Genes on bovine chromosome 18 associated with bilateral convergent strabismus with exophthalmos in German Brown cattle

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**Purpose:** Bilateral convergent strabismus with exophthalmos (BCSE) is a widespread inherited eye defect in several cattle populations. Its progressive condition often leads to blindness in affected cattle and shortens their length of productive life. Furthermore, breeding with BCSE-affected animals is forbidden by the German animal welfare laws. We performed a mutation and association analysis for three candidate genes (troponin T type 1 [TNNT1], retinol dehydrogenase 13 [RDH13], and TCF3 fusion partner [TFPT]), which are located within the previously identified BCSE-linked region on the telomeric end of bovine chromosome 18 (BTA18). In addition, we developed single nucleotide polymorphisms (SNPs) within these three candidate genes and nine other genes that are contained in this genomic BCSE-region to perform association analyses with BCSE in German Brown cattle.

**Methods:** We performed cDNA analyses of all three candidate genes using eye tissues of three affected German Brown cows and three unaffected controls. Furthermore, we screened the exonic and the adjacent genomic sequences of RDH13, TNNT1, and TFPT using four BCSE-affected and four controls of German Brown cattle. Here, we included all exons of RDH13 and those exons of TNNT1 and TFPT for which SNPs were detected by cDNA analyses. In addition, we developed 21 polymerase chain reaction (PCR) products for 17 more genes in the BCSE region and searched them for polymorphisms. All markers detected were genotyped in 48 BCSE-affected German Brown cows and 48 breed and sex matched controls and tested for association with BCSE.

**Results:** In total, we detected 29 SNPs in 12 genes. In the coding sequence of the three candidate genes, we identified 10 exonic SNPs and a new splice variant of TNNT1. Four SNPs were associated with the BCSE phenotype in single marker-trait analyses. These SNPs were located within DHDH (dihydriol dehydrogenase dimeric), CPT1C (carnitine palmitoyltransferase 1C), TNNT1, and NALP7. The marker-trait association for haplotypes including five SNPs of CPT1C, SYT5 (synaptotagmin V), RDH13, and NALP7 (NLR family, pyrin domain containing 7) revealed a significant association with BCSE. We identified three individual haplotypes that were significantly associated with BCSE. These haplotypes spanned the region from 56.05 Mb to 62.87 Mb on BTA18.

**Conclusions:** The haplotype association analysis corroborated the results of the linkage study that the telomeric end of BTA18 harbors a gene responsible for BCSE and further refines the BCSE region to a 6.82 Mb interval ranging from 56.05 Mb to 62.87 Mb on BTA18.

Bilateral convergent strabismus with exophthalmos (BCSE) is a heritable eye defect that occurs in many cattle breeds, e.g., Jersey, German Fleckvieh, German Holstein, and German Brown [1-4]. The incidence of BCSE was estimated to be 0.9% in German Brown cattle [2]. This eye defect is characterized by a progressive, bilateral symmetric anterior-medial rotation of the eyes that is associated with a slight to severe protrusion of the eyeballs. This defect can result in complete blindness. In the development of the bilateral convergent strabismus, a defect in the lateral rectus muscle and the retractor bulbi muscle of the eye or in their appendant nerves (Nervus abducentis and Nervus oculomotorius) might be involved. Histopathological examination of the nuclei of abducentis nerves showed significant differences between BCSE-affected and unaffected cows in the number of nerve cells. BCSE-affected animals had a decreased number of nerve cells in both nuclear regions (Nuclei n. abducentis dexter and sinister), and this may be related with paresis of the M. rectus lateralis and the lateral parts of M. retractor bulbi, which is also involved in lateral eye movement [5]. The histomorphological examination of the lateral and medial rectus muscles of BCSE-affected cows revealed "ragged red fibers," which are indicators for defects in the respiratory chain of muscles [6].

The defect sometimes causes changes in the behavior of the affected animals such as aggressiveness, shying, and panic in everyday situations. The first signs of BCSE can appear as early as the age of six months, but most of the affected animals are not noticed before first breeding. This eye anomaly is incurable [1].

In a previously performed whole genome scan using multipoint non-parametric linkage and haplotype analysis in a total of 159 German Brown cattle, we identified a genomic region harboring a locus responsible for BCSE on bovine...
chromosome 18 (BTA18) [7]. We mapped this BCSE locus to a 6.83 cM interval (MARCU- USDA linkage map) on the telomeric end of BTA18 between the microsatellites, BMS2785 (72.01 cM) and BM6507 (78.84 cM), using linkage and haplotype analysis. The Zmean and LOD score peaked at marker DIK5109 (77.60 cM) [5]. This BCSE region corresponds to a 7.77 Mb interval between 55.23 Mb (BMS2785) and 63.0 Mb (BM6507). These marker positions were determined using BLAST analysis for Btau_4.0 (Bos taurus genome assembly 4.0).

We could identify misinnervation syndromes in humans with similarities in pathology and clinical features to BCSE in cattle. Progressive external ophthalmoplegia (PEO), Duane retraction syndrome (DRS), and congenital fibrosis of the extraocular muscles (CFEOM) belong to this group of diseases in humans. PEOs are characterized by slowly progressive bilateral immobility of the eyes accompanied by ptosis. The three candidate genes, POLG [8], ANTI [9], and C10orf2 [10], for PEO were ruled out as responsible for BCSE [1,11]. CFEOM [12] and DRS [13,14] belong to a group of congenital cranial nerve dysinnervation disorders (CCDD) affecting the eye, eyelid, and/or facial movement [15]. The various forms of CFEOM [12] result from dysinnervation of the ocularmotor nerve innervated ocular muscles and/or trochlear nerve innervated ocular muscles. Genes or loci causing the CFEOM phenotypes include KIF21A (CFEOM1) on centromeric HSA12q12 [16,17], ARIX (CFEOM2) on HSA11q13.3-q13.4 [18], CFEOM3 on HSA16q24.2-q24.3 [19], and CFEOM3A on HSA12p11.2-q12 [20]. The bovine syntenic regions for these genes or loci are on BTA5, 9.7 Mb distally of the Quantitative Trait Locus (QTL) for BCSE (KIF21A), on BTA15 at 51.34 Mb (ARIX), and on BTA18 from 11.5 to 14.0 Mb (CFEOM3). The loci for DRS were mapped to HSA8q13 (DURS1) [21-23] and HSA2q31 (DURS2) [24,25]. The orthologous bovine loci are on BTA14 between 30.2 and 30.7 Mb (DURS1) and BTA2 between 14.7 and 21.3 Mb (DURS2). Therefore, none of these loci or genes identified for CCDD in humans are mapping within the QTL for BCSE.

Comparison of the gene order on the telomeric end of BTA18 (Btau_4.0) with the corresponding region on HSA19 (NCBI Build 36.2) showed two blocks of synteny (Figure 1). The gene order within the first block from LOC540740 to PRKCG is consistent with the human gene order. The second block between EPN1 (epsin 1) and TFPT (TCF3 fusion partner; Btau_4.0) is inverse compared to the gene order of the human genome assembly 36.2. In our analysis, we considered the interval from LOC540740 (54.98 Mb) to TFPT (63.54 Mb), which included the linked BCSE region and its flanking regions on BTA18.

The aim of this study was to identify single nucleotide polymorphisms (SNPs) associated with BCSE within the previously determined BCSE region and within the coding sequence of possible candidate genes contained in this region. Candidate genes were chosen due to their expression profile and their proximity to the microsatellite, DIK5109.

The first candidate gene, troponin T type 1 (TNNT1), is located about 200 kb proximal of DIK5109 at 62.50 Mb. The protein product of TNNT1 is a component of the thin filament of the sarcomere and has the function to prevent actin-myosin interaction in resting muscle. TNNT1 is highly expressed in skeletal muscles [26]. The second candidate gene, retinol dehydrogenase 13 (RDH13), is located in close vicinity to TNNT1 at 62.70 Mb. RDH13 belongs to the short-chain dehydrogenases/reductases (SDR) family and is mostly expressed in cranial nerve tissue and in the retina where it was detected in the inner segment of the photoreceptor cells [27]. Mutations causing strabismus have not yet been reported, but related genes such as RDH5 and RDH12 were shown to cause fundus albipunctatus and retinal dystrophy in human, which can be accompanied by strabismus [28,29]. The third candidate gene, TCF3 fusion partner (TFPT), is ubiquitously expressed mainly in the brain, hematopoietic cell lines, and eye tissue.

**METHODS**

**Animals, phenotypic data, and DNA/RNA extraction:** For our analyses, we collected blood samples from 96 unrelated German Brown cows. Of these animals, 48 were affected by BCSE and showed third or fourth stage BCSE where more than 50% of the eye was filled with sclera [30]. The other 48 German Brown cows were unaffected and more than six years old. Thus, these animals are very unlikely to develop the BCSE phenotype. Genomic DNA from EDTA blood samples was extracted using the QIAamp 96 Spin Blood Kit (Qiagen, Hilden, Germany).

For cDNA analysis, we took biopsies from the retina, N. opticus, and ocular muscles (M. rectus lateralis and M. retractor bulbi) of three unaffected and three severely affected cows (BCSE stage 3) [30]. These samples were taken 15–30 min after the cows were slaughtered.

**Tissue samples were conserved using RNAlater solution (Qiagen).** RNA was extracted from the ocular tissues using the Nucleospin RNA II-Kit (Macherey-Nagel, Düren, Germany) and transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany).

**Gene structure, single nucleotide polymorphisms, polymerase chain reaction, and DNA sequencing:**

**Bioinformatic cDNA analysis**—For cDNA analyses of the candidate genes, we searched the cattle expressed sequence tag (EST) archive for ESTs and the bovine genome for annotated genes by cross-species BLAST searches with the corresponding human reference mRNA sequences for TNNT1 (NM_003283), RDH13 (NM_138412) and TFPT (NM_013342). Table 1 gives an overview of the structure of these human genes and their orthologs in *Bos taurus*. In
addition, we verified the sequence homology between the proteins of the three candidate genes in cattle, mouse, and human using the ClustalW alignment program (Figure 2).

We found a bovine EST (EE371552), isolated from muscle tissue with 89% identity to the human TNNT1 mRNA sequence, and the bovine mRNA of TNNT1 (NM_174474) with an identity of 90% to human TNNT1 mRNA (NM_003283).

For RDH13, we found two overlapping bovine ESTs (DV925005 and DV828503), which cover 77% of the human mRNA sequence with an identity of 88% and the bovine mRNA of RDH13 (NM_001075345). The first EST
Table 1. Candidate genes.

| Gene   | Homo sapiens (36.2) | Bos taurus (4.0) |
|--------|---------------------|-----------------|
|        | DNA (bp) | mRNA (bp) | Number of exons | DNA (bp) | mRNA (bp) | CDS (bp) | Number of exons |
| TNNT1  | HSA       | 19 | 16378 | 980 | 14 | 18 | 9366 | 887 | 9 - 800 (exon 1-exon 13) | 13 |
| RDH13  | 19 | 25191 | 2006 | 8 | 18 | 16985 | 3025 | 119 - 1126 (exon 1-exon 7) | 7 |
| TFPT   | 19 | 8707 | 1077 | 7 | 18 | 7644 | 879 | 62 - 817 (exon 1-exon 6) | 6 |

An overview about the structure of the candidate genes, TNNT1, RDH13, and TFPT of Homo sapiens in comparison to Bos taurus is given.

(DV925005) was isolated from the skin of an embryo and the second (DV828503) from fetal pons.

We found three overlapping bovine ESTs (DV851209, CO881320, and CO873631) that were isolated from brain tissue covering the whole human TFPT mRNA sequence with an identity of 86%. Furthermore, we identified the bovine TFPT employing a genomic BLAST analysis with the bovine mRNA sequence (NM_001075274).

We amplified the cDNA sequence corresponding to the open reading frames (ORF) of the three candidate genes. We used the ESTs and the annotated gene information for primer design with Primer3 software (Table 2).

Genomic DNA sequence analysis for single nucleotide polymorphism detection—For these analyses we employed four BCSE-affected German Brown cows and four controls of the same breed. First, we designed exon flanking intronic primer pairs for the genomic amplification of all exons of RDH13 and the exons of TNNT1 and TFPT, which harbored SNPs detected by cDNA analyses (Table 3). Furthermore, we designed primer pairs for four polymerase chain reaction (PCR) products of these candidate genes for SNP detection within intronic regions (Table 4). To cover the whole region of 8.56 Mb extending between LOC540740 and TFPT, we screened 17 more genes for DNA polymorphisms. A total of 21 amplicons was sequenced (Table 4). We used the DNA of eight German Brown cows (four affected and four controls) for SNP development in the three positional candidate genes and the 17 genes evenly distributed over the QTL region.

Polymerase chain reaction and DNA sequencing—We used 48 BCSE-affected German Brown cows and 48 unaffected cows of the same breed. PCR reactions were performed in a total volume of 30 µl using 2 µl (~20 ng/µl) genomic DNA, 3 µl 10X PCR buffer, 6 µl 10X PCR Enhancer (PeqLab, Erlangen, Germany), 0.6 µl (10 µM) of each primer, 0.6 µl dNTPs (10 mM each), and 0.2 µl (5 U/µl) Taq polymerase (Roche, Mannheim, Germany). The reactions were performed in TProfessional thermocyclers (Biometra, Goettingen, Germany) and started with 5 min initial denaturation at 95 °C followed by 36 cycles at 95 °C for 30 s, optimum annealing temperature (Ta) around 58–60 °C for 1 min, and extension at 72 °C for 45 s. The PCR was completed with a final cooling at 4 °C for 10 min. After purification of the PCR products with MinElute 96 UF Plate (Qiagen), the amplicons were directly sequenced with the DYEEnamic ET Terminator Cycle Sequencing kit (GE Healthcare, Freiburg, Germany) on a MegaBACE 1000 capillary sequencer (GE Healthcare). Sequence data was analyzed using the Sequencher 4.7 program (GeneCodes, Ann Arbor, MI).

We analyzed a total of 41 PCR products within 20 genes (Table 2, Table 3, and Table 4). We genotyped all 20 SNPs detected in the cDNA and genomic sequences of the three candidate genes as well as the nine SNPs detected within the additional genes in the BCSE region to obtain a complete sampling from 48 BCSE-affected German Brown cows and 48 unaffected cows of the same breed (Table 5).

Statistical analyses: A case-control analysis based on χ²-tests for genotypes, alleles, and trend of the alleles was performed using the CASECONTROL procedure of SAS/Genetics (SAS, version 9.1.3; Statistical Analysis System, Cary, NC). The ALLELE procedure of SAS was used for estimation of allele frequencies and tests for Hardy–Weinberg equilibrium (HWE) of genotype frequencies. Statistical calculation of pairwise linkage disequilibrium (LD) was performed and pictured using HAPLOVIEW 4.0 [31]. We used the Tagger algorithm r²≥0.8 [32] to detect SNPs with strong LD among alleles. Subsequently, the association of haplotypes with BCSE was tested using the HAPLOTYPE procedure of SAS/Genetics.

RESULTS

Hardy–Weinberg equilibrium and minor allele frequencies: In total, we developed 29 SNPs within 12 genes. Of these 29 SNPs, 20 were located within the three candidate genes, TNNT1, RDH13, and TFPT. The other nine SNPs were discovered in nine different genes located in the 8.56 Mb interval between LOC540740 (similar to inward rectifier potassium channel) and TFPT. The genotypic distributions of the 27 genotyped SNPs were in Hardy–Weinberg equilibrium.
Figure 2. Alignment of RDH13, TNNT1 and TFPT proteins with known human and mouse orthologs. A: Shown are the protein sequences of RDH13 for cattle, man and mouse. B: Shown are the protein sequences of TNNT1 for cattle, man and mouse. C: Shown are the protein sequences of TFPT for cattle, man and mouse. Bovine protein sequences were derived from our analyzed coding sequences, which were similar to the published bovine protein sequences (NP_001068813.1, NP_776899.1, and NP_001068742.1). The sequences were derived from GenBank entries with the accession numbers NP_612421 (human RDH13), NP_780581 (mouse RDH13), NP_003274 (human TNNT1), NP_035748 (mouse TNNT1), NP_037474 (human TFPT), and NP_076013 (mouse TFPT). Identical residues are indicated by asterisks beneath the alignment. The exons are labeled by different colors.
The PCR primers for the amplification of the cDNA of bovine *TNNT1*, *RDH13*, and *TFPT* are shown.
| Gene | Target | SNP | Primer | Primer sequence (5′→3′) | Annealing temperature (°C) | Product size (bp) |
|------|--------|-----|--------|-------------------------|---------------------------|-------------------|
| TNNT1 | intron 10 | AM930546<br>g.273A>G | TNNT1_SNPex11_F | CAGAGTTGGGGATGGATATG | 58 | 597 |
| | exon 11 | AM930555<br>c.359A>G | TNNT1_SNPex11_R2 | AGACCAGAGGAGTGTTGGG | 58 | 597 |
| | exon 13 | AM930555<br>c.425A>C | TNNT1_SNPex11_R2 | AGACCAGAGGAGTGTTGGG | 58 | 597 |
| RDH13 | 5′UTR | AM930553<br>c.57A>C | RDH13_ex1_F_gen | CCCAGATGGACACACACC | 60 | 381 |
| | exon 1 | AM930553<br>c.103C>G | RDH13_ex1_R_gen | CCGGAAGCAACTAGACCAAA | 60 | 381 |
| | intron 1 | AM930548<br>g.294C>T | RDH13_ex1_R_gen | CCGGAAGCAACTAGACCAAA | 60 | 381 |
| | exon 2 | AM930553<br>c.151C>T | RDH13_ex2_R_gen | CCGGAAGCAACTAGACCAAA | 60 | 381 |
| | intron 2 | AM930549<br>g.8G>A | RDH13_ex3_R_gen | CCGGAAGCAACTAGACCAAA | 60 | 381 |
| | exon 3 | AM930550<br>g.137T>C | RDH13_ex4_R_gen | CCGGAAGCAACTAGACCAAA | 60 | 381 |
| TFPT | exon 2 | AM930551<br>c.337A>T | TFPT_SNPex3_F | GCCACTTCCTGGGTGACATG | 60 | 381 |
| | exon 2 | AM930551<br>c.379G>T | TFPT_SNPex3_F | GCCACTTCCTGGGTGACATG | 60 | 381 |
| | exon 5 | AM930552<br>c.176G>A | TFPT_SNPex3_R | GCCACTTCCTGGGTGACATG | 60 | 381 |
| | exon 5 | AM930552<br>c.176G>A | TFPT_SNPex3_R | GCCACTTCCTGGGTGACATG | 60 | 381 |

The PCR-primers for amplification of the genomic sequences of bovine TNNT1, RDH13, and TFPT genes which contain the SNP markers are shown.
The SNPs within LOC540740 and exon 11 of TNNT1 were not in Hardy–Weinberg equilibrium. Thus, these SNPs were not considered in the subsequent association analyses. The results of the tests for HWE, the observed heterozygosity (HET), polymorphism information content (PIC), and minor allele frequencies for the developed SNPs are shown in Table 5.

**Mutation analysis within bovine TNNT1, RDH13, and TFPT:** We revealed a total of 10 exonic SNPs within the three candidate genes and a new splice variant of TNNT1 (Table 5). Furthermore, we detected 10 SNPs in the intronic sequences of these candidate genes (Figure 3 and Figure 4).

| Gene   | Position HSA19 (Mb) | BLAST hit BTA18 (Mb) | Primer name | Primer sequence (5' -> 3') | Primer location | Product size (bp) |
|--------|---------------------|----------------------|-------------|---------------------------|-----------------|------------------|
| KCNJ14 | 53.65               | 54.98                | KCNJ14_F    | CCAGGGTTGGTGAGAAGACT      | exon 2          | 342              |
|        | 53.65               | 54.98                | KCNJ14_R    | GCTCTTCACTACACCCTCTCGT    | exon 2          | 938              |
|        | 53.65               | 54.98                | CA11_F      | GAAACTTGCTGCAAGAGTG       | intron 3        | 955              |
|        | 53.65               | 54.98                | CA11_R      | CACCAAGCTGGTTCACCCTCTCG   | exon 6          | 556              |
|        | 53.65               | 54.98                | DHDH_F      | AGCTCTTCACTACACCCTCTCGT   | exon 5          | 955              |
|        | 53.65               | 54.98                | DHDH_R      | TCCCTATATGCTCCCCCTCTC     | exon 2          | 652              |
|        | 53.65               | 54.98                | BAX_F       | TCGAGGGTTAGTTGAGTGC       | intron 3        | 556              |
|        | 53.65               | 54.98                | BAX_R       | GTCGACCCAAAACAAAGAGA      | exon 8          | 530              |
|        | 53.65               | 54.98                | HRC_F1      | TGCTGCTGGAAACCTGCTG       | intron 5        | 652              |
|        | 53.65               | 54.98                | HRC_R1      | CAGGGGAGGAGGAAATAAGTG     | exon 7          | 652              |
|        | 53.65               | 54.98                | HRC_F2      | TAACTCTGCTCCCTCTGTCG       | intron 2        | 652              |
|        | 53.65               | 54.98                | HRC_R2      | GAGCAGAGAGTTCGCCGACAG      | exon 4          | 652              |
|        | 53.65               | 54.98                | CPT1C_F     | TGGACCTTTTTTGACCTGACT      | intron 6        | 652              |
|        | 53.65               | 54.98                | CPT1C_R     | GACCTAAAGG66GCTCATCT      | intron 6        | 652              |
|        | 53.65               | 54.98                | KCN3_F      | TGCTCTTCACTACCTGTCCTC     | intron 2        | 337              |
|        | 53.65               | 54.98                | KCN3_R      | AAAAGATCTGCTGGCAAGAAGA     | intron 2        | 619              |
|        | 53.65               | 54.98                | MYBPC2_F    | TGTCCCTCAAGGAGAATAGGCT    | intron 4        | 955              |
|        | 53.65               | 54.98                | MYBPC2_R    | AGCTCTTCACTACCTGTCCTC     | intron 4        | 955              |
|        | 53.65               | 54.98                | KLK6_F      | AGGGGAGAGGAGGAGGAAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | KLK6_R      | AGGGGAGAGGAGGAGGAAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | ETFB_F      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | ETFB_R      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | PRKCG_F     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | PRKCG_R     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TFPT_F      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TFPT_R      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TTYH1_F     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TTYH1_R     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | KIR3DL1_F   | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | KIR3DL1_R   | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | NALP7_F     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | NALP7_R     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | RDH13_F     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | RDH13_R     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TNNT1_F     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TNNT1_R     | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TYR5_F      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | TYR5_R      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | BRK1_F      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | BRK1_R      | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | SUV420H2_F  | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |
|        | 53.65               | 54.98                | SUV420H2_R  | AGGGGAGAGGAGGAAAGAAGAAGA  | intron 4        | 549              |

The gene, its position on HSA19, BLAST hits on BTA18 (Btau_4.0), F and R primers, primer location, and product size are presented. The annealing temperature for all PCR reactions was 58 °C.

The SNPs within LOC540740 and exon 11 of TNNT1 were not in Hardy–Weinberg equilibrium. Thus, these SNPs were not considered in the subsequent association analyses. The results of the tests for HWE, the observed heterozygosity (HET), polymorphism information content (PIC), and minor allele frequencies for the developed SNPs are shown in Table 5.

**Mutation analysis within bovine TNNT1, RDH13, and TFPT:** We revealed a total of 10 exonic SNPs within the three candidate genes and a new splice variant of TNNT1 (Table 5). Furthermore, we detected 10 SNPs in the intronic sequences of these candidate genes (Figure 3 and Figure 4).

**TNNT1:** Two exonic SNPs are located in the coding sequence of TNNT1. One SNP was found in exon 11 and the second in exon 13. Both SNPs did not affect the amino acid sequence. We also identified a deletion of 33 base pairs in the cDNA sequence from eye muscle tissue of all six cows. These 33 base pairs conform to exon 4 of the published bovine mRNA (NM_174474; Figure 4). In contrast, the cDNA isolated from the retina showed that all tested animals were heterozygous.

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### Table 5. SNP Analysis.

| Gene   | SNP          | Location | Number of cases | Minor allele frequencies (%) | HET (%) | PIC (%) | p (HWE) |
|--------|--------------|----------|----------------|-----------------------------|---------|---------|---------|
| KCNJ14 | AM922316     | exon 2   | 32 34          | 50 39.71                    | 34.9    | 37.2    | 0.0165  |
| DHDH   | AM930537     | intron 5 | 38 34          | 16.88 30.88                 | 36.1    | 29.6    | 0.9928  |
| BAX    | AM930538     | intron 3 | 43 40          | 18.6 11.25                  | 25.3    | 22.3    | 0.9197  |
| CPT1C  | AM930539     | intron 15| 43 43          | 22.09 36.05                 | 39.5    | 32.7    | 0.7016  |
| PRKCG  | AM930540     | exon 6   | 43 40          | 32.56 32.5                  | 36.1    | 34.3    | 0.1077  |
| SUV420H2 | AM930545   | intron 4 | 34 41          | 20.59 20.73                 | 33.3    | 27.4    | 0.8861  |
| SYT5   | AM930544     | intron 4 | 39 36          | 7.69 16.67                  | 24.0    | 18.9    | 0.2376  |
| TNNT1  | AM930546     | exon 10  | 43 39          | 6.98 5.13                   | 9.8     | 10.8    | 0.18    |
| TNNT1  | AM930555     | exon 11  | 33 31          | 22.73 4.84                  | 21.6    | 21.3    | <0.0001 |
| RDH13  | AM930553     | 5′UTR    | 48 47          | 39.58 35.11                 | 49.5    | 35.9    | 0.579   |
| RDH13  | AM930553     | c.57A>C  | 48 46          | 22.92 29.35                 | 35.1    | 31.1    | 0.3876  |
| RDH13  | AM930548     | intron 1 | 32 30          | 13.75 11.29                 | 41.7    | 35.9    | 0.8591  |
| RDH13  | AM930553     | c.151C>T | 46 46          | 19.57 13.04                 | 30.4    | 23.6    | 0.2694  |
| RDH13  | AM930549     | g.8G>A   | 46 45          | 11.96 12.22                 | 22.0    | 19.0    | 0.745   |
| RDH13  | AM930550     | intron 3 | 45 40          | 12.22 21.25                 | 25.9    | 23.7    | 0.5842  |
| RDH13  | AM930553     | c.703C>A | 48 45          | 11.46 21.11                 | 23.7    | 23.4    | 0.2257  |
| RDH13  | AM930547     | g.113G>C | 40 38          | 17.5 23.68                  | 30.8    | 27.3    | 0.6181  |
| RDH13  | AM930547     | g.194C>T | 44 43          | 11.36 20.93                 | 27.6    | 23.4    | 0.8409  |
| RDH13  | AM930547     | g.333C>T | 41 43          | 17.07 19.77                 | 8.7     | 8.0     | 0.9192  |
| RDH13  | AM930547     | g.378A>G | 39 42          | 14.29 20.24                 | 19.8    | 25.6    | 0.6617  |
| RDH13  | AM930554     | c.491G>C | 48 43          | 7.29 21.28                  | 44.0    | 29.9    | 0.9942  |
| TNNT1  | AM930555     | exon 7   | 48 43          | 45.35 26.32                 | 23.4    | 18.6    | 0.2833  |
| TNNT1  | AM930543     | intron 4 | 29 35          | 17.24 7.14                  | 23.4    | 18.6    | 0.2833  |
| TYH1   | AM930542     | intron 7 | 29 35          | 17.24 7.14                  | 23.4    | 18.6    | 0.2833  |
| TFPT   | AM930551     | c.337A>T | 7 7           | 7.14 0                     | 7.1     | 6.7     | 0.8898  |
| TFPT   | AM930551     | c.379G>T | 7 7           | 7.14 0                     | 7.1     | 6.7     | 0.8898  |
| TFPT   | AM930541     | intron 3 | 39 42          | 2.56 3.57                   | 6.2     | 5.8     | 0.7744  |
| TFPT   | AM930541     | intron 2 | 48 47          | 8.33 14.89                  | 23.2    | 18.4    | 0.2018  |
| TFPT   | AM930541     | c.176G>A | 7 7           | 7.14 0                     | 7.1     | 6.7     | 0.8898  |

The intragenic single nucleotide polymorphisms (SNPs, n=29) are shown with their number of genotyped cases and controls, minor allele frequencies of genotyped cases and controls, polymorphism information content (PIC), heterozygosity (HET), and test results for Hardy–Weinberg equilibrium (HWE).
for this splice variant. In nerve tissue, all three genotypes were found. In addition, we found one SNP within intron 10.

**RDH13:** In *RDH13*, five exonic SNPs were detected. An A>C transversion (AM930555; c.425A>C) is located in the 5′UTR 14 bases upstream of the start codon. A C>G SNP (AM930555; c.103C>G) is located at position 151 of bovine mRNA (NM_001075345) in exon 1. This SNP changes a CGG triplet to a GGG triplet and thus causes an amino acid exchange from arginine to glycine (p.Arg11Gly). This means there is a change from a charged alkaline amino acid to a nonpolar amino acid. The second SNP (AM930554; c.491G>C), which results in an amino acid exchange from glutamine to glutamate (p.Gln233Glu), was found at position 33 of bovine exon 7. This G>C transversion changes a GAG triplet to a CAG triplet, which has the effect that a polar and uncharged amino acid is replaced by an acidic, nonpolar, and charged amino acid in the primary structure of the protein. In addition, we detected two synonymous SNPs in the coding sequence of exon 2 and 5. Within the introns of *RDH13*, we detected one SNP in introns 1, 2, and 3 and two SNPs each in intron 5 and 6 (Table 5).

**TFPT:** In the coding sequence of *TFPT*, we identified three exonic SNPs. Two of them are located at positions 2 and 44 of exon 2. Both mutations affect the protein structure. The first exon 2 SNP (AM930551; c.337A>T) is an A>T transversion, which causes an amino acid exchange from threonine to serine (p.Thr9Ser). However, both amino acids are polar, uncharged, and differ in only one ─CH<sub>3</sub> side chain. The second SNP (AM930551; c.379G>T) in exon 2 alters the protein structure due to a G>T transversion, which changes a GGC triplet to a TGC triplet (p.Gly23Cys). This means that the nonpolar amino acid, glycine, is exchanged with the polar, sulfur-containing amino acid, cysteine. The third exonic SNP found in the ORF of *TFPT* is a synonymous mutation. This G>A SNP (AM930552; c.176G>A) at position 72 in exon 5 changes a CTG to a CTA triplet, which has no effect on the amino acid sequence of *TFPT*. In addition, we identified one SNP in introns 1 and 2 of *TFPT*.
Association analysis: We detected four SNPs significantly associated with BCSE. These were located in DHDH, CPT1C, TNNT1, and NALP7 (Table 5 and Table 6). An exonic A>G transition (AM930555; c.359A>G) within TNNT1 reached significant results in allele and trend test statistics (Table 6).

The SNPs within the exons and the exon flanking intronic sequences of RDH13 showed no significant results from the $\chi^2$ tests for distribution of genotypes between cases and controls. The $\chi^2$ test statistics for allelic distributions between cases and controls ranged from 0.003 to 3.20 and their error probabilities from 0.07 to 0.97 for the RDH13 SNPs. Four exon SNPs clearly failed the threshold of significance. Only the C>A SNP (AM930553; c.703C>A) in exon 5 with an allelic $\chi^2$ value of 3.20 was close to the threshold of 0.05 (Table 6).

The exonic SNPs of TFPT were not genotyped for the complete sample due to their low minor allele frequency (Table 5), and the other intronic SNPs were not associated with BCSE (Table 6).

Linkage disequilibrium and haplotype association: The r$^2$ values indicated strong linkage disequilibrium (LD) for the SNPs between intron 1 and intron 2 of RDH13. By tagging with threshold r$^2$ ≥0.8, we detected five SNPs in RDH13, which were representative for the total of 12 RDH13 SNPs. Therefore, only these five SNPs of RDH13 were used in the haplotype association analysis. The SNPs within the other genes were not in LD (Figure 5).

We tested the association of haplotypes with BCSE including five SNPs located in the genes, CPT1C (AM930539; g.569A>G), SYT5 (AM930544; g.71G>A), RDH13 (AM930553; c.703C>A and AM930547; g.194C>T), and NALP7 (AM930543; g.103T>G), was significant ($\chi^2$=54.11, p<0.0001). In total, there were eight different haplotypes of these markers that had a frequency of at least 1% (Table 7). Three individual haplotypes were significantly associated with the affected status and occurred with a frequency of more than 5% in our sample. The A-G-C-G haplotype occurred with a frequency of 31.7% in our sample of affected cows and with a frequency of 9.4% in the controls. The A-G-A-T-T haplotype occurred with a frequency of 17.1% in the sample of unaffected cows and with a frequency of 7.0% in the affected cows. The third associated haplotype (G-G-C-C-T) was found with a frequency of 17.0% in our sample of unaffected cows and with a frequency of 7.0% in the affected cows (Table 7). The significantly associated haplotypes spanned the region from CPT1C (56.05 Mb) to NALP7 (62.87 Mb) on the telomeric end of BTA18. Further, two haplotypes adjacent to the proximal and distal region of this aforementioned associated region were tested for association with BCSE. The first haplotype proximally to CPT1C consisted of the SNPs (AM922316; g.141C>G, AM930537; g.351A>C, AM930538; g.493T>C), and the second haplotype distally to NALP7 included the SNPs (AM930542; g.365G>A, AM930541; g.120T>C, AM930541; g.342G>A). Both adjacent haplotypes did not show significant results in marker-trait association tests with BCSE ($\chi^2$=11.7, p =0.07 corresponds to the first haplotype proximally to the BCSE-associated region and $\chi^2$=7.3, p=0.12 corresponds to the second haplotype distally to the BCSE-associated region).
DISCUSSION
We developed a total of 29 intragenic SNPs within an 8.56 Mb region on BTA18 extending from LOC540740 to TFPT.

In a previously performed whole genome scan, this region was found to be linked with BCSE in German Brown dairy cattle [7]. Within the genes, CPT1C and NALP7, two SNPs were
significantly associated with BCSE in association tests for single markers. Within DHDH, TTYH1, and SYT5, three more SNPs reached values close to the significance threshold of $p=0.05$.

Most of the 29 SNPs were detected in the sequences of the potential candidate genes, RDH13, TFPT, and TNNT1. We identified four missense mutations within the coding sequence of these three genes and also detected a new splice variant of TNNT1. Since none of the SNPs within the genes (RDH13, TFPT, and TNNT) were significantly associated with BCSE in association tests for single markers, these genes are unlikely to be causal for this eye defect. However, the detected polymorphisms may be of importance in studies for other bovine diseases especially the SNPs within RDH13. These SNPs could be involved in the genetic pathology of retinal dystrophy or related diseases like the defects reported to be associated to other members of the short-chain dehydrogenases/reductases (SDR) family [27,28].

In cattle, genes influencing the development of strabismus are not yet known. Therefore, neuromuscular eye-disorders in humans with already identified causal genes and even developmental and biological characteristics may be used as candidates for BCSE. However, all potential candidate genes characterized as causal for these syndromes (PEO,
Inclusion of SNPs from the genes CPT1C, SYT5, RDH13, and NALP7. Presence of the haplotype A-G-C-C-G composed of these five SNPs indicated a high probability of an animal to be affected by BCSE later in life whereas the haplotypes A-G-A-T-T and G-G-C-C-T were related with low risk to BCSE. Because the surrounding SNPs did not contribute to the significance of the haplotype association, confirmation has been obtained that this linked BCSE region could be delimited using haplotype analysis. Robustness of the haplotype association was furthermore evident when the surrounding haplotypes were extended with one or three adjacent SNPs from the associated haplotype region. In these cases, the extended haplotypes reached higher \( \chi^2 \)-test statistics and lower error probabilities as more SNPs of the associated region were included. This result was according to our expectation for this region. In conclusion, the haplotype association refined the BCSE region to a 6.82 Mb interval.

To detect the gene responsible for bovine BCSE, further SNPs have to be developed within this BCSE region spanning from 56.05 Mb to 62.87 Mb on BTA18. Haplotype analysis may then be a valuable tool to determine the most likely BCSE causing gene.

Particularly, SNPs within potential candidate genes like CPT1C will be considered. CPT1C is located at 54.89 Mb on HSA19 and specifically expressed within the endoplasmic reticulum (ER) in neurons of the brain [33]. Expression was also detected in the retinal pigment epithelium [34]. The function of this gene is not yet clearly defined. CPT1C is believed to modulate the palmitoyl-CoA pool associated with the ER and therefore to regulate the synthesis of ceramide and sphingolipids. Ceramide and sphingolipids are important for signal transduction, modification of neuronal membranes, and brain plasticity [35-37]. Since one of the BCSE-associated SNPs is located within intron 15 of bovine CPT1C at 56.05 Mb on BTA18, this gene may be a candidate for BCSE.

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