# Chapter 3

# Congenital myasthenia in Brahman calves caused by homozygosity for a *CHRNE* truncating mutation

S. Kraner [1], J. P. Sieb [2], P. N. Thompson [3], O. K. Steinlein [4]

[1] Institute of Human Genetics, Rheinische Friedrich Wilhelms University, Bonn, Germany (Present address: Technical University of Munich, Munich, Germany)

[2] Department of Neurology, Max Planck Institute of Psychiatry, Munich, Germany (Present address: Department of Neurology, Geriatrics and Palliative Care, General Hospital, Stralsund, Germany)

[3] Epidemiology Section, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[4] Institute of Human Genetics, Rheinische Friedrich Wilhelms University, Bonn, Germany (Present address: Institute of Human Genetics, Ludwig Maximillians University, Munich, Germany)

Neurogenetics (2002) 4:87–91

# Abstract

To elucidate the genetic defect in four previously reported related Brahman calves with severe myasthenic weakness, we determined the genomic structure of the gene encoding the bovine ε-subunit (bov*CHRNE*) of the acetylcholine receptor (AChR). Amplification of DNA isolated from paraplast-embedded tissue samples from one of the myasthenic calves and subsequent sequencing of all bov*CHRN*E exons revealed a homozygous 20-bp deletion within exon 5 (470del20). The deletion causes a frame shift followed by a premature stop codon in the predicted bov*CHRNE* protein. Thus, the 470del20 mutation reported here leads to a non-functional allele, explaining the impairment of neuromuscular transmission observed in the affected Brahman calves. With a survival time limited to only several months, the effect on neuromuscular transmission was more pronounced in the calves than that observed in humans homozygous for truncating *CHRNE* mutations. This may be due to a different capacity to express the foetal-type AChR after birth.

# Introduction

Impairment of neuromuscular transmission can be either acquired or inherited. Myasthenia gravis is an autoimmune disorder characterised by fluctuating weakness and fatigability of ocular, facial, bulbar or limb muscles. Congenital myasthenic syndromes are due to gene mutations causing presynaptic, synaptic or postsynaptic defects at the neuromuscular junction (Engel et al., 1999; Sieb et al., 2002). Both myasthenia gravis and the congenital myasthenic syndromes have been studied extensively in humans, and acquired myasthenia has also been described repeatedly in domestic animals (Indrieri et al., 1983; Shelton et al., 1997; Dewey et al., 1999; Lipsitz et al., 1999; Ridyard et al., 2000; Shelton et al., 2001). Myasthenia gravis is one of the most common neuromuscular disorders diagnosed in dogs (Shelton et al., 1997), where some breeds appear to have a genetic predisposition for developing this autoimmune disease (Shelton et al., 1997; Lipsitz et al., 1999). Much less is known about the molecular basis of congenital myasthenia in animals. There are some reports of inherited myasthenic weakness in domestic animals (Flagstad, 1982; Indrieri et al., 1983; Miller et al., 1983; Wallace and Palmer, 1984; Wilkes et al., 1987; Joseph et al., 1988; Flagstad et al., 1989; Thompson, 1998), but so far the molecular basis in these cases has remained elusive. The analysis of such spontaneous animal models can contribute to our understanding of the pathophysiological mechanisms underlying congenital myasthenia in humans. We have therefore determined the genomic structure of the bovine gene for the $\varepsilon$-subunit of the muscular acetylcholine receptor (bov*CHRNE*). This has enabled us to elucidate and describe the genetic defect in calves that suffered from a severe congenital myasthenic syndrome (Thompson, 1998).

# Materials and methods

### *Calves with congenital myasthenia*

The clinical phenotype of one of four South African Red Brahman calves with suspected congenital myasthenia has been published previously in detail (Thompson, 1998). In summary, the calf developed progressive muscle weakness, beginning at 3–4 weeks of age. Within a week it was no longer able to rise without assistance, and

collapsed after standing for between 20 and 60 s. Haematological and serum chemistry examinations were normal, and a muscle biopsy taken from one quadriceps muscle showed no abnormalities (Thompson, 1998). Peripheral nerve stimulation at 27 Hz revealed a decreased response of 15% at the peroneus tertius muscle. Acetylcholinesterase inhibitors improved the condition transiently. At the age of 15 weeks the calf was humanely killed due to severe pneumonia. Another three similarly affected half-siblings were similarly sacrificed at between nine and 21 weeks of age, after showing no clinical improvement. All four calves shared the same sire and maternal grandsire (Figure 1). Tissue samples from the first calf embedded in paraplast were available for molecular studies.

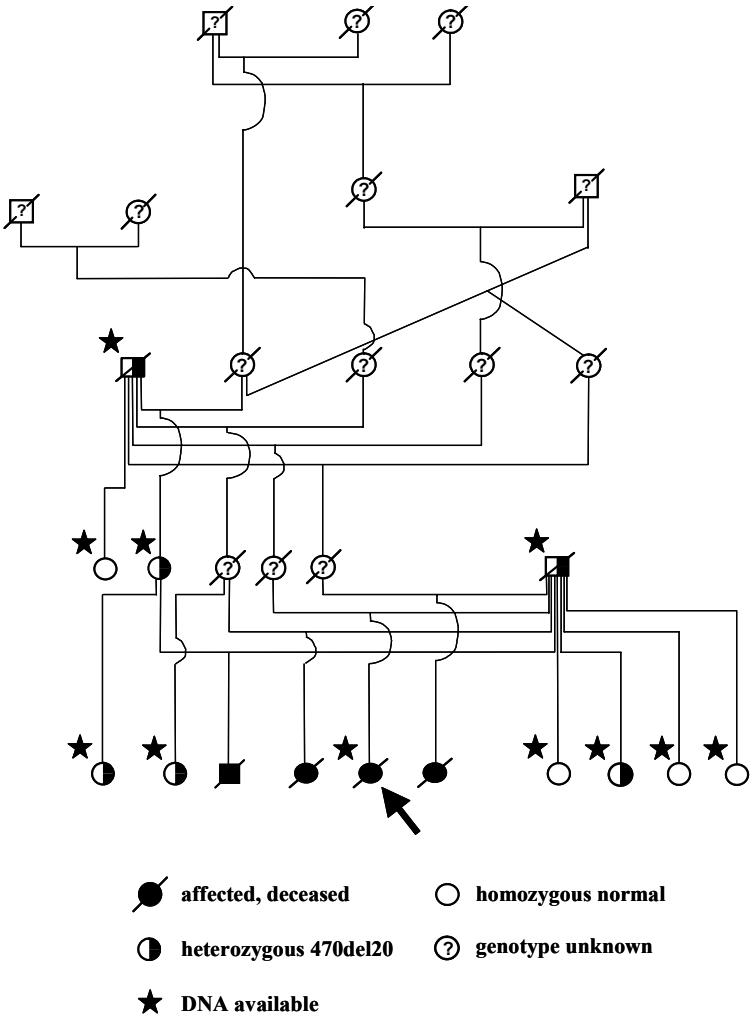


**Figure 1.** Pedigree of the myasthenic calves. The index calf investigated in the present study is marked by an *arrow*. Identification numbers for each individual are given. *Circles*, females; *squares*, males

***Reference bovCHRNE genomic sequence***

The published bov*CHRNE* cDNA sequence (accession number X02597) was used for a BLAST search (Madden et al., 1996). Two genomic clones (accession numbers BE664236 and BE681911) containing parts of bov*CHRNE* were identified. This approach enabled us to identify the boundaries of intron 5 and intron 8. The remaining introns were amplified by exon-exon PCR. For this, the putative positions of exon-exon boundaries were determined by comparison with the human genomic *CHRNE* sequence, and primers were chosen that did not overlap with the predicted boundaries. The template DNA was extracted using the Dneasy Tissue kit (Quiagen) from bovine meat purchased from a local butcher. PCR was carried out in a total volume of 25 $\mu$l in a PTC 200 (MJ Research), containing 50 ng of genomic DNA, 5 pmol of each forward and reverse primers, 200 $\mu$M of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), and 0.1 U Taq DNA polymerase (Invitrogen, Mannheim, Germany). PCR parameters were as follows: denaturation at 95ºC for 5 min followed by 33 cycles of 95ºC for 30 s, annealing at 58–64ºC for 30 s, extension times at 72ºC varying between 30 s and 80 s, followed by a final extension step of 5 min at 72ºC. Approximately 300 bp of each end of the obtained PCR products were directly sequenced on an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA).

***Mutation screening***

Genomic DNA was extracted from paraplast-embedded tissue samples (skeletal muscle and spinal cord) of one of the previously described myasthenic calves (Thompson, 1998), using the Dneasy Tissue kit (Quiagen). The DNA obtained from the tissue samples consisted mostly of fragments shorter than 650 bp. Therefore the amount of DNA used for amplification was increased to 150 ng in a total PCR volume of 25 $\mu$l. The PCR was carried out as described above, except for the following parameters: $MgCl_2$ concentrations 1–2 mM, annealing temperature 58–68ºC, annealing time 30–60 s, and extension times 30–60 s. PCR was performed using primer sets amplifying bov*CHRNE* exons and adjacent exon-intron boundaries (Table 1). Probably due to the high GC-content of intron 9, no suitable forward primer could be designed, and exon 10 was therefore not included in the mutation analysis. The amplification products were directly sequenced as described above.

**Table 1.** Primers used for amplification of bovine *CHRNE*

| Exon | Primer forward | Primer reverse |
|---|---|---|
| 1 | 5′ -ACCTGGGGGACCAGACAGC-3′ | 5′-TCTCAGTCGGTCTCTGTCCTGT-3′ |
| 2 | 5′-TAGACCCGGAGGCAGAGCA-3′ | 5′-AGTTGAGCGCTTGGGGCC-3′ |
| 3 | 5′-TGGCCCCAAGCGCTCAACT-3′ | 5′-TGTGCCGCGCTGCCTGCGA-3′ |
| 4 | 5′-TCGCAGGCAGCGCGGCACA-3′ | 5′-GACCTCGGCTTCGCTCCAGT-3′ |
| 5 | 5′-CCAACTCTCGGTTTCCTGGA-3′ | 5′-GGGCCTGCGAACAAGTAAGT-3′ |
| 6 | 5′-TCGGGGTTGGGATCCTGGCT-3′ | 5′-GTCAGTCAGTCCCAGCATCGA-3′ |
| 7 | 5′-AGAGCTGACTGACCGCGCCT-3′ | 5′-GAGGGAGTGCCTGGTGAGGA-3′ |
| 8 | 5′-GGTGCTGCTCGCCTACTTCCT-3′ | 5′-GCCATGCCCCGCGCAGTCA-3′ |
| 9 | 5′-GAGCAGCTCCACGCCTGCG-3′ | 5′-AAGCCCCACCTCCTTCCCGA-3′ |
| 10 | ND[a] | 5′-TGATCAAGAACCTAGCCCTTGA-3′ |
| 11 | 5′-ACCTGGACGGGTGAGCGGA-3′ | 5′-AGATGCGCAAGAGTCAGGGAT-3′ |
| 12 | 5′-CTTGCGCATCTGGCTCCTACA-3′ | 5′-AATTCTTGTCGGTGTGGCTGTG-3′ |

[a] Not determined

***Unrelated control animals***

When sequence differences were found between the DNA obtained from the myasthenic calf and the reference bov*CHRNE* sequence, a random sample of 23 red or black-and-white German slaughterhouse cattle was obtained. Five Red Brahman and five Grey Brahman cattle from South Africa, unrelated to the myasthenic calves, were also screened as controls. Genomic DNA from the control animals was extracted from 10-ml aliquots of EDTA-anticoagulated blood using a salting-out method (Miller et al., 1988). Exon 5 was amplified using primers given in Table 1, and the PCR products were separated on 10% polyacrylamide gels. The exon 5 amplicon from the DNA of the myasthenic calf was run on the same gel as a positive control for detection of 470del20.

### *Control animals related to the myasthenic calves*

DNA was extracted from stored semen samples of the sire and maternal grandsire of the affected calves, using the Chelex DNA extraction method [for details see Forensic Science Laboratory (FSL) of the South African Police Service (SAPS) Quality Manual no. BIO/WI/C009 Revision 3 1999]. In addition, DNA from eight cattle related to the affected calves was extracted from whole blood using the Wizard Genomic DNA Purification kit (Whitehead Scientific). These ten animals were then screened for the presence of the 470del20 deletion. The positions of the tested individuals within the pedigree are shown in Figure 1.

## Results

### *Genomic structure of bovCHRNE*

The bov*CHRNE* gene consists of 12 exons, encoding a predicted protein of 405 amino acid residues. In silico cloning and exon-exon PCR showed that the localisations of the exon-boundaries are conserved compared with the human *CHRNE* gene structure. The gt/ag rule of conserved splice sites (Breathnach and Chambon, 1981) is followed in all introns. The sizes of bov*CHRNE* introns obtained by sequencing (introns 1–2, 4, 6–7, 9–12) or estimation of fragment sizes from gel electrophoresis (intron 3), were comparable to the known intron sizes of the human *CHRNE* gene (Table 2). The ATG start codon is localised in exon 1, and the TAG stop codon in exon 12. The four transmembrane regions are encoded by exons 7, 8, 9 and 12, respectively. At the amino acid level, the human and bovine *CHRNE* genes have 89% identity and 91% similarity. The genomic bovine *CHRNE* sequences have been submitted to GenBank under accession number AF457656.

### *Detection of a 20-bp deletion*

PCR amplification and subsequent direct sequencing of bov*CHRNE* exons from the DNA of the myasthenic calf revealed a loss of 20 bp within the coding sequence of

exon 5 (Figure 2), between nucleotide 469 and 490 (nucleotide numbering referring to the cDNA sequence published under accession number X02597). The myasthenic calf was homozygous for the mutation 470del20. The sequence change was present in DNA samples extracted from two different tissues (skeletal muscle and spinal cord). It is therefore highly unlikely that the 20-bp deletion was artificially caused by DNA degradation during tissue fixation or DNA preparation. The 470del20 mutation was found neither in 46 chromosomes from German red or black-and-white control cattle, nor in 20 chromosomes from South African Red Brahman or Grey Brahman control animals. Thus the 470del20 is not part of the normal bov*CHRNE* gene sequence, nor is it a variation typically found in Brahman genomes. Mutation screening showed that both the sire and the maternal grandsire shared by all four affected calves were heterozygous for the 470del20 mutation. The heterozygous deletion genotype was also detected in the dam of one of the calves, as well as in three healthy half-sisters. Another three half-sisters and a half-sister of the dam of an affected calf were tested and found to be homozygous for the bov*CHRNE* wild-type allele (Figure 1).

**Table 2.** Sizes of introns[a]

| Intron | Bovine (bp) | Human (bp) |
|---|---|---|
| 1 | 259 | 254 |
| 2 | 119 | 126 |
| 3 | 150–200[b] | 123 |
| 4 | 142 | 139 |
| 5 | ND | 306 |
| 6 | 379 | 334 |
| 7 | 77 | 82 |
| 8 | ND | 1,210 |
| 9 | 71 | 83 |
| 10 | 84 | 90 |
| 12 | 117 | 109 |

[a] Accession number for bov*CHRNE* genomic sequences: AF457656

[b] Estimated by gel electrophoresis of amplified fragments

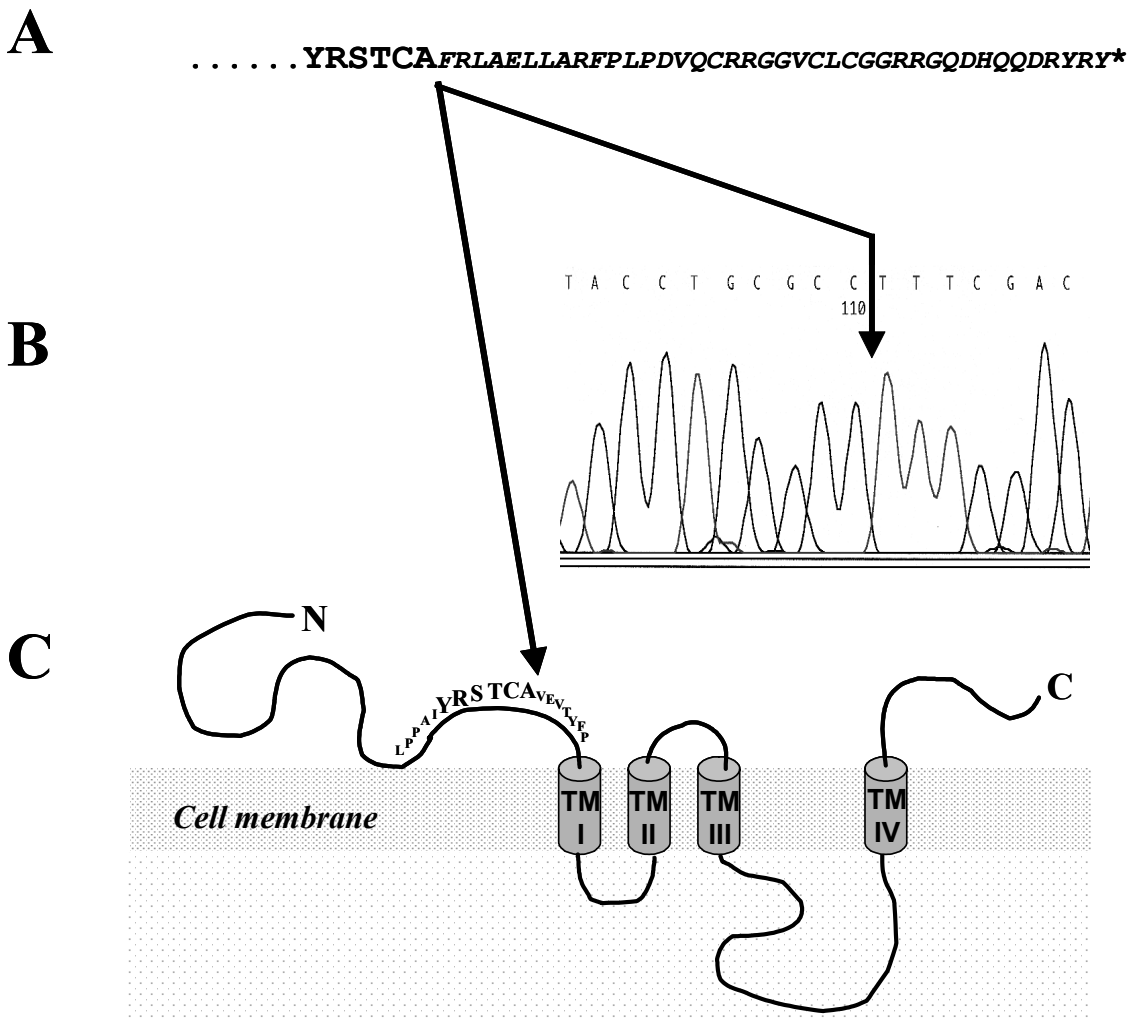


**Figure 2.** Detection of bov*CHRNE*/470del20. A Predicted amino acid sequence of the bov*CHRNE*/470del20 allele. The missense amino acids caused by the deletion are given in *smaller letters*. The premature stop codon is indicated by an *asterisk*. B Sequencing of the affected Brahman calf. The position of the homozygous deletion is indicated by an *arrow*. C Schematic drawing of the putative bov*CHRNE* protein. The four transmembrane regions (TM) are shown, and the position of the 470del20 mutation is indicated by an *arrow*.

***Confirmation of bovCHRNE cDNA sequence***

The cDNA sequence obtained from the cattle control DNA and the affected Brahman calf differed at position bp 317 from the published bov*CHRNE* sequence (accession number X02597). The observed A/C exchange did not affect the predicted amino acid sequence, but deleted a *Dde*I restriction site in our cDNA sequence. The expected fragment sizes of the 193-bp exon 4-PCR product were 105 bp and 88 bp for the published sequence. Only the 193-bp fragment was observed after *Dde*I digestion of amplification products from 90 chromosomes of red, black and white, Red Brahman and Grey Brahman cattle. Thus the published adenine in position bp 317 is either a rare variant or a sequencing error.

## Discussion

Human congenital myasthenic syndromes are a heterogeneous group of disorders which can be caused by presynaptic, synaptic or postsynaptic defects at the neuromuscular junction. Mutations have been found in all four subunits of the muscular nicotinic acetylcholine receptor (AChR), in the choline acetyltransferase gene, and in the *COLQ* gene encoding for the collagen tail of the acetylcholinesterase (Engel et al., 1999; Sieb et al., 2002). Detailed endplate studies including morphology and in vitro electrophysiology were not performed in the Brahman calves. Therefore, it was unclear which subtype of neuromuscular transmission defect was present in the affected animals. However, the beneficial effect of anticholinesterases and the decreased response were consistent with a reduced AChR expression. Thus far in humans, most AChR mutations have been found within the *CHRNE* gene (Engel et al., 1999; Sieb et al., 2002). This guided us to select the bov*CHRNE* gene to start searching for the genetic defect in the myasthenic Brahman calves.

We identified a homozygous 20-bp deletion within exon 5 of the gene. The predicted bov*CHRNE* protein, encoded by the RNA carrying the 470del20 mutation, would be truncated upstream from the four transmembrane domains. Sequence analysis showed that the 470del20 mutation causes a frame shift in the predicted bov*CHRNE* protein after 129 codons, substituting 342 wild-type amino acid residues with 40 aberrant

amino acids, followed by a stop codon. The frame shift occurred 90 amino acids residues N-terminal of the first transmembrane region. Thus, the bov*CHRNE* mutation reported here leads to a non-functional allele, which is likely to be the primary cause for myasthenia in the affected Brahman calves. All four myasthenic calves were related to each other by sharing the same sire and maternal grandsire (Thompson, 1998). Furthermore, the calf investigated in the present study was homozygous for the 470del20 mutation. Thus it is likely that the common ancestors were also related to each other. The prevalence of the 470del20 mutation in the Brahman breed is unknown, and warrants further investigation.

The myasthenic calves do not have a functional copy of the *CHRNE* subunit, and it can therefore be assumed that mature AChR was absent from their neuromuscular junctions. The most likely explanation why the condition did not result in immediate perinatal lethality, would be phenotypic rescue by foetal-type AChR, due to persistent expression of the $\gamma$-AChR subunit encoded by the *CHRNG* gene (Engel et al., 1996). Normally, a switch from *CHRNG* to *CHRNE* gene transcription occurs, either late in pregnancy or in the postnatal phase. Low-level persistent *CHRNG* expression has been demonstrated in healthy humans, as well as in humans carrying two truncating mutations in their *CHRNE* genes (MacLennan et al., 1997; Croxen et al., 2001). Although the true survival times of the affected calves were unknown, as they were killed humanely, all four calves were severely affected at a very young age and would have only been able to survive by hand-feeding (Thompson, 1998). This is in contrast to human myasthenic patients with truncating *CHRNE* mutations, most of whom show a less severe course of the disease (Engel et al., 1996; Ohno et al., 1998; Middleton et al., 1999; Sieb et al., 2000). One possible explanation for this could be a rapid and complete downregulation of bov*CHRNG* gene expression after birth. It would therefore be interesting to study the peri- and postnatal time course of *CHRNG* expression in healthy cattle. Immunohistochemistry of endplates from bovine muscle samples, collected at different ages between the last month before birth and the end of the first six months of life, could be used to compare human and bovine *CHRNG* expression patterns. If no differences are found between humans and cattle, this would argue against a rescuing role of persistent *CHRNG* expression, but for the existence of some other, unknown factors that are able to influence the severity of the phenotype. Breeding of homozygous calves would be helpful to further establish the phenotype-genotype correlation, and would provide the possibility to study the AChR distribution

and composition at the muscular endplate of the affected calves. Thus, future comparative studies including animal models such as the one described, will contribute to our understanding of the complex genotype-phenotype relationship in congenital myasthenic syndromes.

## Acknowledgements

We would like to thank Iris Laufenberg and Pranisha Budaram for excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to O.S.T. and J.P.S. (STE 769/3-2, SI 472/3-1).

## Literature cited

Breathnach, R. and P. Chambon. 1981. Organization and expression of eucaryotic split genes coding for proteins. Annual Review of Biochemistry 50:349–383.

Croxen, R., C. Young, C. Slater, S. Haslam, M. Brydson, A. Vincent, and D. Beeson. 2001. End-plate $\gamma$- and $\varepsilon$-subunit mRNA levels in AChR deficiency syndrome due to $\varepsilon$-subunit null mutations. Brain 124:1362–1372.

Dewey, C. W., J. R. Coates, J. M. Ducote, J. C. Meeks, and J. M. Fradkin. 1999. Azathioprine therapy for acquired myasthenia gravis in five dogs. Journal of the American Animal Hospital Association 35:396–402.

Engel, A. G., K. Ohno, C. Bouzat, S. M. Sine, and R. C. Griggs. 1996. End-plate acetylcholine receptor deficiency due to nonsense mutations in the epsilon subunit. Annals of Neurology 40:810–817.

Engel, A. G., K. Ohno, and S. M. Sine. 1999. Congenital myasthenic syndromes: recent advances. Archives of Neurology 56:163–167.

Flagstad, A. 1982. A new hereditary neuromuscular disease in the dog breed "Gammel Dansk Hønsehund". Genetic investigations. Hereditas 96:211–214.

Flagstad, A., W. Trojaborg, and S. Gammeltoft. 1989. Congenital myasthenic syndrome in the dog breed Gammel Dansk Hønsehund: clinical, electrophysiological, pharmacological and immunological comparison with acquired myasthenia gravis. Acta Veterinaria Scandinavica 30:89–102.

Indrieri, R. J., S. R. Creighton, E. H. Lambert, and V. A. Lennon. 1983. Myasthenia gravis in two cats. Journal of the American Veterinary Medical Association 182:57–60.

Joseph, R. J., J. M. Carrillo, and V. A. Lennon. 1988. Myasthenia gravis in the cat. Journal of Veterinary Internal Medicine 2:75–79.

Lipsitz, D., J. L. Berry, and G. D. Shelton. 1999. Inherited predisposition to myasthenia gravis in Newfoundlands. Journal of the American Veterinary Medical Association 215:956–958.

MacLennan, C., D. Beeson, A. M. Buijs, A. Vincent, and J. Newsom-Davis. 1997. Acetylcholine receptor expression in human extraocular muscles and their susceptibility to myasthenia gravis. Annals of Neurology 41:423–431.

Madden, T. L., R. L. Tatusov, and J. Zhang. 1996. Applications of network BLAST server. Methods in Enzymology 266:131–141.

Middleton, L., K. Ohno, K. Christodoulou, J. Brengman, M. Milone, V. Neocleous, P. Serdaroglu, F. Deymeer, C. Ozdemir, A. Mubaidin, K. Horany, A. Al Shehab, I. Mavromatis, I. Mylonas, M. Tsingis, E. Zamba, M. Pantzaris, K. Kyriallis, and A. G. Engel. 1999. Chromosome 17p-linked myasthenias stem from defects in the acetylcholine receptor epsilon-subunit gene. Neurology 53:1076–1082.

Miller, L. M., V. A. Lennon, E. H. Lambert, S. M. Reed, G. A. Hegreberg, J. B. Miller, and R. L. Ott. 1983. Congenital myasthenia gravis in 13 smooth fox terriers. Journal of the American Veterinary Medical Association 182:694–697.

Miller, S. A., D. D. Dykes, and H. F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 16:1215.

Ohno, K., B. Anlar, E. Ozdirim, J. M. Brengman, J. L. DeBleecker, and A. G. Engel. 1998. Myasthenic syndromes in Turkish kinships due to mutations in the acetylcholine receptor. Annals of Neurology 44:234–241.

Ridyard, A. E., S. M. Rhind, A. T. French, E. A. Munro, and P. B. Hill. 2000. Myasthenia gravis associated with cutaneous lymphoma in a dog. Journal of Small Animal Practice 41:348–351.

Shelton, G. D., A. Schule, and P. H. Kass. 1997. Risk factors for acquired myasthenia gravis in dogs: 1,154 cases (1991–1995). Journal of the American Veterinary Medical Association 211:1428–1431.

Shelton, G. D., G. O. Skeie, P. H. Kass, and J. A. Aarli. 2001. Titin and ryanodine receptor autoantibodies in dogs with thymoma and late-onset myasthenia gravis. Veterinary Immunology and Immunopathology 78:97–105.

Sieb, J. P., S. Kraner, B. Schrank, B. Reitter, T. H. Goebel, S. J. Tzartos, and O. K. Steinlein. 2000. Severe congenital myasthenic syndrome due to homozygosity of the 1293insG epsilon-acetylcholine receptor subunit mutation. Annals of Neurology 48:379–383.

Sieb, J. P., S. Kraner, and O. K. Steinlein. 2002. Congenital myasthenic syndromes. Seminars in Pediatric Neurology 9:108–119.

Thompson, P. N. 1998. Suspected congenital myasthenia gravis in Brahman calves. Veterinary Record 143:526–529.

Wallace, M. E. and A. C. Palmer. 1984. Recessive mode of inheritance in myasthenia gravis in the Jack Russell terrier. Veterinary Record 114:350.

Wilkes, M. K., R. E. McKerrell, R. C. Patterson, and A. C. Palmer. 1987. Ultrastructure of motor endplates in canine congenital myasthenia gravis. Journal of Comparative Pathology 97:247–256.