Silencing neuronal mutant androgen receptor in a mouse model of spinal and bulbar muscular atrophy

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Abstract
Spinal and bulbar muscular atrophy (SBMA), an adult-onset neurodegenerative disease that affects males, results from a CAG triplet repeat/polyglutamine expansions in the androgen receptor (AR) gene. Patients develop progressive muscular weakness and atrophy, and no effective therapy is currently available. The tissue-specific pathogenesis, especially relative pathological contributions between degenerative motor neurons and muscles, remains inconclusive. Though peripheral pathology in skeletal muscle caused by toxic AR protein has been recently reported to play a pivotal role in the pathogenesis of SBMA using mouse models, the role of motor neuron degeneration in SBMA has not been rigorously investigated. Here we exploited synthetic antisense oligonucleotides to inhibit the RNA levels of mutant AR in the central nervous system (CNS) and explore its therapeutic effects in our SBMA mouse model that harbors a mutant AR gene with 97 CAGs expansions and characteristic SBMA-like neurogenic phenotypes. A single intracerebroventricular administration of the antisense oligonucleotides in the presymptomatic phase efficiently suppressed the mutant gene expression in the CNS, and delayed the onset and progression of motor dysfunction, improved body weight gain and survival with the amelioration of neuronal histopathology in motor units such as spinal motor neurons, neuromuscular junctions and skeletal muscle. These findings highlight the importance of the neurotoxicity of mutant AR protein in motor neurons as a therapeutic target.
Introduction

SBMA is a late-onset neurodegenerative disorder which is characterized by progressive motor neuron degeneration in the brainstem and spinal cord, but also elicits skeletal muscle degeneration (1, 2). It is caused by CAG repeat expansion mutations in the AR gene, and usually affects males in adulthood. Testosterone, an AR ligand, facilitates nuclear translocation and intranuclear accumulation of mutant AR with expanded polyglutamine (polyQ) tract. This leads to transcriptional dysregulation, and subsequent defects in pivotal cellular functions, resulting in cell degeneration (3-7). Ligand-dependent nuclear accumulation of mutant AR has been implicated as a key contributor to motor neuron degeneration accounting for SBMA pathogenesis (8-10).

Though the formation of nuclear inclusions is not frequent in patients’ muscle (11, 12), pathogenetic roles of myopathy has also been considered pivotal, since histopathological myopathic changes and elevation of serum creatine kinase levels, indicative of muscle degeneration, are among important SBMA signs (13). Muscle defects would fall short of providing trophic support for innervating motor neurons, which could cause non-cell autonomous neurodegeneration (14, 15). In line with this view, muscle-specific overexpression of trophic factor IGF-1 ameliorates pathologies in an SBMA mouse model expressing human mutant AR with 97 glutamines (AR-97Q) (16), whereas muscle-restricted expression of rat AR induces motor neuron damage in mice (16, 17). Also, a recent report described that genetic inactivation of mutant AR in muscle rescued a bacterial artificial chromosome (BAC) transgenic mouse carrying AR-121Q (BAC fxAR121 mouse) (18). Supporting this finding, systemically administered antisense oligonucleotides (ASOs) that silenced mutant AR
in muscles and peripheral tissues ameliorated behavioral and pathological phenotype of BAC fxAR121 and another knock-in model with AR-113Q (AR113Q mouse), whereas intracerebroventricular (ICV) administration of ASOs silencing mutant AR in the CNS had no beneficial effect (19). These observations suggest a pivotal role of muscular mutant AR in pathogenesis of SBMA.

However, it is argued that model-specific phenotype in BAC fxAR121/AR113Q mice such as marked myopathy might be associated with the observed results in this ASO study (19). Given that profound nuclear inclusions in motor neurons and extensive neuronal loss are the fundamental human SBMA pathology, inherently vulnerability of motor neurons should also be taken into account (10, 20). Neurogenic contraction fasciculation in muscle is one of the initial symptoms of SBMA, and neurogenic abnormalities in electromyogram are evident even in affected male subjects as well as in asymptomatic female carriers (10, 21). Additionally, sensory and autonomic neuropathies are involved in SBMA patients (22-25). Thus the relative importance between motor neuron and muscle lesion in SBMA still remains inconclusive (19).

In our transgenic AR-97Q mouse, SBMA-like neuropathology in motor neurons and neuromuscular junctions is well characterized (26, 27). This further prompted us to address neuropathogenesis in SBMA and explore therapeutic effects of antisense knockdown of mutant AR in the CNS including motor neurons of this model. We demonstrate that ICV ASO injection, suppressing spinal mutant AR levels, ameliorated disease phenotypes of AR-97Q mice including motor dysfunction and neuronal histopathology without overt adverse effects. This effect is achieved through its potential effects against neurotoxicity of mutant AR. This effect was also observed even with concomitant suppression of endogenous Ar in the CNS without altering circulating testosterone levels. Besides myopathic defects, CNS pathology in SBMA
contributes to the pathogenesis and can also be a therapeutic target with lowering mutant AR by ASOs.

Results

**ASO inhibits mutant AR expression in the SBMA mouse CNS**

AR-97Q male mice demonstrate SBMA-like progressive motor dysfunction beginning as early as 8 weeks old with characteristic nuclear accumulation of mutant AR especially in the spinal motor neurons and muscles, and die with a median survival of 13-15 weeks. Females manifest only limited phenotype, recapitulating gender-related differences in human (26). We developed cEt/2′-MOE or 2′-MOE gapmer ASOs, which triggers RNase H cleavage and thus RNA degradation, complementary to both human AR and mouse Ar transcripts or only to human AR transcript (ASO-AR1 or ASO-AR2, respectively) (Supplementary Material, Table S1) (19). Since the blood-brain barrier in mature mice blocks the access of peripherally administered cEt/MOE ASOs to the CNS (28), we used ICV administration to directly distribute ASOs throughout the CNS under cerebrospinal fluid flow. We administered 2.0-6.0 mg/kg body weight of ASO-AR1, 4.0 mg/kg of ASO-AR2 or saline control as a single ICV injection, in 5-week-old males. The ASO effect on AR expression in the spinal cord was analyzed at 7 weeks old, 2 weeks after treatment. Semi-quantitative radioactive RT-PCR revealed that 4.0 mg/kg ASO-AR1 gave 50% and 90% decreases in mutant AR and mouse Ar, respectively, and ASO-AR2 suppressed mutant AR only (Fig. 1A). There was a dose-dependent trend of ASO-AR1 knockdown of mutant AR, and there was a sustained effect at least until 10 weeks old, shown by a time-course study (Fig. 1A). Immunoblot analysis revealed a corresponding reduction in mutant AR levels by ASO-AR1 (Fig. 1B). ASO-AR1 had a similar knockdown effect in brain
but no effect in muscle and liver, consistent with patterns of the ASO-parenchymal uptake (Fig. 1C, 1D, 3B, Supplementary Material, Fig. S1A and S3), as shown previously (19, 28-30).

**ASO ameliorates clinical phenotypes in the SBMA mice**

We observed beneficial effects of both ASOs on disease onset and progression. The 4.0 mg/kg of both ASOs successfully extended the life span, retained motor function measured with serial grip and rotarod-task tests, as well as body weight gain (Fig. 2A-D). A previous report demonstrated that prenatal loss of endogenous Ar enhances motor neuronal pathology through increased testosterone levels in SBMA mice (31). Though ASO-AR1 simultaneously suppresses endogenous Ar, in addition to human mutant AR, serum testosterone levels were maintained at the normal levels in the treated mice (Fig. 2E). Also, ASO-AR1 did not affect motor function, weight gain or survival in wild-type C57BL/6J mice under similar inhibition of mouse Ar in the spinal cord, indicating tolerability of this ASO strategy (Supplementary Material, Fig. S1B and S2). In contrast, 6.0 mg/kg of ASO-AR1 was less beneficial for survival (Fig. 2A), which could be potentially due to toxic effect of high-dose ASO (see below) (28, 32). However, cEt/MOE ASOs minimizes inflammation, in accordance with no marked increase in Aif1 mRNA expression, a macrophage/microglia activation marker (33), in the spinal cord (Supplementary Material, Fig. S1C) (29, 34).

**ASO ameliorates histological phenotypes in the SBMA mice**

**Motor neuron pathology**

Nuclear accumulation of mutant AR is the pathological hallmark of SBMA (10, 12). In ASO-AR1-treated mice, immunohistochemical (IHC) analysis with anti-polyQ
antibody 1C2 showed a decrease in the frequency of α-motor neurons with nuclear mutant AR accumulation at 13 weeks old (Fig. 3A). IHC showed predominant ASO uptake/distribution in the CNS including spinal motor neurons, while almost no cellular uptake in the peripheral tissues such as muscle and heart, though we detected exceptional ASOs in liver sinusoids and renal tubules (Fig. 3B, Supplementary Material, Fig. S3) (28-30). Staining for a functional motor-neuron marker choline acetyltransferase (ChAT) showed prevention of spinal motor neuron shrinkage, indicative of degeneration, in 14-week mice treated with ASO-AR1 (Fig. 3C). Higher ChAT expression in the spinal cord was also detected by immunoblotting (Fig. 3D). Reactive astrogliosis surrounding motor neurons indicates ongoing neuronal degeneration in motor neuron diseases (6), assessed by increased immunoreactivity for glial fibrillary acidic protein (GFAP). IHC showed suppression of GFAP expression in the spinal anterior horn under ASO-AR1 treatment (Fig. 3E). In contrast, the anti-GFAP immunoreactivity was elevated in mice treated with 6.0 mg/kg ASO-AR1 (Supplementary Material, Fig. S4), potentially indicating dose-dependent toxicities of ASO (Fig. 2A) (28, 32).

**Muscle pathology**

We next assessed the ASO effect on muscular defects, one of the key pathologies of SBMA. The number of myocyte with 1C2-positive nuclear accumulation of mutant AR was not substantially decreased by ASO-AR1, which was hardly uptaken in skeletal muscle (Fig. 4A, Supplementary Material, Fig. S3) (28-30). Nevertheless, H&E staining showed the retained myofiber sizes in ASO-AR1-treated mice, contrasting with the smaller myofiber sizes and atrophic fibers seen in control mice at 13 weeks old (Fig. 4B, C). Groups of atrophic fibers, which are associated with
denervation (35), were evident in control mice, but markedly decreased by ASO-AR1 (Fig. 4C). Furthermore, the expression of Myogenin (Myog), a marker of denervated muscle (36), which is increased in SBMA mouse (19) and another motor neuron disease amyotrophic lateral sclerosis mouse (37, 38), was suppressed in ASO-AR1-treated mice (Fig. 4D, E).

Neuromuscular junction pathology

Structural defects in neuromuscular junctions (NMJs) such as fragmented endplates, denervation and increased synaptophysin staining were reported in SBMA mice (5, 27). AR-97Q mice display kyphosis, a sign of axial muscle defects, as seen in mouse models of motor neuron diseases such as ALS and spinal muscular atrophy (SMA). We assessed NMJ structure in longissimus capitis (LC), axial muscle in the neck, in mice that were ICV-injected with 4.0 mg/kg ASO-AR1, at late-stage 15 weeks old. Immunofluorescent NMJ staining showed that ASO-AR1 increased the number of mature pretzel forms of endplate acetylcholine receptor (AChR) and decreased the number of fragmented endplates, that are reported in aged, denervated or dystrophic muscles (Fig. 5A) (30, 39). Moreover, reduction of fully innervated NMJs (see the Materials and Methods) was significantly prevented (Fig. 5B). In contrast, accumulation of synaptophysin staining, labeling for presynaptic terminals, was not apparent in AR-97Q mice (Fig. 5B). Taken together, the present study demonstrated that, even with concomitant knockdown of endogenous Ar, ICV-administered ASO-AR1 had therapeutic effects on the pathology in the CNS including lower motor neurons and delayed its onset, resulting in subsequent amelioration of neurogenic disease phenotypes in the skeletal muscle and NMJs.
Discussion

We demonstrated that ICV administered ASOs against AR, which specifically suppressed mutant AR expression in the CNS, ameliorated SBMA-like phenotypes in our AR-97Q mouse model. The knockdown effect of ASO predominant in the CNS improved motor dysfunction, growth impairment and survival as well as immunohistochemical features of spinal motor neurons such as nuclear accumulation of mutant AR and neuronal shrinkage. The ASO treatment also attenuated the motor-unit pathology including neurogenic muscular atrophy, neuromuscular denervation and fragmented endplates. Though ICV-administered cEt/MOE ASOs distributed throughout the CNS, they were partly cleared out of the CNS and circulated in peripheral tissues (Supplementary Material, Fig. S3). However, because of the low dose, ASO dilution in plasma and tissue compartments and renal excretion, pharmacological effects outside the CNS are likely minimal (19, 28-30, 34). Given that there was indeed no suppression of AR expression or decrease in nuclear accumulation of mutant AR in muscles, the therapeutic property of ASO would act against neuropathology in motor neurons of our mice. This implies that motor neuronal defects, in addition to the muscular pathology (18)-(19), play an important role in the pathogenesis of our SBMA model.

Compared with the therapeutic rescue by repeated systemic ASO administrations for BAC fxAR121 or AR113Q mice (19), the effect of a single ICV administration of ASO in our AR-97Q mice was less striking. The difference of efficacy could be partly attributed to the strategy of regimen such as dosing timing and frequency, as well as the tissue-specific pathogenetic mechanisms in each SBMA model (see below). However, the current results prove compatible to that of ICV ASO intervention in
ALS and SMA mice (34, 40, 41). Further analysis of efficacy of the earlier and/or repeated ICV ASO administration may help to develop this strategy in SBMA mice. We also observed the therapeutic effectiveness of ASO-AR2, which specifically hybridize only to human AR, in AR-97Q mice. This indicates that, the ASO potency, either abrogating endogenous Ar or not, is likely achieved through its mitigation of mutant AR-mediated neurotoxicity in the CNS, while mouse Ar-null background exacerbates disease phenotype by increasing testosterone levels due to androgen insensitivity (31). However, in contrast to the ubiquitous loss of endogenous Ar which begins embryonically, our approach was incomplete and temporary deprivation of Ar exclusively in the CNS at late postnatal stages. Our less potent approach retains circulating testosterone levels (Fig. 2E) and thus may not further promote nuclear accumulation of mutant AR. These findings indicate that, under suppression of mutant AR, endogenous Ar in the CNS may not deleteriously impact on the neurodegeneration, and lowered AR may not lead to CNS dysfunction in the AR-97Q mice. This view is supported by our ASO-knockdown experiments of endogenous Ar in wild-type C57BL/6J mice, which especially demonstrates that no adverse events due to AR decline in the CNS, such as abnormalities in motor function, locomotion and growth (Supplementary Material, Fig. S2) (42, 43). The low dose of ASO was shown pharmacologically tolerable in both SBMA and wild-type mice, and minimized toxic side effects related to ASO chemistry including proinflammatory responses (Supplementary Material, Fig. S1C) (32). Yet, further optimization of the ASO design for CNS application is warranted. A stable ASO serves as a powerful tool for the development of antisense therapeutics as well as for the understanding of toxicity of mutant AR protein in SBMA in vivo. The spatial distribution of ICV-injected ASOs in the CNS leads us to propose that
ASOs would rescue neurogenic pathology at least in the lower motor neurons, although potential effects of ASO in other CNS cells might have an additional benefit. In contrast to our results, ICV injection of ASO-AR1, referred as ASO3, was reported ineffective to improve motor function and survival in a BAC fxAR121 mouse model (19). These inconsistent results between the two studies might in part reflect the difference in the optimal dose and timing of the administration for each model, which may correlate with each overall prognosis. Another important difference between these mouse phenotypes is the involvement of distinct symptoms including urinary tract involvement in AR113Q or BAC fxAR121 mice (19), whereas AR-97Q mice have no defects in urination. This may suggest the possibility that the phenotypic properties of animal models have influence on the effects of ASO against the pathogenic protein. A comparative analysis using different models would further help for better understanding of contribution of peripheral and central lesions in SBMA pathogenesis.

Peripherally administered ASOs partly reach spinal neurons by a retrograde axonal transport (28), but its pharmacological effect in the CNS is limited compared to that obtained by ASOs with direct CNS delivery (30, 44, 45). Our current study and previous report (19) suggest that ASOs targeting to suppress mutant AR expression may prove useful as a potential therapy candidate for SBMA. With its characteristic property of hybridization to RNA sequences, ASOs specifically control expression levels of target genes such as through cleavage of the RNA or modulation of RNA splicing. Recent advances in antisense technology improve in vivo potency, tolerance and stability of ASO chemistry, promoting the development of RNA-targeting therapeutics (32). A clinical study of ASO has been launched in patients with familial ALS (46) and patients with infantile-onset SMA (ClinicalTrials.gov Identifier:...
NCT02193074). The ICV approach in mice using the smaller amount of ASOs administered with a single injection does not affect AR levels in the peripheral tissues and thus can hold an important anabolic function of androgen ligands on skeletal muscle such as maintenance or increase in muscle strength. This advantage would allow the preventive therapeutic application of ASOs for adult-onset SBMA that has a long-term presymptomatic phase, as partly revealed in this study.

**Materials and Methods**

**Oligonucleotides**

cEt/ 2’-MOE/DNA oligonucleotides with a phosphorothioate backbone and all 5-methylcytosines were synthesized and purified as described (19). The sequences of ASOs we used are listed in Supplementary Material, Table S1.

**Animals and mouse physiology**

Mouse protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Nagoya University Animal Experiment Committee. AR-97Q mice were generated, and the rotarod task and grip-strength test were carried out as described (26).

**ICV injection**

Five-week-old mice were anaesthetized with 40 mg/kg pentobarbital sodium and held by the head in a stereotaxic instrument. Five microliters of saline as control or 20 μg/μl of each ASO in saline was injected into the right lateral ventricle. The coordinates for injection were: 1 mm lateral from the sagittal suture, 0.2 mm posterior from the bregma and 3 mm deep from the brain surface.
RNA and protein analyses

For each experimental group, RT-PCR with $\alpha$-$^32$P-dCTP was performed with tissues from three mice. Total RNA extraction with DNase treatment and RT-PCR were performed to analyze human mutant AR, mouse Ar, Aif1 and Gapdh transcripts as described (29). To analyze AR and Ar transcripts, the following PCR primers were used: AR forward/ reverse 5′-CGGAAGCTGAAGAAACTTG-3′/ 5′-ATGGCTTCCAGGACCATT-3′ and Ar forward/ reverse 5′-GGACCATGTTTACCCATCG-3′/ 5′-CGTTTCTGCTGACCATA-3′. The brain cortex, thoracic spinal cord, liver, and quadriceps muscle were used. PCR products were analyzed as described (29). The protein expression in the thoracic spinal cord, liver, and quadriceps muscle was analyzed by Western blotting as described (6, 26). The following antibodies were used: anti-AR (1:2000, Santa Cruz), anti-ChAT (1:1000, Chemicon), anti-Myogenin (1:1000, Abcam) or anti-$\alpha$-tubulin (1:5000, Sigma-Aldrich). One-ml blood was collected by cardiocentesis for an assay of serum testosterone levels using a radioimmunoassay (LSI Medience Corporation).

Mouse histology

Tissue sections for histopathological analysis were prepared and immunostained as described (6, 26, 47). Flash-frozen quadriceps muscle was cut into 12-µm sections for Myogenin and Laminin staining. The following antibodies were used for staining of the thoracic spinal cord and quadriceps muscle: anti-1C2 (1:20000, Chemicon), anti-ChAT (1:1000, Chemicon), anti-GFAP (1:4000, Epitomics), anti-Myogenin (1:100, Hybridoma Bank), or anti-Laminin (1:200, Abcam). Besides, cellular uptake of ASO
in the brain, thoracic spinal cord, heart, liver, kidney, and quadriceps muscle was assessed with antibody against the phosphorothioate backbone.

1C2-positive cells in the thoracic spinal cord and quadriceps muscle were assessed as described (26). The size of ChAT positive motor neurons and the expression levels of GFAP positive glia cells in the anterior horn of thoracic spinal cord were quantified as described (6), using an BZ-X700 microscope (Keyence).

For NMJ staining, after perfusing and post-fixing with 4% paraformaldehyde, longissimus capitis muscle was dissected and teased into layers five to 10 fibers thick. NMJs were immunolabeled with anti-neurofilament (1:2000; Chemicon) for nerves, anti-synaptophysin (1:200; Invitrogen) for presynaptic terminals, and Alexa Fluor 594-conjugated α-bungarotoxin (1:200, Invitrogen) for AChRs. Endplate maturity and fragmentation of the endplates were assessed by AChR topology as described (30). The proportion of fully innervated NMJs was quantified as described (29).

For each sample, at least 100 NMJs were evaluated from random visual fields of the whole mount. Images were acquired with an Axio Imager M1 microscope and an LSM 710 confocal microscope (Carl Zeiss) for bright-field and immunofluorescence imaging, respectively. For NMJs imaging, Z-stacks of images with a 1-µm interval were taken.

**Statistical analysis**

The data was analyzed using two-tailed t tests, and considered p values 0.05 to be statistically significant. Kaplan-Meier survival curves were prepared with Mantel-Cox tests using GraphPad Prism Software. The various histograms and plots show mean
values ± standard deviation. Exceptionally, those for grip-strength and rotarod tests show mean values ± standard error.

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**Conflicts of Interest**

GH and CFB are employees of Isis Pharmaceuticals and own stock options.
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Figure Legends

Figure 1. ASO-induced inhibition of AR expression. (A) Study of dose-response effects of ASO-AR1 and effects of ASO-AR2. RT-PCR shows inhibition of expression of mutant AR (n = 3. *p < 0.05; **p < 0.005) and mouse Ar (n = 3. **p < 0.005; n.s., p > 0.05) in spinal cord of 7-week-old AR-97Q mice. The effect of 4.0 mg/kg ASO-AR1 was sustained at 10 weeks old. (B) Western analysis shows corresponding reduction in mutant AR expression at 10 and 13 weeks old. For monomeric mutant AR, the upper band in each doublet (arrowhead) is quantitated (n = 3. *p < 0.05). (C, D) No effect of ASO-AR1 on muscle or liver shown by RT-PCR (C) and Western analysis (D), at 7 and 10 weeks old, respectively (n = 3. n.s., p > 0.05). For (B–D), 4.0 mg/kg of ASO-AR1 was ICV-injected. We analyzed data using two-tailed t tests.

Figure 2. ASO-mediated amelioration of SBMA-like symptoms. (A) Extended life span by 4.0 mg/kg of ASO-AR1 or ASO-AR2 (*p < 0.05). (B, C) Improved motor function measured with grip strength and rotarod task performance by 4.0 mg/kg of ASO-AR1 or ASO-AR2 (*p < 0.05). (D) Retained body weight. (E) Retained serum testosterone levels in 4.0 mg/kg ASO-AR1-treated mice at 10 weeks old (n = 5. n.s., p > 0.05). ASO-AR1 or ASO-AR2 was ICV-injected at 5 weeks old. We analyzed data using two-tailed t tests, except for the logrank test for survival analysis.

Figure 3. Effects of ASO on motor-neuron histopathology. (A) Reduced nuclear accumulation of mutant AR in spinal motor neurons (arrowhead) (n = 3. **p < 0.005). Immunostaining with 1C2 antibody against expanded polyglutamine in 13-week-old spinal cord. (B) Detection of ASO uptake in 10-week spinal cord cells, including α-
motor neurons. (C) Prevented shrinkage of motor neurons. ChAT staining of 13-week spinal motor neurons (n = 5, >60 neurons per each analyzed. **p < 0.005). (D) Western analysis shows increased levels of ChAT at 13 weeks old (n = 3. *p < 0.05). (E) Prevented reactive astrogliosis. (Left) Glial fibrillary acidic protein (GFAP) staining in the spinal anterior horn at 14 weeks old. (Right) Intensities of immunoreactivity to GFAP (n = 3. **p < 0.005). Bar, 50 µm. 4.0 mg/kg ASO-AR1 was ICV-injected. We analyzed data using two-tailed t tests.

Figure 4. Effects of ASO on muscle histopathology. (A) No substantial reduction in nuclear accumulation in muscle (n = 3. n.s., p > 0.05). 1C2 staining in 10-week quadriceps. (B) Prevented muscle atrophy. Atrophic fibers, including groups of atrophic fibers (asterisk), indicated by H&E. (C) Distribution of fiber sizes in 10-week quadriceps (n = 4). (D) Atrophic fibers manifested by Myog staining. Quadriceps muscle is labeled with anti-Myog (red) and anti-Laminin (green) for the basal lamina. (E) Western analysis shows prevented increase in Myog expression, a marker of denervation-associated muscle atrophy, in 13-week quadriceps (n = 3. **p < 0.005). Bar, 50 µm. 4.0 mg/kg ASO-AR1 was ICV-injected. We analyzed data using two-tailed t tests.

Figure 5. ASO-mediated amelioration of NMJ topology. (A) Increase in the number of mature pretzel forms of endplate and decrease in the number of defective fragmented endplates (arrowhead) in 15-week longissimus capitis (n = 3. *p < 0.05 and **p < 0.005, respectively). (B) Increase in the number of fully innervation (double dagger) and decrease in the number of partial denervation (arrow) (n = 4. *p < 0.05). Fully denervated NMJs are indicated with a dagger. Accumulation of
synaptophysin staining is not apparent in longissimus capitis of control mice. NMJs are labeled with α-bungarotoxin (α-BTX; red) for acetylcholine receptors and anti-neurofilament (NF; green) for nerves, and anti-synaptophysin (Syn; green) for nerve terminals. Bar, 50 µm. 4.0 mg/kg ASO-AR1 was ICV-injected. We analyzed data using two-tailed t tests.

**Abbreviations**

Acetylcholine receptor = AChR; amyotrophic lateral sclerosis = ALS; antisense oligonucleotide = ASO; bacterial artificial chromosome = BAC; choline acetyltransferase = ChAT; glial fibrillary acidic protein = GFAP; intracerebroventricular = ICV; immunohistochemical = IHC; Myogenin = Myog;polyglutamine = polyQ; neuromuscular junction = NMJ; spinal and bulbar muscular atrophy = SBMA; spinal muscular atrophy = SMA; longissimus capitis = LC
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