of rhGAA is e ective at addressing several of the disease manifestations. However, correction of the pathology in the skeletal muscles appears to present a challenge, particularly in patients with more advanced disease that could not be resolved with higher doses or more frequent infusions of the enzyme. In part, this refractoriness may be due to the relatively low e ciency by which the enzyme can be translocated from the circulatory system across the endothelial cells and interstitial tissues to the a ected muscles. e cation-independent mannose 6-phosphate receptor (CI-MPR) on skeletal muscle that is primarily involved in the cellular uptake of rhGAA is also reportedly present in low abundance in this tissue.[16,17] To address this potential limitation of enzyme replacement therapy, we sought to improve the delivery of rhGAA to the a ected muscles.

Uptake of exogenous rhGAA by the skeletal muscles is mediated primarily by the CI-MPR.[18] However, analysis of rhGAA prepared from di erent sources including those from Chinese hamster ovary cells or the milk of transgenic rabbits indicated that they harbored only modest levels of the cognate ligand, mannose 6-phosphate (M6P).[19] Introduction of additional M6P moieties onto rhGAA either by enzymatic engineering (HP-GAA) or chemical conjugation of synthetic oligosaccharides bearing M6P residues (neo-rhGAA) has been shown to improve their binding to the CI-MPR and subsequent uptake by cells in culture.[19–21] Moreover, in the case of neo-rhGAA, administration of the modi ed enzyme to Pompe mice resulted in greater clearance of the aberrant accumulation of lysosomal glycogen when compared with the unmodi ed enzyme.[21] We have extended these ndings and report here our e orts to improve the chemical stability of neo-rhGAA and importantly, to ascertain the e ects of this modi cation on its biological activity. We show that using oxime chemistry to conjugate synthetic oligosaccharides bearing M6P residues onto rhGAA generated a modi ed enzyme (oxime-neo-rhGAA) with higher stability *in vivo* and an improved ability to reduce the burden of glycogen storage in the skeletal and cardiac muscles of Pompe mice. Importantly, we show that associated with these biochemical improvements were signi cant enhancements in motor function in the treated Pompe mice.

RESULTS

Generation of conjugates of rhGAA and synthetic M6P-bearing oligosaccharides with enhanced stability and affinity for the CI-MPR

Previously, we had reported the feasibility of conjugating synthetic oligosaccharides bearing M6P residues (**Figure 1a**) onto rhGAA using carbonyl-coupled hydrazone chemistry.[20,21] Administration of the carbohydrate-modi ed enzyme (neo-rhGAA) into Pompe mice resulted in higher uptake by the a ected muscles with subsequent greater clearance of the accumulated lysosomal glycogen compared to the unmodi ed, parent enzyme. Here, we evaluated an alternate carbonyl-coupled chemistry to generate an oxime bond using an aminooxy-derived glycan.

To assess the relative merits of using an oxime bond in the conjugate, the synthetic M6P-bearing oligosaccharide (**Figure 1a**) containing a hydrazide reactive group was rst converted to an aminooxy reactive group through a single step reaction with *N*-(*t*-BOC)-aminooxyacetic acid tetra uorophenyl ester, followed

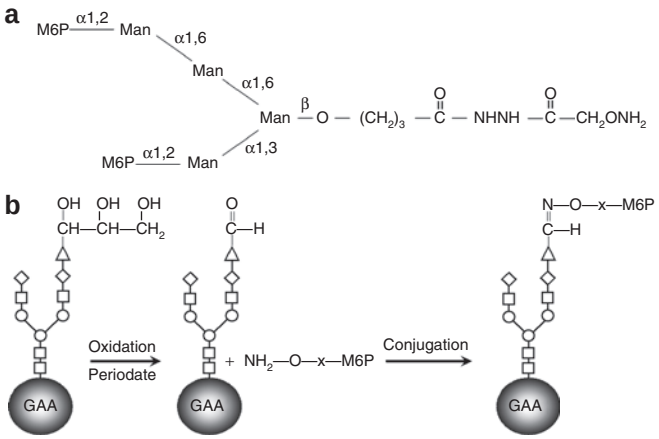

Figure 1 Structure of the synthetic oligosaccharide ligand and scheme for its conjugation to rhGAA. (a) Design of the synthetic oligosaccharide used in the conjugations studies. (b) Scheme used to conjugate the synthetic glycan to rhGAA. The sialic acids on the enzyme were oxidized with periodate before reacting with the reactive group (aminooxy) on the synthetic glycan to generate oxime-neo-rhGAA. Symbols in diagram: open circle, mannose; open square, *N*-acetylglucosamine; open diamond, galactose; open triangle, sialic acid. GAA, acid α-glucosidase; M6P, mannose 6-phosphate; rhGAA, recombinant human acid α-glucosidase.

by deprotection in tri uoroacetic acid. e puri ed M6P-aminooxy ligand was then coupled to periodate-oxidized rhGAA as outlined in **Figure 1b**. e conjugation reaction was very e cient and proceeded to completion when 40–50 µmol/l (~5 mg/ml) oxidized rhGAA was reacted with 0.75 mmol/l (1 mg/ml) M6P-containing glycan. Consistent with the addition of M6P-bearing ligands, the resultant product, oxime-neo-rhGAA, demonstrated an increased molecular mass and a decreased isoelectric point due to the incorporation of additional phosphate groups (data not shown). Subsequent analysis by Dionex chromatography (Dionex, Sunnyvale, CA) indicated a signi cant increase in M6P content in the conjugated enzyme. Based on the number of M6P on oxime-neo-rhGAA and the number of sialic acids on rhGAA, it would suggest that synthetic glycans were appended onto all the sialic acids on rhGAA. e observed increase in molecular weight of oxime-neo-rhGAA (~8 kd when compared with the unmodi ed enzyme) measured using mass spectrometry or sodium dodecyl sulfate-gel electrophoresis (data not shown) was consistent with this assumption. Measurement of the enzymatic activity of oxime-neo-rhGAA showed an increased K_m (~50%) presumably due to steric hindrance by the added oligosaccharide side chains. However, a er the modi ed enzyme was delivered to the lysosomal compartment where the glycans were processed, its enzymatic activity was similar to that of unmodi ed rhGAA (data not shown). e V_{max} of the modi ed enzyme was not a ected. Although our preliminary studies showed that the antibody titers to administration of oxime-neo-rhGAA and unmodi ed enzyme were not di erent (data not shown), further analysis will be required to fully explore the repertoire of antibodies generated against the two enzymes.

Increasing the content of M6P residues on oxime-neo-rhGAA improved its a nity for the CI-MPR when compared to the unmodi ed enzyme. Greater than 95% of oxime-neo-rhGAA bound to a CI-MPR column as compared to 15–30% of

the unmodi ed rhGAA (**Figure 2a**). e addition of M6P onto rhGAA also signi cantly improved enzyme uptake by L6 myoblasts *in vitro* (**Figure 2b**), similar to those reported previously for enzyme conjugates formed using hydrazone chemistry. Stability studies indicated that those formed using oxime chemistry had the added advantage of being signi cantly more stable (**Figure 2c**). No measurable dissociation of the synthetic glycan from oxime-neo-rhGAA was detected a er incubation of the glycoconjugate at 25 °C for 14 days or 4 °C for 90 days. Together, these data indicate that oxime-neo-rhGAA has the desired characteristics that would support its adoption for preclinical development.

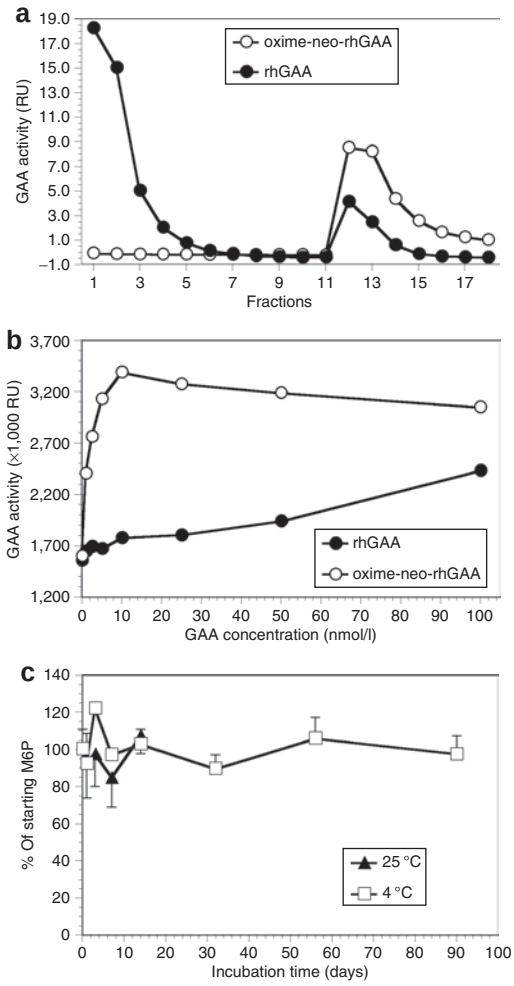

Figure 2 Biochemical characteristics of the unmodified and glycoengineered enzymes. (a) Chromatography of oxime-neo-rhGAA (open circle) and rhGAA (closed circle) over a CI-MPR column. Approximately 5 µg of the different enzymes were loaded onto a 2 ml column. After washing the column with binding buffer, the bound material was eluted (starting at fraction 11) with binding buffer containing 5 mmol/l M6P. Fractions (2 ml) were collected and assayed for GAA activity. (b) Uptake of the enzymes by L6 myoblasts in culture. Increasing amounts of either oxime-neo-rhGAA (open circle) or rhGAA (closed circle) were added to L6 myoblasts and incubated at 37 °C for 18 hours. After washing, the cells were lysed and the enzyme activity in the lysates assayed using the fluorogenic substrate, 4-methylumbelliferyl-α-D-glucopyranoside. Enzyme activity was expressed in relative units (RU). (c) Stability of oxime-neo-rhGAA at 4 °C (open square) and 25 °C (closed triangle). The modified enzyme was incubated at the respective temperatures for the times indicated after which they were subjected to analysis to determine the level of M6P. CI-MPR, cation-independent mannose 6-phosphate receptor; GAA, acid α-glucosidase; M6P, mannose 6-phosphate; rhGAA, recombinant human acid α-glucosidase.

Oxime-neo-rhGAA demonstrates greater efficacy than unmodified rhGAA at reducing glycogen storage in muscles of Pompe mice

To ascertain whether the improved characteristics of oxime-neo-rhGAA observed *in vitro* translated to greater e cacy *in vivo*, 5-month-old Pompe mice were administered four weekly doses of either the modi ed or unmodi ed enzyme. e muscles of Pompe mice at 5 months of age exhibit signi cant lysosomal accumulation of glycogen. Analysis of the tissues 2 weeks a er the last enzyme administration showed that both versions of rhGAA reduced the levels of glycogen in the heart, diaphragm, quadriceps, and triceps in a dose-dependent manner (**Figure 3a**). However, treatment with oxime-neo-rhGAA was more e ective as illustrated by the observation that a comparable reduction in glycogen could be

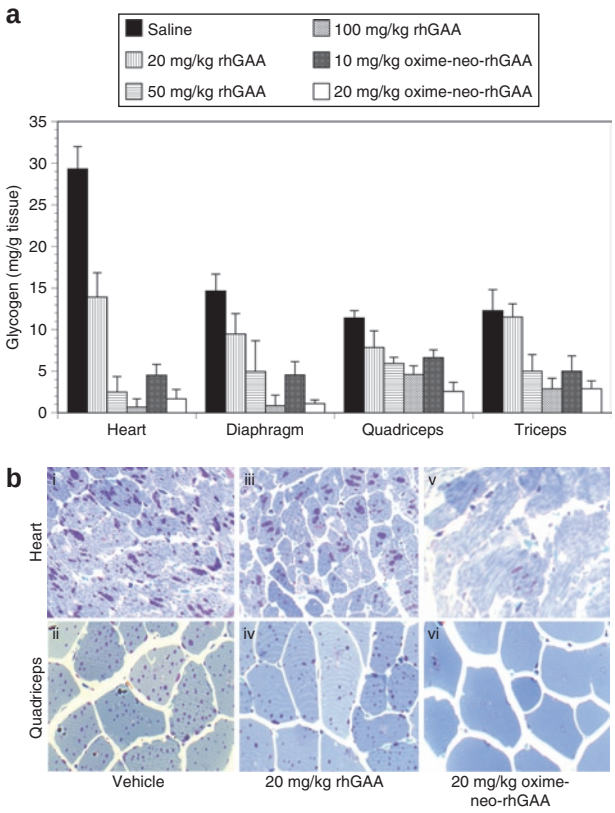

Figure 3 Relative abilities of unmodified and modified rhGAA to reduce tissue glycogen levels in young Pompe mice. (a) Cohorts of 5-month-old Pompe mice were administered increasing amounts of either rhGAA or oxime-neo-rhGAA. The mice (8 animals/group) were treated with four weekly doses of enzyme and killed 2 weeks after the last treatment. Tissues were collected and assayed for glycogen levels using the Amplex Red glucose assay. Data are expressed as means ± SD. (b) Sections of the heart and quadriceps were stained with PAS and then analyzed by high resolution light microscopy. Representative sections from the heart of Pompe mice treated with vehicle (i), 20 mg/kg rhGAA (iii) and 20 mg/kg oxime-neo-rhGAA (v) are shown, as are quadriceps of animals treated with vehicle (ii), 20 mg/kg rhGAA (iv) and 20 mg/kg oxime-neo-rhGAA (vi). Glycogen is visualized as purple-beaded structures within the myocytes. PAS, periodic acid-Schiff; rhGAA, recombinant human acid α-glucosidase.

attained using a vefold lower dose of the modi ed enzyme. For example, the extent of glycogen clearance achieved using 10 and 20 mg/kg of oxime-neo-rhGAA was comparable to that attained using 50 and 100 mg/kg of rhGAA, respectively (**Figure 3a**). e greater e cacy of oxime-neo-rhGAA at reducing tissue glycogen content could also be visualized by examining tissue sections stained for glycogen with periodic acid-Schi reagent (**Figure 3b**, panels iii and iv). Analysis of the heart and quadriceps of animals administered 20 mg/kg rhGAA showed a signi cant decrease in periodic acid-Schi –stained structures compared to these same tissues in untreated mice. However, mice treated with a similar dose of oxime-neo-rhGAA showed an even more complete clearance of glycogen-containing structures from both tissues (**Figure 3b**, panels v and vi).

Motor function improvement was significantly greater in oxime-neo-rhGAA-treated immunotolerant Pompe mice

To determine if the greater ability of oxime-neo-rhGAA to clear lysosomal glycogen deposits from the muscle translated to improved motor function, Pompe mice were also subjected to a variety of functional tests. Because improvements in muscle function could only be visualized over a period of months and because weekly administrations of rhGAA engendered a robust immune response to the human enzyme, Pompe mice were rst immunotolerized to rhGAA. is was accomplished by treating the animals with a recombinant adeno-associated virus (AAV) vector encoding an inactive mutant of rhGAA (D404N) under the transcriptional control of a liver-restricted promoter.[22] is approach of using AAV vectors to confer immunotolerance has been reported for a number of di erent transgene products including GAA.[23–25] Intravenous administration of a recombinant AAV vector encoding the mutant enzyme (AAV8/DC190-GAA$_{D404N}$) into Pompe mice resulted in high-level hepatic expression and secretion of mutant GAA$_{D404N}$ into the systemic circulation. However, as the speci c activity of the mutant GAA was <1% of wild type GAA, there was no measurable decrease in glycogen levels in the Pompe-a ected tissues (**Figure 4a**) compared to naive Pompe mice (data not shown). One month a er dosing mice with AAV8/DC190-GAA$_{D404N}$, subsequent periodic intravenous administrations of rhGAA did not elicit antibodies against the recombinant enzyme (data not shown). In contrast, administration of rhGAA into Pompe mice that had not received AAV8/DC190-GAA$_{D404N}$ developed high titers of antibody to the hydrolase.

To determine the relative ability of the modi ed and unmodied rhGAA to correct the motor function de cits of Pompe mice, the animals were rst administered AAV8/DC190-GAA$_{D404N}$ when they were 4.5 months old. One month later, a er the animals were immunotolerized to the human enzyme, cohorts of 10 mice each were treated biweekly with 20 mg/kg rhGAA, 100 mg/kg rhGAA, 20 mg/kg oxime-neo-rhGAA, or vehicle for 8 months. To evaluate the impact of the di erent treatments on muscle strength and motor coordination, the animals were tasked with performing on a rotarod programmed to rock backwards and forwards (referred to as a rocking rotarod). In this assay, the rotarod was programmed to accelerate to 17.5 rpm within 2.5 seconds a er which the direction of rotation was reversed and this repeated over a period of

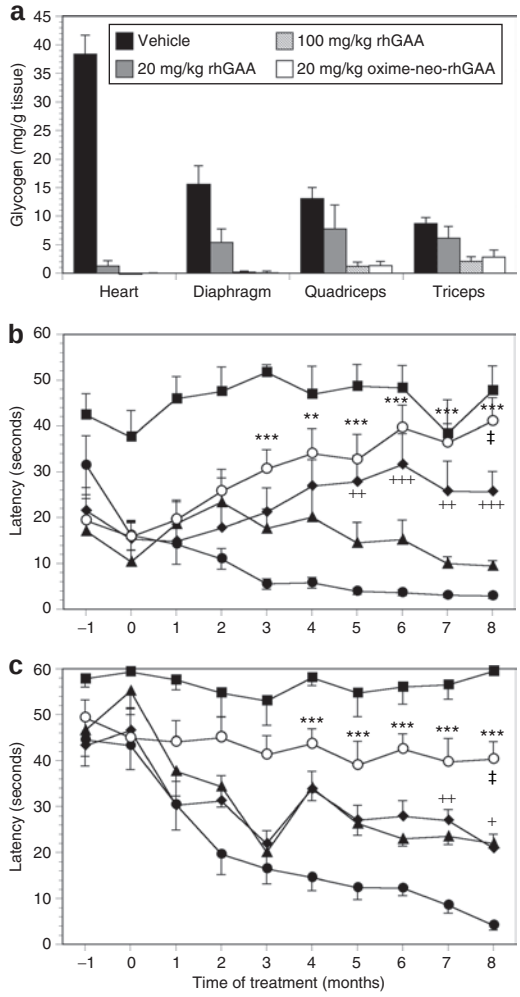

Figure 4 Assessment of motor coordination and muscle strength after enzyme therapy of young Pompe mice. Pompe mice (4.5 months old) were first administered 5E11 drp of AAV8/DC190-GAA$_{D404N}$ to induce immunotolerance to human GAA. One month after dosing with the viral vector, the mice were treated biweekly with injections of different doses of rhGAA or oxime-neo-rhGAA for 8 months. (a) At the end of the study, the mice were killed and their tissues analyzed for glycogen levels. Data are expressed as means ± SD (n = 10 animals per group). Throughout the study, the animals (both wild type and Pompe mice) were subjected to (b) rocking rotarod and (c) wire-hang tests. Age-matched wild type mice (closed square) and Pompe mice treated with vehicle (closed circle), 20 mg/kg rhGAA (closed triangle), 100 mg/kg rhGAA (closed diamond), and 20 mg/kg oxime-neo-rhGAA (open circle) were tested monthly. Statistical analyses were performed between vehicle and enzyme-treated groups (vehicle versus 20 mg/kg oxime-neo-rhGAA, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; vehicle versus 100 mg/kg rhGAA, $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$), as well as between oxime-neo-rhGAA- and rhGAA-treated groups ($^{‡}P < 0.01$)) for each time point. Data are presented as means ± SEM. drp, DNase resistant particle; rhGAA, recombinant human acid α-glucosidase.

60 seconds. In contrast to wild type mice, vehicle-treated Pompe mice displayed a de cit in this assay at the outset (4.5 months old) that became gradually worse as the animals aged (**Figure 4b**). is was indicated by the progressively shorter time the vehicle-treated Pompe mice were able to remain on the rotarod. Pompe mice treated biweekly with 20 mg/kg rhGAA showed a trend toward improvement but this did not reach statistical signi cance

when compared with the vehicle-treated cohort (**Figure 4b**). Mice treated at the higher dose of 100 mg/kg rhGAA showed a marked improvement in motor function that reached statistical signi - cance when compared to the vehicle-treated group a er 5 months of enzyme therapy. e cohort administered 20 mg/kg oxime-neo-rhGAA fared the best, exhibiting statistically signi cant improvements over control animals a er just 2 months on enzyme therapy and reaching levels of performance that were similar to wild type mice a er 8 months of therapy (**Figure 4b**). At 8 months post-treatment, the performance of the cohort administered 20 mg/kg oxime-neo-rhGAA was also signi cantly greater than that treated with 100 mg/kg of unmodi ed enzyme. ese improvements in motor function were consistent with the extent of glycogen clearance attained using the di erent enzyme entities (**Figure 4a**), and indicated that the modi ed oxime-neo-rhGAA was at least ve-fold more e cacious than the unmodi ed version at addressing the motor function de cits in the Pompe mice.

e enhanced therapeutic e ect observed with oxime-neo-rhGAA was further corroborated by the wire-hang assay that measured muscle strength. In this assay, the animals were placed on a wire mesh that was then inverted, and their latency to fall recorded. As with the rotarod assay, measurements were made monthly starting when the animals were 4.5 months old. At this age, all the Pompe mice showed a de cit in muscle strength when compared to wild type mice, as indicated by their decreased latency (**Figure 4c**). As expected, vehicle-treated mice demonstrated a continual decline in muscle strength as the disease progressed. Pompe mice dosed with either 20 or 100 mg/kg of the unmodified enzyme (starting at 5.5 months of age) initially demonstrated a similar decline in performance but appeared to stabilize a er 3 months of treatment. However, these improvements did not reach statistical signi cance until a er 7 months of treatment (**Figure 4c**). In contrast, the cohort of Pompe mice administered 20 mg/kg oxime-neo-rhGAA showed no diminution in muscle strength throughout the duration of the study. Although they did not improve to the level observed in wild type mice, their performance in this assay was signi cantly greater than that observed with both the untreated and rhGAA-treated groups (**Figure 4c**).

is result suggests that animals treated with the modi ed enzyme responded more robustly than those treated with the unmodi ed counterpart, even when administered a vefold lower dose.

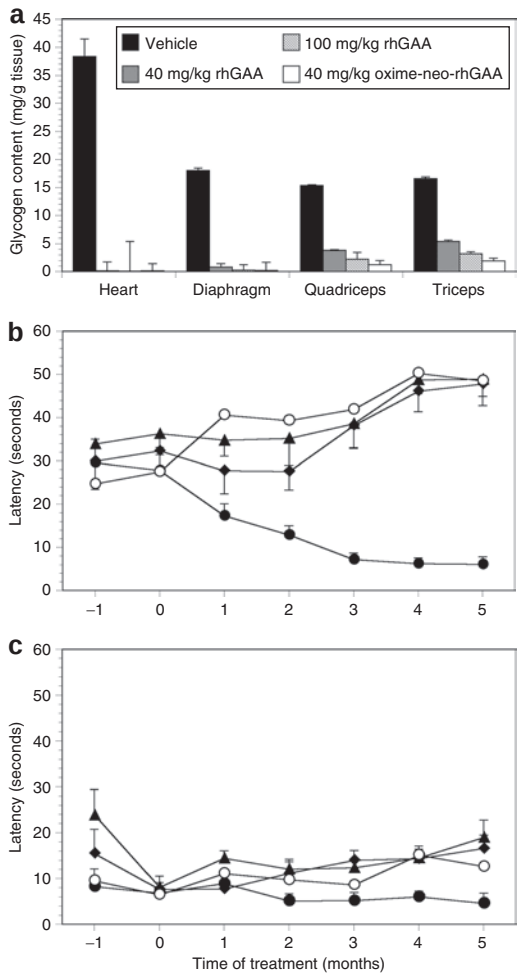

Figure 5 Assessment of motor coordination and muscle strength after enzyme therapy of older, symptomatic Pompe mice. Pompe mice (10 months old) were first immunotolerized to human GAA by administering 7E11 drp AAV8/DC190-GAA$_{D404N}$ and after 1 month they were subjected to weekly injections with varying doses of either rhGAA or oxime-neo-rhGAA for 5 months. (a) At the end of the study, the mice were killed and their tissues analyzed for glycogen levels. Data are expressed as means ± SD (n = 10 animals per group). Throughout the study, the animals were subjected to (b) rocking rotarod and (c) wire-hang tests. Pompe mice treated with vehicle (closed circle), 40 mg/kg rhGAA (closed triangle), 100 mg/kg rhGAA (closed diamond), and 40 mg/kg oxime-neo-rhGAA (open circle) were tested monthly. drp, DNase resistant particle; GAA, acid α-glucosidase; rhGAA, recombinant human acid α-glucosidase.

Reducing lysosomal glycogen levels in older Pompe mice did not translate to significant improvements in motor function

To determine if the virtues shown associated with oxime-neo-rhGAA could be recapitulated in older, more symptomatic Pompe mice, a similar study was performed using mice starting at 10 months of age (instead of 5.5 months). Ten-month-old Pompe mice have higher levels of accumulation of lysosomal glycogen and demonstrate impaired motor function. As with the previous study design, 10-month-old Pompe mice were rst treated with AAV8/DC190-GAA$_{D404N}$ to induce immunotolerance to the human enzyme; 1 month post-treatment with the viral vector, the animals were injected weekly with 40 mg/kg rhGAA, 100 mg/kg rhGAA, 40 mg/kg oxime-neo-rhGAA, or vehicle for 5 months. A higher (40 mg/kg) and more frequent (weekly) dosing regimen was used because older Pompe mice are reportedly more refractory to treatment.[25,26] Analysis of tissue glycogen levels at the end of the study showed near-complete correction of the storage pathology in the heart and diaphragm of animals treated with either form of rhGAA (**Figure 5a**). In the quadriceps and triceps, treatment with 40 mg/kg oxime-neo-rhGAA was as e ective as treatment with 100 mg/kg of the unmodi ed enzyme at reducing glycogen levels (**Figure 5a**), consistent with the observations noted earlier.

e extent of reduction in storage material in the skeletal muscles of treated mice was signi cant but incomplete (~70–90% of stored glycogen was cleared with treatment).

Interestingly, despite a signi cant reduction in glycogen levels in the skeletal muscles, measurements of motor function using

the rotarod and wire-hang assays showed only marginal improvements over the vehicle-treated controls (**Figure 5b,c**). Treatment with either the modi ed or unmodi ed rhGAA resulted in some improvement over the control mice when assayed using the rocking rotarod that had been adjusted to spin at a lower velocity (rotarod was programmed to spin at a speed of 5 rpm/2.5 seconds instead of 17.5 rpm/second). However, we could not detect any di erences in performance between the three enzyme-treated groups (**Figure 5b**). In the wire-hang test, the performance of the enzyme-treated mice was not di erent from that of the vehicle-treated cohort (**Figure 5c**). ese results indicated that a reduction in glycogen storage levels *per se* in older Pompe mice did not necessarily translate to improvements in muscle function.

Early therapeutic intervention with oxime-neo-rhGAA abated the extent of degeneration and regeneration of muscle fibers in Pompe mice

To examine the basis for the observed di erence in functional response to enzyme therapy between animals that were treated earlier versus later, histopathological analysis of the muscles was performed. In healthy muscle bers, as expected, the nuclei were primarily located at the periphery of the cells (**Figure 6a**). A centronuclear appearance is suggestive of a degenerative and regenerative event as may occur a er injury or a serious myopathy. Examination of the quadriceps from 3-month-old Pompe mice showed a similar pattern of peripheral localization of the nuclei as observed in wild type mice (**Figure 6b**). However, when the Pompe animals reached 5 months (**Figure 6c**) or 10 months (**Figure 6d**) of age, progressively greater numbers of myo bers were observed to harbor centrally localized nuclei.

Quantitation of the number of muscle bers with a centronuclear appearance (collated from ~2,000 myo bers from di erent sections of three Pompe mice at each time point) showed an increase in the number of cells with this phenotype as a function of age, re ecting the progressive nature of the disease (**Figure 7a**). e number would appear to stabilize a er 10 months suggesting perhaps a loss of regenerative capacity. In addition to having a larger number of centrally localized nuclei, the sections from the older Pompe mice also displayed evidence of accumulation of glycogen in the cells (visualized as white, punctate spots).

Skeletal muscles from Pompe mice treated with enzyme starting at 5.5 months of age and analyzed 8 months later showed evidence of qualitative and quantitative changes. Pompe mice treated with biweekly infusions of 20 mg/kg rhGAA showed a reduction in the number of glycogen deposits but no di erence in the number muscle bers with a centronuclear appearance (~20%) compared to those noted in untreated, age-matched Pompe mice (**Figures 6e,f** and **7a**). However, those treated with 100 mg/kg rhGAA (**Figure 6g**) or 20 mg/kg oxime-neo-rhGAA (**Figure 6h**) showed a lower number of bers (~12%) with central nuclei and greater clearance of glycogen deposits. e number of bers with a centronuclear appearance in these animals at the conclusion of the study period (8 months post-treatment) was similar to that noted at the start of the therapy, when the animals were 5.5 months old (**Figure 7a**). is suggested that early treatment with either 100 mg/kg of the unmodi ed enzyme or 20 mg/kg of oxime-neo-rhGAA essentially prevented further degeneration and regeneration of the muscle bers. ese results were also consistent with the biochemical and motor function data showing that 20 mg/kg of unmodi ed rhGAA was less e cacious than 100 mg/kg of the same enzyme or 20 mg/kg of oxime-neo-rhGAA.

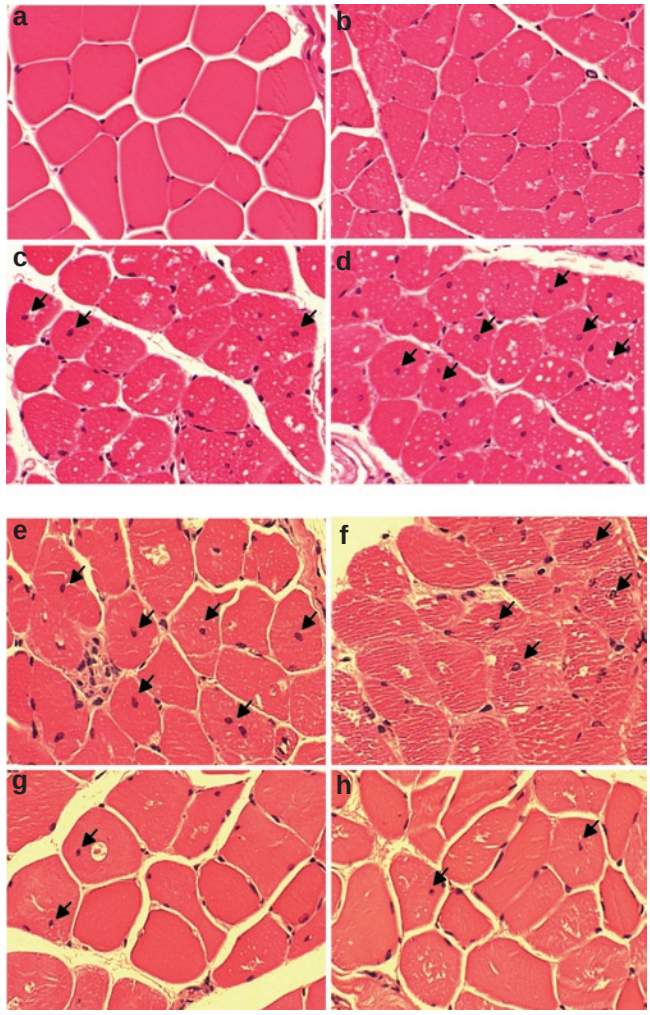

Figure 6 Histopathological analysis of skeletal muscles of Pompe mice. Representative sections of quadriceps from Pompe mice of different ages and after enzyme therapy were fixed in 10% neutral buffer formalin and stained with hematoxylin and eosin. The extent of glycogen accumulation (visualized as white deposits) and centronucleation was noted in tissues obtained from representative tissues (×20 magnification) of (a) 3-month-old wild type mouse, (b) 3-month-old Pompe mouse, (c) 5-month-old Pompe mouse and (d) 10-month-old Pompe mouse. Panel (e) was sectioned from a 13.5-month-old Pompe mouse treated with vehicle for 8 months; (f) was from a 13.5-month-old Pompe mouse that received 20 mg/kg rhGAA beginning at 5.5 months of age for a total of 8 months; panel (g) was obtained from a Pompe mouse that received 100 mg/kg rhGAA for 8 months and panel (h) from one that received 20 mg/kg oxime-neo-rhGAA for 8 months. Arrows denote the location of myofibers with centronucleation. rhGAA, recombinant human acid α-glucosidase.

Analysis of the skeletal muscles of Pompe mice that received enzyme therapy starting at 10 months of age (when the disease was more advanced) showed no change in the frequency of myo- bers with central nuclei when compared to vehicle-treated controls (**Figure 7b**). Irrespective of whether they were administered vehicle, unmodi ed or modi ed enzyme, the number of cells with