Supplementary Information for:

Agonist of growth hormone-releasing hormone improves the disease features of spinal muscular atrophy mice

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SI Materials and Methods

Genotyping

To identify *Smn-/-* mice, a tail fragment was cut at P0 and DNA extracted through a lysis reaction. Briefly, each sample was incubated with 70 µl of lysis buffer (10 mM Tris HCl, 50 mM KCl, 0.01% gelatin, 0.45% IGEPAL, 0.4% Tween-20; Promega Corporation) and 2 µl of proteinase K (PTK) for 30 min at 60 °C. Next, samples underwent a heat shock at 4 °C for 10 min and finally at 95° C for 10 min. SMA mice were identified by reverse transcription polymerase chain reaction (RT-PCR) analysis, using the following primers (Thermo Fisher Scientific): *Smn*, forward 5′-TTTTCTCCCTCTTCAGAGTGAT-3′, *Smn* WT reverse 5′-CTGTTTCAAGGGAGTTGTGGC-3’ and *Smn* TG reverse 5’-GGTAACGCCAGGGTTTTCC-3’, as recommended by Jackson Laboratories. 420 bp and 150 bp products were obtained respectively from the WT and TG alleles.

Behavioral assessment

Specifically designed behavioral tests for neonatal rodents were performed every 2 days (from P2 to P12) in pups of both VHL, MR-1 and MR-2 groups (1). Animals were observed one at a time and kept under a heating lamp until they all had been tested. All pups were then mixed with the cage bedding to minimize maternal rejection. Body weight was measured every day before the tests. Four different behavioral tests were performed following the defined protocols:

- *Tail Suspension Test*: pups were suspended by the tail for 15 sec and a score was assigned based on their hindlimb posture, from 4 (normal hindlimb spread open) to 0 (hindlimb always closed together with clasping).
- *Righting Reflex*: pups were placed on their backs on a flat surface and both the latency and ability in repositioning themselves on the dorsal side up were assessed over 30 sec.
- *Hindlimb suspension test*: this test allowed the evaluation of the proximal hindlimbs muscle strength/weakness and the fatigue in pups. They were head-down suspended by their hindlimbs in a 50 ml centrifuge tube with a cotton ball cushion at the bottom for protection. Their performances were assessed by monitoring the latency to fall into the tube and a score was assigned to the hindlimb posture.
- *Negative Geotaxis*: this test evaluates the motor coordination and vestibular sensitivity of pups, starting from P4. Animals were placed on a 35 °C inclined rough surface with the head facing down. The latency and the ability of the pups to successfully orient themselves and climb upwards was evaluated within 60 sec, assigning a timing of 60 sec to mice that failed the test.

Tissue preparation

Pups were sacrificed at P12, and tissues resected and collected for subsequent analysis. Specifically, for the histological analysis on skeletal muscles, mice were euthanized by cervical dislocation. Fresh quadriceps and gastrocnemius were rapidly collected and embedded in OCT cryostat medium (Killik, Bio-Optica). Transverse section slices (40 µm thick) of muscles were cut on the cryostat and collected onto 4% gelatin-coated glasses. For immunofluorescence analysis on skeletal muscles, mice were deeply anesthetized by gaseous anesthesia and transcardially perfused with 0.1 M phosphate buffer (PBS), pH 7.4, followed by cold 4% paraformaldehyde (PFA)/PBS solution. After postfixation in 4% PFA for 2 h and cryoprotection in 30% sucrose/PBS solution, quadriceps and gastrocnemius were embedded in OCT cryostat medium. Longitudinal section slices (40 µm thick) were prepared and collected directly onto 4% gelatin-coated glasses. For the analysis on spinal cord, mice were deeply anesthetized by gaseous anesthesia and transcardially perfused, as described above. The spinal cord was removed from the vertebral column at the lumbar level (L1-L5) and postfixed in 4% PFA for 2 h. After cryoprotection in 30% sucrose/PBS buffer overnight, tissues were embedded and frozen in OCT cryostat medium. Next, free-floating transverse serial sections (40 µm thick) were collected and stored at -20 °C in an antifreeze solution (30% ethylene glycol, 30% glycerol, 10% PB; 189 mM NaH_2PO_4; 192.5 mM NaOH; pH 7.4) until being used. Finally, for real-time PCR and Western blot analysis, fresh tissues (quadriceps, gastrocnemius, and spinal cord) were collected from animals sacrificed by cervical dislocation, immediately frozen in liquid nitrogen and stored at -80 °C.

Histochemistry

For hematoxylin/eosin (H/E) staining, transverse sections of quadriceps and gastrocnemius muscles were hydrated in distilled water and stained firstly in hematoxylin, then with eosin (Bio-Optica). Lastly, sections were dehydrated in ascending series of ethanol (95–100%), cleared in xylene and cover-slipped with Eukitt mounting medium (Bio-Optica). The sections were drawn and analyzed by Nerolucida software (MicroBrightField Inc.). Data were obtained by data analysis software NeuroExplorer (MicroBrightField Inc.) For Nissl staining, transverse lumbar spinal cord sections were mounted on 4% gelatin-coated Superfrost slides (Bio-Optica) and air-dried overnight. Sections were then hydrated in distilled water, immersed in 0.1% Cresyl violet acetate (Sigma-Aldrich) for 4 min and then cover-slipped with Eukitt.

RT-PCR and real-time PCR

Total RNA extraction and reverse transcription to cDNA (1 µg RNA) from frozen tissues were performed as previously described (2). For real-time PCR, cDNAs were treated with DNA-free DNase (Life technologies) and reaction performed with 50 ng cDNA, 100 nM of each primer and the Luna Universal qPCR Master Mix (New England BioLabs) using the ABI-Prism 7300 (Applied Biosystems). The following primer pairs, designed using Primer3, were used: *MyoD1*, forward 5′-ACTACAGTGGCGACTCAGATGC-3′, reverse 5′-CCGCTGTAATCCATCATGCCATC-3’ (NM_010866.2); *Myog*, forward 5′-TCCCAACCCAGGAGATCATTTGC-3′, reverse 5′-ACGTAAGGGAGTGCAGATTGTGG-3’ (NM_031189.2); *Myh1*, forward 5′-CAACCCATACGACTACGCCT-3′, reverse 5′-CATCAGAAGTGAAGCCCAGAAT-3’ (NM_030679.2); *Myh2* forward 5′-TTCCAGAAGCCTAAGGTGGTC-3′, reverse 5′-GCCAGCCAGTGATGTTGTAAT-3’ (NM_001039545.2); *Myh7* forward 5′-CTTCTACAGGCCTGGGCTTAC-3′, reverse 5′-CTCCTTCTCAGACTTCCGCAG-3’ (NM_080728.3); *Myh8* forward 5′-ACACATCTTGCAGAGGAAGG-3′, reverse 5′-TAAACCCAGAGAGGCAAGTG-3’ (NM_177369.3); *Atrogin-1* forward 5′-CTCTGTACCATGCCGTTCCT-3′, reverse 5′-GGCTGCTGAACAGATTCTCC-3’ (NM_026346.3); *MurF1* forward 5′-TGTCTCACGTGTGAGGTGCCTA-3′, reverse 5′-CACCAGCATGGAGATGCAGTTAC-3’ (NM_001039048.2); *TNF-α* forward 5′-TCTCATCAGTTCTATGGCCC-3′, reverse 5′-GGGAGTAGACAAGGTACAAC-3’ (NM_001278601.1); *IL-1β* forward 5′-CTGCAGCTGGAGAGTGTGGAT-3′, reverse 5′-CTCCACTTTGCTCTTGACTTCTATCTT-3’ (NM_008361.4); *IL-6* forward 5′-TTCCATCCAGTTGCCTTCTTG-3′, reverse 5′-TTGGGAGTGGTATCCTCTGTGA-3’ (NM_031168.2); *18s* rRNA forward 5′-CCCATTCGAACGTCTGCCCTATC-3′, reverse 5′-TGCTGCCTTCCTTGGATGTGGTA-3’ (NR_146144.1). 18s rRNA was used as endogenous control. Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Western blotting

Tissues were lysed in RIPA buffer (Sigma-Aldrich), and protein concentrations were calculated as previously described (2-4).Proteins (75 µg) were resolved in 11% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% BSA in Tris-buffered saline with 0.1% Tween (Sigma-Aldrich) for 1 hour at room temperature, membranes were incubated overnight at 4°C with the specific rabbit polyclonal primary antibody for MuRF1 (1:500; ref: Ab77577, Abcam), GHRH-R (1:500; ref: Ab28692, Abcam) or SMN (1:2000, ref: 610646, BD Biosciences). Blots were reprobed with mouse monoclonal anti-actin antibody (1:500; ref: sc-376421, Santa Cruz Biotechnology) for normalization. Immunoreactive proteins were visualized using horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit (1:4000) antibodies (Southern Biotech) by enhanced chemiluminescence using ChemiDoc XRS (Bio-Rad). Densitometric analysis was carried out with Quantity One software (Bio-Rad).

Immunofluorescence analysis

For immunofluorescence staining, frozen sections were firstly washed in PBS to remove antifreeze solution then incubated overnight at 4 °C with specific primary antibodies diluted in PBS 0.3% Triton X-100 and 2% normal donkey serum (NDS, Sigma-Aldrich). The following primary antibodies were used: anti-glial fibrillary acidic protein (anti-GFAP; rabbit, 1:500; ref: Z0334, DAKO Cytomation) and anti-neurofilament (NF-M; mouse, 1:500; ref: MAB1621, Millipore). Next, the sections were incubated with specific fluorochrome-conjugated secondary antibodies (Cy™ AffiniPure anti-rabbit (1:300), ref: 711165152; Alexafluor anti-mouse 488 (1:200), ref: 715545150; Jackson ImmunoResearch Laboratories; West Grove, PA, USA), diluted in PBS and 2% NDS, washed and cover-slipped with anti-fade mounting medium (Mowiol).

For analysis of NMJs, quadriceps and gastrocnemius slices were treated with a citrate-based buffer (pH 6) for antigen retrieval, followed by incubation for 30 min at room temperature with the α-bungarotoxin (α-BTX) Alexa Fluor 555 conjugated antibody (1:500; Invitrogen) diluted in 0.3% PBS, Triton X-100 buffer (Sigma-Aldrich). 4',6-iamidino-2-Phpenylindole dilactate (DAPI; Sigma-Aldrich) diluted in PBS 1:50 for 3 min was used to stain nuclei. Images were acquired with a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems). For the analysis of astrocyte activation in the spinal cord, confocal images (40 x magnification, acquisition speed 200 Hz, 1024 × 1024 pixels; three fields for animal) of ventral horns section were acquired by Leica TCS-SP5 confocal laser scanning microscope. The percentage of the overall GFAP-positive cells was quantified using ImageJ software.

Morphological and semiquantitative analyses

The morphology of quadriceps and gastrocnemius muscle fibers (100 fibers for each animal) was evaluated in terms of mean fiber area, perimeter and maximum Feret's diameter. Sections were observed using Nikon Eclipse 80i microscope (Nikon), and measures obtained by Neurolucida software analysis (MicroBrightField Inc.). To evaluate NMJ innervation/maturation, longitudinal sections of quadriceps and gastrocnemius were observed with Nikon Eclipse 80i microscope (Nikon). The endplates were classified as "multi-innervated", "mono-innervated" and "denervated". Results were expressed in percentage for each experimental group. For MN counting, spinal cord slices (L4-L5, 1 section every 320 µm) stained with Cresyl violet acetate (Sigma-Aldrich) were analyzed using a stereological technique, the Optical Fractionator, a computer-assisted microscope and the StereoInvestigator software (MicroBrightField Inc.). The guard zones were 5 µm, the counting frame size was 100 x 100 µm and the sample grid size was 150 x 150 µm. Only cells with an area ≥ 80 μm^2, located into the ventral horns and with a visible nucleolus were considered as α-MNs positive and therefore counted. The cell density was reported as MN number/mm^3.

References

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3. D. Gallo *et al.*, GH-Releasing Hormone Promotes Survival and Prevents TNF-alpha-Induced Apoptosis and Atrophy in C2C12 Myotubes. *Endocrinology* **156**, 3239-3252 (2015).
4. R. Granata *et al.*, Growth hormone-releasing hormone promotes survival of cardiac myocytes in vitro and protects against ischaemia-reperfusion injury in rat heart. *Cardiovasc Res* **83**, 303-312 (2009).

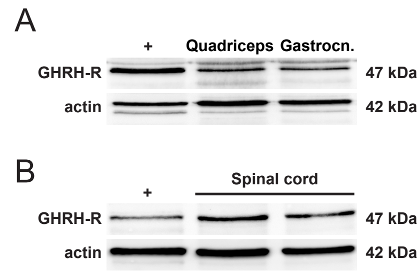

Fig. S1. Protein expression of GHRH-R in muscle and spinal cord of SMA mice. Representative Western blot for GHRH-R in quadriceps and gastrocnemius (Gastrocn.) (A) and spinal cord (B). Mouse brain was used as positive control (+). Equal protein loading was determined by reprobing with antibody to actin (lower panels) (n = 3).

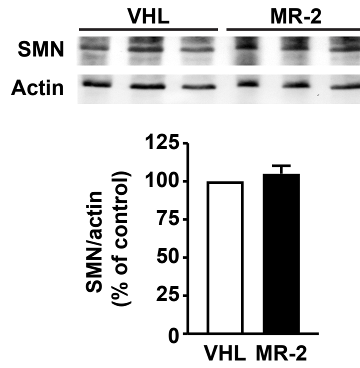

Fig. S2. Protein expression of SMN in the spinal cord of SMA mice. Representative Western blot for SMN in the spinal cord of SMA mice, either untreated (VHL) or treated with MR-2. Equal protein loading was determined by reprobing with antibody to actin (lower panels). Results, expressed as percentage of control (VHL) are mean ± SEM (n = 3 for each group) (n = 3).

Table S1. Quadriceps fiber area, perimeter and maximum Feret's diameter in SMA mice treated with Vehicle (VHL), 1 mg/Kg MR-409 (MR-1) or 2 mg/Kg MR-409 (MR-2) (n = 5 for each group), and in wild type (WT) mice (n = 3). Mean ± SEM. One-way ANOVA and Tukey's post hoc test.

	VHL	**MR-1**	**MR-2**	**WT**
Area (μm^2)	237.13 ± 13.74	354.86 ± 12.36	471.94 ± 42.47	508.72 ± 25.07
Perimeter (μm)	57.57 ± 1.42	70.47 ± 1.43	80.77 ± 3.70	84.76 ± 2.26
Max Feret's diameter (μm)	21.14 ± 0.42	25.64 ± 0.52	29.57 ± 1.41	31.49 ± 1.16

	VHL vs MR-1	**VHL vs MR-2**	**VHL vs WT**	**WT vs MR-1**	**WT vs MR-2**
Area (μm^2)	*$P < 0.05$	***$P < 0.001$	***$P < 0.001$	*$P < 0.05$	$P = 0.8190$
Perimeter (μm)	**$P < 0.01$	***$P < 0.001$	***$P < 0.001$	*$P < 0.05$	$P = 0.7285$
Max Feret's diameter (μm)	*$P < 0.05$	***$P < 0.001$	***$P < 0.001$	**$P < 0.01$	$P = 0.5753$

Table S2. Gastrocnemius fiber area, perimeter and maximum Feret's diameter in SMA mice treated with Vehicle (VHL), 1 mg/Kg MR-409 (MR-1) or 2 mg/Kg MR-409 (MR-2) (n = 5 for each group), and in wild type (WT) mice (n = 3). Mean ± SEM. One-way ANOVA and Tukey's post hoc test.

	VHL	**MR-1**	**MR-2**	**WT**
Area (μm^2)	246.74 ± 7.97	336.43 ± 22.62	430.73 ± 12.04	451.80 ± 3.78
Perimeter (μm)	58.29 ± 0.93	68.57 ± 2.37	77.97 ± 1.07	79.53 ± 0.30
Max Feret's diameter (μm)	21.19 ± 0.35	25.00 ± 0.82	28.65 ± 0.43	29.18 ± 0.09

	VHL vs MR-1	**VHL vs MR-2**	**VHL vs WT**	**WT vs MR-1**	**WT vs MR-2**
Area (μm^2)	**$P < 0.01$	***$P < 0.001$	***$P < 0.001$	**$P < 0.01$	$P = 0.8433$
Perimeter (μm)	**$P < 0.01$	***$P < 0.001$	***$P < 0.001$	**$P < 0.01$	$P = 0.9265$
Max Feret's diameter (μm)	**$P < 0.01$	***$P < 0.001$	***$P < 0.001$	**$P < 0.01$	$P = 0.9341$

Table S3. Analysis of the quadriceps NMJs in SMA mice treated with Vehicle (VHL), 1 mg/Kg MR-409 (MR-1) or 2 mg/Kg MR-409 (MR-2). Results are expressed as percentage of multi-innervated, mono-innervated and denervated endplates (*n* = 5 for each group).

	VHL	**MR-1**	**MR-2**
Multi-innervated endplates (%)	19.47 ± 1.51	19.33 ± 1.72	13.31 ± 0.78
Mono-innervated endplates (%)	62.40 ± 3.64	67.87 ± 3.11	79.08 ± 2.35
Denervated endplates (%)	18.13 ± 2.90	12.80 ± 2.06	7.61 ± 2.37

Table S4. Analysis of the gastrocnemius NMJs in SMA mice treated with Vehicle (VHL), 1 mg/Kg MR-409 (MR-1) or 2 mg/Kg MR-409 (MR-2). Results are expressed as percentage of multi-innervated, mono-innervated and denervated endplates (*n* = 5 for each group).

	VHL	**MR-1**	**MR-2**
Multi-innervated endplates (%)	20.62 ± 2.57	18.67 ± 3.12	13.52 ± 0.71
Mono-innervated endplates (%)	60.05 ± 1.34	68.00 ± 3.03	79.99 ± 2.74
Denervated endplates (%)	19.33 ± 1.73	13.33 ± 0.36	6.49 ± 2.13