Genomic Risk for Severe Canine Compulsive Disorder, a Dog Model of Human OCD

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KEY WORDS: anxiety, obsessive-compulsive disorder, *CDH2*, *CTXN3*, *HTR3C*, *HTR3D*, *HTR3E*, *SLC12A2*, stress response, *Teneurin-3*

ABBREVIATIONS: CCD, canine compulsive disorder; CRF, corticotropin-releasing factor; HPA, hypothalamic-pituitary-adrenal axis; OCD, obsessive-compulsive disorder.

ABSTRACT

Dogs naturally suffer the same complex diseases as humans, including mental illness. The dog is uniquely suited as a model organism to explore the genetics of neuropsychiatric disorders. Historical breed demographics have enriched purebred populations for founder effect mutations with tractable architectures, making genotypic analyses advantageous. Over a pet's lifetime, owners observe the animal's stress tolerance, arousal, and anxiety, and can inform on rich behavioral profiles for phenotypic analyses. Here we leverage these strengths in a search for inherited fac-tors that exacerbate canine compulsive disorder (CCD), the dog counterpart to human obsesssive compulsive disorder (OCD). Our rationale is that identifying pathways that predispose to disease severity will expand therapeutic options, and ultimately bring relief to those patients suffering the most. We have performed a

GWAS of purebred Doberman pinschers that compares severely affected cases to moderately affected cases (24:70). This GWAS identified two statistically sig-nificant risk loci, on *CFA34* and *CFA11*, and a third with suggestive evidence on *CFA16*. The locus on *CFA34* includes a cluster of 5-HT3 receptor genes (*HTR3C*, *HTR3D*, and *HTR3E*) that implicate a serotonergic pathway that is routinely targeted by anti-OCD medications. The locus on *CFA11* is syntenic with human *CTXN3-SLC12A2* (*5q35.1*), an inherited risk factor for schiz-ophrenia. The third locus harbors teneurin-3 (*TENM3*), a modulator of the hypothalamic-pituitary-adrenal (HPA) axis, with effects on stress tolerance and stress-related behavior. We dis-cuss candidate genes and putative functional variants in light of pharmacological responsiveness, psychiatric comorbidity, and the potential for gene-by-environment interactions in the genetic etiology of OCD and CCD.

AUTHOR SUMMARY

We previously identified a locus on canine chromosome 7 that confers susceptibility to obsessive-compulsive disorder in flank and blanket sucking Doberman pinscher dogs. The chromosome 7 locus contains a gene involved in the normal development of glutamate receptors, dysfunction of which is involved in the expression of obsessive-compulsive disorder. This current study is directed at identifying additional genetic factors determining the severity of the condition in our animal model. To this end, we conducted testing in severely affected versus mild-moderately affected dogs to explore genetic differences. We found 2 distinct regions on canine chromosomes 11 and 34 that appear to be genetic modifiers affecting the severity of the condition. The first, a locus on chromosome 11, contains a gene increasing the risk of another psychi-atric condition (schizophrenia) in humans. The second, a locus on chromosome 34 harbors sero-tonin receptor genes. That serotonin genes are involved in determining the severity of the condition seems particularly relevant because drugs targeting the serotonin pathway are routinely used in the treatment of obsessive-compulsive disorder. We hypothesize that the gene on chromosome 7 is essential for susceptibility to compulsive disorder, and that other genes, notably ones affecting the serotonin pathway, affect its severity. These findings have relevance in furthering understanding the pathophysiology of obsessive-compulsive disorder in mammalian species and point the way toward more effective treatments that target both glutamate and serotonin pathways.

INTRODUCTION

Human obsessive-compulsive disorder (OCD) is a mental illness characterized by intrusive, distressing thoughts (obsessions) and time-consuming, repetitive behaviors (compulsions). OCD is one of the most prevalent neuropsychiatric disorders, affecting 1-3% of the worldwide population [1]. The World Health Organization (WHO) lists OCD among the 20 most disabling diseases[2]. Current therapies are not optimally effective and extend medicinal benefit to roughly half of all patients[3]. OCD is a multifactorial disorder with a phenotypic spectrum. Patients suffering from severe OCD report a greater loss of time to persistent compulsions, and experience sub-stantially greater emotional distress and psychological impairment.

Severely affected patients also respond much less frequently to available therapies, and with greatly reduced benefits in quality-of-life outcomes[3, 4]. Understanding the general etiology of OCD may lead to broad improvements in diagnosis, treatment, and possibly prevention. A high clinical priority is to alle-viate disease severity, and to bring relief to those patients who currently have the greatest unmet medical needs. Understanding the genetic basis of severe OCD holds promise for identifying novel pathways, thereby expanding options for improved diagnosis and therapeutic intervention.

The apparent genetic heterogeneity of human OCD has been a major obstacle to genetic studies with human subjects. Additionally, imprecise diagnosis and pheno-

typing, comorbidity and misclassification with other disorders, and societal stigma and privacy concerns further confound human studies. Research to understand genetic risk factors of OCD have met with limited success[5, 6]. A recent large scale GWAS involving thousands of human cases and controls failed to detect any loci significantly associated with OCD[6]. To our knowledge, a replicated locus associated with human OCD remains elusive.

Animal models represent an important complementary strategy for gaining access experimentally to causative mechanisms.[7, 8] It is widely accepted that compulsion is biologically conserved across mammals, and that experimental results with naturally occurring animal models are indeed relevant to human OCD[9, 10, 11]. The literature supports canine compulsive disorder (CCD) as a naturally occurring counterpart of OCD. CCD shares phenomenological aspects with OCD, including the repetitious nature of basic behavioral patterns and the increased anxious state of patients[10, 12, 13]. The early adult onset of OCD in human patients[14] is also observed in peri-pubertal canine patients[13]. The neuroanatomical sites of OCD and CCD also appear to share overlap. Magnetic resonance imaging in dogs showed both anterior cingulate cortex and anterior insula gray matter density reductions, implying altered activity[15]. An fMRI study in humans with OCD and hoarding disorder indicated abnormal activity in these same brain regions[16]. Lastly, human and canine patients respond similarly to therapy. As is clinical practice in human medicine, veterinary medicine combines behavioral modification with anti-OCD drugs for the treatment of CCD[17]. These include drugs developed for human patients, such as fluoxetine, a serotonergic agonist via serotonin reuptake inhibition, and memantine, an NMDA-based glutamatergic antagonist.[4, 18] The effectiveness of these treatments in dogs suggest that clinical trials in veterinary medicine will be predictive of medicinal benefits for human patients. In this way, the dog also has enormous potential as a medical model for improving the diagnosis and treatment of psychiatric disorders in human and canine patients alike.

The genetic basis of CCD is expected to be tractable in breed isolates. Breed predilection implies an inherited predisposition, and breed differences in the specific compulsive behaviors co-opted by CCD further suggests a genetic basis. Examples of breed predisposition to specific compulsions include excessive grooming in certain breeds (i.e., acral lick; [12,19]), repetitive tail chasing in terrier breeds[10, 11, 20], and light-chasing behavior in herding breeds.[21] Thus genetic influences on multiple aspects of CCD can be mapped serially in breeds to exploit independent mutations in diverse populations. Results from multiple breed studies may recapitulate in aggregate the biological complexity observed in human OCD.

Each breed is naturally suited to genetic analysis. Phenotypic variation within a breed is attributable to founder effect variants acting in concert with a relatively constant (purebred) genetic background. This limits phenotypic noise from modifying genes, and increases the relative phenotypic effect of a small number of segregating loci. Each causal variant is located on an ancestral haplotype that is 50- to 100-fold larger than haplotype blocks comprising the human genome. Ancestral haplotypes are readily detectable by GWAS with SNP densities and cohort sizes that are modest relative to human experimental standards.

Three previous studies have addressed the genetics of CCD.[22, 23] The first study identified a locus on chromosome 7 (*CFA7*) that was associated with flank and blanket sucking behavior in Doberman pinschers.[22] The mapped interval spanned several megabases but contained only a single gene, neural cadherin (N-cadherin; *CDH2*). In the second study, *CDH2* was implicated in a different CCD, compulsive tail chasing, in another unrelated breed, Belgian malinois (Cao x, et al, PLOS One, 2014). *CDH2* encodes a cell-adhesion molecule expressed in the

Table 1. *Summary of GWAS results*

p-value	Odds Ratio	Candidate Gene(s)
1.9×10^{-10}	33.4	*HTR3C, HTR3D, HTR3E*
2.7×10^{-8}	11.9	*CTXN3, SLC12A1*
1.2×10^{-5}	5.4	*TENM3*

1 Loci taken from Illumina Canine BeadChipHD array.
2 Physical coordinates from the Boxer reference genome (canFam3).
3 A second SNP (BICF2G630815717) exhibited the same p-value.
This was an adjacent marker at chr16:48162506

hippocampus and cerebellum. N-cadherin influences many neuronal processes but its role in the formation of NMDA receptors at glutamatergic synapses is of particular interest[24]. The risk conferred by this locus appeared dose-dependent, with the risk allele frequency greater among dogs exhibiting multiple compulsive behaviors[22]. Finally, a follow-up study on the Dobermans reanalyzed the primary data using the *MAGIC* algorithm[23], increasing the SNP density fourfold relative to the earlier study. A hybrid mapping approach that incorporated systems biology was used to detect three intervals with genes (*CTNNA2*, *ATXN1*, and *PGCP*) that could be assembled into the *CDH2* network. This network emphasized synaptic function, NMDA receptors, and glutamatergic neurotransmission. Polymorphisms in *CDH2* are also associated with a severe human OCD and Tourette's Syndrome [9], two disorders that show comorbidity. The mechanism by which *CDH2* variation increases risk for CCD, OCD, and Tourette's has not been established. Taken together, the results are compelling as glutamatergic and serotonergic neurotransmission are two pathways routinely targeted using anti-OCD medications[25].

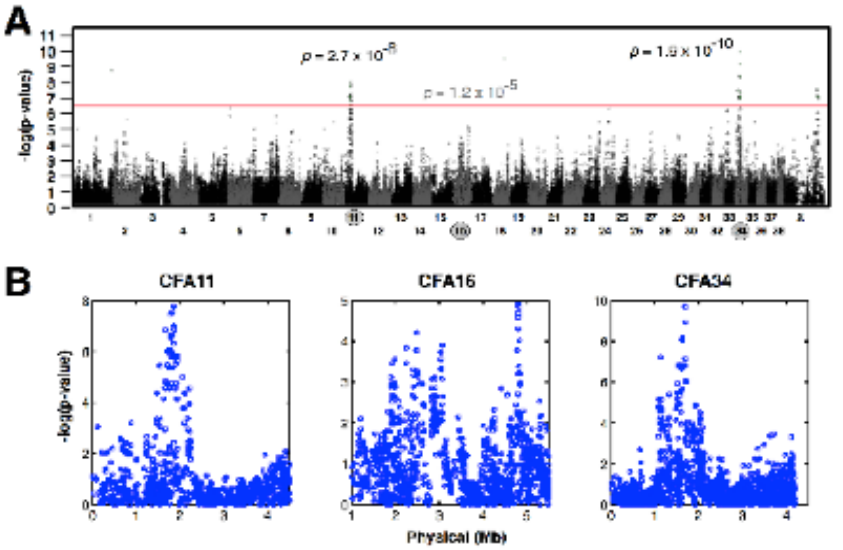
Here we extend experimental observations on CCD by focusing on the genetics of disease severi-ty in the Doberman pinscher breed. By mapping modifiers that exacerbate CCD, we aim to un-cover new factors that implicate novel pathways for therapeutic targeting. We present evidence that 2-3 loci govern CCD severity in Doberman pinscher dogs. Strong candidate genes in the mapped intervals implicate pathways that may explain important and fundamental features of human OCD. These include response to medications that target both the serotonergic and gluta-matergic neural pathways, the comorbidity of neuropsychiatric disorders, and the role that stress tolerance and environmental triggers play in the etiology and severity of CCD and OCD.

RESULTS

GWAS of severe CCD in the Doberman pinscher breed

We performed a GWAS comparing severely affected cases with moderately affected cases (here-after referred to as controls for disease severity). These cohorts were established through a com-bination of clinical evaluation, owner-based surveys, and telephone interviews conducted by a professional veterinary behaviorist. DNA from cases (n = 24) and controls (n = 70) were geno-typed at 174,376 SNPs genome-wide. These genotype data were subjected to conventional QC: 8,628 SNPs were excluded due to poor replication in duplicate genotypings; 2,305 SNPs were excluded for low call rates (< 10%); 75 SNPs were excluded for failing a test of Hardy-Weinberg Equilibrium; and 76,179 SNPs were excluded due to a low minor allele frequency (MAF < 5%). The informative markers that remained (95,817 SNPs) were tested for allelic association. Results from this GWAS are shown in the Manhattan plot in Fig. 1A. Two loci, on *CFA11* and *CFA34*, were detected with statistical significance after correcting for genome-wide testing by permuta-tion

Figure 1A: *Manhattan plot of GWAS for CCD Severity*
Chromosome markers are plotted on the x-axis in order and alternately shaded. The -log10 (p-value) is plotted on the y-axis (and inset). Two loci, on CFA11 and CFA34, showed statistical significance after correcting for genome-wide testing by permutation analysis. A third locus, on CFA16, showed suggestive evidence of association.
Figure 1B: *Localized mapping results for each chromosomal region of interest.*



analysis. A third locus, on *CFA16*, showed suggestive evidence of association. Fig. 1B shows localized mapping results for each chromosomal region of interest. A summary of mapped intervals is provided in Table 1.

Serotonergic genes at the *CFA34* risk locus

The locus most strongly associated with severe CCD was found on *CFA34*. This interval spanned ~1.5 Mb (chr34:16.0-17.5 Mb) and contained 39 annotated genes. The SNP showing the strongest allelic association (BICF2P816458) was 65 kb upstream of a cluster of three paralo-gous genes (*HTR3C*, *HTR3D*, and *HTR3E*), each encoding an isoform subunit of the 5-HT3 re-ceptor. The 5-HT3 receptor is the lone ligand-gated ion channel receptor in the serotonergic sys-tem (26). This pathway is routinely targeted in treatment of both human OCD (12, 27) and ca-nine CCD (28). It is not known if SSRIs are particularly effective in treating severe cases, alt-hough it has been observed that not all patients (human and canine) respond positively to sero-tonergic-based treatment.

A syntenic locus with schizophrenia risk

A second locus, on *CFA11*, also showed significant allelic association. This mapped interval spanned ~2.5 Mb and included 13 annotated genes. The canine locus is syntenic with human *5q35.1*, a chromosomal region recently found associated with human schizophrenia (29). Two adjacent genes (*CTXN3* and *SLC12A2*) in this interval are candidates for schizophrenia risk. *CTXN3*, a member of the cortexin gene family, is involved in intercellular signaling for forebrain development (30). *SLC12A2* encodes a Na-K-Cl symporter involved in maturation of the gam-ma-amino-butyric acid (GABA) signaling pathway (31) GABA is an anxiolytic neurotransmitter (32), and the GABAergic system has been implicated in multiple human mood disorders (33). Though the peak signal for association (SNP BICF2P816458) was nearly a megabase

Table 2. *Risk Allele Frequencies at CDH2* [1]

Source	Cohort	Sample Size	Risk Allele Frequency
This study	Mild/Moderate CCD Controls	70	42%
This study	Severe CCD Cases	24	57%
Dodman et al, 2010	Non-Affected CCD Controls	67	22%
Dodman et al, 2010	Affected CCD Cases, a single compulsion	68	43%
Dodman et al, 2010	Affected CCD Cases, multiple compulsions	20	60%

[1] *Frequency of risk allele at BICF2G630563196; showed strongest association in Dodman et al (2010). Risk allele,T; Alternate allele, C.*

down-stream from the gene tandem, *CTXN3* and *SLC12A2* remained the most compelling candidates in the interval (Fig 1B).

A modulator of the HPA axis and stress tolerance

A third locus, on *CFA16*, showed suggestive evidence for association with severe CCD. The sig-nal for association spanned a large interval (5 Mb). Two adjacent SNPs (BICF2G630815658 and BICF2G630815717) provided equally strong statistical support. The chromosomal region con-tained 36 annotated genes. Among these was *TENM3*, a strong candidate for integrating stress response with stress-related behavior. The teneurins are transmembrane proteins that serve as cell adhesion molecules (34). In addition, the extracellular domain of teneurins is cleaved by pro-teolysis and secreted (35, 36). These C-terminal associated proteins (CTAPs) can counter the ef-fect of corticotropin-releasing factor (CRF) (37, 38) in the stress response pathway, thereby at-tenuating the hypothalamic-pituitary-adrenal (HPA) axis, most likely as an adaptive response to chronic stress (39). Chronic stress and anxiety are believed to be important environmental influ-ences in both CCD and OCD. Two SNP markers showed equally strong statistical support for association, and both were within 100 kb of *TENM3*.

CDH2 **and disease severity**

This GWAS did not detect a significant association at *CDH2*, the locus on *CFA7* previously found associated with CCD (22). This result is consistent with the previously published observa-tion because the present study addressed disease severity. We did find modest evidence for *CDH2* involvement. Specifically, at marker BICF2G630563196, the strongest associated SNP from Dodman et al (22), the risk allele frequency was greater in our severe cases (57%) than in our moderately affected controls (42%). It is thus possible that *CDH2* contributes to disease se-verity, but that additional power

Table 3. *Summary of haplotype analyses*

Locus (Mb)	Haplotype Length (Kb)	Comprising SNPs[1]	Risk Haplo-type[2]	Freq. of Case Haplotypes	Freq. of Control Haplotypes	p-Value[3]
Chr34:16.9	96.8	6	AGGGGG	33.3% (16/48)	2.8% (4/140)	1.6 X 10-14
Chr11:18.6	121.4	10	AGAG-CACGGG	27.1% (13/48)	2.8% (4/140)	6.4 X 10-10
Chr16:48.1	51.1	5	GGGGA	39.6% (19/48)	10.7% (15/140)	1.9 X 10-7

[1] *Information for SNPs comprising each risk haplotype is provided in Table 4.*
[2] *Allelic configuration for inferred risk haplotype.*
[3] *p-value calculated from binomial distribution, as described in Materials and Methods.*

Table 4. *SNP loci in haplotype analyses*

Chromosome (CFA)	Prelim. Position (of 21 SNPs)	Array SNP1	Base Coordinate[2]	Allele 1[3]	Allele 2
11	12	BICF2P962745	18601737	A	G
11	13	BICF2P298471	18607272	G	A
11	14	BICF2P1255374	18617008	A	C
11	15	BICF2P1423859	18645289	G	A
11	16	BICF2S23553865	18662547	C	T
11	17	BICF2P912457	18672446	A	G
11	18	BICF2P1037814	18685096	C	A
11	19	BICF2S23519359	18701992	G	A
11	20	BICF2P1047236	18716127	G	A
11	21	BICF2S23158677	18723056	G	A
16	12	BICF2G630815658	48111441	G	A
16	13	BICF2G630815667	48123834	G	A
16	14	BICF2G630815674	48135347	G	A
16	15	BICF2S23110272	48145249	G	A
16	16	BICF2G630815717	48162506	A	G
34	6	BICF2P1061643	16833175	A	G
34	7	BICF2P69046	16858896	G	A
34	8	G1457f42S203	16885802	G	A
34	9	BICF2S23646017	16905048	G	A
34	10	BICF2S23751509	16911851	G	A
34	11	BICF2P185055	16930008	G	A

[1] SNP loci are taken from the Illumina canineBeadchipHD array.
[2] Physical coordinates from the Boxer reference genome (canFam3).
[3] Specific allele associated with severe CCD risk haplotype.

Table 5. *Summary of whole genome sequencing[1]*

Mapped Locus (Mb)	Interval (Mb)	No. Observed Variants[2]	Variant Freq. (per Kb)	No. Conserved[3]	No. Putatively Functional[4,5]
16.50-19.00	2.5	7,648	3.1	814	116
44.00-49.00	4.0	16,933	4.2	2,006	404
16.00-17.50	1.5	1,341	0.9	193	59

[1] Data generated using the Illumina HiSeq platform with a paired-end library and two lanes of flow cell.
[2] Variants called relative to the Boxer reference genome (canFam3).
[3] Variants with phastCons scores greater than 0.20.
[4] Variants with phastCons scores greater than 0.70, and/or high to moderate SIFT scores.
[5] No variants having high to moderate SIFT scores were detected in candidate genes.
[6] Gene list from within mapped loci that harbor potentially functional sequence variation in Table 9

Table 6. *DNA variants in three chromosomal regions of interest (phastCons > 0.2)*

Chr.	Position	Reference	Variant	phastCon	SIFT Classifier	Proximal Gene	Putatively Functional
CFA11	18,991,950	A	ACTC	0.63			
CFA11	18,991,637	A	C	0.38			
CFA11	18,990,090	T	A	0.64			
CFA11	18,981,368	A	G	0.21			
CFA11	18,979,170	T	C	0.29			
CFA11	18,979,038	TGAGAGA	TGAGA	0.25			
CFA11	18,979,008	CGTGT	C,CATGTGT	0.43			
CFA11	18,979,007	C	A	0.41			
CFA11	18,978,192	A	G	0.23			
CFA11	18,977,799	T	C	0.24			
CFA11	18,976,600	AAC	A	0.31			
CFA11	18,975,866	A	G	0.63			
CFA11	18,973,977	A	G	0.31			
CFA11	18,972,764	C	T	0.21			
CFA11	18,971,599	G	A	0.28			
CFA11	18,965,248	A	G	0.21			
CFA11	18,965,224	G	T	0.23			
CFA11	18,965,183	T	TA	0.31			
CFA11	18,964,693	A	G	0.29			
CFA11	18,964,645	C	T	0.76			x
CFA11	18,964,618	T	C	0.31			
CFA11	18,952,115	A	G	0.73			x

(i.e., larger cohorts) is needed to detect a significant effect. Table 2 shows the comparisons of *CDH2* risk allele frequency among cohorts and across studies.

Haplotypic analysis of risk loci for disease severity

Haplotype signatures facilitate population genetics, genetic epidemiology, and replication stud-ies. In this study, haplotyping was used to select an individual that was genotypically selected for mutation discovery by next-generation sequence analysis. We inferred the risk haplotype at mapped loci using available SNP genotype data. Initially, 21 markers were phased at each of lo-cus. These markers were centered on the strongest associated SNP marker at the locus. Based on initial haplotype lists, we selected a subset of markers to further define a core haplotype, which best differentiated severely affected cases from moderately affected controls. Table 3 summariz-es these results. We applied these multi-marker signatures to infer the presence of causal variants in dogs under consideration for whole genome sequencing.

DNA variants of interest

We performed whole genome sequencing to search for mutations that might influence disease severity in CCD on a case dog that was informative at all three loci of interest. This dog was ho-mozygous for the *CFA34* and the *CFA16* risk haplotypes. This dog was also predicted to be het-erozygous for the *CFA11* risk haplotype. Table 4 shows the parameters for whole genome se-quence analysis. The data comprised a ~32x coverage of the genome. Variants were obtained in comparison to the reference genome that was derived from a purebred Boxer (40). The Boxer breed does not show predilection for CCD. This suggested that causative risk factors in the Do-berman pinscher could be detected as variant alleles relative to this reference genome. Table 5 summarizes DNA variant discovery.

A total of 25,922 DNA variants were detected across chromosomal regions of interest. Of these, roughly 600 variants were likely to have functional effects based

on phylogenetic conser-vation (phastCons > 0.7; n = 579) and/or protein structure/ function informatics (SIFT, moder-ate/high probability scores; n = 64). No protein-coding changes (non-synonymous substitution, frameshifts, etc) were found in candidate genes (Table 6). This implied that causal variants might be located in flanking regions continuing regulatory elements, in introns, or that other ge-nes in these intervals are responsible for the associated risk for severe CCD.

DISCUSSION

The GWAS described here has identified two loci strongly correlated with severe CCD in Doberman pinscher dogs, as well as an additional locus that yielded only suggestive evidence for association. This locus was carried forward, despite modest statistical support, because of the compelling candidate gene in the interval (i.e., *TENM3*), and the implications it may hold for un-derstanding environmental influences on neuropsychiatric disorders. We have interpreted these results to mean that one or more sequence variants within each interval functionally exacerbates CCD.

This GWAS focused on disease severity, and as such, the loci that have been identified likely harboring disease modifying variant, that interact with other loci conferring general risk for CCD[22, 23]. These loci are provisional as they require replication with an independent cohort. This is also true of previously published CCD loci, as these earlier genomic findings were from two studies that utilized the same cohorts[22, 23].

Further studies will identifygene-gene interactions, which are thought to be important in the eti-ology of mental illness, but which have proven difficult to address in human genetic studies.

These loci harbor compelling candidate genes that point to novel physiologic pathways. Previous results emphasized *CDH2*-dependent synaptic function in the glutamatergic system (i.e., *CDH2*, *CTNNA2*, *ATXN1*, and *PGCP*) as the principal pathophysiology of CCD[22, 23]. Our results re-lating to disease severity appear to reflect distinct aspects of CCD/OCD biology that are commonly recognized but poorly understood. These include (i) the efficacy of serotonergic agonists in roughly half of patients with CCD/OCD (*HTR3C*, *HTR3D*, and *HTR3E* on *CFA34*); (ii) comorbidity of OCD with other neuropsychiatric disorders (*CTXN3-SLC12A2* on *CFA11*); and (iii) the influence of environmental factors and chronic stress in exacerbating disease severity (*TENM3* on *CFA16*). Taken together, the evidence strongly suggests that the salient features of human OCD may also be reflected in the genetic susceptibility of dogs to severe CCD.

Serotonin, the 5-HT3 receptor, and the biology of compulsion

To our knowledge, this study is the first to implicate the serotonergic system in inherited suscep-tibility to both OCD and CCD. The seminal role of serotonin as a modulator of human OCD has a long history, dating back to 1972[41, 42]. This early research demonstrated that treatment of OCD with serotonin re-uptake blockers alone was sufficient to generate an anti-obsessional response in many patients. This suggested that low serotonergic signaling was an etiologic factor for OCD. It is now widely accepted that serotonin dysregulation contributes directly to OCD[43].

Serotonergic agonists, particularly SSRIs, are now a mainstay of human OCD treatment[44]. These drugs have been applied in veterinary behavioral medicine for several decades[12], with canine patients showing similar response profiles to human patients[45]. Given the central role of the serotonergic system in OCD and the efficacy of SSRIs in treating CCD, the association of serotonin receptor genes with severe CCD is compelling.

HTR3C, *HTR3D*, and *HTR3E* gene products form multiple isoforms of the 5-HT3 receptor, one of seven receptor subtypes in the serotonergic system. The 5-HT3 receptor is the lone ligand-gated ion channel receptor[26, 46]. Multiple psychiatric conditions have been causally linked to changes in 5-HT3

receptor function[47], and variation in 5-HT3 genes has been shown to have phenotypic effects in human behavior (48, 49). *HTR3C* is an inherited risk factor for autism and HT3RD influences human anxiety[50]. The third paralogous gene in this cluster, *HTR3E*, is res-tricted to myenteric neurons in the peripheral nervous system[51] and thus seems an unlikely candidate for causing CCD. However, Irritable Bowel Syndrome (IBS) shows significant comor-bidity with OCD[52, 53], and gastrointestinal (GI) function is often affected in human OCD pa-tients who also suffer from major depressive illness[54]. Regulatory changes in the HTR3 gene cluster could have pleiotropic effects on the central nervous and GI system. To our knowledge, the comorbidity of CCD and IBS in dogs has not previously been investigated.

The 5-HT3 receptor genes are expressed in brain regions that are functionally abnormal in human OCD patients. Based on neuro-imaging studies, the cingulate, the CA1 region of the hippocam-pus, and amygdaloid complex are aberrant in human patients[55, 56]. *HTR3C* and *HTR3D* are highly expressed in these regions[57], as well as in other brain regions associated with cogni-tion, affect, and modulation of sensory input[58].

The 5-HT3 receptor has also been implicated in the biology of addiction. Clinical studies have shown that 5-HT3 receptor antagonists decrease alcohol consumption in patients with alcoholism[59, 60]. This has led to the suggestion that activation of the 5-HT3 receptor may have rewarding or reinforcing properties. It has been suggested that compulsive behavior is a form of addiction and that known comorbidity of OCD and addictive behavior[1, 61] may stem from shared risk variants acting through the 5-HT3 receptor. Interestingly, treatment of obsessive-compulsive dis-order with odansetron, a specific 5HT3 antagonist, was associated with a significant decrease in the Yale Brown obsessive-compulsive scores in one study of 8 patients[62].

Relevance of OCD comorbidity

Comorbidity is a common and perhaps telling feature of molecular mechanisms underlying neu-ropsychiatric disorders[63]. OCD has a significant comorbidity with several psychiatric disor-ders. This implies shared etiology and common risk factors. Major depressive illness, bipolar di-sorder, Tourette's syndrome, attention deficit disorder, panic disorder, generalized anxiety disor-der, schizoaffective disorder and addiction have all been reported to co-occur with OCD[1, 64]. The locus on *CFA16* (*CTXN3-SLC12A2*) may be relevant to this comorbidity. The orthologous locus in the human genome is an inherited risk factor for schizophrenia[29, 65]; two adjacent genes are candidates for causality. *CTXN3* is a member of the cortexin family, which is involved in cognition, memory, and learning[30] as well as early forebrain development[30, 66])]. *SLC12A2* is a Na-K-Cl symporter involved in GABA signaling. Low GABA neurotransmission is a common finding in mood disorders[33, 66], and there is considerable crosstalk between the GABAergic, glutamatergic, and serotonergic systems[67]. Our results, although tentative, sug-gest that at-risk dogs may suffer from other psychiatric conditions at an increased rate. Whereas no known counterparts for disorders such as major depressive illness or Tourette's syndrome ha-ve been described in the dog, other mood disorders, such as panic disorder and separation an-xiety, are well documented[68, 69]. Comorbidity of these disorders with CCD has not been re-ported.

Integrating nature and nurture

Neuropsychiatric disorders are believed to stem from a combination of genetic and environmen-tal risk factors. The multitude of potentially interacting environmental influences presents an enormous challenge to understanding the role that environment plays in human mental illness. In the etiology of OCD, there is considerable evidence that stress may trigger and/or exacerbate the disorder. Although neuroendocrine control of the stress response is well understood, much less is known of the biology of stress tolerance and of the neural circuitry underlying stress-related behavior. Both are likely to

Table 7. *List of compulsive behaviors observed in Doberman pinschers*

Compulsion	Abbreviation	Description
Blanket Sucking	BS	Excessive mouthing or suckling of soft objects
Flank Sucking	FS	Excessive mouthing of the flank
Object Fixation	OF	Excessive preoccupation with an object or toy
Shopping/Hoarding	SH	Excessive collecting and organizing of objects
Acral Lick[1]	AL	Excessive grooming of the lower extremities

[1] *Self-injurious when severe and intense*.

be important components underlying neuro-psychiatric disorders, in-cluding OCD.

TENM3, a candidate gene at the *CFA16* locus, is involved in integrating stress tolerance with stress-related behavior[70]. *TENM3* encodes teneurin-3, a protein that forms a heterodimer with TENM1 and mediates cell adhesion at synapses. Moreover, the c-terminus of this protein is clea-ved and secreted in the CNS. Exogenous teneurin-3 suppresses stress-related behavior that is induced in the rat by injecting with CRF[38, 71, 72]. Similarly, exogenous TENM1 can counter the effect of CRF to reinstate cocaine-seeking behavior in a rat model of addiction[38, 73]. The inability to cope with chronic stress has been suggested as a risk factor for disease severity in OCD[73, 74]. We suggest that variation in *TENM3* affects a dog's ability to adaptively dampen the stress response, and consequently, worsens disease severity.

An OCD/CCD model that assimilates pharmacological response and genetic risk

Prior pharmacological insights begin to make sense in light of the current results with CCD and OCD that now implicate both glutamatergic and serotonergic mechanisms. The distinct risk con-tributions could explain why response to serotonergic and glutamatergic drugs is variable among patients. The genetic findings in dogs may also explain why a glutamate-blocking strategy more effectively reduces compulsive behavior in an animal model when combined with a SSRI[74].

We propose a working model in which generalized risk stems from variation in several genes that influence the glutamatergic system. *CDH2* influences the assembly and function of NMDA receptors. Drug blockers of NMDA receptors attenuate compulsion in both human and compan-ion animal models[18, 25, 75]. Moreover, the observation that serotonin actually decreases glu-tamate in some brain regions[67] might explain the complementary action of SSRIs and NMDA antagonists in treating OCD.

Breed ancestry, hallmark traits, and pleiotropic effects on CCD

The history of the Doberman pinscher breed is relevant to CCD susceptibility. The breed was constructed in Germany (c.1890) to serve as an energetic and watchful guard dog. Genes from multiple pre-existing breeds were intentionally introgressed to assemble a specific work-related behavioral profile. This profile included high-energy, arousal, and vigilance, all of which are useful for working watchdogs. In this respect, the vigilance of Doberman pinscher dogs is adap-tive and desirable but can also be associated with an anxious or nervous temperament. The sero-tonergic and glutamatergic systems govern anxiety and high arousal, respectively.Anxiety con-tributes to compulsive behavior in dogs[69].

The purpose for which Doberman pinschers were bred also establishes a context for environmen-tal influence in CCD. Although Doberman pinschers were adapted to a demanding and active guard dog lifestyle, most dogs today do not receive this level of environmental engagement. We propose that under-stimulated dogs may be at increased risk for developing severe CCD.

Table 8. *Estimated p-values for informative SNPs in three regions of interest for CCD*

Chromosome (CFA)	Array SNP[1]	Base Coordinate[2]	*p*-value	Odds Ratio
11	BICF2G630294216	15497414	3.62E-06	17.5
11	BICF2P539905	16690377	1.43E-05	7.882
11	BICF2P1310910	16694511	1.43E-05	7.882
11	BICF2G630295301	16757670	1.39E-07	10.81
11	BICF2G630295322	16786128	1.39E-07	10.81
11	BICF2G630295566	17041909	1.00E-06	7.763
11	BICF2G630295619	17110379	3.19E-06	8.798
11	BICF2G630295864	17355025	8.93E-07	10.64
11	BICF2P293356	17406145	4.28E-06	9.529
11	BICF2G630295925	17446652	4.28E-06	9.529
11	BICF2S23548912	17623884	4.28E-06	9.529
11	BICF2S23141428	17626394	4.28E-06	9.529
11	BICF2P1349400	17637729	4.28E-06	9.529
11	BICF2S2303223	17655946	8.93E-07	10.64
11	BICF2P124153	17757867	8.93E-07	10.64
11	BICF2P701	17764798	8.93E-07	10.64
11	BICF2P55387	17871634	4.28E-06	9.529
11	BICF2P461569	17879714	1.53E-06	7.984
11	BICF2P718382	17882504	3.48E-08	13.06
11	BICF2P321292	17926621	2.22E-07	8.533
11	BICF2P878837	17956937	2.87E-07	8.402
11	BICF2P218661	18237383	1.00E-06	7.763
11	BICF2P1088502	18288511	1.68E-06	6.875
11	BICF2G630296074	18300189	2.22E-07	8.533
11	BICF2G630296084	18305904	1.68E-06	6.875
11	BICF2S23643044	18328886	2.75E-08	11.91
11	BICF2G630296192	18422040	4.32E-06	7.043
11	BICF2S23418452	18591044	1.62E-08	NA
11	BICF2P962745	18601737	1.02E-07	10.13
11	BICF2P298471	18607272	4.89E-07	9.194
11	BICF2P912457	18672446	1.39E-07	10.81
11	BICF2P112299	18964693	3.19E-06	8.798
11	BICF2P235304	19098405	1.53E-06	7.984
11	BICF2S24320279	19419232	2.26E-06	8.312
11	BICF2P772375	19502128	1.53E-06	7.984
11	BICF2P1095814	20534589	9.95E-06	7.485
11	BICF2P914727	20546241	9.95E-06	7.485
16	BICF2G630815658	48111441	1.20E-05	5.357

Table 9. *Genes from within mapped loci that harbor potentially functional sequence variation[1,2]*

Chr.	Position[3] (Mb)	Proximal Gene	Molecular Function[4]	Potential Relevance to CCD/OCD
CFA11	16.51	MEGF10	Multiple EGF-Like Domains 10	
CFA11	16.64	PRRC1	Proline-Rich Coiled-Coil 1	
CFA11	16.75	*CTXN3*	Cortexin 3, Kidney- and Brain-expressed	Locus associated with schizophrenia
CFA11	17.25	*SLC12A2*	Solute Carrier Family 12 (Sodium/Potassium/Chloride Transporter), Member A2	Locus associated with schizophrenia
CFA11	17.32	FBN2	Fibrillin 2	
CFA11	17.35	IFBN2	Not Available	
CFA11	17.97	SLC27A6	Solute Carrier Family 27 (Fatty Acid Transporter), Member A6	
CFA11	18.01	IS0C1	Isochorismatase Domain Containing 1	
CFA11	18.39	ADAMTS1 9	A Disintegrin and Metalloproteinase with Thrombospondin Motif	
CFA11	18.51	KIAA1024L	Not Available	
CFA11	18.62	CHSY3	Chondroitin Sulfate Synthase	
CFA16	44.24	**FAT1**	FAT Atypical Cadherin 1	Member of cadherin superfamily highly expressed in brain
CFA16	44.48	F11	Plasma Thromboplastin Antecedent	
CFA16	44.53	CYP4V2	Cytochrome P450, Family 4, Subfamily V, Polypeptide 2	
CFA16	44.58	FAM149A	Family With Sequence Similarity 149, Member A	
CFA16	44.62	H6BA88	Toll-Like Receptor homolog	
CFA16	44.88	SORBS2	Sorbin And SH3 Domain Containing 2	
CFA16	45.14	PDLIM3	PDZ And LIM Domain 3	
CFA16	45.18	CCDC110	Coiled-Coil Domain Containing 110	
CFA16	45.23	ANKRD37	Ankyrin Repeat Domain 37	
CFA16	45.24	LRPSBP	LRP2 Binding Protein	
CFA16	45.24	UFSP2	UFM1-Specific Peptidase 2	

CONCLUSIONS

This study focused on the genetics underlying CCD severity in an innovative animal model. Our aim was to identify novel pathways in OCD/CCD that would ultimately point to more effective therapeutic interventions. To our knowledge, no previous study, in human or canine, has ad-dressed the factors that drive severity in OCD and CCD. To accomplish this, we leveraged the inherent strengths of canine breed genetics, where causal factors are derived from a small number of ancestral mutations. This strategy successfully identified 2-3 novel loci contining candi-date genes that govern CCD severity in the Doberman pinscher breed. These results represent important genetic leads to pursue, and can lead to a better understandings of the molecular mech-anisms underlying CCD and OCD.

MATERIALS AND METHODS

Ethics Statement

Samples were obtained with informed owner consent according to IACUC protocols #G82706 (Cummings School of Veterinary Medicine (CSVM) at Tufts University) and #11-02-002 (Van Andel Research Institute).

Canine Subjects

Severe cases and moderately affected controls were privately owned purebred dogs from the American Kennel Club (AKC) registry. Subjects were recruited by researchers at CSVM, relying both on a clinical program in veterinary behavioral medicine and on a breed community network. A large sample of dogs was enrolled initially (n = 200). Phenotypic assessment was made ac-cording to owners' responses on a phenotype survey questionnaire, the same as used in our previous study (13). All of the dogs studied exhibit flanked sucking or blanket sucking, both closely related, virtually breed-specific CCDs, with or without other compulsions such as object fixation, shopping/hoarding, or acral lick (Table 7). Twenty four dogs exhibited severe CCD as deter-mined by their displaying more than one compulsion (12 dogs), hourly or daily frequency, and owner-estimated duration of >8% of the day. Mild to moderately affected dogs (n = 70) typically engaged in only one compulsion, exhibited the behavior daily of weekly, and engaged in the be-havior <8 of the day (6.4% +/- xxx, n = 59). Time spent per day was only available for 59 dogs, others simply stated "no access," "situational" or "when crated" confirming their mild-moderate status.

Sample Recruitment and DNA

Informed owner consent was obtained at the time of sample submission according to IACUC protocols at TSVM and VARI ((#G82706 and #11-02-002, respectively). Samples were collected randomly from willing participants' dogs across the United States assuring a heterogeneous mix with no geographical or other bias. TSVM samples were collected as blood, and genomic DNA was extracted with conventional methods. VARI samples were collected by buccal swab, saliva kit (Genotek, Ontario, CA), or blood. DNA was extracted from blood or saliva using protocols adapted to an automated workflow (AutogenFlexStar, Holliston, MA) as previously described[76]. Crude extracts were prepared from buccal swabs as previously described[76]. Samples were quantified by nanodrop spectrophotometry. The integrity of genomic DNA (i.e., fragmenta-tion and degradation) was assessed by agarose gel electrophoresis.

Genotyping and Related Analyses

Genome-wide genotyping was performed with the CanineHD BeadChip (Illumina, La Jolla, CA). Briefly, the platform is based on arrayed oligonucleotides, each corresponding to a known physical polymorphism in the dog genome, which can be assayed by enzymatic single-base ex-tension and dual-color fluorescence to report bi-allelic calls. The array offers 174,376 SNP loci at an average density of 70 SNPs per megabase. Raw fluorescence intensity data generated with a BeadArray Reader (Illumina, La Jolla, CA) were converted to curated genotypes using Ge-nomeStudio software with pre-set genotypic cluster algorithms. Fixed array SNP data were ana-lyzed us-

ing PLINK (76). SNP data were filtered to exclude (i) data from individual dogs with total SNP call rates less than 98%; (ii) SNPs with minor allele frequency (MAF) less than 0.05; (iii) individual SNP loci with call rates less than 99.8%; and (iv) individual SNP loci that did not pass the test for Hardy–Weinberg equilibrium (HWE) in the control cohort ($p < 0.05$). Case-control cohorts were tested for population substructure using multidimensional scaling in PLINK. P-values for GWAS were adjusted for multiple tests, and the genome-wide significance threshold was set by permutation of case-control labels (100,000 iterations);[78].

Haplotype analysis was performed using PHASE (v 2.1.1; 79). Phased haplotypes were inferred in two steps. Preliminary haplotypes were inferred with the use of 10 SNPs on each side of the marker that showed the most significant p-value for association. This was performed in each of the three regions of interest (Table 8). A subset of SNPs was then selected to define a core risk haplotype that most strongly differentiated the case group from the control group. These SNPs were re-phased in cases and controls separately. To maximize the likelihood of detecting risk haplotypes in the control group, 25 mock individuals that were homozygous for the risk haplo-type were added to the input genotype data for controls. This 'seeded' the analysis toward detec-tion of the risk haplotype. The estimates of haplotype frequency excluded the results from the mock individuals. The p-values for enrichment of the risk haplotypes in the cases were calculat-ed from a binomial distribution of the haplotype frequency in controls. The p-values in

Table 3 reflect the probability of observing in the cases the same or higher risk haplotype fre-quency that was observed in the controls.

Whole Genome Sequencing and Related Analyses

Whole genome sequencing was performed at Beijing Genome International using two lanes of a HiSeq instrument (Illumina, La Jolla, CA). DNA was isolated from whole blood obtained from a severely affected dog. Raw HiSeq sequence data were aligned to the reference genome (canFam3.0) using the SNAP Sequence Aligner (80). Variants relative to the reference were called using SAM-Tools[81]. Variants were assessed for putative functional impact by SnpEff[82] and SIFT [83], and by assessing conservation across mammalian species (phatsCons scores);[84]. NGS analytics were performed on a cloud cluster (Amazon).

ACKNOWLEDGEMENTS

Three collaborating institutions provided funding and supported this work: Tufts University Cummings School of Veterinary Medicine, the University of Massachusetts Medical School, and the Van Andel Research Institute. We thank the many dog owners who enrolled their pet dogs in this study. We are especially grateful to Kathy Davieds of the Doberman pinscher community. We thank Alex Roemer, Andrew Borgman and Nicole Cottam for technical assistance and Lisa Kefene for performing fixed array genotyping.

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