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Impact of β-galactosidase mutations on the expression of the canine lysosomal multienzyme complex

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ABSTRACT

β-galactosidase (GLB1) forms a functional lysosomal multienzyme complex with lysosomal protective protein (PPCA) and neuraminidase 1 (NEU1) which is important for its intracellular processing and activity. Mutations in the β-galactosidase gene cause the lysosomal storage disease G_{M1}-gangliosidosis. In order to identify additional molecular changes associated with the presence of β-galactosidase mutations, the expression of canine lysosomal multienzyme complex components in *GLB1*$^{+/+}$, *GLB1*$^{+/-}$ and *GLB1*$^{-/-}$ fibroblasts was investigated by quantitative RT-PCR, Western blot and enzymatic assays. Quantitative RT-PCR revealed differential regulation of total β-galactosidase, β-galactosidase variants and protective protein for β-galactosidase gene (*PPGB*) in *GLB1*$^{+/-}$ and *GLB1*$^{-/-}$ compared to *GLB1*$^{+/+}$ fibroblasts. Furthermore, it was shown that *PPGB* levels gradually increased with the number of mutant β-galactosidase alleles while no change in the *NEU1* expression was observed. This is the first study that simultaneously examine the effect of *GLB1*$^{+/+}$, *GLB1*$^{+/-}$ and *GLB1*$^{-/-}$ genotypes on the expression of lysosomal multienzyme complex components. The findings reveal a possible adaptive process in *GLB1* homozygous mutant and heterozygous individuals that could facilitate the design of efficient therapeutic strategies.

1. Introduction

From DNA polymorphisms to protein degradation, eukaryotic gene expression requires chains of complex regulated processes [1]. Due to either structural defects or abnormal processing, impairments in these processes may lead to severe disorders. For several Mendelian diseases, the molecular cause has been successfully identified [2–4]; however, in the majority of the "simple" genetic disorders, a wide phenotypic variability in terms of clinical phenotype and disease severity has been observed [5–7]. Therefore, clinical manifestations of monogenic disorders seem to be analogous to complex traits [7]. One reason for this complexity is the regulation of gene expression at different levels [8,9]. For different genes, certain steps in the regulation of their expression were described after identification of a specific clinico-pathological phenotype. Thus, β-galactosidase (GLB1; EC 3.2.1.23) deficiencies were associated with the lysosomal storage disease G_{M1}-gangliosidosis (OMIM # 230500; OMIA # 00402) which displays three major types (types I, II and III) in humans with respect to the age of onset and clinico-pathological manifestations [10–12]. Other types of β-galactosidase gene (*GLB1*) mutations were also identified in Morquio type B disease characterized by ganglioside accumulation in several organs without central nervous system involvement [13]. Moreover, due to normal alternative splicing, the *GLB1* encodes, additionally to β-galactosidase, the elastin-binding protein (EBP) [14]. As a consequence, structural defects of *GLB1* were associated with impaired elastogenesis [15]. Similar variability of G_{M1}-gangliosidosis has also been described in dogs where the disease resembles either type I (English Springer Spaniel) or type II (Alaskan huskies, Shiba dogs, Portuguese Water Dog, mixed Beagle) human G_{M1}-gangliosidosis [16–18]. Furthermore, structural defects affecting the co- and post-transcriptional processing of *GLB1* like pre-mRNA splicing or generation of premature termination codons (PTC) were identified in humans and dogs [19–21]. Other studies showed that abnormal transcripts can evade the cellular surveillance mechanism when the PTC is located on the last exon [22–24].

After translation, GLB1 forms a functional lysosomal multienzyme complex (LMC) with neuraminidase (NEU1) and lysosomal protective protein (PPCA), which is important for its intracellular processing and activity [25–27]. Defects of either the hydrolytic or the protective components of LMC lead to abnormal phenotypes with varying degrees of clinical severity [28]. Moreover, the senescence-associated GLB1 was identified as being the normal lysosomal GLB1 whose

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activity is increased in physiological conditions related to senescence [29,30].

A 19 bp duplication located in exon 15 of the canine *GLB1* gene was identified in Alaskan huskies with G_{M1}-gangliosidosis [17,31,32]. This genetic modification either disrupts a potential exonic splicing enhancer sequence (ESE) leading to the re-framing of the mRNA by skipping the mutant exon 15 or generates a PTC on the last exon. Therefore, two *GLB1* mRNA populations were identified in diseased homozygous mutant dogs: one mRNA isoform lacking the exon 15 (GLB1ΔEx15) and the other one carrying the abnormal exon 15 with the 19 bp duplication (GLB1dupl) [17,31,32].

For further therapeutic approaches it is important to understand in which 'molecular environment' the therapy will be applied. To define this 'molecular environment' in a canine model of late infantile G_{M1}-gangliosidosis, the regulation of the *GLB1* mRNA expression of normal and abnormal variants and the GLB1 activity in fibroblasts with different *GLB1* genotypes were investigated.

Furthermore, possible mechanisms on how the GLB1 activity is maintained at sufficient levels in heterozygous individuals were analyzed by investigating the relationship between *GLB1* expression and the other LMC components, *PPGB* and *NEU1*.

2. Materials and methods

2.1. Cell culture procedures

Primary canine skin fibroblasts from 26 healthy dogs (10 homozygous $GLB1^{+/+}$ and 14 heterozygous $GLB1^{+/-}$) and two diseased homozygous ($GLB1^{-/-}$) were used [31,33,34]. All experiments were performed with fibroblasts at passage 3.

2.2. RNA extraction, cDNA synthesis and primer design

Total RNA was isolated from primary skin fibroblasts using the RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by a DNase treatment (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The OmniScript™ kit (Qiagen, Hilden, Germany), oligo dT_{12} (Roche, Mannheim, Germany) and 500 ng RNA were used for cDNA synthesis. The primers used for quantification of *GLB1*, *PPGB*, *NEU1* and glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) mRNA expression were designed using Primer 3 v 0.4.0 software and the published sequences [(GenBank™ accession no. DQ196436, NW 876277, XM 538838 and AB038240) [35]. The characteristics of these primers are presented in Table 1.

To distinguish between the different populations of GLB1 mRNAs (total GLB1, GLB1ΔEx15 and GLB1dupl) specific primer pairs were designed as shown in Fig. 1.

2.3. Quantitative RT-PCR (qRT-PCR)

Quantitative RT-PCRs were performed with an Mx3005P™ Instrument (Stratagene, La Jolla, CA, USA) in a total volume of 25 µl. For each reaction, 1 µl cDNA was placed in a 24 µl reaction mixture containing: 0.25 µl *Taq* DNA Polymerase (5 U/µl) (Invitrogen, Karlsruhe, Germany), 2.5 µl supplied 10× buffer, 1.25 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM) (Invitrogen, Karlsruhe, Germany) and 0.5 µl of each primer (10 µM) diluted in distilled water. SYBR® Green I was used as DNA-binding dye while ROX was chosen as reference dye. The cycling temperature profile was identical for all transcripts and consisted of: 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 1 min and extension at 72 °C for 30 s. Melting curves were generated at the end of each amplification reaction.

To generate gene specific standard curves, the PCR products were run on a 2% agarose gel, excised and eluted using the QIAquick Gel Extraction™ kit (Qiagen, Hilden, Germany). The DNA concentration was measured at 260 nm using the GeneQuant Pro™ RNA/DNA calculator (GE Healthcare, Freiburg, Germany). The identity of each amplicon was confirmed by sequencing (Agowa GmbH, Berlin, Germany). All reactions were performed in quadruplicate.

Table 1
Characteristics of the primer pairs used for quantification of GLB1 variants, PPGB and NEU1.

Gene name GeneBank acc. no.	Primer name	Sequence	Positions	Size of PCR product (bp)
GLB1	97fw	5′-TTC ACA ATT GAC TAC AGC CAC-3′	79–100	326
DQ 196436	98rev	5′-TAA TAG CCA AGC AGG TAA TC-3′	384–405	
GLB1ΔEx15	Ex14fw	5′-TCT GAA CAT AAC CGG GAA GG-3′*	1390–1409	107
DQ 196436*	Ex14/16rev	5′-TCC ACA GAC CCT TAA AAT C-3′*	1475–1488	
GLB1dupl	Dupl fw	5′-TTG CCC CAG GAT CCC AGA C-3′*	1697–1715	76
DQ 196436*	Ex15/16	5′-TAA TCC ACA CCT GAC CCT TG-3′*	1754–1773	
PPGB	PPGBfw	5′-CCT ATG AGC AGA ATG ACA ACT CC-3′	687–709	290
NW 876277	PPGBrev	5′-GGC AGG CGA GTG AAG ATG TT-3′	954–983	
NEU1	NEU1fw	5′-CAT GTT GGT GTG GAG CAA AG-3′	507–526	195
XM 538838	NEU1rev	5′-CAC TGA GGA GGC AGA AGA CC-3′	681–700	
GAPDH	GAPDHfw	5′-GCC AAA AGG GTC ATC ATC TC-3′	340–359	229
AB 038240	GAPDHrev	5′-GGC CAT CCA CAG TCT TCT-3′	548–565	

GLB1 — canine β-galactosidase gene; GLB1ΔEx15 — mutant canine β-galactosidase lacking exon 15; GLB1dupl — mutant canine β-galactosidase with the abnormal exon 15 carrying the 19 bp duplication; *PPGB* — protective protein for β-galactosidase gene; *NEU1* — neuraminidase 1 gene; *GAPDH* — glyceraldehyde-3-phosphate-dehydrogenase; fw = sense; rev = antisense; Ex14 = canine *GLB1* exon 14; Ex14/16 = boundary between exons 14 and 16 in the mutant canine *GLB1* lacking exon 15; dupl = 19 bp duplication in exon 15 of canine *GLB1*; * — primers were generated using wild-type (DQ 196436) and mutant specific *GLB1* sequences.

2.4. Quantification of mRNA expression levels

Each assay included two standard curves (one for the gene of interest and one for GAPDH) using serial 10-fold dilutions (ranging from E + 08 to E + 02 copies). The correlation coefficient (R^2) of the standard curves was above 0.985. The slopes of the standard curves were used to determine the reaction efficiency (e) using the equation: $(e) = 10^{(-1/\text{slope})} - 1$ [32]. Thus, obtained amplification efficiencies were above 95%. Between quadruplicates, the median coefficient of variation (CV) was calculated as CV = SD/mean and was under 5%. Due to differences in the amplicon sizes, the CT-values were corrected for PCR efficiency (e) using the following formula: $CT_{e=100\%} = CT_e\ [\log(1+e)/\log(2)]$. The relative number of molecules for each transcript was determined by interpolating the $CT_{e=100\%}$ values of the gene of interest (GOI) to each standard curve and the obtained values were normalized with respect to *GAPDH* for each individual cDNA. The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method using the expression from $GLB1^{+/+}$ fibroblasts as calibrator [36–38]. Statistical differences between groups were assessed by t-test and differences with $p<0.05$ were considered significant.

2.5. β-galactosidase enzymatic assays

GLB1 activities were measured as previously described [18,33,34] and expressed in mU/mg protein.

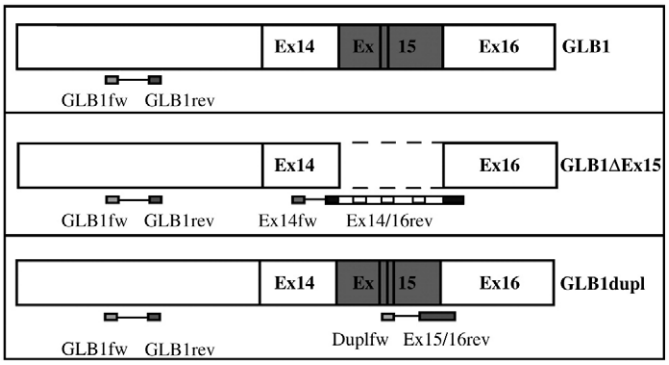

Fig. 1. Design of the primer pairs used to detect different populations of *GLB1* mRNA. Ex14 = canine *GLB1* exon 14; Ex15 = canine *GLB1* exon 15; Ex16 = canine *GLB1* exon 16; fw = sense; rev = antisense primers; GLB1 = wild-type canine *GLB1*; GLB1ΔEx15 = mutant canine *GLB1* lacking exon 15; GLB1dupl = mutant canine *GLB1* with the abnormal exon 15 carrying the 19 bp duplication. Different populations of *GLB1* mRNA: total GLB1, GLB1ΔEx15 and GLB1dupl were specifically detected by using GLB1fw-GLB1rev, Ex14fw-Ex14/16rev and Duplfw-Ex15/16rev primers.

2.6. Western blotting and densitometric analysis

Proteins were extracted and subjected to SDS-PAGE analysis as described [39]. For detection of PPCA (precursor — 54 kD and mature form — 32 and 20 kD) and actin, the polyclonal anti-PPCA (Acris GmbH, Hiddenhausen, Germany) and anti-actin antibody (Santa Cruz Biotechnology, Heidelberg, Germany) were used as primary antibodies. As secondary antibodies the anti-rabbit IgG, HRP-linked antibody (Santa Cruz Biotechnology, Hiddenhausen, Germany) and the polyclonal rabbit anti-mouse IgG HRP-linked antibody (Dako GmbH, Hamburg, Germany) were utilized. All antibodies were diluted 1:1000. The antigen-antibody complexes were visualized with the ECL chemiluminescence system (Amersham) and exposed to a Kodak X-OMAT film. Each set of samples (homozygous $GLB1^{+/+}$, heterozygous $GLB1^{+/-}$ and homozygous $GLB1^{-/-}$) was measured in three independent experiments. The densitometric analysis of the resulted protein bands was performed using the Scion Image Beta Software (Scion Corp., Frederick, MD, USA). The integrated optic densities were normalized to actin and the statistical differences between groups were assessed by *t*-test and differences with $p<0.05$ were considered significant.

3. Results

3.1. GLB1 activities in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts

$GLB1^{+/+}$ and $GLB1^{+/-}$ fibroblasts showed high levels of GLB1 activities whereas $GLB1^{-/-}$ cells had a significant deficiency in the enzymatic activity of GLB1 (Fig. 2). Surprisingly, no significant difference was observed between the GLB1 activities of $GLB1^{+/+}$ and $GLB1^{+/-}$ fibroblasts.

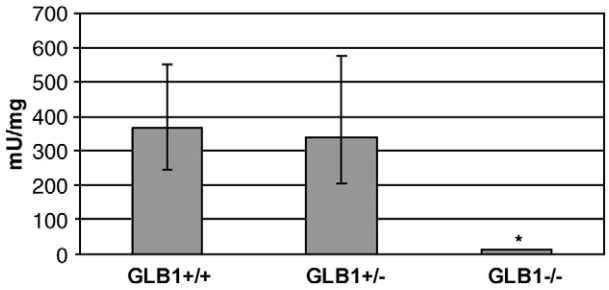

Fig. 2. GLB1 activity in $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ fibroblasts. $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ correspond to GLB1 activities in homozygous dominant ($GLB1^{+/+}$), heterozygous ($GLB1^{+/-}$) and homozygous recessive ($GLB1^{-/-}$) fibroblasts. GLB1 activities are given as mU/mg protein. The GLB1 activity in $GLB1^{-/-}$ fibroblasts represented 2.73% (10 ± 1.43 mU/mg) of those of $GLB1^{+/+}$ and $GLB1^{+/-}$ [365.1 ± 122.33 and 340 ± 127.87 mU/mg respectively; * — statistically significant differences ($p<0.05$)].

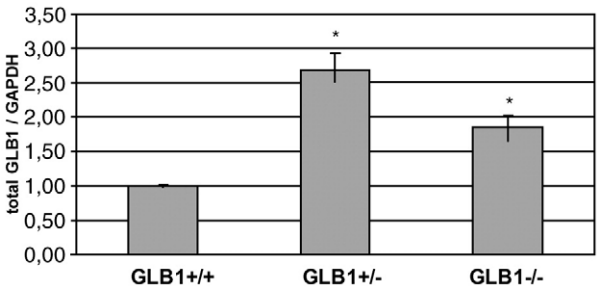

Fig. 3. Expression of total *GLB1* mRNA in $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ fibroblasts. $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ correspond to *GLB1* mRNA expression levels in homozygous dominant ($GLB1^{+/+}$), heterozygous ($GLB1^{+/-}$) and homozygous recessive ($GLB1^{-/-}$) fibroblasts. Columns show relative expression levels of GLB1 normalized to canine GAPDH analyzed by quantitative RT-PCR. Statistically significant differences (* — $p<0.05$) were observed between *GLB1* mRNA levels of $GLB1^{+/+}$ compared to $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts.

3.2. mRNA levels of total GLB1 in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts

To further investigate how GLB1 activity is maintained at similar levels in $GLB1^{+/+}$ and $GLB1^{+/-}$ fibroblasts, *GLB1* mRNA expression was analyzed by qRT-PCRs using a universal GLB1 primer pair (GLB1fw and GLB1rev). These primers were designed to detect the total *GLB1* mRNA expression represented by one (GLB1wild-type) mRNA population in $GLB1^{+/+}$ fibroblasts, three (GLB1wild-type, GLB1ΔEx15 and GLB1dupl) in $GLB1^{+/-}$ and two (GLB1ΔEx15 and GLB1dupl) mRNA populations in $GLB1^{-/-}$ fibroblasts (Fig. 1). Relative quantification revealed the *GLB1* mRNA expression pattern in $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ genotypes (Fig. 3) with a significant increase in total *GLB1* mRNA expression in heterozygous and homozygous recessive fibroblasts. Thus, in $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts total *GLB1* mRNA expression showed a 2.68 and 1.85- up-regulation respectively compared to $GLB1^{+/+}$ fibroblasts.

3.3. mRNA levels of GLB1 variants in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts

To determine which *GLB1* population contributed to the high levels of *GLB1* mRNA in $GLB1^{+/-}$ fibroblasts and to analyze possible alterations in the alternative splicing process between $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts, the expression of *GLB1* variants (GLB1ΔEx15 and GLB1dupl) was further investigated. While, as expected, the $GLB1^{+/+}$ cells showed no GLB1ΔEx15 mRNA expression, the $GLB1^{-/-}$

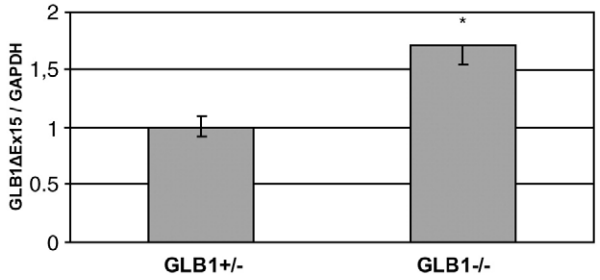

Fig. 4. Expression of GLB1ΔEx15 mRNA (lacking exon 15) in $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts. $GLB1^{+/-}$ and $GLB1^{-/-}$ correspond to GLB1ΔEx15 mRNA expression values in heterozygous ($GLB1^{+/-}$) and homozygous recessive ($GLB1^{-/-}$) fibroblasts. Columns show relative expression levels of GLB1ΔEx15 normalized to canine GAPDH analyzed by quantitative RT-PCR. Statistically significant differences (* — $p<0.05$) of GLB1ΔEx15 mRNA were observed between $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts.

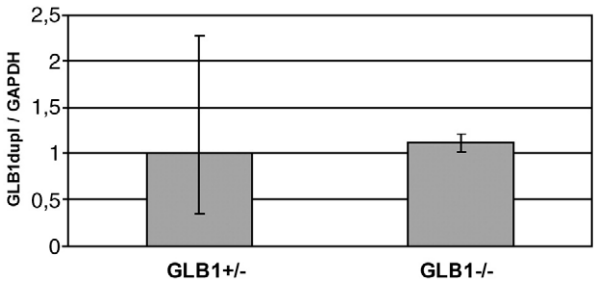

Fig. 5. Expression of GLB1dupl mRNA (with the exon 15 carrying the 19 bp duplication) in $GLB1^{+/-}$, $GLB1^{-/-}$ fibroblasts. $GLB1^{+/-}$ and $GLB1^{-/-}$ correspond to GLB1dupl mRNA expression levels in heterozygous ($GLB1^{+/-}$) and homozygous recessive ($GLB1^{-/-}$) fibroblasts. Columns show relative expression levels of GLB1dupl normalized to canine GAPDH analyzed by quantitative RT-PCR. No significant differences ($p<0.05$) in the expression levels of GLB1dupl mRNA in $GLB1^{+/-}$ and $GLB1^{-/-}$ cells were observed.

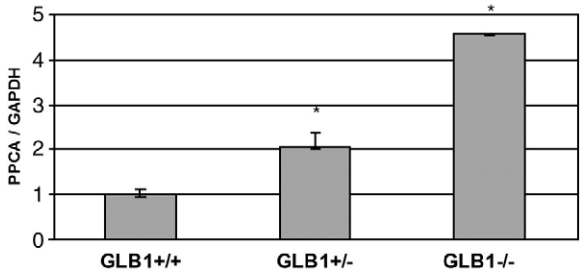

Fig. 7. Expression of *PPGB* mRNA in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts. $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ correspond to *PPGB* mRNA expression levels in homozygous dominant ($GLB1^{+/+}$), heterozygous ($GLB1^{+/-}$) and homozygous recessive ($GLB1^{-/-}$) fibroblasts. Columns show relative expression levels of *PPGB* mRNA normalized to canine GAPDH analyzed by quantitative RT-PCR. Statistically significant differences (* — $p<0.05$) were observed between *PPGB* mRNA levels from $GLB1^{+/+}$ compared to $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts.

fibroblasts expressed significantly higher levels of GLB1ΔEx15 mRNA compared to $GLB1^{+/-}$ cells (Fig. 4). For the GLB1dupl mRNA population, the expression increased slightly in $GLB1^{-/-}$ fibroblasts compared to $GLB1^{+/-}$ fibroblasts but was not statistically significant (Fig. 5). Analysis of the absolute GLB1ΔEx15 and GLB1dupl mRNA levels revealed that the GLB1dupl mRNA exceeded the expression of GLB1ΔEx15 mRNA by 10-fold (E+04 / E+03). Due to great similarities between GLB1wild-type and GLB1dupl sequences, a specific quantification of GLB1wild-type transcript was not possible. Moreover, eventual interference between *GLB1* and *EBP* expression was excluded while a canine *EBP* expression was not detected using primers for the canine *GLB1* similar with those used to amplify the alternatively spliced variant of the human *GLB1* named the human EBP (data not shown).

3.4. mRNA levels of NEU1 in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts

To further investigate whether this increase in total *GLB1* mRNA expression in $GLB1^{+/-}$ fibroblasts was associated with modifications in the expression of *NEU1*, additional qRT-PCRs were performed. The *NEU1* mRNA levels in $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts were not significantly altered compared to $GLB1^{+/+}$ fibroblasts (Fig. 6).

3.5. mRNA and protein levels of PPGB in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts

The investigation of the *PPGB* mRNA expression revealed a gradually increased expression in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts with the highest expression in $GLB1^{-/-}$ (4.57-fold), intermediate expression in $GLB1^{+/-}$ (2.06) and the lowest expression in $GLB1^{+/+}$ fibroblasts (Fig. 7). At the protein level, PPCA precursor (54 kD) showed a similar pattern of expression as *PPGB* mRNA (Fig. 8). Densitometric analysis of the Western blot bands revealed a statistically significant increase in PPCA precursor expression in $GLB1^{-/-}$ (1.14 ± 0.21) compared to $GLB1^{+/+}$ fibroblasts (0.11 ± 0.01). In contrast to PPCA precursor, the mature forms of PPCA (32 and 20 kD bands) were not present in all samples and their appearance did not correlate with the GLB1 genotypes.

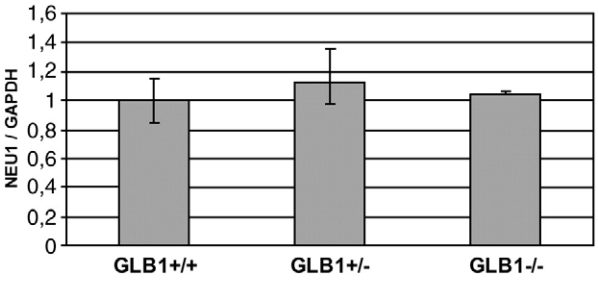

Fig. 6. Expression of *NEU1* mRNA in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts. $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ correspond to *NEU1* mRNA expression levels in homozygous dominant ($GLB1^{+/+}$), heterozygous ($GLB1^{+/-}$) and homozygous recessive ($GLB1^{-/-}$) fibroblasts. Columns show relative expression levels of *NEU1* normalized to canine GAPDH analyzed by quantitative RT-PCR. No significant differences ($p<0.05$) in the expression levels of *NEU1* mRNA in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ cells were observed.

4. Discussion

Over the years, the genetic defects causing several Mendelian disorders have been elucidated and it was shown that different mutations can affect the expression of a single gene at multiple levels. Moreover, single genes are involved in various biological processes. Thus, the phenotypical manifestations of a so-called "simple" genetic defect display variability similar to complex traits. Therefore, even for monogenic diseases, the identification of the molecular cause is only the first step in designing further therapeutic approaches [40].

In the present study, the expression of *GLB1*, *PPGB* and *NEU1* in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ genotypes was comparatively analyzed, to investigate additional molecular changes associated with the presence of *GLB1* mutations. The identification and characterization of such modifications are essential for the development of efficient type-specific therapeutic strategies for the treatment of G_{M1}-gangliosidosis.

The majority of lysosomal genes vary substantially in their expression [41]. Thereby, modifications of the *GLB1* mRNA expression under particular conditions cannot be excluded. Differential expression of *GLB1* was investigated by comparing healthy individuals and patients suffering from G_{M1}-gangliosidosis. However, as for other monogenic diseases, investigating heterozygous $GLB1^{+/-}$ individuals is important not only for the detection of mutant allele carriers but also for developing strategies to antagonize the effect of the mutant allele.

In the present study, the GLB1 activity of $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts was analyzed and, as expected, it was observed that $GLB1^{-/-}$ fibroblasts have a severe deficiency in GLB1 activity compared to $GLB1^{+/+}$ and $GLB1^{+/-}$ fibroblasts. Interestingly, no significant differences between fibroblasts carrying one or two wild-type alleles were identified, suggesting a differential regulation of GLB1 activity in these genotypes. To further elucidate this observation, the total *GLB1* mRNA expression was investigated. In $GLB1^{+/-}$ compared to $GLB1^{+/+}$ fibroblasts a 2.68-fold increase of total *GLB1* mRNA expression was shown. Thus, *GLB1* alleles elevated their expression as a result of a mutation in the *GLB1* gene. In addition, *GLB1* mRNA expression was 1.85-fold increased in $GLB1^{-/-}$. This observation is in contrast to previous reports which showed no difference between the *GLB1* expression in

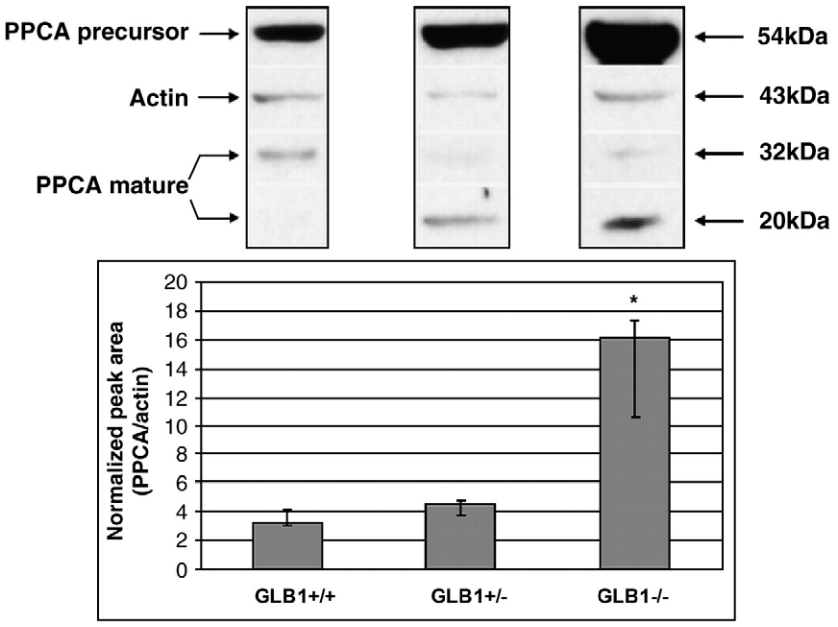

Fig. 8. Expression of PPCA protein in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts. $GLB1^{+/+}$, $GLB1^{+/-}$, $GLB1^{-/-}$ correspond to PPCA protein levels in homozygous dominant ($GLB1^{+/+}$), heterozygous ($GLB1^{+/-}$) and homozygous recessive ($GLB1^{-/-}$) fibroblasts normalized to actin protein. Statistically significant differences (* — $p<0.05$) were observed between the PPCA protein of $GLB1^{-/-}$ compared to $GLB1^{+/-}$and $GLB1^{+/+}$ fibroblasts.

controls and G_{M1}-gangliosidosis patients [4,42]. However, the higher levels of total GLB1 mRNA in $GLB1^{+/-}$ compared to $GLB1^{+/+}$ could also result from an increased expression of each GLB1 transcript. The great similarities between GLB1wild-type and the GLB1dupl sequences (wild-type: 5′- gga**tcccagacttgccccagga**cacc-3′; GLB1dupl: 5′-gga**tccca-gacttgccccagga tcccagacttgccccagga**cacc-3′) did not allow a direct differentiation between these two transcripts. In order to overcome these difficulties the mean β-galactosidase activity was compared between $GLB1^{+/+}$ and $GLB1^{+/-}$ individuals, however no significant differences were found ($p<0.05$). Furthermore, $GLB1^{+/+}$ individuals carry two wild-type alleles which are responsible for the measured β-galactosidase activity (mean value of 365.1 ± 122.33 mU/mg), while $GLB1^{+/-}$ individuals possess only one normal allele which results in a similar mean value of β-galactosidase activity (340 ± 127.87 mU/mg). In addition, only GLB1wild-type mRNA gives rise to an active protein [18]. Thus, it was concluded that the wild-type allele will have higher levels of transcription in $GLB1^{+/-}$ individuals in order to maintain the β-galactosidase activity at levels similar to those detected in $GLB1^{+/+}$ individuals. Moreover, human G_{M1}-gangliosidosis patients carrying splicing defects displayed a decrease in total *GLB1* mRNA expression [4]. As previously mentioned, the 19 bp duplication in canine G_{M1}-gangliosidosis generates a PTC which should trigger nonsense mediated decay (NMD) of the abnormal mRNA. However, due to its special position (less than 50–55 nucleotides upstream the 3′-most exon–exon junction), this PTC confers 'resistance' against NMD [22,24,43]. As a sequel, the mRNA abundance in such NMD-resistant mRNA carriers differs from those lacking these RNAs. This could explain why the *GLB1* mRNA levels were higher in $GLB1^{-/-}$ fibroblasts compared to $GLB1^{+/+}$ Alaskan husky fibroblasts. To prove the presence of NMD-resistant mRNA and to further investigate the distributions of both *GLB1* variants in $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts, qRT-PCR using variant-specific primers was performed. Thus, NMD-resistant mRNA (GLB1dupl) was detected in $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts. In addition, after absolute RT-PCR quantification, it was observed that only a small fraction of this mRNA lacks the mutant exon 15 (GLB1ΔEx15). The amino acid sequence encoded by exon 15 contains a cleavage site important for the correct lysosomal processing of GLB1.

The GLB1 enzymatic activity depends on its correct assembly with PPCA and NEU1 to form the lysosomal multienzyme complex [26,44,45]. Albeit the elevated levels of *GLB1* expression in $GLB1^{+/-}$ fibroblasts a lack of an appropriate amount of the other LMC components could potentially lead to deficiencies in GLB1 activity. Therefore we further investigated the expression of the *PPGB* and *NEU1* genes in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{+/-}$ fibroblasts. Thus, after analysis of *NEU1* mRNA levels in these fibroblasts no significant difference was observed between $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ cells. Furthermore, our previous analyses of the NEU1 enzymatic activity of canine fibroblasts from healthy, carriers and homozygous Alaskan huskies showed no differences among the three genotypes [33]. These results are in concordance with the observation that some human *GLB1* mutant alleles are not associated with modifications in *NEU1* expression [10]. A lack of variation in the *NEU1* expression was also described when sialidosis patients ($NEU1^{-/-}$) were compared to control healthy individuals ($NEU1^{+/+}$) [46].

Housekeeping gene characteristics were initially reported for the *PPGB* promoter; however, the distribution pattern of *PPGB* requires a specific regulation that cannot be anticipated by the housekeeping gene properties of its promoter [39,47]. In the present study, a differential expression of *PPGB* mRNA was observed in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts. This correlates with the increase of total *GLB1* mRNA level in $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts compared to $GLB1^{+/+}$ cells.

Previous studies described two distinct and separate functions of the PPCA: the carboxypeptidase function as mature form and the protective role as precursor [48]. In our experiments, the expression of the PPCA precursor correlates with *GLB1* genotypes (low in $GLB1^{+/+}$, medium in $GLB1^{+/-}$ and high in $GLB1^{-/-}$) while the mature forms showed not only a GLB1 mutation type-dependent expression [10] but also individual variations. These observations suggest a role of PPCA precursor in GLB1 protection in $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts. Therefore, the GLB1 activity in $GLB1^{+/-}$ fibroblasts seems to be maintained at high levels by a simultaneous increase of GLB1 and PPCA precursor expression. Interestingly, the observation that PPCA precursor expression in $GLB1^{-/-}$ fibroblasts was significantly higher compared to $GLB1^{+/-}$ fibroblasts, indicates a mechanism whereby the PPCA precursor level is increased in order to antagonize the lack of GLB1 activity. Further studies are necessary to decipher this mechanism and its clinical relevance.

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