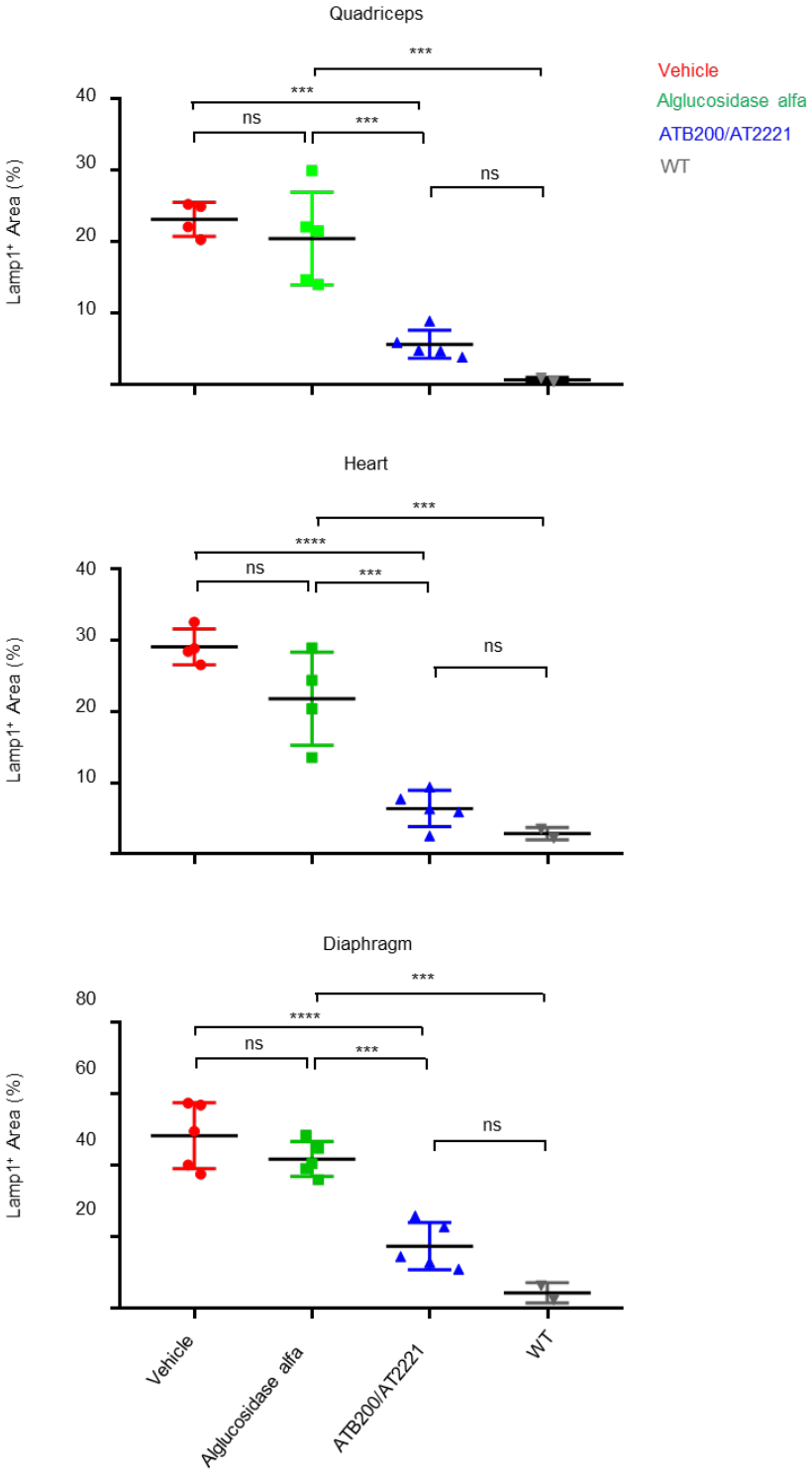
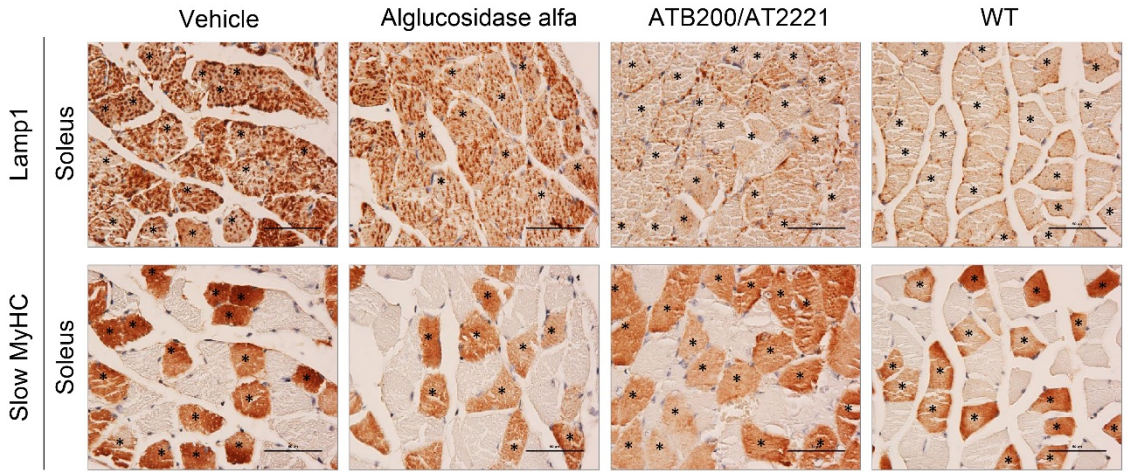
Supplemental Figures

Figure S1



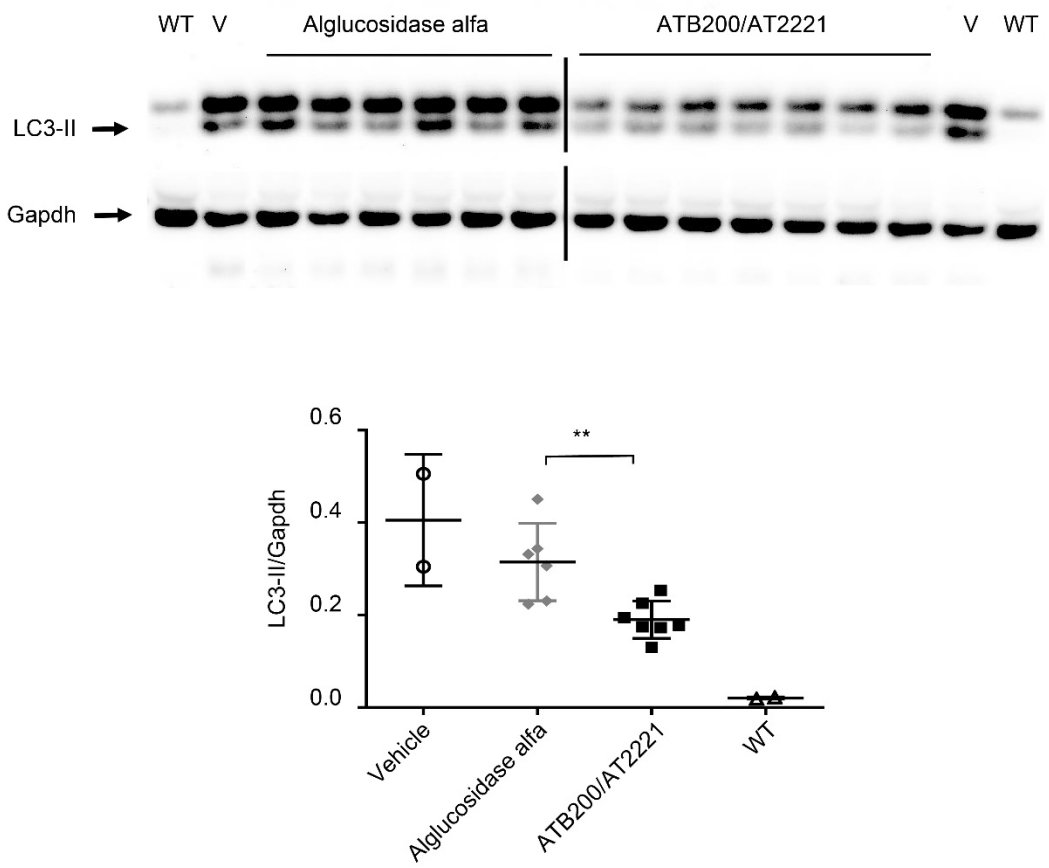
Sixteeen-week-old male *Gaa* KO mice received two biweekly IV administrations of vehicle, 20 mg/kg alglucosidase alfa, or 20 mg/kg ATB200 with 10 mg/kg of AT2221 administered orally 30 minutes prior to ATB200 injections (ATB200/AT2221). Tissues were collected 14 days after the second administration. The area encompassed by the Lamp1 signal shown in **Figure 3A** was quantitated using ImagePro software. The analysis was performed on one representative image per animal. For muscle (quadriceps), at least 100 fibers were analyzed for each mouse (~3-5% fibers in a cross section). For the diaphragm, ~ 50 fibers were analyzed for each animal (~5% of the total area). For the heart, a total area of at least 0.15 mm^2 was analyzed for each mouse (~4% of the total area). A dramatic decrease in the areas occupied by Lamp1-positive structures is observed in ATB200/AT2221-treated, but not in alglucosidase alfa-treated mice. Individual values and mean ± SD are shown; n = 4-5 per group (n = 2 for WT); ns: not significant; *** $P<0.001$; **** $P<0.0001$; Tukey's multiple comparison under one-way ANOVA.

Figure S2



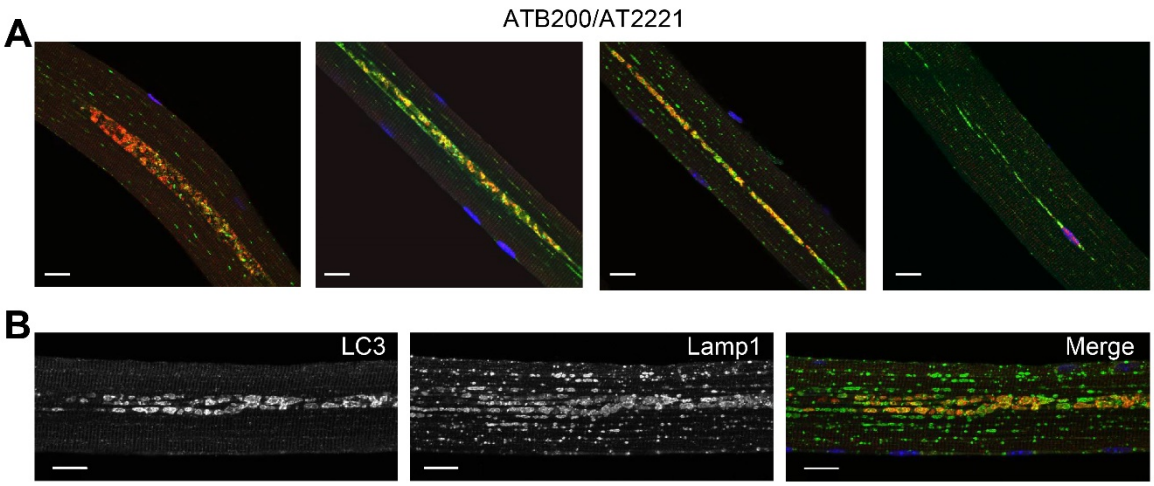
Representative images of muscle samples stained with Lamp1 (top) and slow myosin heavy chain (MyHC, bottom) on adjacent paraffin sections of soleus muscle. Sixteen-week-old male *Gaa* KO mice received two biweekly IV administrations of vehicle, 20 mg/kg alglucosidase alfa, or 20 mg/kg ATB200 with 10 mg/kg of AT2221 administered orally 30 minutes prior to ATB200 injections (ATB200/AT2221). Tissues were collected 14 days after the second administration. The effect of alglucosidase alfa is more pronounced in type I slow oxidative muscle, whereas ATB200/AT2221 appears to reduce Lamp1 staining in both type I and type II glycolytic muscles. n = 3-5 animals per group. Magnification: 400×. Bar: 50 μm.

Figure S3.



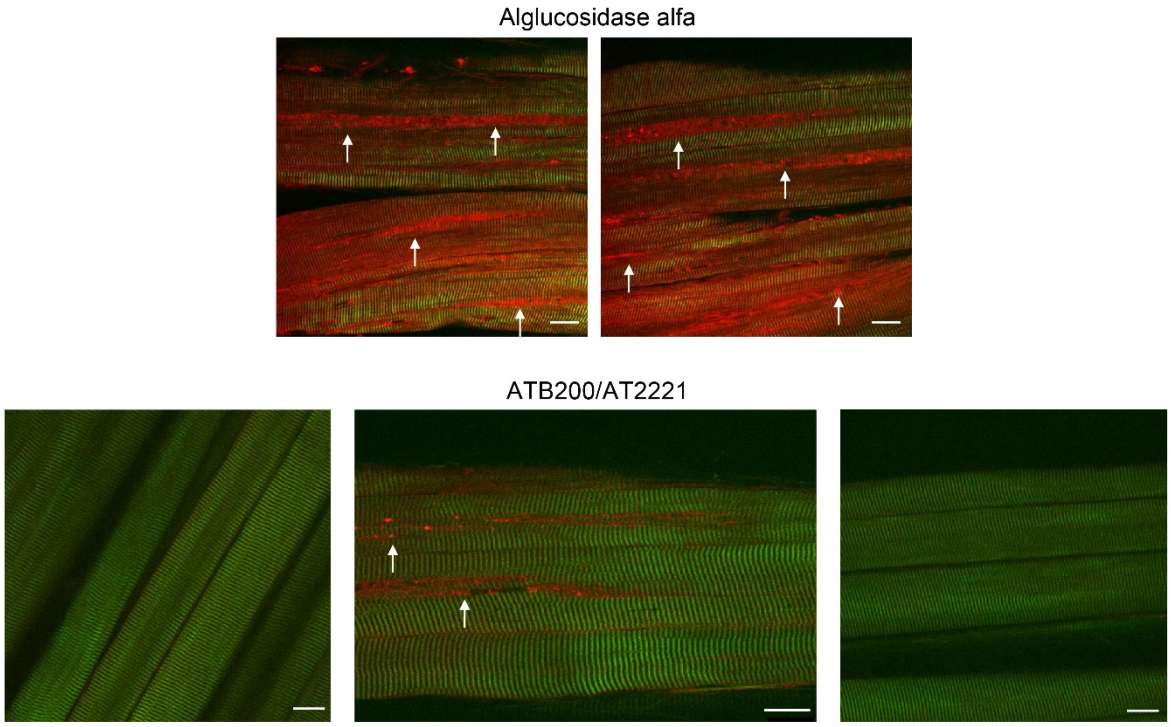
Representative images of western blot analyses of whole muscle lysates from vehicle (V)-, alglucosidase alfa-, or ATB200/AT2221-treated *Gaa* KO, and untreated WT mice with an anti-LC3 antibody. Gadph was used as a loading control. Sixteen-week-old male *Gaa* KO mice received two biweekly IV administrations of vehicle, 20 mg/kg alglucosidase alfa, or 20 mg/kg ATB200 with 10 mg/kg of AT2221 administered orally 30 minutes prior to ATB200 injections (ATB200/AT2221). Quadriceps femoris muscles were collected 14 days after the second administration. A significant decrease in the autophagosomal marker LC3-II is observed in ATB200/AT2221-treated mice compared with alglucosidase alfa-treated mice. The graph shows densitometric quantification of the LC3-II/Gapdh ratio. n = 6-7 animals per group (n = 2 for vehicle and WT); ** $P<0.01$; 2-sided t-test between alglucosidase alfa and ATB200/AT2221.

Figure S4.



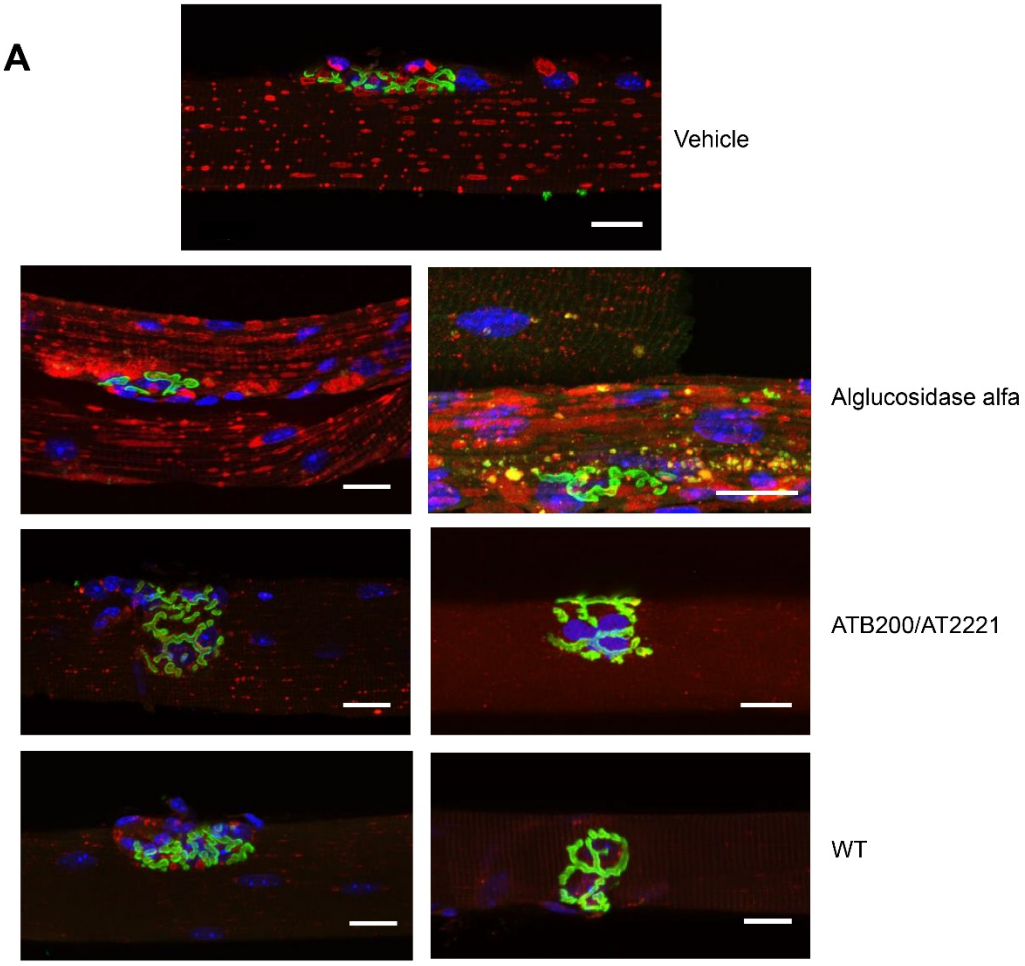
Additional images of muscle fibers from ATB200/AT2221-treated *Gaa* KO mice. Sixteen-week-old male mice received four biweekly IV injections of 20 mg/kg ATB200 with 10 mg/kg of AT2221 administered orally 30 minutes prior to ATB200 injections (ATB200/AT2221). Muscle samples (white part of gastrocnemius) were obtained 2 weeks after the last administration and single fibers were stained with markers for lysosomes (Lamp1; green), autophagosomes (LC3; red), and nuclei (Hoechst dye; blue). (**A**) The images show different stages of the resolution of autophagic buildup. (**B**) Well-defined LC3-positive autophagosomes (left panel) and Lamp1-positive lysosomes (middle panel) extensively co-localize, indicating efficient lysosomal-autophagosomal fusion; this pattern may represent a stage prior to the resolution of the autophagic buildup in the diseased muscle. Bar: 20 μm.

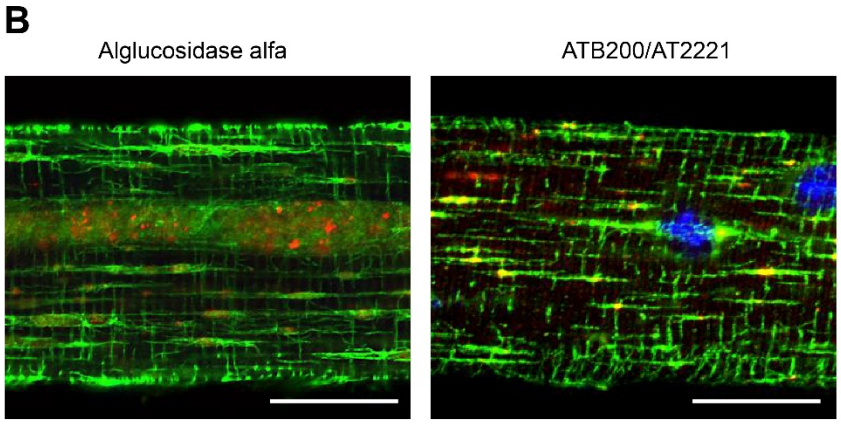
Figure S5.



Additional images of muscle fibers from alglucosidase alfa- or ATB200/AT2221-treated *Gaa* KO mice. Sixteen-week-old male mice received four biweekly IV injections of 20 mg/kg alglucosidase alfa or 20 mg/kg ATB200 with 10 mg/kg of AT2221 administered orally 30 minutes prior to ATB200 injections (ATB200/AT2221). The samples were obtained 2 weeks after the last administration. 2PEF (red) and SHG (green) images were recorded from bundles of fibers from the white part of gastrocnemius muscles. The images show profound defects in each fiber of alglucosidase alfa-treated mice; the areas with no SHG signal were filled with autofluorescent particles (arrows) that correspond to the regions of autophagic buildup. In contrast, most fibers from ATB200/AT2221-treated mice appear normal or near normal. Bar: 25μm.

Figure S6.


A
Vehicle
Alglucosidase alfa
ATB200/AT2221
WT


B
Alglucosidase alfa
ATB200/AT2221

(**A**) Single fibers from gastrocnemius muscles of vehicle-, alglucosidase alfa-, or ATB200/AT2221-treated *Gaa* KO mice were stained with the lysosomal marker Lamp1 (red) and fluorescent α-bungarotoxin (green), which selectively binds to acetylcholine receptors (AChR). Sixteen-week-old male *Gaa* KO mice received four biweekly IV administrations of vehicle, 20 mg/kg alglucosidase alfa, or 20 mg/kg ATB200 with 10 mg/kg of AT2221 administered orally 30 minutes prior to ATB200 injections (ATB200/AT2221). Muscle samples were obtained 2 weeks after the last injection. The pattern of postsynaptic-labeled AChR clusters in alglucosidase alfa-treated fibers appears similar to that in vehicle-treated muscle. In contrast, the elaborate shape of the clusters in ATB200/AT2221-treated fibers is no different from that in the WT. Note the absence of enlarged Lamp1-positive lysosomes in ATB200/AT2221-treated fibers. (**B**) *Gaa* KO mice were treated as in **A.** Single fibers from gastrocnemius muscles were stained for lysosomal marker Lamp1 (red) and alpha-tubulin (green). Altered distribution of microtubules in the area of autophagic buildup is a typical feature in muscle fibers from *Gaa* KO mice. The disruption of the microtubule network in muscle persists in alglucosidase alfa-treated *Gaa* KO mice since the vast majority of fibers still contain autophagic buildup. The resolution of the buildup in the majority of fibers from ATB200/AT2221-treated mice is associated with the restoration of the microtubule network. Bar: 20 μm.

Supplemental Table 1. Number of fibers from quadriceps examined for fiber size analysis

Animal	Group			
	Vehicle	**Alglucosidase alfa**	**ATB200/AT2221**	**WT**
1	4695	5729	3092	3725
2	3429	4782	4223	2998
3	5262	4249	3447	2844
4	2842	3936	2189	3029
5	4250	2166	4543	3986
6	2832		3706	
Total	23310	20862	21200	16582

Number of fibers from quadriceps examined for fiber size analysis as shown in Figure 8 C&D. Sixteen-week-old male *Gaa* KO mice received twelve biweekly IV administrations of vehicle, 20 mg/kg alglucosidase alfa, or 20 mg/kg ATB200 with 10 mg/kg of AT2221 administered orally 30 minutes prior to ATB200 injections (ATB200/AT2221). Quadriceps were collected 2 weeks after the last injection. One cross section from each mouse were stained with a laminin antibody and subjected to morphometric analysis. The fibers were from *vastus lateralis* region.