Identification of a Mutation in the \textit{CHAT} Gene of Old Danish Pointing Dogs Affected with Congenital Myasthenic Syndrome

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\textbf{Abstract}

The presence of a recessive inherited muscle disease in Old Danish Pointing Dogs has been well known for years. Comparisons of this disease with myasthenic diseases of other dog breeds and humans have pointed toward a defect in the synthesis of the neurotransmitter acetylcholine possibly due to decreased activity of the enzyme choline acetyltransferase. We sequenced exons 5–18 of the gene encoding choline acetyltransferase (\textit{CHAT}) in 2 affected and 2 unaffected dogs and identified a G to A missense mutation in exon 6. The mutation causes a valine to methionine substitution and segregates in agreement with the inheritance of the disease. The mutation was not detected in 50 dogs representing 25 other dog breeds. A DNA test has been developed and is now available to the breeders of Old Danish Pointing Dogs.

An autosomal recessive neuromuscular transmission defect known as congenital myasthenic syndrome (CMS) has been observed in the Danish dog breed Old Danish Pointing Dog since 1977 (Flagstad 1982). Affected dogs are able to run normally for 5–30 min after which they take shorter and shorter strides and eventually fall down with flexed fore- and hindlegs. After some minutes rest, they are able to walk and run again for variable periods of time before the signs reappear. Myasthenic diseases are usually referred to as either acquired or congenital myasthenia gravis. The acquired myasthenia gravis is an autoimmune disease characterized by circulating autoantibodies directed toward the acetylcholine receptors (Drachman et al. 1978). No antibodies toward acetylcholine receptors have been detected in the plasma of affected Old Danish Pointing Dogs, thus distinguishing the syndrome from the acquired form (Flagstad et al. 1989). In the congenital myasthenia gravis, there is a decreased number of acetylcholine receptors as demonstrated in smooth fox terriers (Jenkins et al. 1976), Jack Russel terriers (Wallace and Palmer 1984), and smooth-haired miniature dachshunds (Dickinson et al. 2005). In contrast to this, a normal number of acetylcholine receptors have been shown in affected Old Danish Pointing Dogs (Engel AG, personal communication). In most cases of myasthenic diseases, the administration of anticholinesterases have a dramatic effect on generalized weakness, and daily oral treatment improves the condition (Johnson et al. 1975; Miller et al. 1983; Dickinson et al. 2005). Neither edrophonium nor neostigmine administered intravenously during attacks of weakness or prolonged oral neostigmine treatment were shown to have any clinical or electrophysiological effect on affected Old Danish Pointing Dogs (Flagstad et al. 1989).

The diagnosis of the CMS in Old Danish Pointing Dogs has been based on an electrophysiological test to examine neuromuscular function using repetitive stimulation. The electrophysiological test showed a myasthenic decrement using 3-Hz stimulation also seen in acquired and congenital myasthenia gravis. However, the decrement at 3 Hz did not occur until fatigue had been induced by a long stimulation train. (Trojaborg and Flagstad 1982). A similar stimulation technique is necessary to demonstrate a myasthenic decrease in humans affected with congenital myasthenic syndrome with episodic apnea (CMS-EA) (Engel and Sine 2005). Also, the synthesis of acetylcholine has been evaluated in...
myasthenic, clinically normal heterozygous, and unaffected control dogs. This study indicated that the synthesis of acetylcholine in affected Old Danish Pointing Dogs was decreased compared with the other groups (Flagstad A, in preparation). All these results point to a presynaptic defect related to synthesis of acetylcholine in affected Old Danish Pointing Dogs. The best characterized presynaptic defect in humans is caused by mutations in choline acetyltransferase (\textit{CHAT}), the gene encoding the enzyme choline acetyltransferase, resulting in impaired resynthesis of acetylcholine (Maselli et al. 2001; Ohno et al. 2001). In the present study, the canine ortholog of the human \textit{CHAT} gene was sequenced in affected and unaffected Old Danish Pointing Dogs and searched for polymorphisms.

### Materials and Methods

#### Animals and Diagnostics

Three litters of Old Danish Pointing Dogs comprising 5 parental animals and 13 offspring were included in the study (Figure 1). All animals were diagnosed affected or unaffected by an electrophysiological examination as previously described (Flagstad 1993). The 13 offspring were distributed as 6 affected and 7 unaffected. Additional animal material comprised 50 dogs representing 25 different dog breeds.

#### DNA Extraction and Sequence Analysis

Ethylendiaminetetraacetic acid–stabilized blood from all individuals was available, and DNA was extracted using a salting-out protocol (Miller et al. 1988). The human \textit{CHAT} gene has been mapped to HSA10q11.2 (Viegas-Pequignot et al. 1991). A BLASTN search with the human mRNA sequence against the canine genome showed the highest identity to CFA28 (96% identity, Genbank accession number NW_876285.1). The \textit{CHAT} gene consists of 18 exons; R, N, M, S, and 5–18. Translation of the first 4 exons has not been described in animals, but a transcript including exon M and S have been amplified from a human spinal cord cDNA library (Ohno et al. 2001). Human sequence (Genbank accession numbers AF305895–AF305906) was used to localize the exons in the canine genome, and primers for each exon were subsequently designed from intron sequence. Primer sequences and locations are listed in Table 1. Exons 5–18 representing the translated parts of the canine \textit{CHAT} gene were polymerase chain reaction (PCR) amplified in 2 affected and 2 unaffected dogs. Each reaction consisted of 25 l containing 2 l of genomic DNA, 1.5 mM MgCl2 (2.5 mM MgCl2 for exon 15 and 16), and 0.5 units of TEMPase Hot Start DNA polymerase (Ampliqon/Bie & Berntsen A-S, Herlev, Denmark). The cycling conditions were 1 cycle of denaturation at 95 °C/10 min, followed by

### Table 1

Sequences of primers used for amplification and sequencing exon 5–18 of the canine \textit{CHAT} gene. The position of each primer in megabases is referring to the \textit{canis familiaris} chromosome 28 genomic contig, whole-genome shotgun sequence, NW_876285.1, build 2.1.

<table>
<thead>
<tr>
<th>Exon number</th>
<th>Forward primer</th>
<th>Reward primer</th>
<th>Position of primers, forward–reward</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>AGATTGGGACCTGACGTGAG</td>
<td>CTCCTAGCCCAAGAGAGGAC</td>
<td>1.481.086–1.481.482</td>
</tr>
<tr>
<td>6</td>
<td>TGATGGGAGACACCAACTTGA</td>
<td>CCTACCTGGCCCTTCATCAG</td>
<td>1.483.344–1.483.770</td>
</tr>
<tr>
<td>7</td>
<td>ATGGGAGTGGCAGCTTTCAC</td>
<td>CTCTGCTCTCAGAGGGGCAATTG</td>
<td>1.484.321–1.484.663</td>
</tr>
<tr>
<td>8</td>
<td>CTCTGGAAGACTGGGCAGCA</td>
<td>GGAGTCTACCTGCGCTGTGA</td>
<td>1.485.772–1.486.118</td>
</tr>
<tr>
<td>9</td>
<td>GTGGCTCTTTGACTGCTGCA</td>
<td>TCACGTAAGTCTCGAAGG</td>
<td>1.487.366–1.487.771</td>
</tr>
<tr>
<td>10</td>
<td>AGGTCCCAACTTGTTAGCTG</td>
<td>CTATGGCAATTGGGATGAG</td>
<td>1.489.524–1.489.950</td>
</tr>
<tr>
<td>11</td>
<td>CCACGTGATTGTGAGAGAAA</td>
<td>ACTTTCTAGTCGCTTCAGC</td>
<td>1.508.660–1.509.084</td>
</tr>
<tr>
<td>12</td>
<td>CTCAGGCTATGGGAAATTTGAC</td>
<td>CACCCACATTTTTCCTGTG</td>
<td>1.511.137–1.511.734</td>
</tr>
<tr>
<td>13</td>
<td>TGAGAAGCTCCACGACTGAGG</td>
<td>TGATGGGAGACACCACTCTGGA</td>
<td>1.512.440–1.512.877</td>
</tr>
<tr>
<td>14</td>
<td>CCAGTGACTGAGGACATCA</td>
<td>AAGTGGTCCTCGCTGCTGCAG</td>
<td>1.514.999–1.515.329</td>
</tr>
<tr>
<td>15 and 16</td>
<td>GCTGGACAGGGTATTTCAGTT</td>
<td>TATGCTGACAGCCACC</td>
<td>1.518.077–1.518.585</td>
</tr>
<tr>
<td>17</td>
<td>AGCGTGTCGGCCCTAGTGA</td>
<td>AGCCAGTGCCCTGTAAGAG</td>
<td>1.525.075–1.525.430</td>
</tr>
<tr>
<td>18</td>
<td>AGGCTCAGGGTGCTTATGAA</td>
<td>GCCTCAAGTGCTGCTGCTG</td>
<td>1.526.982–1.527.482</td>
</tr>
</tbody>
</table>
**Figure 2.** Alignment of the amino acid sequence of human 83-kDa ChAT and of dog, pig, mouse, and rat ChAT. Dots indicate residues identical to the human ChAT. The position of the valine that is exchanged with a methionine in the affected dogs is indicated with an arrowhead.
were homozygous for the mutation, whereas all unaffected Pointing Dogs. All the affected Old Danish Pointing Dogs had a mutation in the CHAT gene. We identified a mutation in exon 6, position 29, of the CHAT gene. It was a G to A substitution in exon 6 of the 2 affected dogs and a C to A substitution in exon 6 of the 2 affected and 2 unaffected Old Danish Pointing Dogs. The single-base substitution resulted in a methionine residue instead of a valine residue. At least 14 different mutations have been identified in the CHAT gene of human patients with CMS-EA (Ohno et al. 2001; Kraner et al. 2003; Maselli et al. 2003; Schmidt et al. 2003). Two of the mutations abolish ChAT expression either through a frameshift mutation or through insertion of a stop codon. The other 12 mutations affect expression levels or kinetic parameters. A frequently reported effect is an increase of the Michaelis–Menten constant (Km) of ChAT leading to decreased affinity between enzyme and substrate (Ohno et al. 2001; Cai et al. 2004). The 3-dimensional structure of the ChAT protein with the position of 12 human mutations have been published (Cai et al. 2004). With respect to the 3-dimensional structure, the closest human mutations are V194L (Maselli et al. 2003), E441K, and R560H (Ohno et al. 2001). All 3 human mutations alter the acetylcoenzyme A or choline-binding sites (Cai et al. 2004), but further studies have to be carried out to confirm or disconfirm a similar effect of the canine mutation on enzyme-substrate binding.

A routine test for genotyping has been developed using a TaqMan SNP assay (Roche Molecular Systems Inc., Alameda, CA), and the test is now offered to the Danish breeders of Old Danish Pointing Dogs. Testing the dogs before breeding is essential in order to avoid mating between heterozygous carriers. Previously, a breeding program was based on carrier detection using the electrophysiological test (Flagstad et al. 1993). With the development of a DNA test, the diagnostic procedure has become cheaper, easier to perform, and more accurate with respect to detection of carriers. The Old Danish Pointing Dog is one of the national Danish dog breeds; thus, the Danish breeders feel a special obligation to keep the breed healthy. The breed is numerically small and has gone through a severe bottleneck around 1950. In addition, it is not possible to import new breeding animals from other countries. In concordance with the experience from introducing a DNA-based breeding program in Danish Bedlington terriers (Proschowsky et al. 2003), and to avoid a dramatic reduction of the number of available breeding dogs, the Danish breeders association of Old Danish Pointing Dogs have decided to include heterozygous carriers for controlled matings with homozygous normal individuals for a period of time.

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**References**


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