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Research

DRD2 is associated with fear in some dog breeds

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ABSTRACT

Behavioral problems occur frequently in dogs and represent a significant threat to dog welfare. Anxiety, phobias, and fears comprise most of the canine behavioral conditions. The identification of an association between specific behavioral phenotypes and genetic variants of candidate genes would be a valuable tool in selection for dogs less susceptible to anxiety and fear, which may improve animal welfare. The DRD2 gene encodes the dopamine receptor 2. In this study, we found 8 SNPs in the DRD2 gene of the Havanese, a breed that shows large variation in a behavioral phenotype that manifests itself as a tendency to react fearfully by withdrawing in social situations. Significant associations were detected between 2 SNPs in exon 2 of the DRD2 gene and increased social fear in Havanese dogs (n = 158), as evaluated through observation by an external evaluator (respective allelic odds ratio: 4.35, 4.07) and through owner questionnaires (respective allelic odds ratio: 1.96, 2.2). Because different types of fear-related behavioral disorders commonly co-occur, the SNPs in exon 2 were also investigated for possible association to noise reactivity in 5 breeds: Havanese (n = 121), collie (n = 94), Irish soft-coated wheaten terrier (n = 44), Nova Scotia duck tolling retriever (n = 33), and standard poodle (n = 29). Significant associations were detected between SNPs in exon 2 of the DRD2 gene and noise reactivity in the Irish soft-coated wheaten terrier (respective allelic odds ratio: 2.64, 2.88) and collie (allelic odds ratio: 3.03). The same SNP alleles were associated with the beneficial phenotypes in the 3 breeds.
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Introduction

Behavioral problems occur frequently in dogs (Bamberger and Houpt, 2006) and represent a significant threat to dog welfare. Behavioral problems are an important cause of both dog abandonment (Scarlett et al., 1999) and euthanasia (Houpt et al., 1996). In 1 study from the United States, at least 1 behavioral reason was recorded for 40% of relinquished dogs and behavioral reasons accounted for 27% of single-reason canine relinquishments (Scarlett et al., 1999). In 1 set of UK shelters, problematic behavior was responsible for 34.2% of relinquishments (Diesel et al., 2010), which is similar to the 35% calculated for purely behavioral relinquishments in the United States (Dolan et al., 2015). The most recent data indicate that 20% of 3.3 million shelter dogs in the United States are euthanized (ASPCA, 2018), with dogs with behavioral concerns especially at risk. Marston et al., 2004 reported that 54% of 4,846 relinquished dogs in 3 shelters in Australia were euthanized for temperament, aggression, or other behavioral problems. Behavioral problems pose the single largest health and longevity threat to modern pet dogs (Dreschel, 2010).

Various types of anxiety constitute a large portion of these behavioral issues, including anxiety in general, various phobias, separation anxiety, noise reactivity, and various social and environmental fears. Bamberger and Houpt, 2006 found that anxiety, phobias, and fears comprise well over 20% of cases presented at a large university behavioral clinic. Fear aggression toward owners (5.2%) and strangers (16.8%) were also common complaints. Being fearful is a welfare issue in itself, but anxious dogs might also be subject to secondary welfare issues such as isolation or unethical training methods. Anxiety-related issues are also of relevance to

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society, as aggression resulting from anxiety can create unpleasant or dangerous situations for people or other dogs. Studies show that different types of anxiety commonly co-occur in both clinical studies (Overall et al., 2001) and survey studies of the general population (Tiira et al., 2016).

Some behavioral traits have high heritability in laboratory animals, humans, and other species studied. A large genetic influence has been detected for personality traits of shyness, inhibition, and fear in people (Eley et al., 2003), and heritability estimates for anxiety disorders, in general, are often high in humans (Davies et al., 2015). One study (Saetre et al., 2006) on behavioral traits in dogs estimated the heritability of the shyness/boldness aspect of a dog's personality to be 0.25. The heritability of fearfulness has been estimated to be 0.5 in one study of guide dogs (Goddard and Beilharz, 1982), whereas fearlessness has been reported to have a heritability estimate of 0.20 in a study of rough collies (Arvelius et al., 2014). One study reported a heritability estimate of 0.56 for gun shyness in Labrador retrievers (van der Waaij et al., 2008).

In a well-known selection study in foxes (Trut et al., 2009), systematic selection was performed to improve tameability. The clear selection response can be considered as evidence that tameness has a high realized heritability, indicating that it is possible to reduce aggressive-avoidance responses through breeding. In an open-field study (DeFries et al., 1978), mice were categorized as fearful or not fearful based on their activity level, allowing the establishment of 3 selection lines (fearful, not fearful, and controls). A strong selection response was shown in both the fearful and the not-fearful lines. A similar strong selection response has also been shown for dogs when breeding for either anxious or outgoing temperament in English pointers (Murphree et al., 1974).

Neurotransmitters are chemical compounds that transfer signals from neurons, by binding to the neurotransmitter receptors on surrounding neurons. A number of neurotransmitters, including dopamine, adrenaline, noradrenaline, serotonin, acetylcholine, and glutamate, are known to influence behavior and mood through regional brain and neurochemical effects.

Regulation of the amount, release, and reuptake/termination of these neurotransmitters is crucial for optimal neurological and mental function. Each neurotransmitter is regulated by various mechanisms, including high numbers of different receptors, transporters, and reuptake systems that work together in complex interactions. Each of the receptors is encoded by specific genes, and different genetic variants in these genes may influence the function of the receptor and "success" of neurotransmission.

Dopamine levels in the amygdala can influence individual differences in anxious temperament in humans (Kienast et al., 2008). Low dopamine reuptake by neurons is associated with increased anxiety and irritability (Laakso et al., 2003). Genes related to dopamine regulation may also have an association with anxiety and behavioral issues in dogs (Lit et al., 2013a, b, c). DRD2 is one of the several dopamine presynaptic receptors, which functions as an autoreceptor to ensure negative feedback when dopamine levels are elevated (Stahl, 2008). A polymorphism in the 3′UTR-region of the human gene has been associated with dopamine receptor density and anxiety, in close interaction with the dopamine transporter gene DAT (Kulikova et al., 2008).

There is large interest in animal models in human psychiatry, to understand molecular contributions to psychiatric disorders, including those related to anxiety. The identification of associations of specific behavioral phenotypes with genetic variants of candidate genes would be an important step in an increased understanding of etiology and could improve psychopharmacological application and aid new drug development. For dogs, identification of genetic variants could also improve animal welfare by improving selection for less fearful and anxious individuals.

Breeds represent pools of canalized genetic variation. Dog breeds can be important sources of information about behavioral phenotypes and genetic variants. In 2013, the Norwegian Havanese Club conducted a survey on health and behavior in their breed, in which 18.6% of owners reported that their dog was either "nervous" or "very nervous" (unpublished results). The survey indicated that there are interesting phenotypic variations within the breed, motivating further investigations. In the preliminary research for this study, owner interviews were conducted to obtain more detailed information on the phenotype of these "nervous" dogs. We found that the dogs functioned relatively well in everyday life and at home but had an exaggerated tendency to react fearfully in certain social situations with unfamiliar dogs and people. This behavior represents a major deviation from the typical and desired behavior of the Havanese, as these dogs are considered to generally have a very sociable and outgoing personality.

The main goal of this study was to investigate potential associations between the DRD2 gene and an increased tendency in several dog breeds to react fearfully to social or environmental stimuli, in the absence of truly threatening circumstances. Fear can be normal and adaptive in context. For the purposes of this study, pathological fear was defined as responses to stimuli (social or physical) that are characterized by active (backing or turning away, escape, hiding, flight) or passive (lowered/hunched body posture, tail tucked/down, ears back) avoidance/withdrawal behaviors associated with sympathetic physiological signs (increased heart rate/respiration, shaking, trembling, salivation, mydriasis) (Overall, 2013).

One form of environmental fear is noise phobia/reactivity. Noise-phobic dogs are characterized by a profound, nongraded, extreme response to noise, that manifest as intense avoidance, escape, or anxiety, associated with the sympathetic branch of the autonomic nervous system. Dogs that are characteristically distressed when exposed to specified noises but that do not meet the criteria for a "phobia" may be classified as 'reactive' in the absence of more specific provocative information (Overall et al., 2001; Scheifele et al., 2016).

We investigated dopamine gene variants with respect to fear in social situations and noise phobia and reactivity.

Materials and methods

Dogs

Data on social fears in Havanese and noise reactivity in 5 breeds (including Havanese) were collected from privately owned dogs in collaboration with breed clubs and owners (Table 1). First, a candidate gene study on DRD2 and social fear was conducted in the Havanese. Cases and controls from 5 breeds (Table 2) was then tested to look for associations of noise reactivity to the identified

Table 1
Breeds and number of dogs included in the study

Breed (abbreviation)	Number of dogs (females, males)
Havanese	158 (92, 66)[a]
Collie (smooth and rough)	94 (62, 32)
Irish soft-coated wheaten terrier	44 (27, 17)
Nova Scotia duck tolling retriever	33 (17, 16)
Standard poodle	29 (19, 10)
Total	358 (217, 141)

[a] Number of individual Havanese where information on at least one phenotype (provocative behavioral evaluation [PBE], owner score on social fear and/or classification for noise reactivity) was available. For most of the dogs, there was information on all 3 phenotypes, but a portion of dogs were classified as intermediate and therefore excluded—see detailed information in the text.

Table 2
Breed and number of cases and controls for noise reactivity

Breed (abbreviation)	Number of dogs (cases, controls)
Havanese	121 (25, 96)
Collie	94 (49, 45)
Irish soft-coated wheaten terrier	44 (20, 24)
Nova Scotia duck tolling retriever	33 (16, 17)
Standard poodle	29 (15, 14)

SNPs. Owners were contacted through the respective breed clubs and the Norwegian Kennel Club (including adverts in the Norwegian Kennel Clubs journal and in dog shows), and samples were collected from all dogs whose owner responded and allowed DNA sampling.

EDTA-blood samples were collected from all dogs by certified veterinarians, with owner's consent, in agreement with the provisions enforced by the Norwegian Animal Research Authority. Genomic DNA was extracted using E.Z.N.A blood DNA kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's recommendations and subsequently stored at −20°C. The samples were collected according to rules for ethical approval for collecting blood samples (FOR-2010-07-08-1085, FOR-1996-01-15-23, Regulation on Animal Experimentation). Performagene buccal swabs (DNA Genotek Inc) were used when blood sampling was impossible due to geographic distance. DNA was extracted following the manufacturer's recommendations.

Behavioral classification for social fear in Havanese

In Havanese, the dogs' tendencies to react fearfully in social situations were classified both through a PBE and through a questionnaire (owner score).

Inclusion criteria for each classification system (behavioral parameters and survey questions, respectively) were based on the phenotypic characteristics described in owner interviews during the initial planning of the study. An increased tendency to act fearfully towards unfamiliar dogs and people, displayed as active (backing or turning away, escape, hiding, flight) or passive (lowered/ hunched body posture, tail tucked/down, ears back) avoidance behaviors, were the main complaint and therefore the main focus of the Havanese study.

Lower body and tail posture has been associated with fearful, withdrawn, or uncertain behaviors (Beerda et al., 1999). Owner interviews revealed that "dropping the tail" was an important indicator of fear in Havanese. For this reason, tail position was also registered in the PBE.

Provocative behavioral evaluation

A standardized evaluation of the dogs' behavior was performed for each dog. The evaluator first presented herself to the owner, ignoring the dog. The evaluator then approached the dog directly by bending down, holding one hand forward, and calling the dog. Finally, the dogs' reaction to gentle restraint at an examination table before DNA sampling was registered. Tail position was noted at the time of initial greeting.

The dogs were observed and classified for 3 criteria (contact seeking, tail position, and reaction to gentle restraint that physically supported and stabilized the dog). Dogs that displayed fearful behavior in all criteria were classified as cases and dogs that displayed no fearful and only affiliative behavior in all criteria were classified as controls. The same person (K.K.L.B.) evaluated all the dogs that were included in the study. DNA samples were obtained in the home of the owner after finishing the behavior evaluation.

Questionnaire (owner score)

A questionnaire was sent to all owners of dogs that participated in the provocative behavioral evaluation. It was also sent to Havanese owners who were not able to participate in the provocative behavioral evaluation due to geographic distance, which explains the difference in sample size.

The questionnaire consisted of 9 questions concerning the dogs' tendency to react fearfully in social situations (Supplemental Table 1). The owners were asked to what degree they could agree with various statements on the dogs' behavior. Answers were given on a 5-point scale, representing high to low levels of fear. The average of all answers was then calculated to indicate the individuals' general tendency to react fearfully in social situations (owner score [OS]).

Behavioral classification for noise reactivity determined by short questionnaire across breeds

Owners of collies (smooth and rough), Irish soft-coated wheaten terriers (ISWTs), Nova Scotia duck tolling retrievers (NSDRs), standard poodles, and Havanese answered 4 questions concerning reactions to loud noises including gunshots, fireworks, thunderstorms, and heavy traffic (Supplemental Table 2). Answers were given on a 5-point scale, indicating high or low levels of noise reactivity. Cases were defined as dogs with a score of ≤2 in at least 1 of the 4 categories and controls had a score of ≥4 in all categories. This methodology mirrors that of published studies (Overall et al., 2006, 2014; Scheifele et al., 2016).

Selection of candidate genes

DRD2 is an interesting gene that has been associated with a large variety of behavioral traits in humans (Munafo et al., 2007; Markett et al., 2011; Takeuchi et al., 2015). Several studies have found associations between the DRD2 gene and personality traits of apprehension and neuroticism (Kulikova et al., 2008; Kazantseva et al., 2011; Montag et al., 2012). Because the phenotype of interest in the Havanese was a general "nervousness" and increased tendency to react fearfully, we chose DRD2 as the candidate gene for this study.

Primers

Primers embracing all exons and UTRs were designed based on the reference dog genome (CanFam3.1), using Primer3plus (https://primer3plus.com/). Amplification was successful for all parts except for exon 1 and parts of the 3′UTR. Optimal temperatures were detected using a temperature gradient PCR program with temperatures ranging from 54°C to 64°C.

Sequencing of the PCR products was performed following a standard Sanger method on an ABI 3500 XL DNA analyzer (Applied Biosystems, Life Technologies of Thermo Fisher Scientific), followed by manual inspection using the Sequencher software from Gene Codes Corporations, at the Norwegian University of Life Sciences. Primers and optimal temperatures are listed in the Supplemental Table 3.

Statistical analyses

For the association testing, odds ratios were calculated according to Altman (Altman, 1991) and the *P*-values were calculated according to Sheskin (Sheskin, 2004). The correlation between the 2 means of classification for social fear in Havanese was calculated using Pearson correlation coefficient, and the intrarater reliability of the questionnaire was calculated using Cronbach's alpha, both in

JMP Pro v. 14. The positive and negative predictive value (PPV/NPV) of the owner questionnaire (OQ) compared to the PBE was calculated using the formulas (true case/classified as case using questionnaire) and (true control/classified as control using questionnaire), respectively.

Results

Provocative behavioral evaluation

A total of 104 Havanese underwent a provocative observational classification (Table 3). Of these, 28 dogs were classified as cases and 33 were classified as controls. Forty-three dogs did not meet the criteria for either cases or controls (indicating an intermediate phenotype) and were therefore excluded.

Questionnaire (owner score)

Owners of 150 dogs responded to the questionnaire. Dogs with more than 2 missing answers were excluded (n = 3). The lowest recorded individual owner score (most fearful dog score) was 1.22 and the highest (least fearful dog score) was 5.0. The average score was 4.12 (Figure). Cutoff for cases was set as 0.5 σ below average OS and the cutoff for controls was set as 0.5 σ above average OS. Forty-three dogs were classified as cases and 60 dogs were classified as controls.

Correlation and predictive value between questionnaire and provocative behavior test for social fear

The correlation between the 2 means of classification for social fear in Havanese was calculated using Pearson correlation coefficient and was good ($\rho = 0.738$, P-value < 0.001). All Havanese dogs that were classified as cases using the questionnaire were also classified as cases in the PBE. Four dogs that were classified as controls using the questionnaire were classified as cases in the PBE. Based on the dogs that had results from both evaluations, the PPV/NPV of the OQ compared to the PBE were estimated to 1.0 (case in OQ also case in PBE) and 0.88 (control in PBE also control in OQ), respectively.

Questionnaire reliability

Most of the questions included in our questionnaire were identically worded as questions included in a health survey conducted in the Havanese breed a few months later. Because some owners (n = 35) had answered both questionnaires, we were able to calculate an estimate of intrarater reliability using Cronbach's alpha. We found good reliability for survey scores on both social fear and noise reactivity ($\alpha = 0.82$ and $\alpha = 0.80$, respectively). Other studies on questionnaires designed in a similar way also indicate that OQs on behavior have acceptable reliability (Hsu and Serpell, 2003).

Table 3
Criteria for observed phenotype classification in the provocative behavioral evaluation

Observation	Case (N = 28)	Control (N = 33)
Reaction to visitor	Avoiding contact with visitor, hiding behind owner	Actively contact seeking, does not pull away when petted
Tail position	Down	Up
Reaction to gentle restraint on examination table before DNA sampling	Strong avoidance, climbing on to owner, frantic escape behavior, or vocalization	No or only mild avoidance calmly accepting gentle restraint or positive reaction

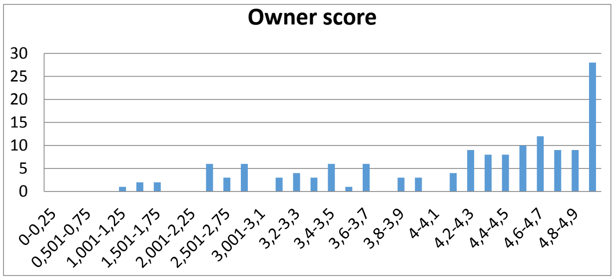

Figure. Distribution of average owner scores; x axis is score; y axis is frequency.

Detection of SNPs

All exons were initially sequenced for a small group of 8 unrelated Havanese to identify regions with variation. Eight SNPs were identified in the DRD2 gene—3 located in introns, 3 located in exons, and 2 located in the 3′UTR (Table 4). The 3 exonic SNPs were synonymous.

Exon 2

The 2 synonymous SNPs in exon 2 were evaluated for association to phenotype. First, we evaluated these with respect to social fear in Havanese and then to noise reactivity in the breeds collie, ISWT, NSDR, standard poodle, and Havanese. The allele frequencies varied between the breeds and can be found in Table 5.

Social fear in Havanese

Genetic assessment using the PBE

Significant association was detected between 2 SNPs in exon 2 of the DRD2 gene and social fear in Havanese dogs, classified through a PBE. In the first SNP (5:19782667), the allelic odds ratio was 4.35 (P-value 0.0008) (T = beneficial allele). In the second SNP (5:19782829), the allelic odds ratio was 4.07 (P-value 0.0010) (C = beneficial allele).

Genetic assessment using OQ

Significant association was detected between the 2 SNPs in exon 2 of the DRD2 gene and social fear in Havanese dogs, classified through an OQ (owner score). In the first SNP (5:19782667), the allelic odds ratio was 1.96 (P-value 0.0283) (T = beneficial allele). In the second SNP (5:19782829), the allelic odds ratio was 2.22 (P-value 0.0095) (C = beneficial allele). The average behavioral score of each genotype can be found in Table 6.

Table 4
SNPs identified in the DRD2 gene (Havanese)

Index	Intron/exon	Location (CanFam 3.1)	Alleles (CanFam3.1 in bold)	Amino acid change
1	Intron 1	5:19782497	**G**/A	-
2	Exon 2	5:19782666	**C**/T	Synonymous
3	Exon 2	5:19782828	**T**/C	Synonymous
4	Intron 4	5:19787766	**T**/C	-
5	Intron 4	5:19787788	**C**/T	-
6	Exon 7	5:19791794	**C**/T	Synonymous
7	Exon 8, 3′UTR	5:19794262	**A**/G	-
8	Exon 8, 3′UTR	5:19794287	**T**/C	-

Table 5
Allele frequencies (%) of the SNPs in exon 2 in the DRD2 gene

SNP chromosome and position[a]	Alleles	Havanese	Collie	ISWT	NSDR	Standard poodle
5:19782666	C/T	64/36	0/100	61/39	55/45	36/64
5:19782828	T/C	63/37	13/87	60/40	50/50	48/52

[a] Canfam 3.1.

Noise reactivity across breeds

Significant association between noise reactivity and the DRD2 gene was detected for the ISWT and collie. Significant association was found between the first SNP (5:19782667) and noise reactivity in the ISWT (this SNP showed no variation in the collie), with allelic odds ratio of 2.64 (P-value 0.0371) (T = beneficial allele). Association between noise reactivity and the second SNP (5:19782829) was significant in the ISWT with allelic odds ratio of 2.88 (P-value 0.0227) and in the collie with allelic odds ratio of 3.03 (P-value 0.0319) (C = beneficial allele).

Discussion

Significant associations were detected between SNPs in exon 2 of the DRD2 gene and social fears in Havanese and noise reactivity in ISWT and collie. Because the SNPs are synonymous, the functional effect associated with the SNPs is most likely due to the effect of variation in linked sequences/modifications. We found no association to noise reactivity in the Havanese, but the observed portion of dogs with noise reactivity in this breed was very low compared to the other breeds (Table 2), which may indicate that noise reactivity is not an issue of large importance in this specific breed.

The level of social fear in the Havanese dogs was classified through both an OQ and a PBE by an external evaluator. Observations made by the owner and observations made by an external evaluator have different strengths and weaknesses (Spady and Ostrander, 2008).

A challenge when working on behavioral traits is consistent recording of traits and describing them correctly. To obtain a consistent characterization and diminish misclassification, we used strict inclusion criteria for a dog to be classified as a case or control in the PBE. Because of the stated definitional and inclusion criteria, we believe that our records are consistent, which is important for a reliable analysis of the described and studied phenotype. This test will identify fearful dogs, although they will not all be equally fearful, and some may be less affected than others.

Previous studies have shown that complex behavioral patterns in dogs can be reliably evaluated by an experienced person and that a few, well selected characteristics may be sufficient to describe the differences between dogs (Wilsson and Sundgren, 1997).

The major weakness of owner evaluation may be that owners may evaluate dogs differently based on their skills and frame of reference. Owners may also not be objective because they are reluctant to classify their dog as fearful. One study showed that owners are less likely to report unfavorable behavioral traits in a nonconfidential survey, compared to a confidential survey (Segurson et al., 2005). Owners also may recognize or understand only the easiest to detect signs of any fear- or anxiety-related condition and so underestimate the presence of the condition if their dogs show different signs (Mariti et al., 2012). Finally, some behavioral signs are simply less apparent than others if constant monitoring of the dog is not occurring, suggesting that false negatives may be a risk (Overall et al., 2016). This issue is further discussed, below.

Table 6
The average behavioral score of each genotype

5:19782667 (beneficial allele in bold)	Average OS	5:19782829 (beneficial allele in bold)	Average OS
CC	4.00	TT	3.94
CT	4.15	T**C**	4.18
TT	4.39	**CC**	4.42

OS, owner score.

Subjective bias in owner evaluation (e.g., systematic underreporting of fear) could be a challenge if one owner/breeder was reporting several dogs from a certain line/genotype, which could lead to a false association. The number of Havanese per owner in this study was 2.08, and therefore, we do not believe that the owner classification represents a systematic problem. Most owners did, however, rate their dogs quite high, indicating low levels of fear.

Another challenge using owner ratings by questionnaires is that dogs may change by age and that the level of challenges/exposure (e.g., time of exposure, and loudness/type of fireworks) the dog have met at the time the owner replies to a questionnaire may influence results. If dogs are not exposed or not witnessed to react, owners would report a potential false negative (Overall et al., 2016). We note that in the survey-based classification for social fear in Havanese, there was no significant age difference between the case and control groups.

To reduce the risk of misclassification, the survey questions were based on wording frequently used by owners, to ensure a mutual understanding of the terminology. In addition, the distinct phenotypic variations in the Havanese breed combined with relatively strict inclusion criteria for cases and controls should help reduce the risk of misclassification. Studies show that questionnaires designed in a similar matter have acceptable validity (Duffy et al., 2014; Hsu and Serpell, 2003).

In the owner-based classification, we observed that the criteria/threshold for inclusion of dogs as cases/controls could have a marked influence on the results. This demonstrates the challenge of a biologically correct behavioral phenotyping and may, together with genetic heterogeneity, explain the variable reproducibility of many studies on genetics of behavior. To reduce the frequency of misclassification and obtain a clear difference between cases and controls, we were conservative and cutoffs were set as 0.5 σ above and below average owner score.

We found good correlation between the survey-based and observational classification of the Havanese ($\rho = 0.738$, P-value < 0.001). All Havanese dogs that were classified as cases using the questionnaire were also classified as cases in the PBE. Four of the dogs that were classified as controls using the questionnaire were classified as cases in the PBE, suggesting that the concern with false negatives on owner-based assessments is real (PPV = 1.0; NPV = 0.88), but minor. This result confirms that there is generally very good concordance between the 2 classification systems but also underpins our hypothesis of a slight underreporting/underobserving of fear in the OQ producing the occasional false negative as has been noted in other studies (Overall et al., 2016). That owners appear to produce false negatives suggests that more studies should validate questionnaire results with behavioral test results within the same population under study, something especially important for genetic studies.

In this initial study, we did not identify functional mutations in any of the successfully sequenced exons of DRD2, as the identified SNPs associated with fear were both synonymous. However, some studies have shown the likely effect of synonymous mutations in DRD2 on RNA stability (Duan et al., 2003). If DRD2 is functionally involved, the functional effects may also be due to closely linked

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variants in gene regulatory regions (3′ or 5′ to the gene, including promoter regions) or to epigenetic effects.

It is also possible that the functional effect is caused by variants in closely linked genes. The region 3′ to the gene involves genes including NCAM1-TTC12-ANKK1 and is frequently discussed in behavioral issues (Savitz et al., 2013). Functional interactions with other relevant genes are also reported (Montag et al., 2010). On the 5′-side of DRD2 are HTR3A and HTR3B, 2 serotonin receptors and potential candidate genes (Jajodia et al., 2015; Kondo et al., 2015), within 300K distance. This is a region with many candidate genes reported to be associated with anxiety, which supports that the present results may indicate important functional variants in the region.

Exon 2 was prioritized in the association testing and sequenced for all individuals. Exon 7 was only sequenced for a small group of dogs to look for variation. Because the SNPs in exon 2 are located only 8966 base pairs away from the SNP in exon 7, the probability of recombinations between them is negligible. We expect a large degree of LD between all markers within the gene, i.e., variation in exon 7 would be covered by the variation in exon 2. The SNPs in exon 2 were prioritized over the SNP in exon 7, partly because of a higher estimated MAF.

Conclusion

SNPs in exon 2 of the DRD2 gene are significantly associated with an increased tendency to react fearfully in social situations in Havanese and noise reactivity in Irish soft-coated wheaten terrier and collie. The same alleles were associated with the beneficial phenotypes in the 3 breeds. There was no significant association between noise reactivity and the SNPs in the Havanese, NSDR, or standard poodle. Because the SNPs are synonymous, the functional effect associated with the SNPs is most likely due to linked mutations and/or epigenetic effects.

Acknowledgment

The study was funded by the Norwegian University of Life Sciences, The Norwegian Kennel Club, and The Research Council of Norway.

Ethical considerations

EDTA-blood samples were collected by certified veterinarians, with owner's consent, in agreement with the provisions enforced by the Norwegian Animal Research Authority. The samples were collected according to rules for ethical approval for collecting blood samples (FOR-2010-07-08-1085, FOR-1996-01-15-23, Regulation on Animal Experimentation).

Conflict of interest

Karen L. Overall is the Editor-in-Chief of Journal of Veterinary Behavior: Clinical Applications and Research. Another editor, Christel Moons, was appointed to manage all steps of this submission to ensure that the highest ethical standards were upheld by the Journal.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jveb.2018.07.008.

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