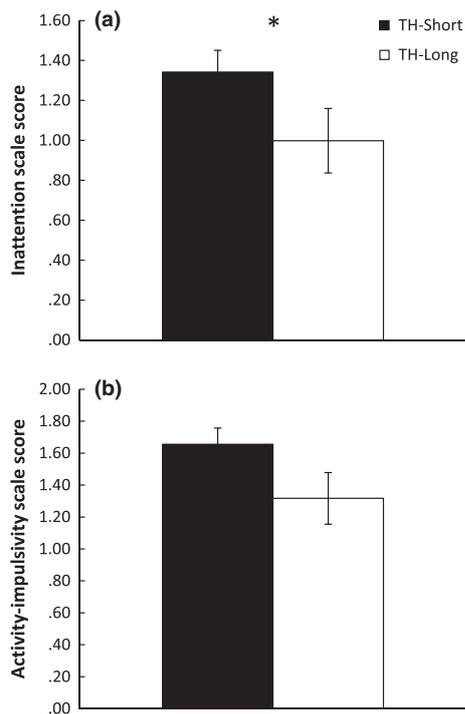


Figure 2 Associations between *TH* genotype and (a) inattention ($M \pm SE$) scale scores and (b) activity-impulsivity scale scores ($M \pm SE$) from the questionnaire. Siberian Huskies from Europe with two short alleles received significantly higher inattention ratings ($P = 0.04$) and marginally higher activity-impulsivity ratings ($P = 0.05$). Model-fitted values account for sex, age and owner identity. * P -value < 0.05.

significantly more so than individuals possessing long *DRD4* alleles and either short or long *TH* alleles.

Discussion

In this study, we investigated the relationship between dopaminergic genes and measures of activity, impulsivity and inattention in racing Siberian Huskies. Recent reports (Hejjas *et al.* 2007b; Kubinyi *et al.* 2012) had established that these genes are associated with activity-impulsivity in GSDs. However, the results on GSDs may not be generalisable to other breeds due to the genetic isolation of each breed and allelic heterogeneity between breeds. The Siberian Husky was selected for this study, because it has higher genetic diversity than more recent breeds (Vonholdt *et al.* 2010) and has undergone selection for enhanced endurance (Huson *et al.* 2010), which can be considered an activity-related trait.

We found that the Siberian Husky breed possesses more variants of the *DRD4* exon 3 polymorphism than do all other breeds previously tested (Niimi *et al.* 2001; Hejjas *et al.* 2007a). A total of seven variants were found, among them the longest known allele, described only in wolves thus far (Hejjas *et al.* 2007a), supporting suggestions that the breed admixed with wolves, has ancient origins and

displays high genetic diversity (Vonholdt *et al.* 2010). Previous studies reported only two variants in GSDs, Belgian Tervuerens, Belgian Groenendaels, Belgian Malinois and Golden Retrievers, whereas three variants were reported in Shetland Sheepdogs, four in Beagles and five in Shiba Inus (Niimi *et al.* 2001; Hejjas *et al.* 2007a). Interestingly, the Shiba Inu, with the second highest number of variants, is also considered a breed with ancient origins and high genetic variability (Parker *et al.* 2004).

We found that the behaviour test captured consistency in activity levels and movement across two subtests. Activity level in the greeting subtest was associated with the activity level, duration of leg movement and number of head movements in the spontaneous activity scenario. The test battery therefore appears to be a useful tool in characterising canine activity level and could be used in future studies. Because the total duration of the test is less than 5 min, the test is feasible to conduct when experimenters face time constraints. However, test-retest reliability and external validity should be further assessed to determine whether the behaviour observed in the test generalises to other situations and reveals the dog's traits.

By including participants from different continents, we were able to increase our sample size and increase the likelihood of detecting gene-behaviour associations. As others have noted, the combination of samples from different continents can be a useful method of increasing power without necessarily increasing the rate of false positive associations (Quignon *et al.* 2007). To address possible population stratification effects, we accounted for the dogs' area of origin in our analyses of data from the full sample. After additionally controlling for the effects of owner and the dogs' sex and age, we demonstrated that short alleles of the *DRD4* and *TH* repeat polymorphisms are associated with greater activity and impulsivity in racing Siberian Huskies. Dogs possessing at least one short *DRD4* allele (labelled as 1, 2, 3a, 3b, 4) displayed higher activity levels in the behavioural test than did those possessing the longer variants (5, 8). In addition, dogs from Europe possessing two short *TH* alleles (labelled as 1) were scored as more inattentive by their owners on the Dog-ADHD Rating Scale. Finally, dogs with short *DRD4* or *TH* alleles were rated as more active-impulsive than were dogs possessing long alleles at both sites, whereas dogs with the particular combination of short *DRD4* and long *TH* alleles were more active in the behavioural test than were individuals possessing long *DRD4* alleles and either short or long *TH* alleles. Significant associations were not uncovered when the North American sample was analysed alone; smaller sample sizes in this group could explain these results.

Because each gene was associated with a different activity-impulsivity related measure, it is possible that the *DRD4* and *TH* genes are differentially influential on the behaviours characterised by the measures or that each measure captures a different behaviour. The lack of strong

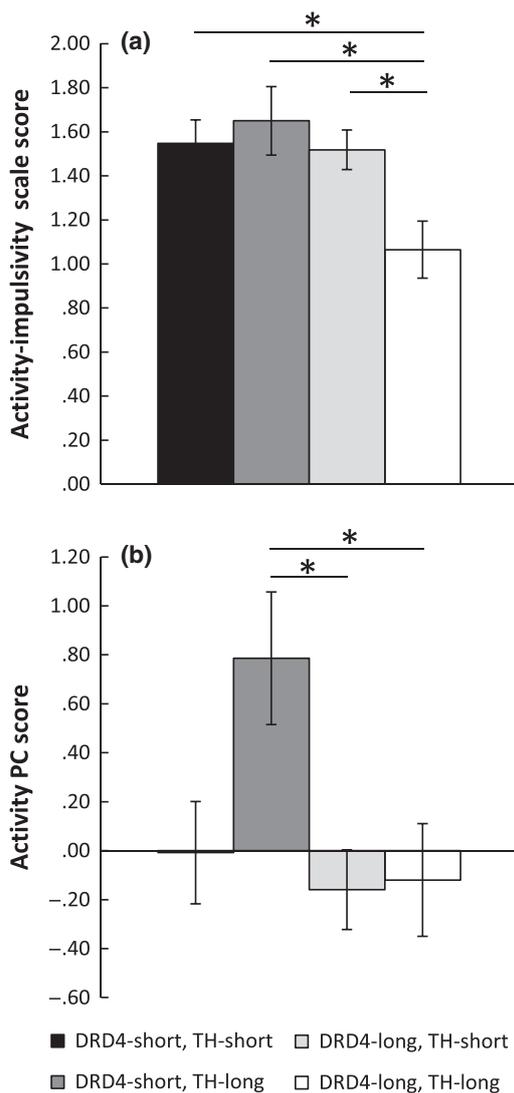


Figure 3 Associations between combined-genotype group and (a) activity-impulsivity scores ($M \pm SE$) from questionnaire and (b) activity PC scores ($M \pm SE$) from the behavioural test. Siberian Huskies with long alleles at both sites received lower activity-impulsivity ratings than did each of the other groups ($P < 0.025$ for all comparisons). Siberian Huskies with the combination of short *DRD4* and long *TH* alleles were significantly more active than were those with long *DRD4* alleles and either short ($P = 0.008$) or long *TH* alleles ($P = 0.032$). Model-fitted values account for sex, age, continent of origin and owner identity. * P -value < 0.05 .

correlations between the behavioural test and questionnaire scores suggests that the two types of measures were divergent. The behavioural test may be a context-specific characterisation of activity in a public setting, whereas the questionnaire reflects general activity and attention levels as perceived by the owner. Therefore, the behaviour test should continue to be used in conjunction with the questionnaire or other measures in order to assess activity-impulsivity related behaviour.

Previous studies in GSDs also had demonstrated associations between short alleles of dopaminergic genes and

increased activity-impulsivity. For example, GSDs with the short *TH* allele received higher activity-impulsivity scores (Kubinyi *et al.* 2012). However, the patterns of associations for the *DRD4* gene in Siberian Huskies and GSDs were not aligned. Although we found that short *DRD4* alleles (1, 2, 3a, 3b, 4) in Siberian Huskies were associated with greater activity than were long alleles (5, 8), the 2 allele in GSDs has been associated with lower activity-impulsivity and social impulsivity than has the 3a allele (Hejjas *et al.* 2007b, 2009). Unfortunately, we could not directly compare the 2 and 3a alleles in Siberian Huskies, because the 2 allele was uncommon in this breed. Likewise, the longer 5 and 8 alleles and, in fact, any alleles other than the 2 and 3a are very rare in GSDs. Population differences in allelic frequencies of the *DRD4* exon 3 polymorphism, as well as their associations with behaviour, have also been documented in humans. The seven-repeat variant is considered the long form of the *DRD4* polymorphism and has been associated with ADHD in many studies involving European samples (e.g. Holmes *et al.* 2000; Curran *et al.* 2001; Langley *et al.* 2009). In contrast, the seven-repeat variant is extremely rare in East Asian samples (Chang *et al.* 1996), and the two-repeat variant has instead been linked with ADHD in at least one study with Chinese participants (Leung *et al.* 2005). Given these results, the discrepancy between GSDs and Siberian Huskies in *DRD4* associations is not entirely surprising and underlines the importance of exploring gene-behaviour relationships in multiple breeds.

Research on the differences in function between shorter and longer alleles of dopaminergic genes in dogs is needed to establish that the assignment of alleles into short and long groups has a functional basis and to clarify discrepancies in results between breeds. Although we have provided evidence that short alleles of dopaminergic genes are associated with increased activity in Siberian Huskies, the function of the polymorphisms in dogs is still unclear. In humans, the number of repeats in exon 3 of *DRD4* affects the length of the third intracellular loop of the dopamine receptor D4. The four-repeat variant, the most common form in European populations, has been shown to be more potent than the seven-repeat variant, requiring a dopamine concentration three times lower to achieve the same reduction in cyclic AMP (Asghari *et al.* 1995; Jovanovic *et al.* 1999). Also in humans, the VNTR in intron A of *TH* may be directly involved in transcriptional regulation of the gene (Meloni *et al.* 1998). To determine whether the polymorphisms in canines have similar functions, further work is clearly needed.

Overall, our results in dogs showing associations between activity-impulsivity related measures and *DRD4* and *TH* polymorphisms support relevant findings in humans. The relationship between *DRD4* and activity-impulsivity traits in humans has been well established (Gizer *et al.* 2009; Varga *et al.* 2012). In the case of *TH*, direct links with activity and impulsivity traits have not been reported, but neuroticism

and extraversion, which are associated with impulsivity (Whiteside & Lynam 2001), have been linked to *TH* polymorphisms (Persson *et al.* 2000; Tochigi *et al.* 2006). Our results add to evidence showing that the dog is a useful model species for the study of genetic influence on human behaviour and personality (Takeuchi *et al.* 2009; Dodman *et al.* 2010; Konno *et al.* 2011). Although these association studies are still in their early stages, it is evident that these methods can offer outstanding possibilities for those who are looking for the genetic mechanisms underlying typical behavioural variation, as well as behavioural disorders.

Moreover, association studies on working dogs, such as racing Siberian Huskies in the present study, may lead to earlier and more predictive selection of suitable individuals. By understanding the genetics behind working dog behaviour, breeders may be able to more effectively target the traits that working dogs need. For example, desired traits in sled dogs include endurance, speed and work ethic (Huson *et al.* 2010), whereas guide dogs must display a lack of fearfulness, aggressiveness and distractibility (Goddard & Beilharz 1983). Traditional methods of selection, such as puppy temperament tests, do not predict the success of adult working dogs (Goddard & Beilharz 1986; Wilsson & Sundgren 1998; Olson *et al.* 2004), and guide dog organisations have already begun to collect DNA in order to determine the genotypes that could be associated with desired behavioural phenotypes (Olson *et al.* 2004; Takeuchi *et al.* 2009). Breeders can currently conduct genetic testing for a wide range of physical disorders (Mellersh 2012), such as progressive retinal atrophy, and may one day conduct similar testing for behavioural purposes.

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Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Dog-ADHD Rating Scale.

Appendix S2 Supplementary analyses: Demographic variables of dogs.

Appendix S3 Supplementary analyses: Comparison of Siberian Huskies and German Shepherd Dogs.

Figure S1 Activity–impulsivity and inattention scale scores ($M \pm SE$) from questionnaire according to *TH* genotype and breed (GSD = German Shepherd Dog, SH = Siberian Husky).

Table S1 Parameter estimates for linear mixed models on German Shepherd and Siberian Husky data from Europe with fixed effects of age, sex, and *TH* genotype.

Table S2 Parameter estimates for linear mixed models performed on Siberian Husky data from Europe with fixed effects of age, sex, and genotype and random effects for owner.

Table S3 Parameter estimates for linear mixed models performed on Siberian Husky data from Europe and North America with fixed effects of age, sex, and genotype and random effects of owner and continent.