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Antibody formation and mannose-6-phosphate receptor expression impact the efficacy of muscle-specific transgene expression in murine Pompe disease

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Abstract

BACKGROUND—Lysosomal storage disorders such as Pompe disease can be more effectively treated, if immune tolerance to enzyme or gene replacement therapy can be achieved.

Alternatively, immune responses against acid α -glucosidase (GAA) might be evaded in Pompe disease through muscle-specific expression of GAA with adeno-associated virus (AAV) vectors.

METHODS—An AAV vector containing the MHCK7 regulatory cassette to drive muscle-specific GAA expression was administered to GAA knockout (KO) mice, immune tolerant GAA-KO mice, and mannose-6-phosphate deficient GAA-KO mice. GAA activity and glycogen content were analyzed in striated muscle to determine biochemical efficacy.

RESULTS—The biochemical efficacy from GAA expression was slightly reduced in GAA-KO mice, as demonstrated by higher residual glycogen content in skeletal muscles. Next immune tolerance to GAA was induced in GAA-KO mice by co-administration of a second AAV vector encoding liver-specific GAA along with the AAV vector encoding muscle-specific GAA. Antibody formation was prevented by liver-specific GAA, and the biochemical efficacy of GAA expression was improved in absence of antibodies as evidenced by significantly reduced glycogen content in the diaphragm. Efficacy was reduced in old GAA-KO mice despite the absence of antibodies. The greatest impact upon gene therapy was observed in GAA-KO mice lacking the mannose-6-phosphate receptor in muscle. The clearance of stored glycogen was markedly impaired despite high GAA expression in receptor-deficient Pompe disease mice.

CONCLUSIONS—Overall, antibody formation had a subtle effect upon efficacy, while the absence of mannose-6-phosphate receptors markedly impaired muscle-targeted gene therapy in murine Pompe disease.

Keywords

Glycogen storage disease type II; gene therapy; antibody formation; adeno-associated virus; acid alpha-glucosidase; acid maltase; Pompe disease

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INTRODUCTION

Glycogen storage disease type II (GSD-II; Pompe disease; MIM 232300) results from the inherited deficiency of lysosomal acid α -glucosidase (GAA; acid maltase; EC 3.2.1.20). The disease is characterized by the massive accumulation of lysosomal glycogen in striated muscle with an accompanying disruption of cellular functions. While enzyme replacement (ERT) has shown promise in patients with both infantile-onset and late-onset Pompe disease, no curative therapy is available. Efforts to develop gene therapy in Pompe disease are justified by the limitations of ERT, which include the requirement for frequent intravenous infusions of high levels of GAA to achieve efficacy and the risk of neutralizing antibodies [1]. High level GAA replacement (40–100 mg/kg/dose) reduced the glycogen content of heart and skeletal muscle in immune tolerant GAA-knockout (KO) mouse models [2, 3]. Similarly, doses of 20–40 mg/kg were required to improve clinical endpoints in clinical trials of ERT in patients with Pompe disease [4, 5]. The amount of GAA required to achieve efficacy is approximately 30 to 100-fold greater than the doses used for ERT in other lysosomal disorders [6].

Pompe disease patients who lacked any residual GAA protein by Western blot analysis of skin fibroblasts are deemed cross-reacting immune material negative (CRIM-negative). CRIM-negative Pompe disease subjects produced anti-GAA antibodies and demonstrated markedly reduced efficacy from ERT [1, 7]. In the first pilot study of ERT using CHO cell-derived recombinant GAA, both CRIM-negative Pompe disease subjects produced the highest titers of anti-GAA antibodies and demonstrated markedly reduced efficacy from ERT [1]. The similarity with regard to neutralizing antibody response in GAA-KO mice and in CRIM-negative Pompe disease patients could be linked to the lack of residual GAA protein expression. If GAA deficiency is caused by an underlying null mutation(s), the immune system is likely to react to introduced GAA by forming neutralizing antibodies. Neutralizing antibodies occurred in CRIM-negative Pompe disease patients, and reduced the efficacy of ERT in those subjects.

Wide variability in the response in different organs to ERT has been observed in mice with Pompe disease, with liver and heart muscle taking up GAA more efficiently than skeletal muscle [8]. Barriers to the uptake of GAA in muscle fibers include not only the endothelial barrier, but also the endomysium. Type II myofibers were resistant to correction by ERT, in comparison with type I myofibers, which explained the resistance of the quadriceps and gastrocnemius to over-expression from the liver-specific transgene [3]. Poor uptake of GAA by skeletal muscle was also linked to the low abundance of the cation-independent mannose-6-phosphate receptor in skeletal muscle compared to heart [2, 9, 10].

The availability of GAA-KO mice has aided the development of gene therapy with AAV vectors, because the immune responses in GAA-KO mice to GAA are similar to those of CRIM-negative Pompe disease patients. GAA-KO mice feature no residual GAA expression, and therefore respond to recombinant hGAA with antibody formation and very low efficacy [Raben et al., 2003]. GAA-KO mice resemble CRIM-negative patients with regard to the response to ERT by forming high-titer antibodies and failing to demonstrate efficacy [2, 11]. Liver-expressing transgenic GAA-KO mice express very low levels of GAA only in liver, mimicking untreated CRIM-positive patients by not forming antibodies in response to recombinant GAA; therefore, these mice have been termed tolerant GAA-KO mice. The levels of GAA in cardiac and skeletal muscles for tolerant GAA-KO mice remain extremely low, and glycogen accumulates to high levels in striated muscle of tolerant GAA-KO mice just as it does in CRIM-positive patients with Pompe disease (Raben et al., 2003). A third strain of GAA-KO mice features an additional knock-out of the cation-independent mannose-6-phosphate receptor (double (D)KO), and might result in a CRIM-negative

phenotype with impaired receptor-mediated uptake of recombinant GAA [12]. Thus, the DKO mouse could be useful when evaluating the complicating factor of deficient mannose-6-phosphate receptor upon ERT in Pompe disease.

Major obstacles to therapy in Pompe disease have been identified over the last several years. Consequently, we have more thoroughly investigated the effects of antibody formation, age, and mannose-6-phosphate receptor availability upon the response to muscle-targeted gene therapy in GAA-KO mice.

RESULTS

The most efficient approach to gene therapy in Pompe disease might be the expression of GAA in directly transduced myofibers, because the main therapeutic target is striated muscle. The question of whether immune tolerance would impact the efficacy of muscle-targeted gene therapy within muscle was further investigated by administration of an AAV2/8 vector expressing muscle-restricted GAA, AAV-MHCK7hGAA, to GAA-KO mice. We previously demonstrated that AAV-MHCK7hGAA provoked antibody formation, but not cytotoxic T lymphocyte (CTL) responses, and achieved long-term biochemical correction in GAA-KO mice [13].

AAV2/8-MHCK7hGAA was administered to mice that featured immune tolerance to human GAA or its absence. AAV2/8-MCHK7hGAA (7×10^{11} vector particles (vp)) was administered to transgenic GAA-KO mice that were immune tolerant to GAA due to the liver-specific expression of low levels of GAA, termed tolerant GAA-KO mice [14], and to GAA-KO mice. In both strains of Pompe disease mice the vector significantly elevated GAA activity in the heart and the majority of skeletal muscles analyzed, in comparison with untreated mice (Figure 1A; $p < 0.01$ for each muscle). Only in the gastrocnemius of tolerant GAA-KO did AAV2/8-MHCK7hGAA fail to significantly elevate GAA activity, in comparison with untreated GAA-KO mice. Furthermore, AAV2/8-MCHK7hGAA significantly lowered glycogen content in the heart and skeletal muscle of both tolerant GAA-KO and GAA-KO mice (Figure 1B; $p < 0.01$ for each muscle), with the exception of the gastrocnemius in GAA-KO mice ($p=0.06$). However, the glycogen content of the gastrocnemius was significantly elevated in GAA-KO mice, in comparison with tolerant GAA-KO mice, following AAV2/8-MHCK7hGAA administration. The presence of higher GAA activity in the muscle of GAA-KO mice following vector administration ($p=0.007$ for each muscle), in comparison with tolerant GAA-KO mice, did not result in lower glycogen content in GAA-KO mice. Tolerant GAA-KO mice did not form anti-GAA antibodies, whereas GAA-KO mice uniformly produced antibodies in response to AAV-MCHK7hGAA administration (Figure 1C). These data demonstrated that immune responses impacted the reduction of glycogen storage in the skeletal muscle of Pompe disease mice.

The question of whether eliminating immune tolerance would impair efficacy in GAA-KO mice was investigated by administration of an AAV2/8 vector that ubiquitously expressed GAA, AAV-CBhGAA, simultaneously with AAV-MHCK7hGAA. This experiment evaluated whether adding a second vector that expressed GAA but provoked CTL, in the form of AAV-CBhGAA, would affect the efficacy from AAV-MHCK7hGAA. AAV-CBhGAA (1×10^{11} vp) was administered simultaneously with AAV-MHCK7hGAA (7×10^{11} vp) in GAA-KO mice to provoke immune responses against GAA. Administration of both vectors significantly elevated GAA activity in the heart, gastrocnemius, and extensor digitorum longus (EDL), in comparison with untreated GAA-KO mice (Figure 2A; $p < 0.005$ for each). However, efficacy was low in GAA-KO mice following administration of both vectors, as reflected by a lack of significantly reduced glycogen content in the gastrocnemius and EDL muscles. In contrast, the administration of AAV-MHCK7hGAA

alone in tolerant GAA-KO mice significantly reduced glycogen content in the heart and all skeletal muscles (Figure 2B). Moreover, even though the GAA activity was significantly elevated in the gastrocnemius of GAA-KO mice treated with both vectors in comparison with tolerant mice treated with only AAV-MHCK7hGAA (Figure 2A; $p=0.006$), the glycogen content of gastrocnemius trended lower in the latter group of mice (Figure 2B; $p=0.05$). Only GAA-KO mice formed antibodies following administration of AAV-CBhGAA, whereas tolerant GAA-KO mice did not form antibodies (Figure 2C). These data confirmed the criticality of immune tolerance to GAA with regard to correction of GAA deficiency, because simply adding another vector that expressed GAA ubiquitously at higher levels did not enhance the correction of glycogen storage in the skeletal muscle of Pompe disease mice.

Histomorphological analysis performed 18 weeks following vector administration revealed improved clearance of glycogen vacuoles in the gastrocnemius of AAV-MHCK7hGAA-treated tolerant GAA-KO mice, in comparison with GAA-KO mice treated with AAV-MHCK7hGAA and AAV-CBhGAA (Figure 3A–C). CTL was not observed in mice following administration of AAV-CBhGAA, because this immune response has been observed within the first 3 weeks following vector administration [15]. The size of vacuolated glycogen accumulations was reduced in AAV-MHCK7hGAA-treated tolerant GAA-KO mice. The reductions in glycogen vacuoles in multiple tissues of GAA-KO mice in absence of antibody formation supported the role of immune tolerance in modulating the efficacy of gene therapy in Pompe disease.

The data presented above were obtained in two different GAA-KO mouse strains; moreover, tolerant GAA-KO mice express GAA in liver that notably increased GAA activity and decreased glycogen content in the heart in absence of vector administration, in comparison with sham-treated GAA-KO mice (Figure 1). To further investigate the effects of immune tolerance upon efficacy in a single strain of mice with Pompe disease, GAA-KO mice were treated with an AAV vector, AAV2/8-LSPhGAA, that induced immune tolerance to hGAA in GAA-KO mice [11, 16]. GAA-KO mice were treated with both AAV2/9-MHCK7hGAA (1×10^{11} vp) and AAV2/8-LSPhGAA (1×10^{11} vp), or with AAV2/9-MHCK7hGAA (1×10^{11} vp) alone. AAV-MHCK7hGAA was pseudotyped as AAV9 to increase the transduction of striated muscle, as demonstrated previously [13] Indeed, GAA expression was similarly elevated for both groups of vector-treated mice, in comparison with sham-treated GAA-KO mice (Figure 4A). However, the glycogen content was significantly reduced in the diaphragm of dual vector treated GAA-KO mice, in comparison with GAA-KO mice that were treated with AAV2/9-MHCK7hGAA alone (Figure 4B; $p=0.05$). The diaphragm is a critical therapeutic target in Pompe disease, and therefore the improved correction of glycogen storage in that muscle is an important marker for efficacy [17]. Evidence for a more global correction of glycogen storage was demonstrated in the brain of GAA-KO mice treated with both AAV2/9-MHCK7hGAA and AAV2/8-LSPhGAA, which trended toward decreased glycogen content in the cerebrum ($p=0.08$), in comparison with AAV2/9-MHCK7hGAA-treated GAA-KO mice (Figure 3D–E). The basis for improved efficacy from dual vector treatment was revealed by Western blot analysis of plasma, which revealed the presence of circulating 110 kD GAA precursor that could be taken up through mannose-6-phosphate receptors by untransduced cells (Figure 5A). Furthermore, the presence of transgene expression in the liver was detected only dual-vector treated mice (Figure 5B). The presence of increased GAA in the quadriceps confirmed that liver expression from AAV2/8-LSPhGAApA was responsible for improved biochemical correction in dual vector treated mice, through secretion of GAA from the liver and receptor-mediated uptake in skeletal muscle (Figure 5B).

The absence of antibody formation in GAA-KO mice treated with both AAV2/9-MHCK7hGAA and AAV2/8-LSPhGAA demonstrated that immune tolerance had been established by hepatic expression of GAA (Figure 6A). Further evidence for efficacy was demonstrated by increased Rotarod latency in dual vector-treated GAA-KO mice, in comparison with GAA-KO mice that were treated with AAV2/9-MHCK7hGAA alone (Figure 6B).

The effectiveness of dual vector administration was evaluated in a challenging model for gene therapy, namely older GAA-KO mice. The intractability of advanced Pompe disease to biochemical correction has been emphasized by ERT and gene therapy experiments [18–21]. While GAA activity was elevated by dual vector administration in the heart and diaphragm (Figure 7A), glycogen content was reduced only in the heart of these old GAA-KO mice, in contrast with GAA-KO mice treated at a young age where glycogen storage was also reduced in skeletal muscles (Figure 7B vs. Figure 4B). Thus, treatment of advanced Pompe disease remains a significant obstacle for the development of new therapy in Pompe disease.

The role of receptor-mediated uptake and lysosomal targeting of GAA was investigated in DKO mice that were deficient in both GAA and GAA uptake, which were generated by crossing GAA-KO mice with muscle-specific cation-independent mannose-6-phosphate receptor KO mice. The AAV8-pseudotyped vectors, AAV-MHCK7hGAA (1×10^{11} vector particles/mouse) and AAV-LSPhGAA (2×10^{10} vector particles/mouse) were administered simultaneously in an attempt to prevent the formation of anti-GAA antibodies observed following AAV-MHCK7hGAA with fewer vector particles of AAV-LSPhGAA; however, anti-GAA antibodies were detected in all mice following dual vector administration (not shown). Nonetheless, significantly elevated GAA activity was observed in the muscles of mice following vector administration, in comparison with untreated GAA-KO mice (Figure 8A). As expected, DKO mice had equivalently elevated glycogen content in striated muscle to that observed in GAA-KO mice (not shown). Surprisingly, no significant difference in GAA activity between DKO and GAA-KO striated muscle was observed following vector administration, except in the gastrocnemius muscle that contained significantly higher GAA activity in DKO mice than in GAA-KO mice (Table 1). The latter, unexpected result was attributed to random variation, because uptake and expression of GAA would not be adversely affected by the presence of mannose-6-phosphate receptor. Consistent with expectations, glycogen was significantly increased in the majority of muscles from DKO mice, in comparison with GAA-KO mice following vector administration (Figure 8B). The only exception was for the quadriceps, which demonstrated approximately equal glycogen content in both DKO and GAA-KO mice (Table 1). The role of the mannose-6-phosphate receptor was implicated not only in the uptake of GAA, but also in the intracellular trafficking to lysosomes by these data.

DISCUSSION

Three factors involved in the response to muscle-targeted gene therapy were evaluated in Pompe disease mice: antibody formation, age, and mannose-6-phosphate receptor availability. Each of these has been implicated in previous research, either preclinical or clinical, as impacting GAA replacement therapy in Pompe disease [3, 7, 19]. Of these factors, antibody formation was least relevant, apparently due to antibodies being limited to the extracellular space such that muscle transduction was unaffected. Suppression of antibody formation did not improve the response of old GAA-KO mice to gene therapy. Mannose-6-phosphate receptor availability was implicated as a critical factor in the response to gene therapy, because glycogen clearance was dependent upon mannose-6-phosphate receptor expression despite the presence of markedly elevated muscle GAA activity.

The advantages of muscle-restricted expression with an AAV vector have been demonstrated in mouse models of muscular dystrophy. AAV vectors evaded cellular immune responses against foreign proteins expressed in muscle in normal mice, possibly because dendritic cells were transduced inefficiently [22]; however, cellular immune responses were demonstrated to ubiquitously expressed foreign proteins in mice with muscular dystrophy. Dystrophin-deficient mdx mice had increased immune responses to β -galactosidase expression driven by a CMV regulatory cassette compared to non-deficient mice, and substitution of an MCK regulatory cassette/enhancer delayed the onset of the immune reaction in mdx mice [23]. The lymphocytic infiltration in response to MCK regulatory cassette-driven β -galactosidase was predominantly CD4⁺ lymphocytes in mdx mice [23], as was the lymphocytic response to MCK-driven GAA in Pompe disease mice [15]. Substitution of an MCK regulatory cassette/enhancer for a CMV regulatory cassette/enhancer in an AAV vector encoding γ -sarcoglycan reduced the immune response and increased levels of transgene expression in γ -sarcoglycan-deficient mice; moreover, CTL assays were positive against LacZ and not against γ -sarcoglycan, when each was expressed with the CMV regulatory cassette [24, 25].

Investigating the role of antibody formation in determining the efficacy of muscle-targeted gene therapy was inspired by knowing the relevance of cell-mediated responses to gene therapy in Pompe disease mice. We described the avoidance of a cellular immune response against muscle-targeted gene therapy in GAA-KO mice by muscle-restricted expression of GAA with an AAV vector pseudotyped as AAV2/6 (AAV-MCKhGAA) [15]. In contrast to the analogous vector containing the hybrid CB regulatory cassette (AAV-CBhGAA), which expressed GAA only transiently, AAV-MCKhGAA expressed GAA at high levels 6 weeks after intramuscular administration [15]. The presence of anti-GAA antibodies did not prevent the correction of glycogen content in the skeletal muscle of GAA-KO mice, presumably related to the stable transduction of individual myofibers. Glycogen content was significantly reduced in the injected gastrocnemius with AAV-MCKhGAA, whereas it was not reduced with AAV-CBhGAA. Similarly, an improved vector containing the MHCK7 regulatory cassette to drive GAA transduced skeletal muscle efficiently but provoked anti-GAA antibody formation without preventing glycogen clearance [13]. However, it remained unclear whether antibody formation also impaired the response to muscle-specific GAA expression in GAA-KO mice, and current studies indicate that the impact of antibody formation is subtle.

Previous studies had suggested that the transduction of skeletal muscle following systemic delivery of myotrophic AAV vectors encoding GAA could circumvent the obstacles to enzyme replacement therapy and liver-targeted gene therapy in Pompe disease, including anti-GAA antibodies and mannose-6-phosphate receptor down-regulation in mature skeletal muscle [2, 3]. AAV2/6 vectors transduced striated muscle with high efficiency following intravenous administration in mice, if the vector is co-administered with vascular endothelium growth vector (VEGF) to increase vascular permeability [26]. At higher particle numbers an AAV2/6 vector containing the CMV regulatory cassette to drive β -galactosidase expression effectively transduce striated muscle in absence of VEGF, and the proportion of transduced myofibers was much reduced compared to the level achieved by VEGF co-administration [26]. An AAV2/6 vector containing an abbreviated MCK regulatory cassette/enhancer (CK6 regulatory cassette) transduced skeletal muscle with lower efficiency, in comparison to an analogous vector containing a CMV regulatory cassette/enhancer; however, the MCK-driven β -galactosidase expression persisted longer than CMV-driven expression. In contrast to abbreviated expression with the CMV regulatory cassette, the CK6 regulatory cassette prolonged the expression of dystrophin in mdx mice for at least 6 weeks and enhanced the efficacy of muscle-targeted GAA expression [27]. These studies implied that muscle-specific transgene expression would

more stably achieve biochemical correction in Pompe disease. However, the current study has revealed that direct muscle transduction failed to circumvent the limitation posed by low mannose-6-phosphate receptor expression.

Muscle-targeted gene therapy has distinct advantages for gene therapy in Pompe disease, including stable transduction in absence of cell-mediated immune responses against GAA. Remaining issues that should drive future research include reducing the vector dose requirements, which remain high. Importantly, the limitations posed by reduced mannose-6-phosphate receptor availability in type II myofibers should be addressed by new therapeutic strategies.

MATERIALS AND METHODS

Preparation of AAV 2/8 vector

Briefly, 293 cells were transfected with the vector plasmid, the AAV packaging plasmid p5E18-VD 2/8 [28], and pAdHelper (Stratagene, La Jolla, CA). The liver-specific regulatory cassette in pAAV-LSPhGAApA (subcloned from pAV-LSP-cFIX, courtesy of Dr. Inder Verma, Salk Institute, La Jolla, CA; sequence available upon request) contains a thyroid hormone-binding globulin promoter sequence downstream from 2 copies of a $\alpha 1$ -microglobulin/bikunin enhancer sequence [Ill et al. 1997], and previously achieved long-term efficacy in hemophilia B mice within an AAV vector encoding coagulation factor IX [29]. The AAV-MHCK7hGAApA vector plasmid contains the muscle specific regulatory cassette from α MHCKChAP [30](provided by Dr. Stephen Hauschka, University of Washington, Seattle, WA) and expressed high level human GAA in the heart and skeletal muscle [13]. The pAAV-CBhGAApA vector plasmid contains the cytomegalovirus early immediated enhancer and chicken β -actin promoter to drive human GAA expression [16]. Cell lysate was harvested 48 hours following infection and freeze-thawed 3 times, and isolated by sucrose cushion pelleting followed by 2 cesium chloride gradient centrifugation steps. AAV stocks were dialyzed against 3 changes of Hanks buffer, and aliquots were stored at -80°C . The number of vector DNA containing-particles was determined by DNase I digestion, DNA extraction, and Southern blot analysis. All viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the NIH.

In vivo analysis of AAV vector

All mice were genotyped to confirm GAA-KO and transgene status. Tolerant GAA-KO mice were genotyped as described [14]. Muscle specific cation-independent mannose-6-phosphate receptor KO mice were generated using tissue specific promoter (creatine kinase; CK) and the cre/loxP conditional knock out system as described previously [Wylie et al. 2003]. The muscle-specific mannose-6-phosphate KO mice were crossed with GAA-KO mice to generate DKO mice. GAA-KO genotyping was done with a PCR-based assay to determine the deletion of exon 6 in GAA-KO mice. Genomic mouse DNA (100 ng) was used as template in a PCR reaction (35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute) using the primers: exon5 F(5'-CCTTTCTACCTGGCACTGGAGGAC-3'), exon7 R (GGACAATGGCGGTCGAGGAGTA-3') and neomycin F (5'-CCTCGTGCTTTACGGTATCGC-3'). This genotyping was carried out till GAA knock out mice colony was successfully established. Mouse CI-MPR Genotyping was done with a PCR-based assay to determine the presence of the loxP site in *M6P/IGF2R* intron 9. 100 ng of mouse genomic DNA was used as template in a PCR reaction (35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute) using the primers INT9F2 (5'-CCTTCCCTCCAGGCCGTTAC-3') and INT9R1 (5'-AGGTCTCCATCTGAGTACC-3'). Routine genotyping of this colony was needed until the muscle-CI-MPR-KO mouse colony

was successfully established. Mouse Cre genotyping was done with a PCR-based assay was used to determine presence of Cre recombinase expressed in specific tissue. Every DKO colony litter was genotyped using 100 ng of mouse genomic DNA as template in a PCR reaction (35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute) using the primers: Cre F (5'- ATGTCCAATTTACTGACCG-3'), Cre R(5'- CGCCGCATA ACCAGTGAAA-3'). Mouse testing positive for MCK-Cre were used for experimentation.

The glycogen storage in the heart and skeletal muscles of DKO mice was equivalent to that observed in GAA-KO mice (not shown). The vector stocks were administered intravenously (via the retroorbital sinus) in 3 month-old and 15 month-old GAA-KO mice, and to 3 month-old tolerant GAA-KO and DKO mice. At the indicated time points post-injection, plasma or tissue samples were obtained and processed as described below. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Rotarod testing was performed as described [31]. GAA activity and glycogen content were analyzed as described [32]. A *P* value of <0.05 indicated a significant difference between the observed values for each group of GAA-KO mice following AAV vector administration and the control group of PBS-injected GAA-KO mice.

Western blotting of hGAA was performed as described [31] using the hGAA monoclonal antibody (courtesy of Genzyme Corp., Framingham, MA). The ELISA was performed as described [31]. All samples yielded absorbance values that were within the linear range of the assay at this dilution.

Statistical analyses

Comparison of two groups was assessed by a homoscedastic Student T-test. The significance of differences between multiple groups was tested using a two-sided Wilcoxon rank sum test for continuous variables, and was conducted with Stata 10 (StataCorp LLC, College Station, TX). A *p*-value <0.05 was considered to be statistically significant.

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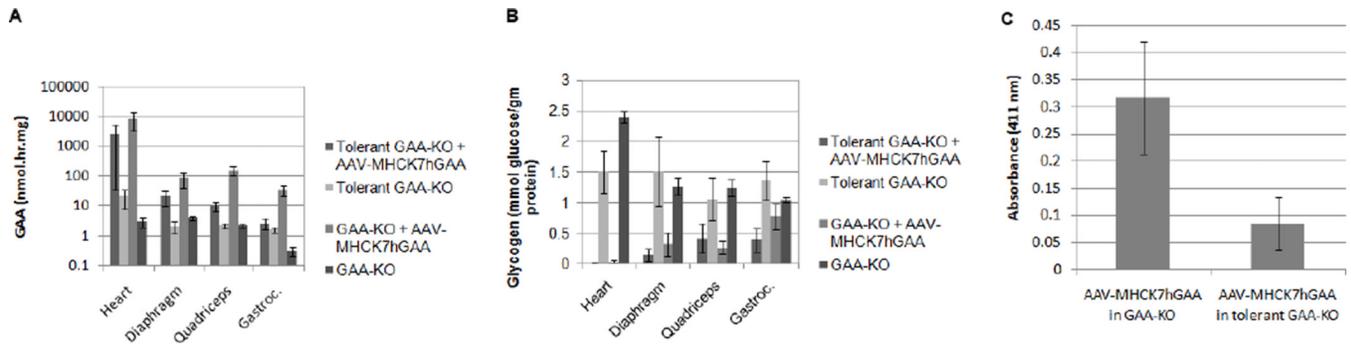


Fig. 1. Biochemical correction of striated muscle following administration of AAV2/8-MHCK7hGAA to GAA-KO or tolerant GAA-KO mice

GAA activity and glycogen content in the indicated striated muscles, 18 weeks following AAV2/8 vector administration. AAV2/8-MHCK7hGAA (7×10^{11} vp) was administered intravenously at 3 months of age to GAA-KO mice ($n=7$) and to tolerant GAA-KO mice ($n=5$). Sham-treated GAA-KO mice ($n=4$) and tolerant GAA-KO mice ($n=4$) were negative controls. Mean \pm standard error are shown. (A) GAA activity in the heart, diaphragm, quadriceps, and gastrocnemius. (B) Glycogen content for striated muscles of GAA-KO mice in A. (C) ELISA for anti-GAA IgG1 at 18 weeks following vector administration.

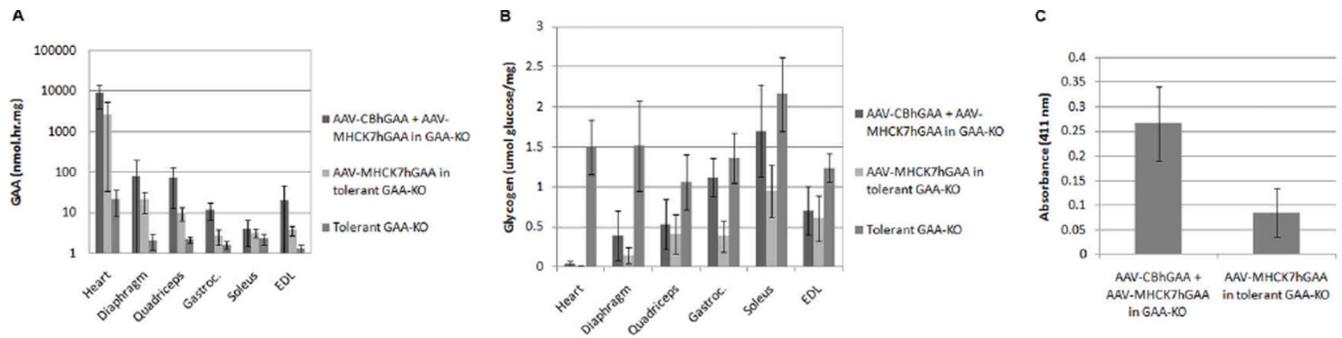


Fig. 2. Biochemical correction of striated muscle following administration of AAV-CBhGAA and AAV2/8-MCHK7hGAA to GAA-KO mice or AAV2/8-MCHK7hGAA to tolerant GAA-KO mice GAA activity and glycogen content in the indicated striated muscles, 18 weeks following AAV2/8 vector administration. AAV2/8-CBhGAA (1×10^{11} vp) and AAV2/8-MHCK7hGAA (7×10^{11} vp) were administered intravenously at 3 months of age to GAA-KO mice ($n=4$) and AAV2/8-MHCK7hGAA (7×10^{11} vp) was administered to tolerant GAA-KO mice ($n=5$). Sham-treated tolerant GAA-KO mice were negative controls ($n=4$). Mean \pm standard error are shown. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus, and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in A. (C) ELISA for anti-GAA IgG1 at 18 weeks following vector administration.

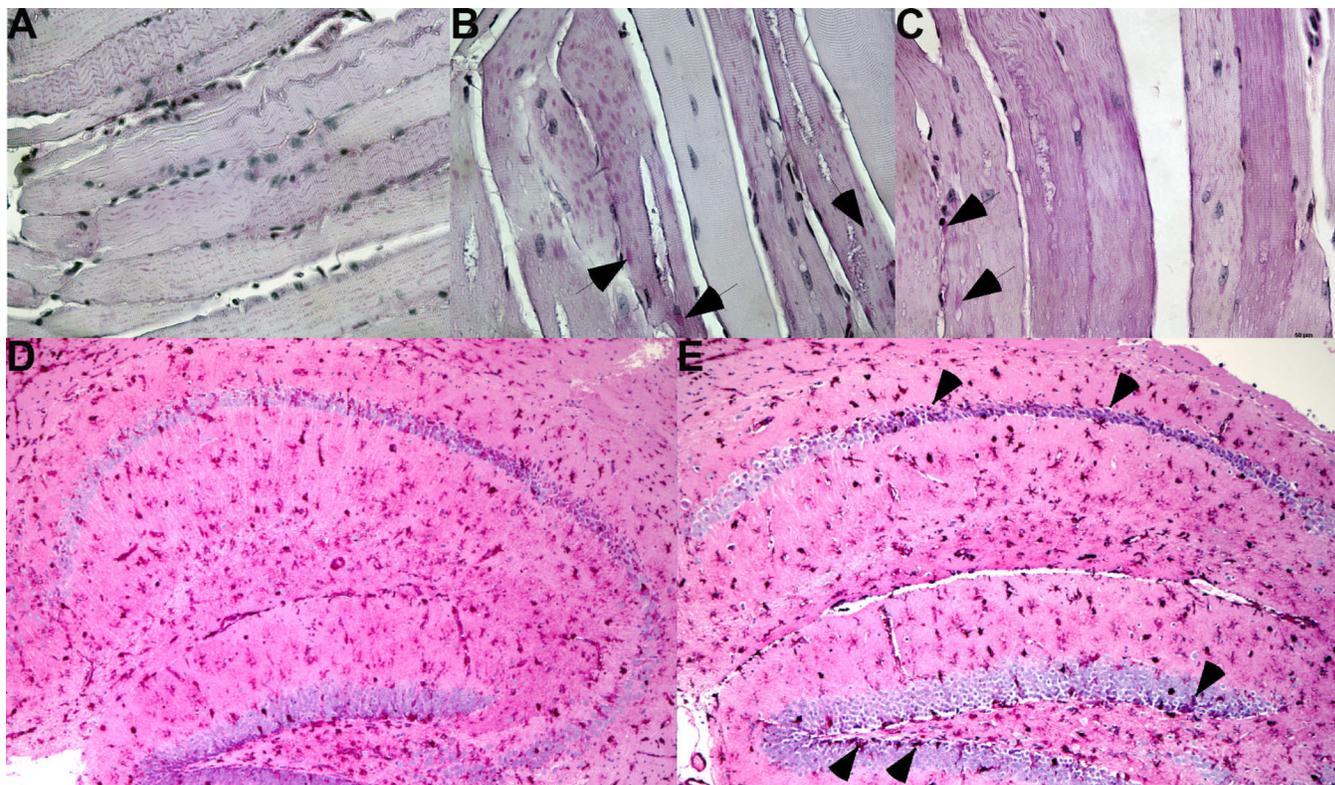


Fig. 3. Glycogen staining of gastrocnemius muscle

Periodic acid-Schiff staining of tissues 18 weeks following intravenous administration of vectors to GAA-KO mice. (A) Gastrocnemius following administration of dual vectors, both AAV2/8-CBhGAA (1×10^{11} vp) and AAV2/8-MHCK7hGAA (7×10^{11} vp). (B) Gastrocnemius following administration of AAV2/8-MHCK7hGAA (7×10^{11} vp). (C) Gastrocnemius for sham-treated, age-matched GAA-KO mouse sample shown for comparison. (D) Hippocampus following administration of dual vectors, both AAV2/8-CBhGAA (1×10^{11} vp) and AAV2/8-MHCK7hGAA (7×10^{11} vp). (E) Hippocampus for sham-treated, age-matched GAA-KO mouse sample shown for comparison. Glycogen-filled vacuoles representing accumulated lysosomal glycogen indicated (arrows). Focal areas of glycogen staining indicated (arrowheads). Original magnification for gastrocnemius: 400. Original magnification for hippocampus: 100.

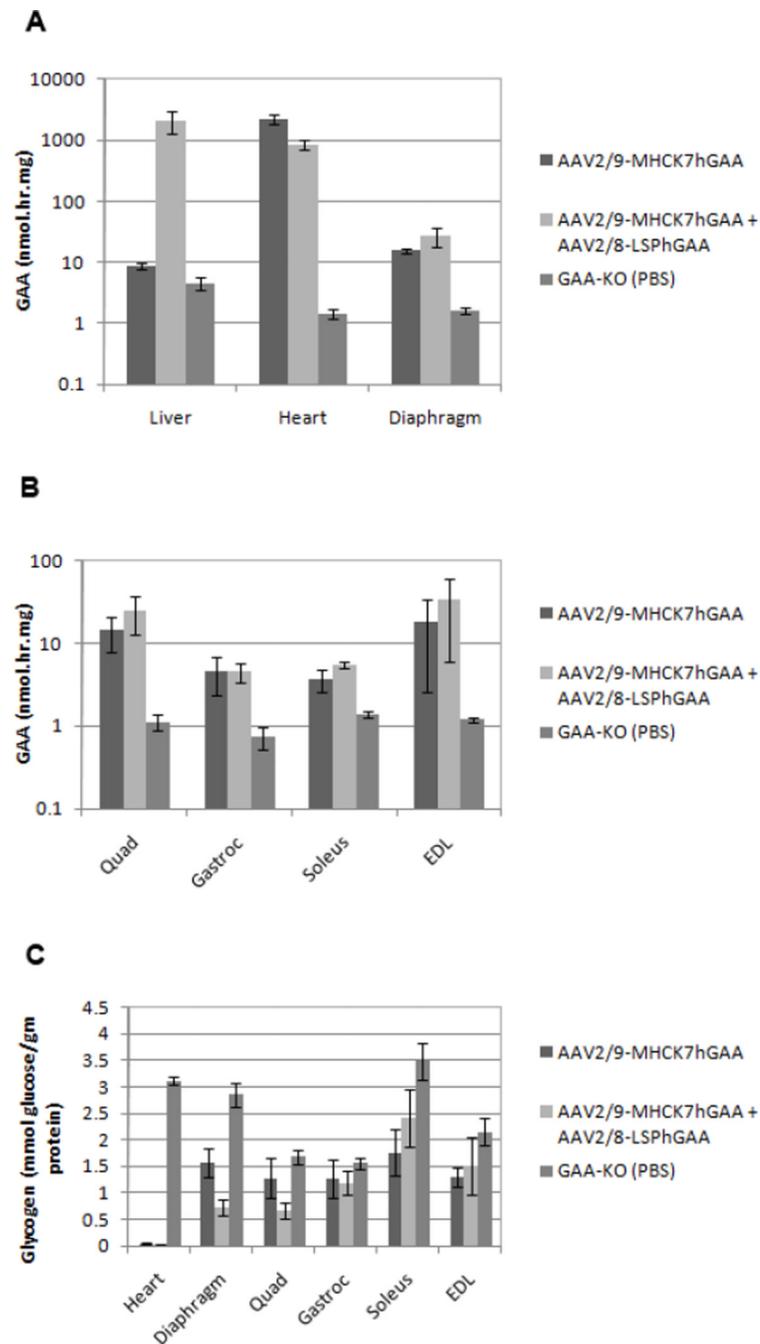


Fig. 4. Biochemical correction of striated muscle following administration of AAV2/9-MHCK7hGAA +/- AAV2/8-LSPhGAA

GAA activity and glycogen content in the indicated striated muscle following AAV vector administration. Either AAV2/9-MHCK7hGAA (1×10^{11} vp; $n=4$), or both AAV2/8-LSPhGAA (1×10^{11} vp) and AAV2/9-MHCK7hGAA (1×10^{11} vp; $n=6$) were administered to GAA-KO mice at 3 months of age, and tissues were analyzed 18 weeks later. One mouse treated with both vectors died of unknown causes before the end of the experiment. Mock-treated GAA-KO mice were negative controls ($n=4$). Mean \pm standard error are shown. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus, and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in A.

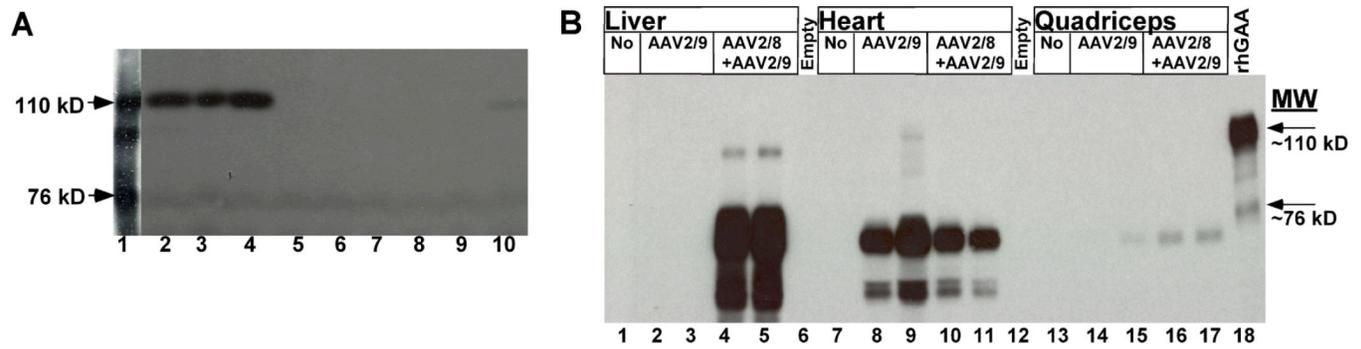


Fig. 5. Western blot detection of GAA

Each lane represents an individual mouse. (A) Plasma samples from GAA-KO mice (lanes 2–4) following administration of dual vectors, AAV2/8-LSPhGAA (1×10^{11} vp) and AAV2/9-MHCK7hGAA (1×10^{11} vp); tolerant GAA-KO mice (lanes 5–7) following administration of AAV2/9-MHCK7hGAA (1×10^{11} vp); and sham treated GAA-KO mice (lanes 8–10). Comparison with recombinant human GAA standard (lane 1) revealed the 110 kD precursor GAA was present in dual vector treated GAA-KO mice (lanes 2–4), whereas no GAA was detected in tolerant mice treated with AAV2/9-MHCK7hGAA (lanes 5–7) or in GAA-KO mice treated with AAV2/9-MHCK7hGAA (not shown). (B) Detection of GAA in the indicated tissues, following administration of AAV2/9-MHCK7hGAA (AAV2/9), dual vectors AAV2/8 + AAV2/9, or no vector (No). The 110 kD precursor GAA was present in the liver of dual vector treated GAA-KO mice, whereas processed GAA of ~70 kD was present in the heart and skeletal muscle of both vector-treated groups.

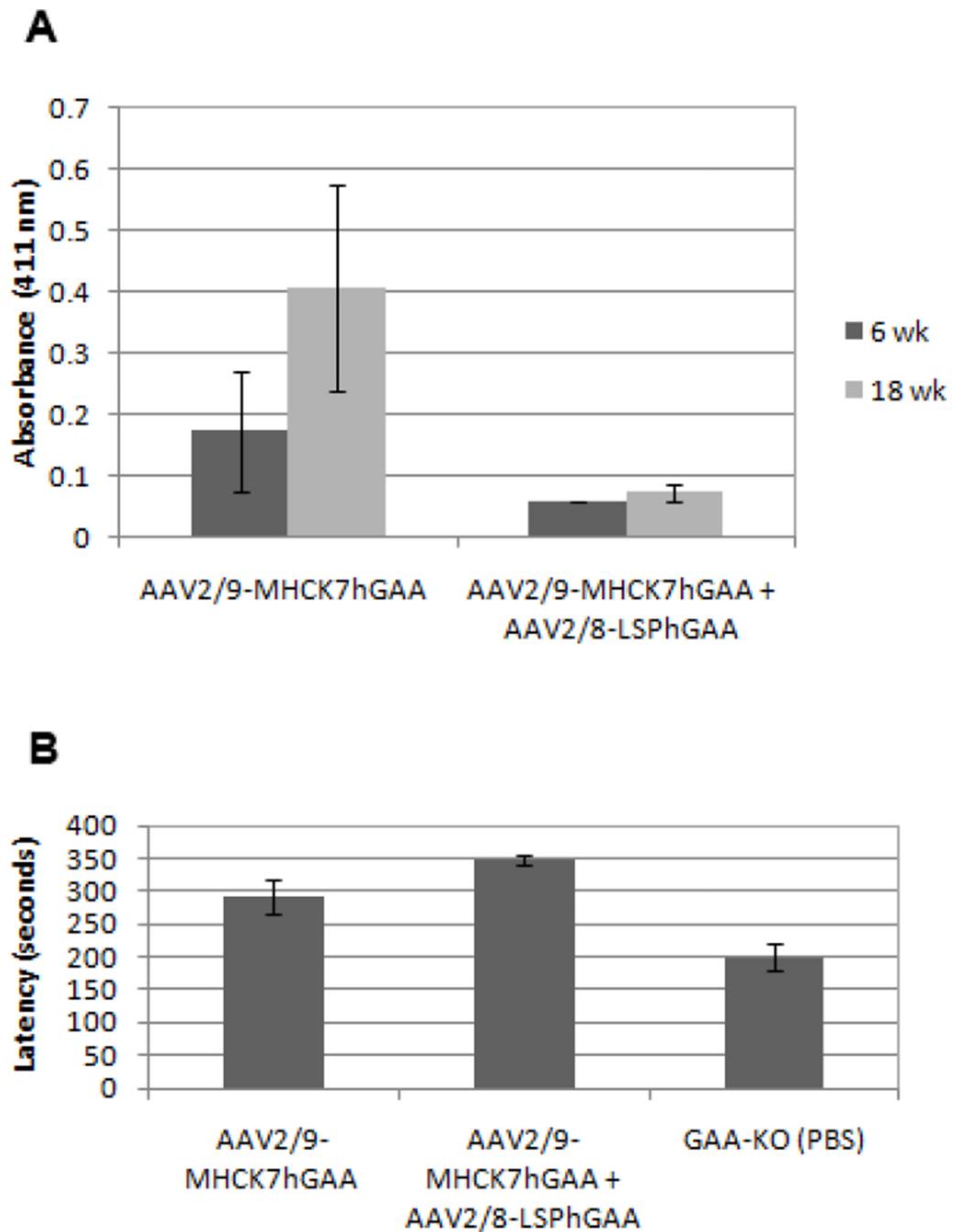


Fig. 6. Humoral response and muscle function testing following AAV vector administration
 Mean and standard error are shown. (A) ELISA for anti-GAA IgG1 at 6 and 18 weeks following vector administration. (B) Rotarod latency at 6 months of age. GAA-KO mice were treated with AAV2/8-LSPhGAA and AAV2/9-MHCK7hGAA (n=5), AAV2/9-MHCK7hGAA (n=4), or no vector (GAA-KO; n=4).

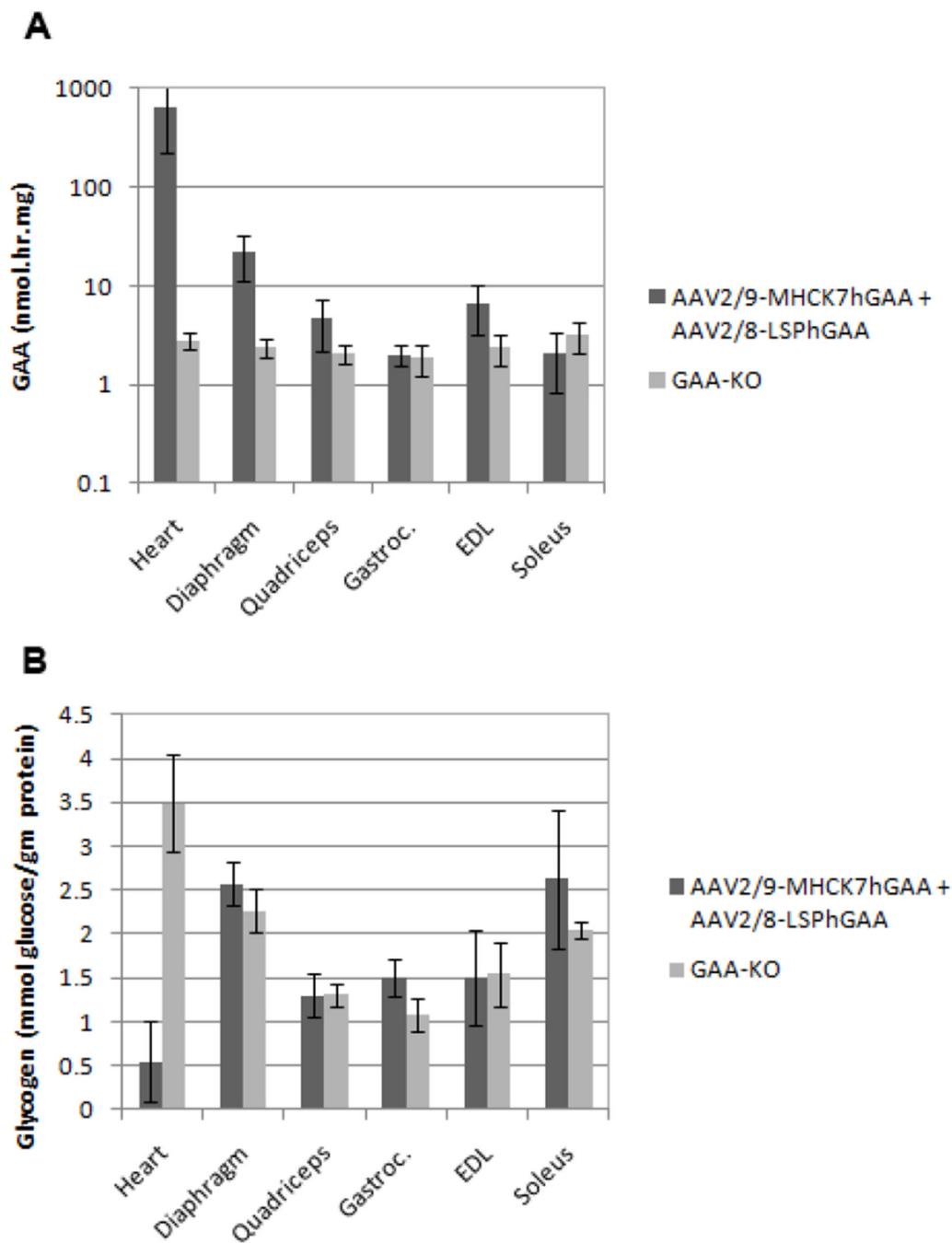


Fig. 7. Biochemical correction of striated muscle following administration of AAV2/9-MHCK7hGAA +/- AAV2/8-LSPhGAA to old Pompe disease mice

GAA activity and glycogen content in the indicated striated muscles following AAV vector administration. AAV2/8-LSPhGAA (1×10^{11} vp) and AAV2/9-MHCK7hGAA (1×10^{11} vp) were injected intravenously at 15 months of age and tissues were analyzed 12 weeks later ($n=7$). Three mice died of unknown causes, most likely age-related, before the end of the experiment. Sham-treated, 11 month old mice were analyzed as controls for old GAA-KO mice ($n=3$). Mean \pm standard deviation are shown. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus, and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in A.

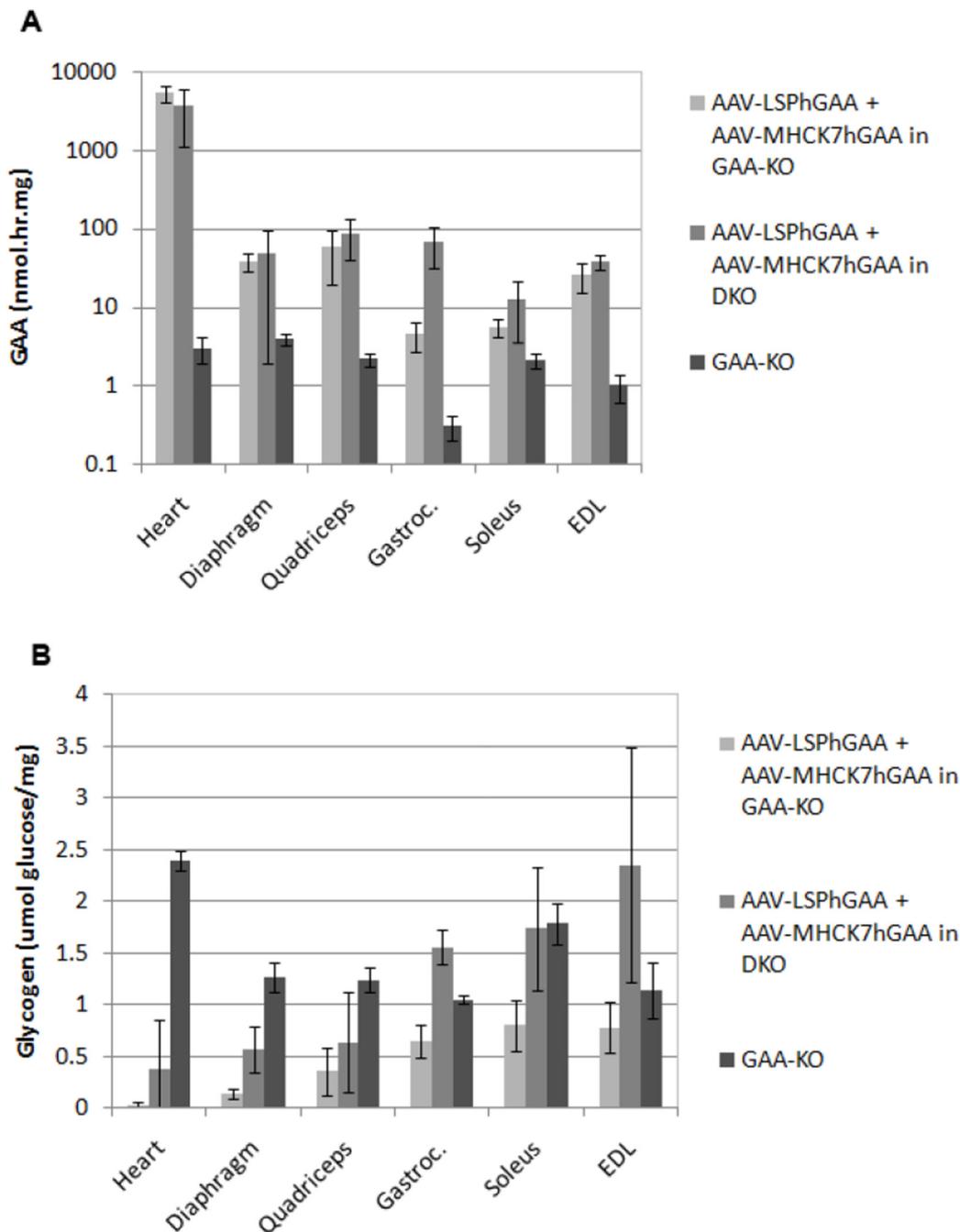


Fig. 8. Impaired biochemical correction of striated muscle in absence of mannose-6-phosphate receptor expression following administration of AAV2/8-MHCK7hGAA and AAV2/8-LSPhGAA
 GAA activity and glycogen content in the indicated striated muscle following AAV vector administration. AAV2/8-MHCK7hGAA (7×10^{11} vp) and AAV2/8-LSPhGAA (2×10^{10} vp) were injected intravenously at 3 months to DKO mice ($n=5$) and to GAA-KO mice ($n=4$), and tissues were analyzed 18 weeks later. Mock-treated GAA-KO mice were negative controls (GAA-KO; $n=4$). Mean \pm standard error are shown. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus, and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in A.

Table 1

Comparison of dual vector administration in DKO and GAA-KO mice

Muscle	GAA decrease in DKO, versus GAA-KO (%)	GAA (p, DKO versus GAA-KO)	Glycogen increase in DKO, versus GAA-KO (%)	Glycogen (p, DKO versus GAA-KO)
Heart	32	0.3	1300	0.20
Diaphragm	-26	0.7	410	0.008
Quadriceps	-49	0.4	180	0.3
Gastrocnemius	-1400	0.01	240	0.00006
Soleus	-120	0.2	220	0.02
EDL	-50	0.08	303	0.03