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ORIGINAL RESEARCH

Evaluation of the Serotonergic Genes *htr1A*, *htr1B*, *htr2A*, and *slc6A4* in Aggressive Behavior of Golden Retriever Dogs

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Abstract Aggressive behavior displays a high heritability in our study group of Golden Retriever dogs. Alterations in brain serotonin metabolism have been described in aggressive dogs before. Here, we evaluate whether four genes of the canine serotonergic system, coding for the serotonin receptors 1A, 1B, and 2A, and the serotonin transporter, could play a major role in aggression in Golden Retrievers. We performed mutation screens, linkage analysis, an association study, and a quantitative genetic analysis. There was no systematic difference between the coding DNA sequence of the candidate genes in aggressive and non-aggressive Golden Retrievers. An affecteds-only parametric linkage analysis revealed no strong major locus effect on human-directed aggression related to the candidate genes. An analysis of 41 single nucleotide polymorphisms (SNPs) in the 1 Mb regions flanking the genes in 49 unrelated human-directed aggressive and 49 unrelated non-aggressive dogs did not show association of SNP alleles, genotypes, or haplotypes with aggression at the candidate loci. We completed our analyses with a study of the effect of variation in the candidate genes on a collection of aggression-related phenotypic measures. The effects of the candidate gene haplotypes were estimated using the Restricted Maximum Likelihood method, with the haplotypes included as fixed effects in a linear animal model. We observed no effect of the candidate gene haplotypes on a range of aggression-related phenotypes, thus extending our conclusions to several types of aggressive behavior. We conclude that it is unlikely that these genes play a major role in the variation in aggression in the Golden Retrievers that we studied. Smaller phenotypic effects of these loci could not be ruled out with our sample size.

Keywords Dog · Aggression · Serotonin · Candidate gene · Linkage · Association

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Introduction

Dogs have been living in close proximity to humans for at least 15,000 years (Clutton-Brock 1995). Behavior has

been a strong selective factor in the domestication and breeding of dogs. According to the breed standard, Golden Retriever dogs should have a friendly character (http://www.goldenretrieverclub.nl; link accessed March 2007). However, there are reports of very aggressive Golden Retrievers (Galac and Knol 1997; Heath 1991). We recently described the behavioral phenotype of 110 Golden Retrievers referred to our clinic for aggression problems and 118 Golden Retrievers that were recruited because they were related to one or more of the aggressive dogs (van den Berg et al. 2006). The phenotypes were based on mail questionnaires and on personal interviews with dog owners. In a quantitative genetic study including 325 Golden Retrievers, we found a heritability of 0.8 for the traits of human-directed aggression and dog-directed aggression (Liinamo et al. 2007).

The influence of serotonin (5-hydroxytryptamine, 5-HT) on aggressive behavior has been studied extensively (reviewed by Berman and Coccaro 1998; Gingrich and Hen 2001; Lesch and Merschdorf 2000). There is evidence for a role of the 5-HT system in canine aggression as well. For instance, Reisner and colleagues (1996) reported decreased levels of 5-hydroxyindoleacetic acid (the major metabolite of 5-HT) in cerebrospinal fluid of dominant aggressive dogs. Badino et al. (2004) found modifications of 5-HT receptor concentrations in brains of aggressive dogs. Domestication of silver foxes, which are taxonomically close relatives of dogs, seems to cause modifications in the 5-HT system (see Trut 2001 for a review). The role of 5-HT in canine aggression is further supported by two small clinical studies, where pharmacological or dietary intervention in the 5-HT system was shown to modulate aggressive behavior (DeNapoli et al. 2000; Dodman et al. 1996).

Four genes that code for factors involved in serotonergic neurotransmission are particularly good candidates for the regulation of aggressive behavior: the serotonin receptor genes 1A (*htr1A*), 1B (*htr1B*), and 2A (*htr2A*), and the serotonin transporter gene (*slc6A4*). Serotonin receptor 1A plays a role in anxiety, stress response, and aggression (Olivier et al. 1995). *Htr1A* knockout mice show increased anxiety and stress response and an antidepressant-like phenotype (Heisler et al. 1998; Ramboz et al. 1998). In the above-mentioned studies of silver foxes, the researchers observed a lower density of 5-HT_{1A} receptors in the hypothalamus of tame foxes compared to their wild counterparts (Popova et al. 1991). Many studies have suggested involvement of *htr1B* in the etiology of mental disorders. For instance, Huang et al. (2003) and Sanders et al. (2002) reported an association between one of the polymorphisms in the human *HTR1B* gene and alcoholism, suicidality, and obsessive-compulsive disorder. Knockout mice lacking *htr1B* display increased aggression (Saudou et al. 1994). A mutation in the human *HTR2A* gene is associated with altered 5-HT binding, which has been implicated in schizophrenia, suicidal behavior, impaired impulse control, and aggression history (Abdolmaleky et al. 2004; Bjork et al. 2002; Khait et al. 2005). Peremans and colleagues (2003) found an increased binding index of serotonin 2A receptors in cortical brain regions of impulsive aggressive dogs. A polymorphism in the promoter region of *SLC6A4* influences serotonin transporter density in the brain and is associated with mental disorders in humans (Anguelova et al. 2003; Hariri et al. 2002; Lesch et al. 1996). *Slc6A4* knockout mice show reduced aggression (Holmes et al. 2003).

In this paper, we test the hypothesis that there is a strong effect of variation in these genes on the variation in aggression in Golden Retrievers. We performed mutation screens of the coding DNA sequence in unrelated aggressive Golden Retrievers. In addition, we used linkage analysis to determine the likelihood of the presence of a strong aggression locus in or close to the genes in several dog families. Third, we used 50 unrelated aggressive Golden Retrievers and 50 unrelated non-aggressive Golden Retrievers to search for association of alleles of 41 SNPs flanking the candidate genes with the trait of human-directed aggression. To complete our analyses, we evaluated the effects of variation in the genes on a range of aggression-related phenotypes using the same models as in Liinamo et al. (2007), extended to include the effects of the most common candidate gene haplotypes.

Materials and methods

Animals, DNA isolation, and phenotyping

We have collected behavioral information of 328 privately owned Golden Retrievers. This group includes 162 dogs that were referred to our clinic because of their aggressive behavior ("probands") and 166 relatives of 36 probands. DNA samples were available for 281 of these dogs. In addition, we collected DNA of a cohort of random privately owned Goldens that were born between July 2002 and February 2003. No phenotypes were available for these random dogs. Genomic DNA was isolated from whole blood leucocytes using a standard protocol (Miller et al.1988). For each type of analysis (mutation analysis, linkage analysis, association analysis, and quantitative genetic analysis) we selected a study group that was suited for the study design. The study groups are described in the Supplementary Information I.

We have collected various quantitative measures of aggressiveness for the dogs (van den Berg et al. 2003a, 2006). In the linkage and association analysis we focused

on one of these measures: the dog owners impression on human-directed aggression. Owner impressions were collected in a personal interview. We asked the owners if their dog was aggressive towards humans and the status of the dog was coded in three classes: non-aggressive (score 1), threatens (score 2), or bites (score 3). We focused on human-directed aggression because the majority of the probands were referred to our clinic for human-directed aggressive behavior. Owner impressions were available for all dogs and the quantitative genetic analyses showed that the heritability of this trait was high in our population of dogs (Liinamo et al. 2007).

Mutation screening

We analyzed the coding DNA sequence (CDS) of the four candidate genes in seven (*htr1A* and *htr1B*) or eight (*htr2A* and *slc6A4*) probands. The CDS were amplified and sequenced using overlapping primer pairs as described previously (van den Berg et al. 2004, 2005). Possible functional effects of polymorphisms were predicted with POLYPHEN (http://www.genetics.bwh.harvard.edu/cgi-bin/pph/polyphen.cgi). Effects of polymorphisms close to splice sites were predicted with three splice prediction programs: NetGene2 (Brunak et al. 1991), Splice Prediction by Neural Network (Reese et al. 1997), and SpliceSiteFinder (Shapiro and Senapathy 1987).

Linkage analysis

We used nine families for linkage analysis (Figs. S1–S9 of Supplementary Information I). DNA samples were available for 31 affected and 65 unaffected dogs from these families. We converted the owner impression about human-directed aggression into a dichotomous variable for the linkage analysis (see Supplementary Information I). We have described polymorphic markers for the candidate genes before (van den Berg et al. 2003b, 2004, 2005). We selected three microsatellite markers and seven single nucleotide polymorphisms for linkage analysis (see Table 1). Microsatellite markers were genotyped after PCR on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). PCR conditions were described by van den Berg et al. (2004, 2005). GENESCAN 3.7 software was used for genotype assessment. Single nucleotide polymorphism genotyping was performed by DNA sequencing of PCR products on the ABI 3100 Genetic Analyzer (van den Berg et al. 2004, 2005). The DNA sequence chromatograms were inspected using LASERGENE software (DNASTAR, Inc., Madison, WI USA). We combined several markers into haplotypes for the genes *htr1B*, *htr2A*, and *slc6A4*. For dogs that were heterozygous for multiple markers, we deduced the haplotypes from the data of relatives. If this was not possible, we assigned the most frequently observed possible haplotypes to these dogs.

We performed a parametric affecteds-only linkage analysis to determine whether the candidate gene haplotypes were linked to aggressive behavior in the Golden Retriever families. Marker haplotype frequencies were determined in a group of 27 (*htr1A* and *htr2A*), 31 (*htr1B*), or 26 (*slc6A4*) parent dogs (see Supplementary Information I). The mode of inheritance of the aggressive phenotype in our families is unclear. We therefore analyzed the data under both autosomal dominant and autosomal recessive models. The penetrance of the genotype at risk was set at 0.01. In this way, affected dogs are assumed to have the risk allele and the software calculated likelihood that aggressive dogs share alleles by descent from a common ancestor. Unaffected dogs with the genotype at risk have no effect on the outcome of the calculation. We assumed that there were no phenocopies in the families and we assumed genetic homogeneity because all probands were related to each other within a limited number of generations (not shown). The frequency of the aggression allele was set at 0.1 to allow for multiple transmitting ancestors in the pedigrees. SUPERLINK software was used to calculate two-point logarithm of the odds (LOD) scores (Fishelson and Geiger 2002, 2004).

In order to estimate the power of the pedigrees, we calculated the maximum obtainable LOD scores. Affected individuals were assigned haplotypes 2/2 in these calculations; unaffected parents were assigned haplotypes 1/2; and other unaffected individuals were assigned haplotypes 1/1. We assigned haplotypes 0/0 (unknown) to dogs from which we did not have a DNA sample. We assumed that there were four alleles of the hypothetical marker with equal allele frequencies.

Association study

To test for a more complex genetic effect of variants of the candidate genes, we performed an association study. Fifty aggressive Golden Retrievers were selected from our database. The main selection criteria were high estimated breeding values for human-directed aggression and as little interrelationship among the cases as possible. Non-aggressive dogs were selected for low estimated breeding values for both human- and dog-directed aggression. We avoided an excess of relationship within either the case or the control group. The non-aggressive group was completed with 25 dogs from the random group. A more

Table 1 Markers used for linkage analysis

Gene[a]	Type of marker[b]	Position of marker[c]	Alleles observed (allele frequency)[d]	Haplotypes observed (haplotype frequency)[d]
htr1A	$(CA)_n$	*7370	297 (0.5)	–
	(UU160O12)		303 (0.5)	
htr1B	A/C SNP[e]	157	A (0.58)	143-A-G-T-G (0.20)
			C (0.42)	143-C-A-T-G (0.42)
	G/A SNP	246	G (0.58)	143-A-G-C-C (0.06)
			A (0.42)	139-A-G-C-C (0.24)
	T/C SNP	955	T (0.69)	139-A-G-T-G (0.05)
			C (0.31)	147-A-G-T-G (0.03)
	G/C SNP	1146	G (0.69)	
			C (0.31)	
	$(GA)_n$	−68395	139 (0.29)	
	(UU18L8)		143 (0.68)	
			147 (0.03)	
htr2A	C/T SNP	IVS 2-10	C (0.85)	128-C (0.07)
			T (0.15)	130-C (0.41)
	$(CA)_n$	IVS2 + 1439	128 (0.07)	132-C (0.37)
	(UUHTR2AEX2)		130 (0.41)	132-T (0.15)
			132 (0.52)	
slc6A4	C/T SNP	411	C (0.75)	C-G (0.75)
			T (0.25)	T-A (0.25)
	G/A SNP	IVS9-12	G (0.75)	
			A (0.25)	

[a] *htr1A*, *htr1B*, *htr2A* = respectively serotonin receptor 1A, 1B, and 2A gene; *slc6A4* = serotonin transporter gene

[b] SNP = single nucleotide polymorphism. Names of microsatellite markers have been included in brackets

[c] Position refers to the coding sequence of the canine gene. We used the nomenclature recommended by den Dunnen and Antonarakis (2001): the A of the ATG start codon is designated number 1, the nucleotide 5′ to this A is numbered −1, and the nucleotide 3′ of the translation termination codon is *1. Positions in introns refer to the nearest exon. The nomenclature of the introns is based on the human gene structure. IVS = intervening sequence

[d] Allele and haplotype frequencies were determined in a group of 27 (*htr1A* and *htr2A*), 31 (*htr1B*), or 26 (*slc6A4*) parents

[e] This polymorphism is nonsynonymous

detailed description of cases and controls is provided in Supplementary Information I.

We genotyped a total number of 60,073 SNPs in these 100 Golden Retrievers using customized Affymetrix Genotyping Arrays. The SNPs were selected for the chip using a scoring system that optimized the SNPs accounting for low repeat content, low likelihood of SNPs in the assay probe sequences and their distribution over the genome as a whole (Lindblad-Toh 2007; personal communication). Twenty 32-mer probes interrogated each locus with genotyping calls made using the algorithm BRLMM (http://www.affymetrix.com) which analyses intensities for sets of probes that interrogate both forward and reverse sequences with perfect match and mismatched probes. Dogs with call rates of lower than 50% were discarded from the analysis. The total set of SNPs was filtered for genotype call probability, heterozygosity rate, and call rate across a large set of dogs, reducing the dataset to 26,625 SNPs. From this set, only SNPs within 1 Mb of the four candidate genes were used for the analysis described in this paper. There were 29 SNPs within 1 Mb of *htr1A*, 20 within 1 Mb of *htr1B*, 25 within 1 Mb of *htr2A*, and 20 within 1 Mb of *slc6A4*. From these SNPs, we selected 43 SNPs with a minor allele frequency >0.05 and call rates of >0.75.

We used Haploview software version 4.0 (Barrett et al. 2005) for the analysis of the presence of Hardy-Weinberg equilibrium (HWE), the local association analysis, and the calculation of pairwise linkage disequilibrium (LD) between the SNPs. A HWE *P*-value cutoff of 0.001 was used. We used Bonferonni correction to account for multiple testing in the association analysis. Genotype frequencies in cases and controls were compared with Chi square tests using SPSS software. Two

tailed Fisher's exact tests were used when the number of expected cases was less than 5 in more than 20% of the categories. Haplotype blocks were formed using three methods in Haploview (confidence intervals, four gamete rule, and solid spine of LD). For all possible combinations we performed 10,000 permutations to obtain empirical P values for haplotype association tests. We used the genetic power calculator prepared by Purcell to estimate the power of the association analysis (http://www.pngu.mgh.harvard.edu/~purcell/gpc/). The following assumptions were made: high-risk allele frequency = 0.1; prevalence = 0.01. The mean pairwise D′ between the SNPs flanking a candidate gene was used as an estimate of the local D′ in these estimations. Calculations were performed for two different genotype relative risks: 2 (genotype relative risk Aa = 2; genotype relative risk AA = 4) and 5 (genotype relative risk Aa = 5; genotype relative risk AA = 10).

Quantitative genetic analysis

In addition to the owner impression on human-directed aggression and dog-directed aggression, we collected a variety of other aggression-related behavioral measures using the canine behavioral assessment and research questionnaire (CBARQ; Hsu and Serpell 2003). As described in Liinamo et al. (2007), these measures were of three types: original CBARQ items (27 items on the aggressiveness of the dog in various everyday situations), shortened CBARQ scores (scores based on questions that addressed stranger-directed, owner-directed, and strange dog-directed aggression), and CBARQ factors (scores based on questions about stranger-directed, owner-directed, and strange dog-directed, and familiar dog-directed aggression). For further explanation of the measures and the difference between shortened CBARQ scores and CBARQ factors, see Liinamo et al. (2007).

The effects of the haplotypes of the serotonergic genes on the different aggression measures were estimated with Restricted Maximum Likelihood (REML) method (Patterson and Thompson 1971), using univariate analyses and an animal model with the VCE4.2.4 software (Groeneveld 1997). The analyses were an extension of the analyses outlined in Liinamo et al. (2007), using similar linear animal model methodology, but this time also including the haplotype classes of the dogs for the four studied loci as additional fixed effects in the model. For instance, the linear animal model that was assumed in the analyses for owner impression traits was:

$$y_{ijklmno} = \mu + sex_i + age_j + htr1a_k + htr1b_l + htr2a_m + slc6a4_n + a_o + e_o$$

where $y_{ijklmno}$ is the observed value for the owner impression score for animal o; μ the general mean in the population; sex_i the fixed effect of the reproductive status (i = 1–4, with 1 = intact male, 2 = castrated male, 3 = intact female, and 4 = castrated female); age_j the fixed effect of the age j (j = 1–11, with 1 = 0.5–1 year old, 2 = 1–2 years old,…,10 = 9–10 years old, and 11 = over 10 years old); $htr1a_k$, $htr1b_l$, $htr2a_m$, and $slc6a4_n$ the fixed effects of the respective haplotype classes, a_o the random additive genetic effect (i.e., polygenic breeding value) of the animal o, and e_o the random residual effect related to the animal o. The age and reproductive status of the dogs had been recorded at the same time as the owner impressions. The haplotype classes were formed so that the most common haplotypes formed separate classes, the very rare haplotypes were all combined in one class, and the unknown haplotypes were classified as a separate class (see Table 4).

Results

Mutation screening of the coding DNA sequence

The coding DNA sequence of each candidate gene was scanned for mutations in seven (*htr1A* and *htr1B*) or eight (*htr2A* and *slc6A4*) aggressive Golden Retrievers. Analysis of the CDS in non-aggressive Golden Retrievers has been described by van den Berg et al. (2004; 2005). There was no variation in the CDS of *htr1A* and *htr2A* in the Golden Retrievers. We observed five SNPs in the CDS of *htr1B* and one SNP in the CDS of *slc6A4*. The allele distribution of these SNPs in the two groups of Golden Retrievers did not indicate a role in aggressive behavior. In conclusion, there seems to be no systematic difference between the CDS of the candidate genes in aggressive and non-aggressive Golden Retrievers.

Linkage analysis

We observed two alleles for *htr1A* marker UU160O12 (Table 1). The four SNPs in *htr1B* were fully in LD (D′ = 1). The SNPs displayed six haplotypes in the Goldens, three of which were rare (frequency <0.1). We detected four haplotypes of *htr2A* in the Golden Retrievers. The two SNPs in *slc6A4* were fully in LD and formed two haplotypes in the dogs. In the nine families that we used for linkage analysis, haplotypes were deduced with certainty in 86% of the dogs for *htr1B*, 100% for *htr2A*, and 87% for *slc6A4*. We calculated the maximum achievable LOD score using hypothetical genotypes. The maximum LOD

Table 2 Results from the ODDS (LOD) scores[a]

Gene	Autosomal dominant	Autosomal recessive
htr1A	0.26	−0.30
htr1B	−0.72	−2.3
htr2A	−1.0	−2.1
slc6A4	0.030	−1.2
Maximum	2.8	5.3

[a] LOD scores were calculated with the following assumptions: frequency of the "aggression allele" = 0.1; penetrance of the "aggression allele" = 0.01; $\theta = 0$. Marker haplotype frequencies were deduced from a group of parents

score generated by our pedigrees was 2.8 at recombination fraction $\theta = 0$ assuming a dominant mode of inheritance (Table 2). Under a recessive model, the maximum LOD score was 5.3 at $\theta = 0$. The families are therefore theoretically powerful enough to prove linkage under a recessive model and powerful enough to provide a good indication of the presence of linkage under a dominant model. There was no significant linkage of any of the candidate genes with the aggressive phenotype (Table 2). LOD scores varied from −1.0 to +0.26 assuming dominant inheritance and from −2.3 to −0.30 assuming recessive inheritance. The highest LOD scores were obtained for *htr1A* (+0.26 under a dominant model and −0.30 under a recessive model).

Association study

Two dogs (one case and one control) were discarded from the association analysis because they had call rates lower than 50%. Mean call rates in the other 98 dogs were 92% for SNPs flanking *htr1A*, 94% for *htr1B*, 93% for *htr2A*, and 93% for *slc6A4*. There were 43 SNPs with a minor allele frequency >0.05 and call rates of >0.75 that occurred within 1 Mb of the candidate genes. The genotype frequencies of BICF2P1093362 for *htr1B* and BICF2P969902 for *slc6A4* deviated from HWE in control dogs ($P = 4.42E\text{-}14$ and $P = 5.242E\text{-}12$, respectively). All but one dog in both case and control group had heterozygous genotypes for these SNPs. We concluded that the data for these two SNPs was artefactual and they were excluded from further analyses. The observed genotype frequencies of the other 41 SNPs were in HWE in controls (P values are listed in Table S2 in Supplementary Information II). The final SNP set used for the association analysis consisted of 12 SNPs flanking *htr1A*, 11 flanking *htr1B*, 8 flanking *htr2A*, and 10 flanking *slc6A4* (Table 3).

The power of our association analysis depends on the local extent of LD. The mean r^2 between the SNPs flanking *htr1A* was 0.36 and the mean D′ between these SNPs was 0.89. Mean r^2 values were 0.23, 0.25, and 0.21 for *htr1B*, *htr2A*, and *slc6A4*, respectively. Mean D′ values were 0.80, 0.72, and 0.88 for *htr1B*, *htr2A*, and *slc6A4*, respectively. When the marker allele frequency is 0.1, the power to detect a variant with a relative risk of 5 with 49 cases and 49 controls would be 0.91 for *htr1A*, 0.85 for *htr1B*, 0.78 for *htr2A*, and 0.90 for *slc6A4*. Additional power estimations are provided in Fig. S10 in Supplementary Information II.

The allele frequencies of the SNPs did not differ significantly between cases and controls after correction for multiple testing (Table 3). Genotype frequencies also did not display significant differences between cases and controls (see Table S2 in Supplementary Information II). We also analyzed the association of haplotypes with the phenotype. No significant associations were found (data not shown). In conclusion, there seemed to be no association between alleles, genotypes or haplotypes of SNPs that flank the candidate genes and human-directed aggression in the Golden Retrievers.

Quantitative genetic analysis

We completed our analyses with a study of the effect of variation in the candidate genes on a collection of aggression-related phenotypic measures. The haplotype effects were studied on owner impressions on human- and dog-directed aggression, the original CBARQ items related to stranger- and owner-directed aggression, the shortened CBARQ scores and the CBARQ factors. The haplotypes did not have a significant effect on any of the studied measures, i.e. the heritability estimates of the measures remained similar to the results presented in Liinamo et al. (2007) in spite of incorporation of the haplotypes in the mixed model. The results for owner impressions on human- and dog-directed aggression, which are the most reliable estimates due to the largest number of observations, are presented in Table 4. In conclusion, the large genetic variability between the dogs could not be explained by the serotonergic genes studied in this paper.

Discussion

We collected behavioral information and DNA samples of 281 dogs over a period of 10 years. Dogs were selected from this collection to evaluate four genes involved in serotonin metabolism by four methods: DNA sequence analysis of the coding region of the genes, genetic linkage analysis, genetic association analysis, and quantitative genetic analysis. The results indicate that it is unlikely that there is a major locus effect of one of the genes on aggression in the Golden Retrievers that we studied.

Table 3 Single nucleotide polymorphisms (SNPs) used for the association study, their allele frequencies in 49 aggressive cases and 49 control dogs, and results of chi-square tests for comparisons of case and control allele frequencies

SNP name[a]	Chromosomal location	Chromosomal position (Mb)[b]	Minor allele frequency in controls	Corresponding allele frequency in cases	χ^2	*P*-value
BICF2P546848	2	51.80	0.29	0.30	0.044	0.83
BICF2P1051894	2	51.96	0.073	0.028	1.6	0.21
BICF2P1398268	2	52.01	0.33	0.33	0.011	0.92
BICF2S23127755	2	52.14	0.23	0.21	0.078	0.78
BICF2P1200391	2	52.22	0.30	0.33	0.21	0.64
BICF2P590055	2	52.35	0.24	0.27	0.19	0.66
BICF2S22939125	2	52.44	0.48	0.53	0.54	0.46
BICF2S23215863	2	52.48	0.30	0.31	0.025	0.87
BICF2P25993	2	52.73	0.49	0.45	0.19	0.66
BICF2S23442706	2	52.76	0.19	0.28	1.8	0.18
Htr1A	*2*	*52.88–52.88*	–	–	–	–
BICF2P519607	2	53.22	0.27	0.24	0.17	0.68
BICF2P1341930	2	53.62	0.49	0.54	0.52	0.47
BICF2P1159241	12	41.11	0.12	0.13	0.012	0.91
BICF2P555130	12	41.59	0.13	0.21	2.1	0.15
BICF2S23326229	12	41.63	0.24	0.32	1.3	0.25
Htr1B	*12*	*41.65–41.66*	–	–		–
BICF2P670331	12	41.80	0.15	0.23	2.2	0.14
BICF2S23153760	12	41.84	0.039	0.12	3.2	0.073
BICF2P1426522	12	42.29	0.12	0.12	0.013	0.91
BICF2P27571	12	42.34	0.12	0.10	0.21	0.65
BICF2S23444066	12	42.39	0.12	0.11	0.032	0.86
TIGRP2P164447_rs8805986	12	42.51	0.038	0.12	3.6	0.059
BICF2P812153	12	42.57	0.033	0.12	4.8	0.029
BICF2P855402	12	42.62	0.021	0.12	6.9	0.0086
BICF2G630315581	22	6.383	0.10	0.14	0.83	0.36
BICF2G630315746	22	6.611	0.10	0.14	0.45	0.50
BICF2P1168502	22	6.973	0.10	0.11	0.0020	0.96
Htr2A	*22*	*7.395–7.453*	–	–	–	–
BICF2P164280	22	7.509	0.43	0.54	2.0	0.16

Table 3 continued

SNP name[a]	Chromosomal location	Chromosomal position (Mb)[b]	Minor allele frequency in controls	Corresponding allele frequency in cases	χ^2	*P*-value
BICF2G630316047	22	7.709	0.44	0.53	1.7	0.19
BICF2S23125159	22	7.866	0.19	0.21	0.047	0.83
BICF2S23661838	22	8.207	0.31	0.36	0.59	0.44
BICF2S22954191	22	8.470	0.23	0.27	0.35	0.55
BICF2P813837	9	46.93	0.39	0.30	1.5	0.22
BICF2S23018060	9	47.06	0.33	0.37	0.22	0.64
BICF2S23551918	9	47.46	0.34	0.38	0.24	0.62
Slc6A4	*9*	*47.55–47.57*				–
BICF2S23325050	9	47.64	0.11	0.15	0.53	0.47
BICF2S23124809	9	47.79	0.18	0.22	0.38	0.54
BICF2P950384	9	47.88	0.11	0.12	0.032	0.86
BICF2S245135	9	48.02	0.27	0.25	0.074	0.79
BICF2S2347312	9	48.09	0.22	0.28	0.84	0.36
BICF2S23154457	9	48.13	0.31	0.24	1.2	0.28
BICF2S23141984	9	48.26	0.36	0.36	0.0070	0.93

[a] *htr1A*, *htr1B*, *htr2A* = respectively serotonin receptor 1A, 1B, and 2A gene; *slc6A4* = serotonin transporter gene. The genes are included in the table to show their position relative to the SNPs

[b] SNP positions are based on the second version of the dog genome assembly, released in May 2005 (CanFam2.0) as can be viewed on http://www.broad.mit.edu/ftp/pub/papers/dog_genome/snps_canfam2/

Positions of the genes are based on the second version of the dog genome assembly (CanFam2.0) as displayed in NCBI Map Viewer for *Canis familiaris* http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9615

Table 4 The effects of the studied genotypes (*htr1A*) or haplotypes (*htr1B, htr2A*, and *slc6A4*) on owner impressions of human-directed aggression and dog-directed aggression in 320 dogs

Genotype or haplotype class[a]	Number of animals	Effect relative to class 'unknown'	
		Human-directed aggression	Dog-directed aggression
htr1A			
Unknown	44	0.00	0.00
297/297	74	0.028	0.11
297/303	111	0.24	0.054
297/305	15	0.012	0.089
303/303	62	0.18	0.085
303/305	10	−0.11	−0.067
Other	4	−0.046	−0.17
htr1B			
Unknown	166	0.00	0.00
143-A-G-T-G/143-A-G-T-G	20	−0.73	0.28
143-A-G-T-G /143-C-A-T-G	8	−0.63	−0.19
143-A-G-T-G /139-A-G-C-C	19	0.11	0.19
143-C-A-T-G/143-C-A-T-G	41	−0.17	0.13
143-C-A-T-G /143-A-G-C-C	10	−0.021	0.059
143-C-A-T-G /139-A-G-C-C	30	0.0006	0.32
139-A-G-C-C/139-A-G-C-C	9	0.24	0.11
Other	17	0.15	0.36
htr2A			
Unknown	114	0.00	0.00
132-C/132-C	40	−0.15	−0.19
132-C/130-C	60	−0.27	−0.023
132-C/132-T	28	−0.22	0.073
132-C/128-C	15	−0.38	−0.43
130-C/130-C	21	−0.33	−0.072
130-C/132-T	22	0.10	−0.19
130-C/128-C	9	−0.52	−0.29
Other	11	0.33	−0.036
slc6A4			
Unknown	103	0.00	0.00
C-G/C-G	116	0.025	−0.23
C-G/T-A	94	0.14	−0.23
Other	7	0.079	−0.10

None of the effects was significant

[a] Note that there are additional alleles compared to Table 1 as a result of the larger study group. The *htr1A* class "other" contains genotypes 295/297 ($n = 2$ dogs) and 305/305 ($n = 1$ dog). For *htr1B*, the class "other" contains haplotypes 143-A-G-T-G/143-A-G-C-C ($n = 3$ dogs), 143-A-G-T-G/139-A-G-T-G ($n = 1$ dog), 143-A-G-T-G/147-A-G-T-G ($n = 2$ dogs), 143-A-G-C-C /139-A-G-C-C ($n = 3$ dogs), 143-A-G-C-C/147-A-G-T-G ($n = 1$ dog), 139-A-G-C-C / 139-A-G-T-G ($n = 3$ dogs), and 143-C-A-T-G/139-A-G-T-G ($n = 4$ dogs). For *htr2A*, "other" contains haplotypes 0/130-C ($n = 2$ dogs), 0/132-T ($n = 1$ dog), 132-T /132-T ($n = 6$ dogs), and 132-T/128-C ($n = 2$ dogs). For *slc6A4*, "other" contains C-G/T-G ($n = 1$ dog) and T-A/T-A ($n = 6$ dogs). Unknown genotypes and haplotypes are the result of failure of genotyping or of the absence of a DNA sample ($n = 44$ dogs)

The genetic study of the variation of aggression in Golden Retrievers is a promising tool to identify the molecular systems involved in aggression. The relative ease to find disease loci in the dog genome compared to the human genome is the result of the population structure of dog breeds. Within a breed, Lindblad-Toh and colleagues (2005) observed a limited number of common haplotypes per genomic region. In addition, LD in dog breeds extends over at least 50-fold greater distances than in human populations. These characteristics make the dog highly suited for molecular genetic studies of complex traits (Sutter and Ostrander 2004).

We did not detect mutations in the CDS of the genes specific for aggressive dogs. All SNPs except A157C in *htr1B* were synonymous. This A157C variation, resulting in an isoleucine/leucine polymorphism of amino acid 53, was predicted to be functionally insignificant by POLYPHEN. The genes *htr2A* and *slc6A4* contain SNPs close to splice sites (at position IVS2-10 and IVS9-12, respectively) that could theoretically affect splicing. However, the polymorphisms did not have a large effect on splice site prediction by three software programs. We performed mutation screening in a limited number of dogs and it is possible that we have missed rare alterations in the genes.

Apart from this limitation, we conclude that there is no common variant acting on protein structure that contributes to the variation in aggression in our Golden Retriever sample.

We used linkage analysis to evaluate the likelihood that there is a major aggression-influencing variant in the chromosomal regions surrounding the coding exons. The affecteds only parameters that we used in the calculations are a simplification with the assumption that all affected dogs of a family have the genotype at risk but unaffected dogs can have any genotype at the aggression locus. A LOD score of 3 is usually considered as evidence for linkage, whereas LOD scores below −2 exclude the gene. A power calculation with simulated genotypes was not feasible in this study due to the complexity of some pedigrees with multiple loops. Instead, we calculated the maximum achievable LOD scores to get an impression of the power embodied in the pedigrees. We assumed full informativeness of the markers in these calculations, but in reality, we expect the markers to have limited informativeness. Realistic obtainable LOD scores would then be lower than the maximum values that we presented in Table 2. These LOD scores are too low to obtain significant results, but they provide a means to set the obtained results into perspective. The LOD scores for *htr1B* and *htr2A* were low compared to the maximum obtainable scores. A major role of these genes is unlikely. The results for *htr1A* and *slc6A4* are less conclusive. This is probably the result of the poor informativeness of the markers. For both genes, we observed only two alleles or haplotypes with high frequencies. Typing of additional markers might help to definitively exclude the genes. However, in the light of the observed low level of variation it is unlikely that *htr1A* and *slc6A4* have a strong effect on aggression in the Golden Retriever families.

Our linkage analysis does not account for genetic heterogeneity or phenocopies. We have thus only tested for a very strong major locus effect. In reality, the aggressiveness in the Golden Retrievers may be more complex. We therefore used a third study design to investigate the candidate genes: association analysis. For this analysis, we used data from a large-scale genotyping project in 100 Golden Retrievers. Our power calculations demonstrate that this sample size is expected to be sufficient to detect variants that confer a high relative risk for a range of marker allele frequencies. From the total set of 60,073 SNP genotypes, we selected 41 SNPs that flank the candidate genes. We found no association between alleles, genotypes or haplotypes of these SNPs flanking the candidate genes and human-directed aggression of the Golden Retrievers.

We focused on human-directed aggression in the affecteds-only linkage analysis and the association study. In our quantitative genetic analysis, we studied additional types of aggression. There is no consensus in the literature on how aggression should be subdivided (Houpt and Willis 2001; Jacobs et al. 2003; Serpell and Jagoe 1995). There are indications that various types of aggression have a distinct genetic basis. For instance, selection of rats and silver foxes for reduced fear-induced aggression towards humans did not change predatory or inter-male aggression (Naumenko et al. 1989; Popova et al. 1993). This suggests that molecular genetic studies of aggressive behavior should focus on specific classes of aggression. However, reduced aggressiveness towards man in the rats and foxes was accompanied by reduced fear of novelties and irritable aggression, indicating that there is overlap between classes. As long as the genetic roots of aggressive behavior are poorly understood, it will remain impossible to design a classification that reflects the genetic basis.

In conclusion, none of the four methods of analysis provided evidence for a strong effect of variants of the candidate genes on aggression in the Golden Retrievers that we studied. These results seem to contradict reports of the involvement of the candidate genes in the regulation of aggressive behavior. However, the study designs that we used are not powerful enough to detect variants of small effect. We can therefore not rule out the possibility that variation in the candidate genes has a smaller genetic effect on aggression. In addition, our results cannot be construed as evidence against a major role for these genes in aggression in other dog breeds. Possibly, other genes in the serotonin pathway play a role. With the completion of the dog genome project, genome-wide association studies have become feasible in dogs (Lindblad-Toh et al. 2005). This opens the opportunity for finding genes that have not been associated with aggression up to date. Such studies are in progress.

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