The nSMase2/Smpd3 gene modulates the severity of muscular dystrophy and the emotional stress response in mdx mice

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Abstract

Background: Duchenne muscular dystrophy (DMD) is a progressive, degenerative muscular disorder and cognitive dysfunction caused by mutations in the dystrophin gene. It is characterized by excess inflammatory responses in the muscle and repeated degeneration and regeneration cycles. Neutral sphingomyelinase 2/sphingomyelin phosphodiesterase 3 (nSMase2/Smpd3) hydrolyzes sphingomyelin in lipid rafts. This protein thus modulates inflammatory responses, cell survival or apoptosis pathways, and the secretion of extracellular vesicles in a Ca²⁺-dependent manner. However, its roles in dystrophic pathology have not yet been clarified.

Methods: To investigate the effects of the loss of nSMase2/Smpd3 on dystrophic muscles and its role in the abnormal behavior observed in DMD patients, we generated mdx mice lacking the nSMase2/Smpd3 gene (mdx:Smpd3 double knockout [DKO] mice).

Results: Young mdx:Smpd3 DKO mice exhibited reduced muscular degeneration and decreased inflammation responses, but later on they showed exacerbated muscular necrosis. In addition, the abnormal stress response displayed by mdx mice was improved in the mdx:Smpd3 DKO mice, with the recovery of brain-derived neurotrophic factor (Bdnf) expression in the hippocampus.

Conclusions: nSMase2/Smpd3-modulated lipid raft integrity is a potential therapeutic target for DMD.

Keywords: Duchenne muscular dystrophy, Neutral sphingomyelinase 2/sphingomyelin phosphodiesterase 3, CRISPR-Cas9, Inflammatory cytokine, Monocytes/macrophages, Membrane permeability, Muscle performance, microRNA, Brain-derived neurotrophic factor, Anxiety behavior

Background

Duchenne muscular dystrophy (DMD) is an X-linked, recessive, inherited, and debilitating disorder affecting one in 3500 males in Japan. It is caused by loss-of-function mutations in the dystrophin gene on chromosome Xp21 [1]. Disruption of the dystrophin–glycoprotein complex (DGC) on the cell membrane causes cytosolic Ca²⁺ influx, resulting in protease activation, mitochondrial dysfunction, progressive myofiber degeneration, chronic inflammation, muscle wasting, and fragility. The latter phenomena are caused by the replacement of functional myofibers with fibrotic connective tissue and adipose tissue [2–5]. However, the myofiber-specific loss of dystroglycan (DG), a DGC component, in mice does not result in dystrophia-like muscle degeneration, but mice with stem cell-specific deletion of DG do show markedly delayed...
muscle regeneration [6, 7]. This suggests that myofiber instability is not the only cause of dystrophic degeneration, but rather that the phenotype might be caused by multiple factors, including stem cell and myofiber functions.

In addition to the function of dystrophin in the structural integrity of myofibers described above, a novel function of asymmetric cell division in satellite cells (SC) has been revealed, in which SCs lacking dystrophin show a marked increase in abnormal nonpolarized mitotic divisions and reduced asymmetric cell divisions and myogenic progenitors [7–9]. Thus, the continuing cycles of degeneration and regeneration in the initial stages of the dystrophic pathology exacerbate the phenotype. This exacerbation is thought to be caused by the misregulation of SC fate between differentiation and self-renewing proliferation during the regeneration of degenerated muscles [10, 11]. However, the mechanisms by which SC dysfunction is involved in muscular dystrophy have not yet been elucidated. In addition to muscle degeneration, the loss of dystrophin in the brain has often been associated with nonprogressive cognitive deficits, behavioral disabilities, and enhanced fearfulness [12–14]. However, an effective treatment for these abnormalities has not yet been established.

Neutral sphingomyelinase 2/Sphingomyelin phosphodiesterase 3 (nSMase2/Smpd3) is a membrane-associated enzyme that hydrolyzes sphingomyelin and affects membrane trafficking, receptor clustering, and signal transduction [15, 16]. nSMase2/Smpd3 is activated by inflammatory cytokines such as TNF-alpha, IL-1beta, and interferon-gamma (IFN-gamma), and it in turn activates caspase-12 and calpain in a Ca²⁺-dependent manner [17–19]. In addition, nSMase2/Smpd3 deficiency reduces the inflammatory response through decreased macrophage infiltration and lipid deposition [20]. However, the involvement of nSMase2/Smpd3 in muscular dystrophy and the abnormal behavior observed in DMD patients has not yet been elucidated.

In this study, we test the hypothesis that the pathogenesis in the dystrophic muscles and brains of mdx mice is affected by the nSMase2/Smpd3 protein through an inflammation response, as well as regeneration, differentiation, and signaling pathways. Deletion of the nSMase2/Smpd3 gene from mdx mice resulted in decreased inflammation and increased muscle regeneration in the skeletal muscle (SM) in early stages of the dystrophic process, but caused adverse effects in later stages. Furthermore, loss of the nSMase2/Smpd3 gene in mdx mice suppressed abnormal emotional behavior, such as the stress-induced anxiety response, as well as the recovery of hippocampal Bdnf expression. Thus, these findings suggest that the nSMase2/Smpd3 protein is a potential therapeutic target for muscular dystrophy and abnormal behavior.

Methods

Cell culture

The murine skeletal myoblast C2C12 cell line were maintained in a proliferation medium that Dulbecco’s modified Eagle’s medium (DMEM; Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Cell Culture Technologies, Lugano, Switzerland) and 1% (v/v) penicillin/streptomycin (Wako Pure Chemical Industries, Osaka, Japan) at 37°C in controlled humidified air with 5% CO₂ in six-well plates (BM Equipment, Tokyo, Japan). Before reaching confluency, the cells were rinsed with PBS, and the proliferation medium was changed to a differentiation medium (DMEM containing 2% heat-inactivated horse serum (HS) and 1% penicillin/streptomycin) until myotube formation was completed.

Dystrophin-deficient H2K myoblasts, which were derived from H-2 kb-tsA58 transgenic mice [21], were seeded at a density of 5 × 10⁴ cells/well in a 75 cm² flask and grown at 33°C in DMEM with GlutaMAX, IFN-gamma at a concentration of 20 U/mL, and 20% (v/v) fetal bovine serum [22]. After treatment, the cells were differentiated into myotubes by incubating them in DMEM with GlutaMAX containing 5% (v/v) HS at 37°C. At this plating density, the cells must be passaged once or twice a week, when they reach approximately 10⁴ per cm², and the medium must be changed twice weekly to fresh medium and fresh IFN-gamma. Four days after the initiation of differentiation, which is induced by a high cell density (≥10⁴ per cm²), the myotubes were used for experiments [23].

Extraction and quantification of transcripts

As described previously [23], for total RNA extraction, differentiated C2C12 myotube cells at the indicated times and mouse tissue samples were homogenized. DNA-free RNA was obtained by using the Purelink total RNA extraction kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. Specific complementary DNA (cDNA) was synthesized from the purified total RNA with random primers, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed by the StepOne Real-Time Polymerase Chain Reaction (PCR) System (ABI, Foster City, CA, USA) with gene-specific primers, according to the manufacturer’s instructions (Additional file 1: Table S1). Relative expression of each genes was calculated using SDS 2.1 real-time PCR data analysis software (ABI) and 2^(-ΔΔCt) method. Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (gapdh) were used as reference genes for normalization. The gene expression data from triplicate data per one sample are presented as median ± standard error of the mean (SEM).
Western blotting analysis
The cell lysates were prepared from the differentiated C2C12 cells and mouse tissues on ice in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Nonident P-40, and protease inhibitor cocktail). Expression of proteins was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% mini protein precast gel (Bio-Rad Laboratory, Hercules, CA, USA). The protein band was transferred to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, USA). Then, the membrane was incubated in gene-specific antibodies (1:1000 in a mixture of tris-buffered saline and polysorbate 20 [TBST]) (1:1000 in TBST) for 1 h at RT with gentle shaking. Then, the membrane was washed for 3 times with TBST having 0.5% milk. An anti-rat immunoglobulin G (IgG)-conjugated to horseradish peroxidase (HRP) antibody (1:1000 in TBST) was used for visualization as secondary antibody (1:1000 in TBST). The membrane was incubated for 1 h at RT with gentle shaking. Then, the membrane was washed for 3 times with TBST having 0.5% milk. For signal detection of specific band, the membrane was analyzed on the LAS-3000 Imager (Fujifilm, Tokyo, Japan).

Measurement of SMase enzymatic activity
SMase enzymatic activity was measured using the Sphingomyelinase Activity Colorimetric Assay Kit according to the manufacturer’s protocol (BioVision). Briefly, GAS muscles were removed from wt, mdx, and mdx: Smpd3<sup>−/−</sup> (#238 and #11) mice, and then SMase Assay Buffer and SMase Extraction Detergent with Protease Inhibitor Cocktail (Promega, Madison, WI, USA) were added. To extract the tissue lysate, GAS muscles were homogenized on ice, and supernatants were collected after centrifugation at 10,000g for 5 min. Next, 5 µL of supernatant was added to a 96-well plate with 45 µL of SMase Assay Buffer. The reaction mix, containing 32 µL of SMase Assay Buffer, 2 µL of SMase Enzyme Mix I, 10 µL of SMase Enzyme Mix II, 4 µL of SMase Substrate, and 2 µL of SMase Probe, was added to each well containing the sample solution. After mixing well, the reaction solution was incubated for 30 min at 37°C and the absorbance at 570 nm was measured.

Generation of nSMase2/Smpd3 knockdown mice
Under specific pathogen-free (SPF) condition, all mice lines used in this study were maintained at 21°C on a 12:12 h light to dark cycle at the National Institute of Neuroscience (NCNP), Japan, and treated in accordance with the guidelines provided by the Ethics Committee for the Treatment of Laboratory Animals of the National Center of Neurology and Psychiatry (approval ID: 2015006), which has adopted the three fundamental principles of replacement, reduction, and refinement. Cages were always enriched with crinkle paper and animals were allowed to drink water and eat food ad libitum. Consistent with the protocol stipulated by the research permit, all efforts were made to minimize the suffering and discomfort experienced by the animals. To design sgRNAs against the nSMase2/Smpd3 gene, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Design Tool (http://crispr.mit.edu) was used to search for candidate nucleotide sequences, and we selected three candidate target sequences based on the prediction scores of the CRISPR Design Tool (Additional file 2: Table S2). The plasmids expressing CRISPR-associated protein 9 (Cas9) and the sgRNAs were constructed by ligating three oligonucleotide pairs against each sgRNA with the BbsI-digested pX330 plasmid (Addgene, Watertown, MA, USA) according to the manufacturer’s instructions [24]. In brief, to prepare for the ligation of the sgRNAs into the BbsI site of the pX330 plasmid, three oligonucleotide pairs were added using four tagged nucleotide sequences, i.e., 5′-cacc-3′ at the 5′-end of the forward oligonucleotide and 5′-caca-3′ at the 3′-end of the reverse oligonucleotide, together with 0.1 µM of forward and reverse oligonucleotides in tris-ethylenediaminetetraacetic acid (TE) buffer, and the oligonucleotides were annealed using a thermal cycler, as follows: one cycle of 95°C for 5 min, 60°C for 5 min, and 25°C for 60 min. The ligation solution was prepared as follows: annealed oligonucleotides, BbsI-digested pX330, and Ligation high buffer, and this was incubated for 1 h at 16°C. The plasmid DNA-ligated sgRNA was transformed into a competent Escherichia coli strain (Competent Quick DH5alpha, TOYOBO, Tokyo, Japan), and incubated on Luria-Bertani (LB) containing 100 µg/mL ampicillin at 37°C for 14 h.

Individual colonies on the plate were picked up and incubated in LB liquid culture containing 100 µg/mL ampicillin with shaking overnight at 37°C for 16 h. The plasmid DNA was purified from the liquid cultures using a spin column from a commercially available kit (PureLinkR HiPure Plasmid Maxiprep Kit, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s
instructions, and integration of the sgRNA into the BbsI site of the pX330 vector was confirmed by direct sequencing. SgRNA-pX330 plasmid DNA in their circular form were injected directly into the pronuclei of zygotes collected from the oviducts of wt female B6C3F1 mice mated to wt male B6C3F1 mice, to reduce their integration into the host mouse genome, although the transgenic efficiency with the circular form of the plasmid DNA is approximately 10 times lower than that of the linear form [24, 25].

To screen for target mutant mice, genomic DNA was extracted from tissue samples taken from the tails of pups that developed from the microinjected eggs, by incubating them in lysis buffer at 50 °C for 17 h followed by phenol/chloroform purification. The extracted DNA was then genotyped by PCR amplification, the amplified PCR fragments were purified using DNA purification spin columns, and nucleotides were determined by sequencing with the indicated primer set (Additional file 3: Table S3). To determine the integration of Cas9 nucleotides into the genome DNA of the mice targeted by the sgRNAs, PCR amplification was performed using a pair of primers for the Cas9 nucleotide sequence. To check for off-target effects, a homology search for the three sgRNAs with a mouse genome sequence was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The resulting matching genomic sequences were amplified and their nucleotide sequences were determined via PCR amplification and sequenced using region-specific primer sets. To generate nSMase2/Smpd3-null, dystrophin-deficient DKO mice, we crossed nSMase2/Smpd3 KO mice with mdx mice. The genetic background of the mdx:nSMase/Smpd3 DKO mice used in this study is a mix that was created by mating mice from the B6C3F1 and B6j backgrounds and used after 4–5 generations of inbreeding.

**Tissue preparation**

Mice were sacrificed by cervical dislocation. Body and wet muscle weight were measured. The TA, GAS, and diaphragm muscles were collected using standard dissection methods, as described previously [26]. Some muscle samples were frozen in isopentane cooled by liquid nitrogen for histological analysis, and the remaining muscle samples were frozen in liquid nitrogen for RNA or protein isolation, and stored at 80 °C as described previously [26, 27]. Transverse cryosections (20 μm thick) of each muscle were stained with H&E, as described previously [28].

**Patients**

A total of 119 unrelated Japanese patients with DMD, BMD, myotonic dystrophy 1, distal myopathy with rimmed vacuoles (DMRV), facioscapulohumeral muscular dystrophy (FSHD), limb-girdle muscular dystrophy (LGMD), or limb-girdle muscular dystrophy 2B (LGMD2B) were participated in this study (Additional file 4: Table S4). Informed consent form with the patient’s signature was obtained from all participants after the details of the study had been explained to them and prior to the collection of peripheral blood. The protocol was approved by Research Ethics Committee of the NCNP (approval ID: A2011-113), in accordance with the regulations of the Declaration of Helsinki. All results were treated with standard medical confidentiality, and confidentially was maintained to the extent stipulated by the law.

**Measurement of CK activity**

From the blood samples, the supernatant was removed by microcentrifugation after incubation at room temperature. Serum CK assays were carried out using a commercially available Fuji Dri-Chem system (Fujifilm Medical, Tokyo, Japan) according to the manufacturer’s protocol, as described previously [23]. Serum (10 μL) was incubated at 37 °C on a Fuji Dri-Chem slide, and the dye absorbance was measured spectrophotometrically for 5 min at 540 nm. The values were calculated according to the installed formula, and data are expressed as units per liter (U/L).

**Muscle Evans blue dye uptake experiments**

To assess muscle damage, EBD (Nacalai Tesque, Tokyo, Japan) was dissolved in phosphate buffered salts (PBS) and sterilized by filtration. Twenty-four hours before sacrifice, mice were injected intraperitoneally with 1% EBD as described previously [29]. The muscle tissues were collected, and then frozen in melting isopentane. The sectioned muscle was created, and incubated in acetone (ice-cold) for 10 min. After wash the sections three times for 10 min with PBS, the flat-mount muscle was created by Vectashield mounting medium. Fluorescence microscopy was used for evaluation of the presence of EBD in myofibers.

**Treadmill and grip strength tests**

The treadmill muscle performance test was performed as described previously [30].

Briefly, mice were placed on a motor-driven flat MK-680S treadmill system (Muromachi Kikai, Tokyo, Japan) and forced to run for 5 min at a speed of 5 m/min. After 5 min, the speed was accelerated by 1 m/min every min. The test was stopped when the mouse was exhausted and did not attempt to remount the treadmill, and the time to exhaustion was recorded.

The forelimb grip strength of the mice was monitored using a grip strength meter (MK-380 M, Muromachi Kikai) with the investigator blinded to genotype. The mice were held 2 cm from the base of the tail, allowed to grab a woven metal wire with their forelimbs, and were
pulled gently in the horizontal plane until they released their grip. The force at the time of release was recorded as peak tension. Five sequential tests were carried out for each mouse, at 5 s intervals. The average peak tension in these attempts was defined as forelimb grip strength.

**Extraction and quantification of miRNA**

Total RNA isolation from serum or tissues was performed according to the manufacturer instructions for the PureLink™ RNA Mini Kit (Ambion, Austin, TX, USA) as previously described [31, 32]. The cDNA derived from the total RNA was prepared using a TaqMan miRNA Reverse Transcription (RT) kit (ABI, Foster City, CA, USA) and miRNA-specific stem-loop primers (part of the TaqMan miRNA assay kit; ABI) as previously described [23, 33]. For the real-time PCR of the miRNA, we used individual miRNA-specific primers (part of the TaqMan miRNA assay kit; ABI) with the StepOne Real-Time PCR System (ABI) according to the manufacturer’s protocol. Each miRNA was assayed in triplicate and data are presented as median values with the standard deviation. The relative expression levels for each miRNA were normalized using by endogenous and exogenous controls that are miR-16 and cel-miR-39. SDS 2.1 real-time PCR data analysis software (ABI) was used for the data analysis.

**Cell viability analysis**

The C2C12 or H2K cell line was seeded in 96-well plates (BM Equipment, Tokyo, Japan). Ten microliters of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium salt from the Cell Counting Kit-8 (CCK-8) (DOJINDO Laboratories, Kumamoto, Japan) was added to each well to detect cell proliferation and cell toxicity based on the quantity of formazan dye generated by the dehydrogenases in the cells, as described previously [23]. The cells continued to be cultured for 2 h, and then the absorbance at 450 nm of each well was measured using a Model 680-well microplate reader (Bio-Rad Laboratory).

**Proteome profiler cytokine array**

The skeletal and diaphragm muscles of mdx:Smpd3−/− (#238) and mdx mice were excised and homogenized in PBS with protease. After homogenization, Triton X-100 was added to a final concentration of 1%. To remove cellular debris, the tissue lysates were centrifuged at 10,000×g for 5 min. The protein concentration of the lysates was determined, and the relative expression of the cytokines and chemokines in the lysates was quantified using the Proteome Profiler Array (Mouse Cytokine Array, Panel A; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Briefly, 2.0 mL of Array Buffer was added into each well of the 4-well multi-dish as a blocking buffer. Each membrane was incubated for 1 h in a well of a 4-well multi-dish plate on a platform shaker. 1.5 µL of reconstituted Mouse Cytokine Array Panel A Detection Antibody Cocktail was added to each prepared sample, and the samples were then incubated at room temperature for 1 h. After blocking, the Array Buffer was aspirated from the wells. The sample/antibody mixtures were placed on the 4-well multi-dish and incubated overnight at 4 °C on a rocking platform shaker. Each membrane was washed thrice with 1× Wash Buffer for 10 min on a rocking platform shaker. The membrane was incubated with diluted Streptavidin-HPR for 30 min at room temperature on a rocking platform shaker. Then, after the membrane had been washed, signal detection was performed using the Chemi Reagent Mix and analyzed on the LAS-3000 Imager (Fujifilm Corporation).

**Measurement of cathepsin B activity using an in vivo imaging system**

ProSense 680 is a small peptide substrate for activated cathepsin B (CTSB). When cleaved by the CTSB enzyme, two caged fluorophores are released, with peak excitation at 680 nm and emission in the near-infrared range (700 nm). ProSense 680 (0.75 or 1.5 nmol) was intraperitoneally injected into 5-week-old wt, mdx, mdx: Smpd3−/−, and Smpd3−/− mice, which were then placed on a heating pad under anesthesia to keep their body temperature constant. Imaging was performed using a single excitation/emission filter pair optimal for the wavelength of the probe.

**Apoptosis assay**

Caspase-3 and caspase-9 activities were determined using Caspase-3/CPP32 or Caspase-9 colorimetric Assay Kit (BioVision), respectively, according to the manufacturer’s protocol. Briefly, GAS and TA muscles and cerebellum of mdx:Smpd3−/−, mdx, and wt mice were homogenized, and then lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Nonident P-40, and protease inhibitor cocktail) was added. After incubation on ice for 10 min, the tissue lysates were centrifuged at 10,000×g for 5 min for removal of cellular debris. The DEVD-pNA substrate was added into the sample solution adjusted the protein concentration and incubated at 37 °C for 2 h. The absorbance of each well at 405 nm was determined using a Model 680-well microplate reader (Bio-Rad Laboratory).

**Restraint test in mice**

Mice were restrained by the experimenter by placing the neck between the thumb and index finger and putting the tail between the third and little fingers [12]. After 10
s, the mouse was released into a cage (24 cm × 17 cm, surrounded by a 12 cm-high wall) containing wood chips (illuminated at 80 lx). A camera on the ceiling of the cage recorded a video and saved it to a personal computer. Locomotion and freezing were calculated from the image files obtained during the 5 min after the restraint using Image OF (O’Hara & Co., Tokyo, Japan), which is a modified version of the public-domain NIH Image program (developed at the US National Institutes of Health and available from http://rsb.info.nih.gov/nih-image/). Complete immobilization of the mouse, except for respiration, was regarded as a freezing response.

Sample size and statistical power
To determine the effective sample size needed to ensure the required statistical power in tests involving mdx and mdx:Smpd3+/− or mdx:Smpd3−/− mice and DMD and BMD patients, we calculated sample sizes based on the means of populations 1 and 2 and the common standard deviation, using the Power/Sample Size Calculator (available from https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html) (Additional files 5, 6: Table S5, S6).

Results
Expression of nSMase/Smpd-family genes during C2C12 differentiation and generation of nSMase2/Smpd3 knockdown mice in the mdx genetic background
To investigate the expression patterns of nSMase2/Smpd3 genes in response to the induction of myogenic differentiation in C2C12 mouse myoblasts, we quantified the mRNA expression levels of acid sphingomyelinase (aSMase)/Smpd1, nSMase/Smpd2, and nSMase2/Smpd3 during the course of C2C12 differentiation. All three genes were upregulated as early as 4 days after induction with differentiation medium, when the cells became phase-bright fused myotubes, and the upregulated levels were sustained for 6 days (Additional file 7: Fig. S1A). The expression level of the adult myosin heavy chain (Myh1) gene was used as a differentiation marker for C2C12 myoblasts, similar to the expression pattern of nSMase2/Smpd3 (Additional file 7: Fig. S1A). Western blotting showed that the nSMase2/Smpd3 protein was upregulated until day six (Additional file 7: Fig. S1B). On the other hand, the Bdnf protein was upregulated at day one, peaked on days two and three, then decreased until day six (Additional file 7: Fig. S1B). These findings indicate that nSMase2/Smpd3 is essential for myoblast differentiation.

To define the role of nSMase2/Smpd3 in the dystrophic process, we generated nSMase2/Smpd3 DKO mice using the CRISPR-Cas9 system with three single guide RNAs (sgRNAs) located in a large insertion region (LIR) between catalytic domain 1 (CD1) and CD2 of the nSMase2/Smpd3 protein (Additional file 7: Fig. S1C: lower panel). To reduce the possibility of off-target effects, we searched the mouse genome for regions homologous to the three sgRNA target nucleotide sequences using a BLAST search. We examined a total of 82 nucleotide sequences from homologous genomic regions for off-target effects using PCR-amplification and direct sequencing of the genomic DNA of each nSMase2/Smpd3 DKO mouse line generated (data not shown). In addition, the genomic DNA was analyzed to ensure that the Cas9 transgene (Tg). After the mouse lines with off-target effects or the Cas9 Tg had been eliminated, six independent nSMase2/Smpd3 DKO mouse lines were established, of which one (#20) had a premature stop codon at the amino acid (AA) at position 224 of the nSMase2/Smpd3 protein, with the last seven AAs included as substitutions (Additional file 7: Fig. S1C and D). The other five lines showed AA deletions within the nSMase2/Smpd3 protein at AA positions 219–220 (#11) or 243–245 (#26), a deletion of AA 246 with a substitution at position 245 from Arg to His (#28), a deletion of AAs 245–246 with a substitution at AA position 244 from Ile to Arg (#30), or a deletion from AA 191–273 with 30 AA insertions (#238) (Additional file 7: Fig. S1C and D).

To generate nSMase2/Smpd3-null, dystrophin-deficient double-mutant (DM) mice, we crossed nSMase2/Smpd3 DKO mice with mdx mice (Additional file 8: Fig. S2A and B). These founders were not born in a Mendelian ratio (Additional file 9: Table S7A and B), but there were no differences in organ weights between them and mdx mice (data not shown). Immunoblot analysis indicated that the nSMase2/Smpd3 protein was expressed in the gastrocnemius (GAS) muscle of both mdx:Smpd3−/− and mdx:Smpd3+/− mice at 14 weeks of age (Additional file 10: Fig. S3A and B), in the cerebellum of wild-type (wt), mdx, and mdx:Smpd3−/− DKO (#238) mice at 14 weeks of age (Additional file 10: Fig. S3B), and in the hippocampus of both mdx and mdx:Smpd3−/− DKO mice at 5 and 9 weeks of age (Additional file 10: Fig. S3C). In addition, the hippocampi of two of the mdx:Smpd3−/− DKO mouse lines, namely #11 (at 12 weeks) and #30 (at 18 weeks), expressed nSMase2/Smpd3 proteins, but in mouse line #28 at both 6 and 13 months, and in line #20 at 19 weeks, the specific band for nSMase2/Smpd3 could not be detected (Additional file 10: Fig. S3C). In addition, analysis of lysates of the SM and hippocampi of wt, mdx, and mdx:Smpd3−/− DKO (#238 and #11) mice showed that there was no difference in nSMase enzymatic activity between the #238 line and mdx mice in either the SM or the hippocampus (Additional file 10: Fig. S3C). However, the nSMase activity in the #11 line was significantly lower in the SM (Additional file 10: Fig. S3D) and somewhat lower in the hippocampus (Additional file 10: Fig. S3E) than in the #238 line and mdx mice. These results indicated that the LIR, in part, regulates nSMase/Smpd3 activity in vivo.
Deletion of the nSMase2/Smpd3 gene in mdx mice reduces inflammation in dystrophic muscles

To characterize the contribution of the nSMase2/Smpd3 gene to muscular inflammation in dystrophic muscles, cross sections of tibialis anterior (TA) muscles from 6-week-old mice were stained with hematoxylin and eosin (H&E). The area of inflammatory infiltration was significantly smaller in 6-week-old \textit{mdx}:\textit{Smpd3}^{+/−} (#11) mice than in \textit{mdx} mice (Fig. 1a, b, \(p < 0.05\)). To quantify the inflammation in the limb muscles of live animals, cathepsin-B enzyme activity was measured as an indicator of inflammation. The hindlimb muscles of \textit{mdx} mice exhibited higher levels of cathepsin-B activity than those of \textit{wt} mice and \textit{mdx}:\textit{Smpd3}^{−/−} mice, and activity levels in the muscles of \textit{Smpd3}^{−/−} mice were significantly lower than in \textit{wt} mice (Fig. 1c, d, \(p < 0.01\)).

Next, to analyze the protein expression levels of inflammatory cytokines that can be used as cellular markers, we performed multiple cytokine/chemokine protein array analyses on GAS and diaphragm muscles extracted from \textit{mdx}:\textit{Smpd3}^{−/−} and \textit{mdx} mice (Additional file 11: Fig. S4). The expression of \textit{Trem1} was lower in \textit{mdx}:\textit{Smpd3}^{−/−} mice than in \textit{mdx} mice (Fig. 2a: left). The levels of markers of activated macrophages, such as \textit{Ccl2} (M1 and M2a macrophages), \textit{Cxcl13} (M1 macrophages), \textit{IL-1beta} (M1 and M2b macrophages), \textit{IL-6} (M1 and M2b macrophages), and \textit{IL-12} (M1 and M2b macrophages) were also measured.
Fig. 2 Analysis of inflammation-related gene expression. **a** Expression levels of the cytokines and chemokines in the gastrocnemius (GAS) muscle (left) and diaphragm (right) of 12-week-old mdx and mdx:Smpd3−/− mice. **b** Expression of inflammation-related genes in the GAS of wt, mdx, and mdx:Smpd3+−/− mice at 12 weeks of age was measured by real-time RT-PCR (n = 3 per genotype). *p < 0.05, **p < 0.01
macrophages), Ccl1 (M2a and M2b macrophages), IL-1ra (M2a macrophages) and IL-10 (M2a, M2b, M2c, and M2d macrophages), and TNF-alpha (M1, M2b, and M2d macrophages) were lower in \textit{mdx:Smpd3}\textsuperscript{−/−} mice than in \textit{mdx} mice (Fig. 2a: left). In the diaphragm muscle, activated macrophage markers such as Cxcl13, Ccl1, IL-1beta, Ccl2, and IL-10 were also lower in \textit{mdx:Smpd3}\textsuperscript{−/−} mice than in \textit{mdx} mice (Fig. 2a: right).

Next, transcription levels of inflammatory cytokines in \textit{mdx:Smpd3 DKO} and \textit{mdx} mice were analyzed using real-time quantitative polymerase chain reaction (qPCR) analysis. In the GAS of 12-week-old \textit{mdx:Smpd3}\textsuperscript{+/−} mice, levels of TNF-alpha, CD68, CD45, Ccr5, IL-1ra, and IL-6 were significantly lower than in \textit{mdx} mice (Fig. 3b). However, Ccl9 and F4/80 expression levels in the GAS of \textit{mdx:Smpd3}\textsuperscript{−/−} mice were significantly higher than in \textit{mdx} mice (Fig. 2b, \(p<0.05\)). In addition, the diaphragm muscle of 12-week-old \textit{mdx:Smpd3}\textsuperscript{+/−} (\#238) mice had significantly lower levels of MyoD than that of \textit{mdx} mice (Additional file 12: Fig. S5 \(p<0.05\)). TNF-alpha levels in the GAS of 12-week-old \textit{mdx:Smpd3}\textsuperscript{−/−} mice were significantly lower than in \textit{mdx} mice (\#238, \(p<0.01\); \#11, \(p<0.001\)), whereas at 18 weeks old, levels in the \#238 line were similar to those in \textit{mdx} mice (Additional file 13: Fig. S6). In addition, IL-6 levels in \textit{mdx:Smpd3}\textsuperscript{−/−} (\#238 and \#11) mice at 12 weeks and the \#238 line at 18 weeks were significantly lower than in \textit{mdx} mice (Additional file 13: Fig. S6, \(p<0.05\)).

To characterize the inflammatory cell populations within the SM of \textit{mdx} and \textit{mdx:Smpd3}\textsuperscript{−/−} mice, we used real-time qPCR to compare the mRNA expression levels of molecular markers of inflammation in the GAS muscles of 12-week-old \textit{mdx} and \textit{mdx:Smpd3}\textsuperscript{−/−} (\#238 and \#11) mice. The expression levels of CD45 (monocytes) and Dectin-1 (M2a macrophages) in \textit{mdx:Smpd3}\textsuperscript{−/−} (\#238) mice were significantly lower than in \textit{mdx} mice (Additional file 13: Fig. S6, \(p<0.01\) and \(p<0.05\), respectively). In addition, the levels of Fizz/Retnla (M2a macrophages), IL-10 (M2a macrophages), and Ccr5 (monocytes) in \textit{mdx:Smpd3}\textsuperscript{−/−} (\#238) mice were significantly lower than in \textit{mdx} mice (Additional file 13: Fig. S6, \(p<0.05\)).

**Fig. 3** Smpd3 ablation reduces sarcolemmal instability in the muscles of young \textit{mdx} mice but exacerbates instability in older mice. \textbf{a} Serum creatine kinase (CK) levels of \textit{mdx, mdx:Smpd3}\textsuperscript{+/−}, \textit{mdx:Smpd3}\textsuperscript{−/−}, \textit{Smpd3}\textsuperscript{−/−}, and wt mice at 12 and 28 weeks of age. \textbf{b} Representative Evans blue dye (EBD) uptake in the tibialis anterior (TA) muscle of \textit{mdx} and \textit{mdx:Smpd3}\textsuperscript{−/−} mice at 12 weeks of age and \textit{mdx} and \textit{mdx:Smpd3}\textsuperscript{−/−} (\#11 and \#238) mice at 20 weeks of age. The number of EBD-positive fibers per arbitrary unit area was counted (right). The number of animals used is indicated in parentheses. Numbers preceded by # represent mouse lines. *\(p<0.05\)
somewhat lower than in mdx mice (Additional file 13: Fig. S6). We also analyzed molecular markers for monocytes/macrophages in the GAS of mdx:Smpd3−/− mouse lines #238 and #11. The level of CD11b (monocytes/macrophages) was significantly lower in the #238 line, but not in the #11 line, than in mdx mice (Additional file 13: Fig. S6, p < 0.05). In contrast, the expression level of F4/80 (a mature macrophage marker) in mdx:Smpd3−/− (#11) mice was approximately 10-fold that in mdx mice (Additional file 13: Fig. S6). In addition, expression levels of the nNOS gene in the GAS of mdx:Smpd3−/− (#238) mice at 9 and 12 weeks of age were higher than in mdx mice (Additional file 13: Fig. S6, ns and p < 0.05, respectively). In addition, expression of the Cox-2 gene at 12 weeks of age in the GAS of mdx:Smpd3−/− mice was significantly lower than in mdx mice (Additional file 13: Fig. S6, p < 0.05). To assess the effects of nSMase2/Smpd3 on the phosphorylation of Ser536 of p65, we performed a western blot analysis on the GAS of mdx:Smpd3−/− and mdx mice. However, we found no obvious differences in the expression of p65 or phosho-p65 between mdx and mdx:Smpd3−/− mice (data not shown). At 17 weeks, expression of Foxp3 in the GAS of mdx:Smpd3−/− mice was significantly lower than in mdx mice (Additional file 13: Fig. S6, p < 0.01), whereas at 19 weeks it was significantly higher (Additional file 13: Fig. S6, p < 0.05). Combined, these data suggest that depletion of nSMase2/Smpd3 may reduce inflammatory cytokine expression in monocytes/macrophages in the dystrophic process.

Disruption of the nSMase2/Smpd3 gene attenuates muscle membrane permeability in dystrophic mdx mice early on, but exacerbates it later

To assess the potential contribution of nSMase2/Smpd3 ablation on myofiber membrane permeability in the dystrophic SM of mdx mice, we measured the levels of serum creatine kinase (CK) as a serum biomarker for dystrophic muscle in wt, mdx, and mdx:Smpd3 DKO mice. In 6-week-old mdx:Smpd3−/− mice, serum CK levels were approximately 67% lower than those in mdx mice (Additional file 14: Fig. S7A). Similarly, at 8–10 weeks of age, serum CK levels in two mdx:Smpd3−/− mice groups (#20, #28, and #35) and (#11), and mdx:Smpd3−/− (#11) mice were significantly lower than in mdx mice (Additional file 14: Fig. S7B, p < 0.05 for mdx:Smpd3−/− mice #20, #28, and #35, p < 0.01 for mdx:Smpd3−/− mice #11, and p < 0.01 for mdx:Smpd3−/− mouse # 11). We also observed significantly lower levels of serum CK in mdx:Smpd3−/− (#28 and #11) mice at 12 weeks old (Fig. 3a: left, p < 0.01) and mdx:Smpd3−/− (#30) mice at 18 weeks old (Additional file 14: Fig. S7C, p < 0.001) than in mdx mice. However, at 28 weeks, we observed somewhat (#238) or significantly (#11; p < 0.05) higher serum CK levels in mdx:Smpd3−/− mice than in mdx mice (Fig. 3a: right).

Next, to visualize and quantify the degree of myofiber damage, we analyzed uptake of Evans blue dye (EBD) in the TA hind limb and diaphragm muscles. At 12 weeks of age, there were significantly fewer EBD-positive muscle fibers in the TA of mdx:Smpd3−/− mice than in mdx mice (Fig. 3b: upper, p < 0.05), but there was no difference between the diaphragm muscles of mdx:Smpd3−/− and mdx mice (data not shown). The number of EBD-positive muscle fibers in the TA of line #238 mdx:Smpd3−/− mice at 20 weeks of age was also substantially lower than in mdx mice, but it was higher in line #11 mdx:Smpd3−/− mice (Fig. 3b: bottom). These results suggest that early in life, nSMase2/Smpd3 ablation may have beneficial effects with respect to myofiber membrane degeneration in mdx mice, but that later on it may have adverse effects.

Genetic ablation of nSMase2/Smpd3 improves muscle performance in mice with dystrophic phenotypes

To further assess whether the reduction in muscle membrane permeability caused by nSMase2/Smpd3 KO in mdx mice improved muscle performance, we assessed muscle force and endurance. The grip strength test demonstrated that at 12 weeks of age, the muscle strength of mdx mice was significantly lower than that of wt mice (p < 0.001), but that of mdx:Smpd3−/− mice was significantly higher than that of mdx mice (Fig. 4a, p < 0.05). However, the mdx:Smpd3−/− mice also weighed significantly more than the mdx mice (Fig. 4b, p < 0.01), and when grip strength was scaled to body weight, there was no difference between mdx:Smpd3−/− and mdx mice (data not shown). An inclined treadmill running test indicated that mdx:Smpd3−/− mice tended to run further than mdx mice, but the difference was not statistically significant (Fig. 4c). However, mdx:Smpd3−/− mice ran for significantly longer than mdx mice at 16 (Fig. 4d, p < 0.001) and 60 (Additional file 15: Fig. S8, p < 0.05) weeks of age. These data show that loss of the nSMase2/Smpd3 gene may improve dystrophic muscle function in mdx mice.

nSMase2/Smpd3 deletion modulates fiber size in dystrophic muscles of mdx mice

We analyzed muscle fiber diameter and central nucleation in wt, mdx, and mdx:Smpd3−/− (#238 and #11) mice at 6 and 20 weeks of age. The muscle fibers were narrower in both lines of mdx:Smpd3−/− mice than in mdx mice at 6 weeks (Fig. 5a), but were thicker at 20 weeks (Fig. 5b). At 6 weeks, there were significantly fewer centrally nucleated fibers (CNFs) containing two nuclei per fiber in both lines of mdx:Smpd3−/− mice than in mdx mice (Fig. 5c, p < 0.05), but significantly more without central nuclei (Fig. 5c, p < 0.05). However, at 20
weeks there were no significant differences in the numbers of CNFs (Fig. 5d). These findings suggest that the loss of the nSMase2/Smpd3 gene may affect fiber size via fiber fusion.

**Effects of the nSMase2/Smpd3 gene on the survival, proliferation, and differentiation of myogenic cells in mdx mice**

Next, we hypothesized that nSMase2/Smpd3 regulates muscle regeneration via the survival and apoptosis of dystrophic myofibers. To test this hypothesis, we measured caspase-3 and caspase-9 activity in the SM and cerebellum of wt, mdx, and mdx:Smpd3−/− (#238) mice. At 12 weeks, caspase-3 activity in the GAS and TA muscles of mdx:Smpd3−/− mice was significantly lower than in mdx mice (Additional file 16: S9B–E), but there were no differences between them for the GAS muscle at 9 or 18 weeks or for the cerebellum at 18 weeks (Additional file 16: S9A). There were also no differences between these lines in terms of caspase-9 activity in the GAS at either 9 or 12 weeks of age (Additional file 16: S9A). The expression levels of Bdnf protein in the GAS muscles of mdx:Smpd3−/− mice at 5 (ns), 9 (p < 0.05), 12 (ns), and 14 (p < 0.05) weeks of age were lower than in mdx mice (Additional file 16: S9A), but there was no difference at 18 weeks (Additional file 16: S9F). We also performed a western blot analysis using an anti-caveolin-3 antibody as a lipid raft marker protein. At 14 weeks, caveolin-3 expression in the GAS muscle of mdx mice was significantly higher than in both wt and mdx:Smpd3−/− mice (Additional file 16: S9E, p < 0.05).

To further investigate the effects of the loss of nSMase2/Smpd3 in the degenerative muscles of mdx
mice, we analyzed expression of various genes in the GAS muscles of wt, mdx, and mdx:Smpd3+/- mice. The expression levels of Pax7 at 14 weeks (p < 0.05) and MuRF1 at 12 weeks (p < 0.001) in mdx:Smpd3+/- mice were significantly lower than in mdx mice (Additional file 16: Fig. S9G). At 9 weeks, dysferlin C2A (Dysf_C2A) and integrin alpha5 (Itga5) in mdx:Smpd3-/- mice were significantly upregulated, approximately 6-fold and 2-fold, respectively, relative to mdx mice (Additional file 16: Fig. S9H, p < 0.01), but at 12 and 19 weeks, these differences had disappeared (Additional file 16: Fig. S9H). At 19 weeks, Tead1 expression in the GAS of mdx:Smpd3-/- mice was significantly downregulated relative to mdx mice (Additional file 16: Fig. S9H, p < 0.05). On the other hand, at 12 weeks, Igf-1 expression in the GAS of mdx:Smpd3-/- (#11) mice was significantly lower (p < 0.05) than in mdx mice, but in line #238, it was somewhat higher (ns; Additional file 16: Fig. S9H). At 18 weeks, mdx:Smpd3-/- mice exhibited significantly lower Gata-2 expression than mdx mice (Additional file 16: Fig. S9H, p < 0.05). The expression of Hsp72 in mdx:Smpd3-/- (#238) mice was significantly upregulated relative to mdx mice (Additional file 16: Fig. S9H, p < 0.05), but not in line #11 mice (Additional file 16: Fig. S9H). In addition, at 12 weeks, the expression of Has2 in the GAS of mdx:Smpd3-/- (#238) mice was significantly upregulated relative to mdx mice (Additional file 16: Fig. S9H, p < 0.05).

The expression of the sarcolipin (Sln) gene, which encodes the SERCA-inhibitory peptide, in mdx:Smpd3-/- (#238) mice was lower than in mdx mice at 9 weeks but higher at 14 weeks (Additional file 16: Fig. S9H). At 12 weeks, mdx:Smpd3-/- mice exhibited significantly lower expression of the proapoptotic protein Bax than mdx mice (Additional file 16: Fig. S9H, p < 0.05). At 9 weeks, mdx:Smpd3-/- (#238) mice exhibited significantly higher cyclin D1 expression than mdx mice (p < 0.01), whereas at 12 (p < 0.01) and 19 (p < 0.05) weeks the cyclin D1 expression levels in both #238 and #11 mice were substantially lower than in mdx mice (Additional file 16: Fig. S9H).

At 14 weeks of age, expression of the myogenic differentiation marker embryonic myosin heavy chain (eMHC)/myosin, heavy polypeptide 3 (Myh3) was significantly higher in the GAS of mdx:Smpd3-/- (#238) mice than in mdx mice (p < 0.05), but not in #11 mice, although their expression level was significantly lower at 19 weeks (Additional file 16: Fig. S9H, p < 0.05). In addition, at 9 weeks, expression of myogenin (MyoG), another myogenic differentiation marker, in mdx:Smpd3-/- (#238) mice was significantly higher than in

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**Fig. 5** nSMase2/Smpd3 regulates fiber size and fusion in vivo. a, b Fiber size distribution in wt, mdx, and mdx:Smpd3-/- (#11 and #238) mice at 6 (a) and 20 (b) weeks of age. c, d The proportion of fibers containing the indicated number of nuclei per fiber in wt, mdx, and mdx:Smpd3-/- (#11 and #238) mice at 6 weeks of age (c) and wt, mdx, and mdx:Smpd3-/- (#11 and #238) mice at 20 weeks of age (d).
mdx mice (p < 0.05), whereas at 19 weeks it was significantly lower (Additional file 16: Fig. S9H, p < 0.01). At 9 weeks, Mef2d expression in the GAS of mdx: Smpd3−/− mice was significantly higher than in mdx mice (p < 0.05), but at 19 weeks, their expression of Mef2c was significantly lower than that of mdx mice (Additional file 16: Fig. S9H, p < 0.05). At 14 weeks, the expression of Pax7 in mdx:Smpd3−/− (#238) was significantly lower than in mdx mice (Additional file 16: Fig. S9H, p < 0.05). At 12 weeks of age, the mitochondrial marker Tfam was significantly upregulated in mdx: Smpd3−/− (#238) mice relative to mdx mice, but the reverse was true at 19 weeks (Additional file 16: Fig. S9H, p < 0.05). Expression of myoglobin at 9 and 12 weeks of age was higher in mdx:Smpd3−/− (Additional file 16: Fig. S9H, p < 0.05). The expression of Myf5 and CD34, the earliest markers of myogenic commitment, was slightly higher in mdx:Smpd3−/− (#238 and #11) mice at 14 weeks of age than in mdx mice (Additional file 16: Fig. S9H). To test the specification of muscle fiber types, the expression of myosin heavy chain (MyHC) isoforms (types I, IIa, Iib, and IIX) were quantified using real-time PCR. At 12 weeks, expression of slow/oxidative type I and fast/oxidative type IIa fibers were unaffected by the loss of nSMase2/Smpd3 in the mdx genetic background, but that of fast/glycolytic type Iib and fast/intermediate type IIX was higher in mdx:Smpd3−/− mice than in mdx mice (Additional file 16: Fig. S9H). In 9-week-old mdx: Smpd3−/− mice, levels of Pgc-1alpha and troponin 1 (Tnnt1) were significantly lower than in mdx mice (Additional file 16: Fig. S9H, p < 0.05). The atrophy-associated genes Foxo1, Foxo3, MuRF, and Gdf8 were also upregulated in mdx:Smpd3−/− mice relative to mdx mice (Additional file 16: Fig. S9H). However, at 12 weeks, expression of the Klfl5 gene was significantly lower in mdx:Smpd3−/− (#11) mice than in mdx mice (Additional file 16: Fig. S9H, p < 0.001). Expression of the Hgf gene in the GAS of mdx:Smpd3−/− mice was higher than in mdx mice, but that of Jag1, Notch1, and Notch3 was lower (Additional file 16: Fig. S9H).

Combined, these findings suggest that nSMase2/Smpd3 knockdown might induce the regeneration of dystrophic muscle early in life, but that the opposite effect might be induced later on.

**Effects of expression of muscle-specific miRNAs on muscle health**

It was recently reported that treatment of 10 DMD patients with an antisense oligonucleotide (NS-065/NCNP-01) that induces skipping of exon 53 in mutated dystrophin transcripts increased the dystrophin/spectrin ratio [34]. Using the sera of 10 DMD patients who had been administered with intravenous NS-065/NCNP-01 for 12 weeks, we quantified their expression of a set of muscle-abundant miRNAs (myomiRs) before and after this treatment. The median expression levels of miR-1, miR-133a, and miR-206 in all 10 patients after treatment were approximately 25%, 67%, and 20% lower than before treatment, respectively, but these results were not significant; only that of miR-133a approached significance (p = 0.08; Additional file 17: Fig. S10A–C).

We then divided the 10 patients into three groups according to the dose of NS-065/NCNP-01 they received (1.25, 5, or 20 mg/kg weekly). We compared the fold-change in the miRNA levels before (“pre”) and after (“post”) treatment and calculated these as log_2[post/pre]. At a weekly dose of 5 mg/kg, the levels of all three miRNAs after treatment were lower than before treatment, and levels of miR-133a were reduced after treatment in all three groups (Additional file 17: Fig. S10D–F).

It has been shown that recovery of the dystrophin protein via the skipping of exon 23 in the mature dystrophin transcript normalizes serum myomiR levels [35, 36]. Given that NS-06/NCNP-01 induces the skipping of exon 53 in the dystrophin transcript and increases dystrophin protein expression, our results suggest that the restoration of extracellular myomiR levels is associated with the pathology of dystrophic muscles. To assess the association of serum extracellular vesicle (EV) content with muscular damage, we analyzed serum EV content in patients with seven different types of muscular disorder, and found that it was substantially lower in DMD patients than in Becker muscular dystrophy (BMD) patients (Additional file 17: Fig. S10G).

We then investigated these effects in our mouse lines. To assess whether deletion of the nSMase2/Smpd3 gene in mdx mice restores myomiR levels, we quantified serum miR-1, miR-133a, and miR-206 levels in mdx: Smpd3−/−, mdx:Smpd3−/−, and mdx mice. Serum miR-1 and miR-206 levels (Additional file 17: Fig. S10H) and exosomal miR-133a levels (Additional file 17: Fig. S10I) in mdx:Smpd3−/− mice were lower than in mdx mice. This was not the case for mdx:Smpd3−/− mice at 9 and 14 weeks of age (Additional file 17: Fig. S10J–L), but their serum miR-133a levels were significantly higher than those of mdx mice at 19 weeks (Additional file 17: Fig. S10L). However, at 9, 14, and 19 weeks, serum levels of the muscular regeneration marker miR-31 in mdx: Smpd3−/− mice were significantly higher than in mdx mice (Additional file 17: Fig. S10M).

Next, to analyze whether loss of the nSMase2/Smpd3 gene in the mdx background affects muscular dystrophy via muscle-abundant miRNAs, we quantified the levels of the myomiRs miR-1, miR-133a, and miR-206 in the GAS muscles of mdx and mdx:Smpd3−/− (#11 and #238) mice. The expression levels of miR-1 and miR-133a in
mdx mice were lower than in wt mice, but that of miR-206 was higher (Additional file 18: Fig. S11). In contrast, levels of miR-1 and miR-133a were substantially higher in mdx:Smpd3−/− (#238) mice than in mdx mice, while those of miR-206 were lower. mdx:Smpd3−/− (#11) mice, on the other hand, exhibited no substantial differences in this respect from mdx mice (Additional file 18: Fig. S11).

We then investigated whether the benefits of the miRNA regulated by the nSMase2/Smpd3 gene could lead to a recovery in the survival of myogenic cells. To address this issue, we incubated H2K cells in serum-depleted medium with or without the nSMase inhibitor GW4869 (0, 5, 10, 15, 20, 25, or 30 μM). Survival rates of myoblasts incubated for 24 h with 10–30 μM of GW4869 were significantly higher than in cells incubated without GW4869 (Additional file 19: Fig. S12A). We then assessed the effect of the nSMase2/Smpd3 protein on H2K cell survival under the overexpression of myomiRs by culturing H2K cells that had or had not been transfected with myomiRs. The survival of H2K cells transfected with miR-1 was significantly poorer than that of cells without it, whereas significantly more of the H2K cells cultured with miR-206 survived than those without it (Additional file 19: Fig. S12B). We then assessed the effect of the nSMase2/Smpd3 protein on H2K cell survival under the overexpression of myomiRs by culturing H2K cells that had or had not been transfected with miR-1, miR-133a, or miR-206 with GW4869. The survival of those transfected with miR-133a was better than that of non-transfected cells (Additional file 19: Fig. S12C). In addition, the number of EVs in the supernatant of H2K cells was significantly higher than that in the supernatant of C2C12 cells (Additional file 19: Fig. S12D). The protein content of serum-derived EVs from mdx:Smpd3−/− mice was significantly lower than that of mdx mice (Additional file 19: Fig. S12E and F).

Loss of the nSMase2/Smpd3 gene in mdx mice modulates anxiety behavior
To investigate the effects of the nSMase2/Smpd3 gene on emotional responses, mdx and mdx:Smpd3−/− mice at 11–12 weeks of age were restrained for 10 s as an emotionally aversive stimulus. The mice were then released into a cage, and their behavior was monitored for 5 min for freezing vs locomotion. All the mdx mice froze for at least half of the 5-min period, whereas half of the mdx:Smpd3−/− (#238) mice froze for much less than half of the time (Fig. 6a). Thus, the defense response was partially rescued in the mdx:Smpd3−/− (#238) mice. We also investigated anxiety, emotionality, and the adaptive stress response to a novel environment using the home-board test. The mdx mice performed significantly fewer head dips and exhibited significantly greater head-dip latency than both wt and mdx:Smpd3−/− (#238) mice (Fig. 6b, c). The mdx mice also traveled a substantially shorter total distance than both of the other lines, although this difference was only significant in the comparison with the wt mice (Fig. 6d).

Next, to assess the role of the nSMase2/Smpd3 gene in spontaneous behavior and reactivity in a novel environment, the open-field test was performed on 10-week-old wt, mdx, mdx:Smpd3−/− (#238), and Smpd3−/− (#238) mice. Although the mdx and Smpd3−/− (#238) mice moved significantly more slowly and covered less distance than the wt mice, there was no significant difference between the mdx and mdx:Smpd3−/− (#238) mice (Fig. 6e, f). However, in the first bin, the mdx:Smpd3−/− (#238) mice covered substantially less distance than the mdx mice (Fig. 6g), and overall, they and the Smpd3−/− mice covered significantly less distance and moved for significantly less time than the mdx mice (Fig. 6h, i). However, all three of these lines covered significantly shorter distances and spent less time in motion than wt mice (Fig. 6h, i). There were no significant differences in movement time, total time spent in the center of the field, or average speed among any of the four lines (Fig. 6j–l). Combined, these findings suggest that ablation of the nSMase2/Smpd3 gene in mdx mice modulates their anxiety behavior and stress response.

Roles of Bdnf, miRNA, and anxiety-associated genes in the abnormal behavior of mdx mice
To analyze whether nSMase2/Smpd3 plays a role in the regulation of Bdnf expression in the hippocampus, we performed western blotting using an anti-Bdnf antibody on samples from mdx and mdx:Smpd3−/− mice at various ages. There were no significant differences in Bdnf protein levels between mdx and mdx:Smpd3−/− mice aged 5, 12, or 19 weeks (Additional file 20: Fig. S13A, C, and D). However, at 9 weeks, the Bdnf protein level in mdx mice was significantly lower than that of both wt and mdx:Smpd3−/− mice (Additional file 20: Fig. S13B).

Next, to assess the effects of the nSMase2/Smpd3 protein on the transcription levels of the Bdnf isoforms in the hippocampus of mdx mice, we performed real-time qPCR using nine exon-specific primers in wt, mdx, and mdx:Smpd3−/− mice aged 12 weeks (Additional file 20: Fig. S13E). In mdx mice, expression levels of five isoforms of Bdnf, exons I, II, V, VI, and VIII, were lower than in wt mice (Additional file 20: Fig. S13F). In other hand, Bdnf protein expression levels of all isoforms excluding exon IX were higher than in mdx mice (Additional file 20: Fig. S13G).

To assess myomiR-mediated regulation of Bdnf expression levels, we analyzed the transcriptional levels of myomiRs in the hippocampus. Although there were no significant differences in precursor-microRNA-1 levels between mdx and mdx:Smpd3−/− mice at 5–19 weeks of age (Additional file 20: Fig. S13H), primary-microRNA-1
levels in \textit{mdx}:\textit{Smpd3}^{+/-} (#238 and #11) mice at 12 weeks were significantly lower than in \textit{mdx} mice (Additional file 20: Fig. S13I). Although there were no significant differences in precursor-microRNA-133a levels between \textit{mdx} and \textit{mdx}:\textit{Smpd3}^{+/-} mice at 12 weeks of age (Additional file 20: Fig. S13J), the precursor-microRNA-206 levels of \textit{mdx}:\textit{Smpd3}^{−/−} (#11) mice at this age were significantly higher than those of \textit{mdx} mice (Additional file 20: Fig. S13K). To investigate the expression levels of genes induced in the hippocampus, we performed an expression analysis of 12-week-old \textit{wt}, \textit{mdx}, \textit{mdx}:\textit{Smpd3}^{+/-}, and \textit{Smpd3}^{−/−} mice using real-time qRT-PCR. The expression levels of dopamine receptor d1 (Drd1), cholecystokinin (Cck), the ionotropic glutamate receptor NMDA2B (NR2B), and postsynaptic density protein 95 (PSD-95) genes in \textit{mdx}:\textit{Smpd3}^{+/-} mice were significantly lower than in \textit{mdx} mice (Additional file 20: Fig. S13L, \textit{p} < 0.05). In addition, the expression levels of Erg1 and Arc genes in \textit{mdx}:\textit{Smpd3}^{−/−} (#238) mice were slightly higher than those in \textit{mdx} mice (Additional file 20: Fig. S13M). Expression levels of the dopamine receptor d2 (Drd2) and GIT Arf-GAP 1 (Git1) genes were higher in \textit{mdx}:\textit{Smpd3}^{−/−} (lines #238 and #11, respectively) mice than in \textit{mdx} mice.
These findings suggest that the loss of the nSMase2/Smpd3 gene may regulate the anxiety phenotype of mdx mice via the regulation of Bdnf expression.

**Discussion**

In this study, to investigate the role of the nSMase2/Smpd3 gene in the dystrophic phenotypes of mdx mice, mutant mice with a deletion in the LIR of the nSMase2/Smpd3 protein were crossed with mdx mice to produce nSMase2/Smpd3 dystrophin DKO mice. The DKO mice exhibited a reduced inflammation responses and less muscular degeneration in the early stages of the dystrophic process, but had exacerbated muscular necrosis in the later stages. We also found that the Bdnf pathway modulated the anxiety and stress responses of these DKO mice.

The nSMase/Smpd3 family of enzymes comprises four members: nSMase1, nSMase2, nSMase3, and mitochondria-associated nSMase. With the exception of nSMase3, these enzymes possess a DNase I-type catalytic core, suggesting a common mechanism for sphingomyelin catalysis [37]. Further, it has previously been shown that the mRNA expression level of nSMase3 in brain is higher than that of nSMase1 or nSMase2 [38]. We observed a relatively large degree of variation in SMease activity in the hippocampi of line #11 mdx:Smpd3−/− mice. This may have been due to the functional redundancy of other SMases.

We found less inflammation in the SM of mdx: Smpd3−/− mice than in mdx mice. The acute phase of the pathology of mdx muscles (prior to four weeks of age) involves muscle inflammation with a bias toward M1 macrophages, which contributes to oxidative stress and muscle fiber lysis via the production of iNOS-derived NO, and promotes inflammation and myoblast proliferation via the production of Th1 cytokines [28, 39]. Later, at the age of 3 months, the arginase-expressing M2a macrophages that compete with iNOS for arginine can begin to reduce M1 macrophage cytotoxicity, and the muscles enter the regenerative phase.

The SM of dystrophin-deficient (DMDmdx) rats has been shown to be infiltrated by leukocytes, whose kinetics during the pathological course were parallel to those of serum CK levels in the SM, and the numbers of CD45+ mononuclear leukocytes at 4–16 weeks of age were significantly higher than in wt rats [27]. Of these increased muscle CD45+ mononuclear cells in Dmdmdx rats, approximately 90% were CD68+ macrophages. In addition, the mononuclear cells from these rats expressed higher levels of transcripts for the cytokine TNF-alpha, which is associated with early muscle damage, than the same cells from wt rats. The inflammatory cytokines TNF-alpha, IL-6, and IL-1beta lead to increased endothelium permeability and promote early recruitment of innate immune cells, such as neutrophils and monocytes, which differentiate locally into inflammatory macrophages within injured tissues. Thus, leukocyte infiltration in the muscles of DMD model animals is associated with damaged muscle fibers and elevated serum CK levels.

In our study, expression levels of CD68, CD45, IL-6, and TNF-alpha in the SM of 12-week-old mdx:Smpd3 DM mice were significantly lower than in mdx mice. This was also true for serum CK levels in 6–18-week-old mdx:Smpd3 DM mice. These findings may suggest lower numbers of infiltrating mononuclear cells in mdx:Smpd3 DM mice. In addition, the tissue recruitment of monocytes is mainly mediated by the chemokine Ccl2 and the chemokine receptor CCR2 or CCR5 via the blood circulation [40]. A deficiency in CCR2 in the SM of mdx:CCR2 KO mice markedly and persistently reduced the infiltration of Ly6Chigh inflammatory monocytes, which enter tissues in response to injury and differentiate into inflammatory M1 macrophages within inflamed tissues, relative to mdx controls, leading to a reduction in muscle fiber necrosis and endomysial fibrosis at 14 weeks of age [41]. On the other hand, in mdx:CCR2 KO mice the abundance of intramuscular Ly6Cflow macrophages, which patrol the vascular space with homing properties under steady-state conditions and comprise over 90% of resident macrophages, was significantly lower than in mdx controls during the early stages (4–9 weeks of age), but from 14 weeks to 6 months, it was similar to the mdx mice. The improvement in necrosis and fibrosis in the limb muscles of the mdx:CCR2 KO mice was not sustained during the latter phase, possibly because of unsuppressed levels of profibrotic growth factors such as osteopontin.

It is likely that a mixture of monocyte-derived and tissue-resident macrophages accumulate in chronically inflamed tissues. However, the origins of the Ly6Chigh and Ly6Clow monocytes/macrophages in dystrophic muscles are unclear. Although bone marrow has been thought to be the principal source of monocytes, it was recently reported that splenic Ly6Chigh monocytes, which are outsourced from the bone marrow, contribute to recruitment and infiltration in dystrophic limb muscles, and to muscle fiber necrosis, during the early stages of the disease [42]. A reduction in infiltrated CD45+ cells in mdx mice improved muscle fiber necrosis and increased eMHC-positive regenerating fibers under a lack of splenic monocytes induced by splenectomy during the early phases of the condition. However, during the late stages, dystrophic muscle regeneration is impeded by reduced angiogenesis and increased fibrosis. This causes a delay in the phenotypic shift from proinflammatory to proregenerative macrophages, which affects the tissue cytokine environment. Thus, the optimal response to chronic tissue injury in dystrophic muscle depends on
the fine balance between the phenotypes of macrophage types, and the cytokine environment may be critical for the progression of the pathology.

It has also been shown that nSMase2/Smpd3 deficiency or inhibition strongly suppresses M1 macrophage infiltration and differentiation, and inhibits inflammation, in a mouse model of atherosclerosis via Nrf2 (NF-E2-related factor 2)/HO-1 pathway activation. In this pathway, the rapid nuclear translocation and accumulation of Nrf2 protein was promoted to inhibit early cytokine response, such as IL-1beta and IL-6. This may occur via persistent Akt phosphorylation through the reduction of the ceramide-induced activation of PP2A (protein phosphatase 2) activity and the suppression of the expression of inflammatory and adhesion genes such as Cc2l and ICAM-1 and/or ceramide [20, 43]. Given these reports, the beneficial effects in the early stages and adverse effects in later stages in our mdx:Smpd3 DKO mice may have been caused partially by the inhibition of monocyte/macrophage recruitment and inflammatory responses. Furthermore, older mdx:Smpd3 DKO mice might display more severe dystrophic phenotypes, such as necrosis and fibrosis, which could more clearly be distinguished from the effects seen in younger mdx:Smpd3 DKO mice, such as the increased serum CK levels in 28-week-old mdx:Smpd3 DKO (#238 and #11) mice and the increased number of EBD-positive fibers in 20-week-old mdx:Smpd3 DKO (#11) mice.

Splenectomized mdx and mdx:CCR2 DM mice do not show improvements in muscle strength at 14 weeks or 6 months [41, 42]. On the other hand, in our study, the running performance of the mdx:Smpd3 DM mice at 16 and even 60 weeks in the treadmill exhaustion test was better than that of the mdx mice. Combined, this evidence suggests that suppression of the inflammation of the monocytes/macrophages may not contribute to the improvement in muscle performance in the nSMase2/ Smpd3-deficient mdx mice.

In general, degenerative/necrotic lesions in the dystrophic muscles of mdx mice exist in small clusters, possibly due to an imbalanced tissue environment caused by the repeated degeneration–regeneration cycles, during which inflammatory cells such as monocytes and macrophages are recruited and infiltrate muscle cells. The pathologic changes in mdx:Smpd3 mice during the early stages were spatiotemporally restricted to specific areas, suggesting that the main cause of the pathologic changes in the SM of mdx:Smpd3 mice may be the inhibition of monocyte/macrophage recruitment into the dystrophic lesions. However, during the later stages, the dystrophic lesions in the SM of mdx:Smpd3 mice were sparse but worsened despite the improvements in muscle performance. This indicates that the changes in the dystrophic pathology within their SM, such as susceptibility to exercise-induced injury, oxidative stress, and impaired regeneration capacity, may have been caused by multifactorial pathways. Possible candidates are decreased cytosolic and mitochondrial calcium concentrations, calpain inactivation [17], and inhibition of phosphorylation of the Stat1/Stat3 transcriptional factor, in addition to the sustained inhibition of Lyc6C<sup>high</sup> monocyte/macrophage recruitment and suppressed anti-inflammatory differentiation. Additionally, the dual CCR2/CCR5 chemokine receptor antagonist has been shown to reduce macrophage infiltration and decrease the prevalence of regenerated CNFs, which are thought to be a marker of previous necrosis–regeneration events, in the diaphragm of mdx mice [44]. Thus, the significant reduction in CNFs containing multiple nuclei in the mdx:Smpd3 DM mice may also have been caused by the inhibition of monocyte/macrophage infiltration.

The inhibition of TRPV2 (transient receptor potential cation channel), which is a principal Ca<sup>2+</sup>-entry route, leads to a sustained Ca<sup>2+</sup> increase and muscle degeneration in two DMD mouse models [45]. Dystrophic pathologies, such as increased abundance of CNFs, variability in fiber size, increased Ca<sup>2+</sup> levels in muscle fibers, elevated serum CK levels, and reduced muscle performance, are all ameliorated by the inhibition of TRPV2 in the early stages (4–10 weeks), when the degeneration–regeneration cycles are ongoing. However, in old mice (>26 weeks), when the potential for such cycles may have been exhausted, the improvements in abundance of CNFs and fiber size variability are slight [45]. Also, since necrosis could play a critical role in mediating the myocyte and myofiber loss associated with calcium dysregulation, the inhibition of cyclophilin D which directly regulates the changes in mitochondrial permeability that depend on calcium and reactive oxygen species causes noticeable improvements in muscular dystrophies, such as reductions in CNFs, fibrosis, and myofiber necrosis [46].

Dystrophic phenotypes such as the increased abundance of CNFs, fibrosis, calpain activation, and serum CK levels that are seen in mdx mice at 6 weeks to 3 months have been shown to be improved by SM-specific overexpression of sarcoplasmic reticulum Ca<sup>2+</sup> ATPase 1 (SERCA1). This enzyme reverses a defect in sarcoplasmic reticulum Ca<sup>2+</sup> reuptake that causes dystrophic fibers and reduced total cytosolic Ca<sup>2+</sup> [31]. In addition, reducing sarcolipin, which is an inhibitor of SERCA and is abnormally elevated in both the slow- and the fast-twitch SM of DMD patients and animal models, mitigates dystrophic phenotypes and improves muscle regeneration via the restoration of SERCA function [47]. Increased expression of intramuscular heat shock protein 72 (Hsp72) also ameliorates the dystrophic pathway and preserves muscle strength, via an interaction with SERCA that enhances its function [48]. On the other
hand, nSMase2/Smpd3 deficiency suppresses the ceramide-dependent activation of protein phosphatase 2a (PP2a) that maintains Akt phosphorylation, thereby inducing hyaluronan synthase 2 (HAS2) and Hsp72 expression [49, 50]. The amelioration of dystrophic phenotypes in young mdx:Smd3 DM mice may therefore contribute to protection from mitochondrial Ca^{2+}-overload-induced myofiber necrosis.

It has also been shown that C5a, sICAM-I, IL-1ra, IL-16, Ccl2, TIMP-1, and TNF-alpha levels are higher in mdx mice than in wt mice [32]. The levels of all seven of these cytokines and chemokines in the GAS muscle of the mdx:Smpd3/− mice were lower than in the mdx mice, as were the expression levels of TNF-alpha and IL-6. A C5a inhibitor rescued the decreased force and increased the abundance of necrotic fibers in the mdx mice via a change to fast-twitch fibers, and also increased the maturation of macrophages [32]. A coordinated balance between pro-inflammatory and anti-inflammatory macrophages is important for successful muscle repair. However, in dystrophic muscle, this balance of inflammatory responses might be disrupted. Bdnf is produced by immune cells such as CD4⁺ and CD8⁺ T lymphocytes and monocytes/macrophages. It is modulated by TNF-alpha and IL-6, which are located near regenerating fibers that are positive for p75NTR, and CD56/NCAM owing to the repair of tissue in inflamed muscle [51, 52]. The Bdnf–p75NTR axis positively regulates the tissue-protection response [52].

The crosstalk between immune and muscle cells, such as macrophages and SCs, can positively regulate homeostasis, proliferation, and the repair of myogenic cells via chemokines and cytokines that originate from infiltrated monocytes/macrophages [52]. The macrophages play either a supportive or a deleterious role in cells via the amplification or downregulation, respectively, of inflammatory responses that promote the elimination of myogenic debris and prevent excessive tissue damage. The role they play depends on their activation state, which is affected by changes in their environment. However, their role in either the promotion or mitigation of the pathogenesis of dystrophy is unclear. In our study, Bdnf protein expression was higher in the GAS of mdx mice than in both wt and mdx:Smpd3/− mice at 9 and 14 weeks of age. Thus, the infiltration of circulating monocytes might be reduced in mdx:Smpd3/− mice.

It has been shown that repression of Bdnf synthesis depends on cell differentiation in the SM [53]. Bdnf was highly expressed in muscle Pax7-positive SCs and myoblasts in culture, whereas its expression was absent or very low in myofibers, and was repressed after myogenic differentiation. In addition, the overall levels of Bdnf mRNA are strongly correlated with those of a progenitor marker, Pax3, in mature muscles. By reducing endogenous levels of Bdnf, myoblasts engage in early myogenic differentiation, despite the presence of growth media. This evidence indicates that the primary role of Bdnf in SM is to maintain the population of SCs by preventing their myogenic differentiation.

The expression levels of the Myog and eMHC/Myh3 genes in the GAS of the mdx:Smpd3/− mice were higher in the early stages and lower in the later stages than in the mdx mice. In addition, expression levels of the early myogenic markers Myf5 and CD34 were higher in the GAS of mdx:Smpd3/− mice than in mdx mice. However, Pax7 expression in the GAS of mdx:Smpd3/− mice was significantly lower than in mdx mice. Also, it has been shown that Pax7 expression is directed by the SM-specific miR-431, which promotes myogenic differentiation via the upregulation of MyoG and mitochondrial transcription factor A (Tfam) [54]. However, the expression level of Myf5 was not affected by miR-431. This suggests that Pax7 expression in mdx:Smpd3 DKO mice may be regulated by miR-431.

These results imply that the severity of the dystrophy in mdx:Smpd3/− mice may be ameliorated via modulation of the differentiation balance of SCs in dystrophic muscles. It has been shown that chronic overactivation of Notch signaling occurs in severely dystrophic muscles with impaired muscle regeneration [55]. In this study, mdx:Smpd3/− mice exhibited downregulation of the Jag1, Notch1, and Notch3 genes relative to mdx mice. Jag1, induced by IL-1beta, suppresses the muscle regeneration capacity of DMD muscles, probably through Notch3 activation [56]. This evidence suggests that nSMase2/Smpd3 regulates muscle regeneration with SCs via the Notch and Pax7 pathways.

We also analyzed the defensive behavior of the mdx: Smpd3/− mice and found that the abnormal behavior exhibited by mdx mice was completely reversed in half of the DKO mice observed. In addition, the mdx: Smpd3/− mice performed significantly fewer head-dips (a marker of anxiety behavior in the hole-board test) than the mdx mice. In the CA1 region of the hippocampus of mdx mice, abnormal synaptic plasticity caused by a reduction in gamma-aminobutyric acid (GABA) efficacy has been reported [14, 57]. nSMase2/Smpd3 inhibition blocks TNF-alpha-induced excitatory postsynaptic currents from CA1 pyramidal cells [29]. In our study, Bdnf protein expression in the hippocampus of mdx mice was significantly lower than in wt mice, but loss of the nSMase2/Smpd3 gene ameliorated this effect. Bdnf secretion promotes GABAergic synaptogenesis [58]. Synaptic GABA_{A}R's are sensitive to benzodiazepines, drugs with robust anti-anxiety effects that bind exclusively to GABA_{A}R's. They are predominantly located within non-lipid raft fractions and enhance the potentiating effects of benzodiazepines by impairing lipid raft integrity. This
suggests that the localization of receptors in lipid rafts affects the potency and efficacy of neurotransmitter signaling and that this plays a role in neurological disorders [59, 60]. Thus, the association of lipid rafts with GABA<sub>A</sub>-R appears to be a downregulatory mechanism for selective synaptic transmission and plasticity. In addition, the disruption of lipid rafts blocks the potentiating inhibitory effects of Bdnf in GABA<sub>A</sub>-R signaling via Bdnf-induced recruitment of the TrkB receptor into neuronal lipid rafts, creating selective synaptic plasticity [61, 62].

**Conclusion**

In summary, our study shows that ablation of the nSmase2/Smpd3 gene in mdx mice ameliorates membrane instability in the sarcolemma, improves muscle force and performance, and reduces excess inflammation in the early stages. Furthermore, the abnormal stress response of mdx mice was modulated by the loss of the nSmase2/Smpd3 gene, possibly via Bdnf signaling. These findings suggest that signaling pathways modulated by the nSmase2/Smpd3 protein through lipid rafts might be novel therapeutic targets for DMD, via the stage-specific regulation of the expression levels of this protein and transcript, for example through exosomal transfer.

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**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12916-020-01805-5.

Additional file 1: Table S1. PCR primers for real-time PCR.

Additional file 2: Table S2. Nucleotide sequences of guide RNA for the nSmase2/Smpd3 gene.

Additional file 3: Table S3. Oligonucleotide sequences for genotyping the nSmase2/Smpd3 and the dystrophin genes.

Additional file 4: Table S4. Muscular disease patients analyzed in this study.

Additional file 5: Table S5. Smase activity, inflammation area from TA sections, live-animal optical imaging, serum CK levels, number of EBD-positive fibers, force in grip test, body weight, running times in treadmill test, caspase-3 activity, caspase-9 activity, myofibers levels in ASO-treated DMD patients, exosome levels in patients of muscular diseases, freezing times in restrain test, hole board test, and open field test.

Additional file 6: Table S6. Sample sizes and statistical powers.

Additional file 7: Fig. S1. Expression of the Smase/Smpd gene family during C<sub>2</sub>C<sub>12</sub> differentiation, and generation of nSmase2/Smpd3 KO mice in the mdx genetic background using the CRISPR-Cas9 system. (A) Expression of nSmase2/Smpd1, nSmase2/Smpd2, nSmase2/Smpd3, and Myh1 in C<sub>2</sub>C<sub>12</sub> myotubes differentiated from day 1 to day 6, measured by real-time RT-PCR, which were normalized to glyceraldehyde 3-phosphate dehydrogenase (gapdh) gene expression. (B) Expression of the nSmase2/Smpd3 and Bdnf proteins in C<sub>2</sub>C<sub>12</sub> myotubes differentiated from day 1 to day 6, detected by western blotting. Heat shock protein 90 (Hsp90) and gapdh were included as loading controls. Expression levels of nSmase2/Smpd3 and Bdnf were normalized by the expression level of Gapdh at each timepoint, and arbitrary units (AU) represent the band intensities in the western blot (n = 2). (C) Top: Amino acid sequences of the wt nSmase2/Smpd3 proteins and the six deletion mutants generated by the CRISPR-Cas9 system (#11, #20, #26, #28, #30, and #238). Amino acid deletions and substitutions are indicated in red. Phosphoserine sites are shown in green. Numbers on the top line show the amino acid positions. The asterisk represents a stop codon. Bottom: The domain structure of the nSmase2/Smpd3 protein, consisting of two N-terminal hydrophobic segments (H51 and H52: 1–84), a cytoplasmic juxtamembrane region (JX: 85–118), two catalytic domains (CD1 and CD2: 119–175 and 340–651), and a large insertion region (175–340). Red triangles indicate the targets of the sgRNAs. (D) Nucleotide sequences of the genomic region of exon 3 of the Smpd3 gene in wt (bottom) and six deletion and/or insertion mutant mice (#11, #20, #28, #30, and #238) derived from (upper) the CRISPR-Cas9 system. Red and blue reverse triangles show deletion or/and insertion start and end sites, respectively. The identity of each mutant mouse line is indicated by the number preceded by # in the upper left corner of each nucleotide sequence.

Additional file 8: Fig. S2. Generation of nSmase2/Smpd3 KO mice in the mdx genetic background using the CRISPR-Cas9 system. (A) Genotyping results of the dystrophin gene in wt and mdx mice using mutation-specific primers that can detect the point mutation in the dystrophin gene. (B) Levels of the dystrophin protein in the tibia anterior of wt and mdx mice, which were mated with Smpd3<sup>−/−</sup> mice to produce mdx:Smpd3<sup>−/−</sup> mice.

Additional file 9: Table S9. Founder mice.

Additional file 10: Fig. S3. (A, B) Levels of the nSmase2/Smpd3 protein and the unrelated protein Gapdh in the gastrocnemius muscle (GAS) (A) and cerebellum (B) of wt, mdx, and mdx:Smpd3<sup>−/−</sup> mice detected by western blotting. (C) Levels of the nSmase2/Smpd3 protein and the unrelated protein Gapdh in the hippocampi of wt, mdx, and mdx:Smpd3<sup>−/−</sup> mice at the indicated ages, detected by western blotting. (D, E) nSmase2/Smpd3 enzymatic activities from the skeletal muscle (SM) (D) and hippocampus (E) of wt, mdx, and mdx:Smpd3<sup>−/−</sup> mice from the indicated mouse lines (n = 3). * p < 0.05.

Additional file 11: Fig. S4. Total array images of the expression of cytokines and chemokines in gastrocnemius (GAS) muscle (A) and diaphragm (B) of 12-week-old mdx (upper) and mdx:Smpd3<sup>−/−</sup> (lower) mice. (C) The corresponding positions of each molecule within the array.

Additional file 12: Fig. S5. The expression of inflammation-related genes in the diaphragm of wt, mdx, and mdx:Smpd3<sup>−/−</sup> mice at 12 weeks of age was measured via real-time RT-PCR (n = 3 per genotype). * p < 0.05, ** p < 0.01.

Additional file 13: Fig. S6. Real-time PCR analysis of inflammation gene markers in the gastrocnemius (GAS) muscle of mdx and mdx:Smpd3<sup>−/−</sup> (#11 and #238) mice at the indicated ages. The number of animals used is indicated in parentheses. * p < 0.05, ** p < 0.01.

Additional file 14: Fig. S7. Smpd3 ablation reduces sarcolemmal instability in the muscles of young mdx mice but exacerbates it in older mice. (A–C) Serum CK levels of wt, mdx, mdx:Smpd3<sup>−/−</sup> (#11, #20, #26, #28, and #35), mdx:Smpd3<sup>−/−</sup> (#30), Smpd3<sup>−/−</sup> (#11, #26, #28, and #35), and Smpd3<sup>−/−</sup> (#11) mice at 6 (A #26), 10 (#26, #20, #28, and #35), and 18 (C) #30) weeks of age. The number of animals used is indicated in parentheses. * p < 0.05, ** p < 0.01, *** p < 0.001.

Additional file 15: Fig. S8. Mice lacking the nSmase2/Smpd3 gene in the mdx genetic background showed enhanced muscle performance. The average time spent running on the treadmill for wt, mdx, mdx:Smpd3<sup>−/−</sup> (#398), and Smpd3<sup>−/−</sup> (#29) mice at 60 weeks of age are shown. The number of animals used is indicated in parentheses. * p < 0.05, ** p < 0.01.

Additional file 16: Fig. S9. Effects of loss of the nSmase2/Smpd3 gene in mdx mice on the proliferation, differentiation, and survival of myogenic cells. (A) Caspase-3 activity in the gastrocnemius (GAS; blue circles) and tibialis anterior (TA; yellow circles) muscles and cerebellum (orange circles); and caspase-9 activity in the GAS muscle (blue triangles) were measured in wt, mdx, and mdx:Smpd3<sup>−/−</sup> mice at the indicated ages. Representative western blot analysis (upper) and quantitation of Bdnf (B–F) and caveolin-3 proteins (E) (lower) in the gastrocnemius (GAS) muscle of mdx and mdx:Smpd3<sup>−/−</sup> mice at 5 (B, 9), 12 (D, 14), and 18 (F) weeks of age. (G) Expression in the GAS of wt, mdx, and mdx:Smpd3<sup>−/−</sup> mice at 12 weeks of age was measured via real-time RT-PCR (n = 3 per
Additional file 17: Fig. S10. miRNA levels in the sera of muscular dystrophy patients and mdx mice as diagnostic markers. miR-1 (A), miR-133a (B), and miR-206 (C) levels in the serum of Duchenne muscular dystrophy (DMD) patients before (pre) and after (post) treatment with the antisense oligonucleotides NS-065/NCNP-01 that induce exon skipping to correct the frame-shift. Post-treatment expression levels expressed as fold-changes relative to pre-treatment expression levels (based on A–C) of miR-1 (D), miR-133a (E), and miR-206 (F) at doses of 1.25 mg/kg (cohort 1), 5 mg/kg (cohort 2), and 20 mg/kg (cohort 3) in patients administered weekly with NS-065/NCNP-01 for 12 weeks. Expression levels were normalized to those of miR-16. The number of patients analyzed is indicated in parentheses. Extracellular vesicles (EVs) were extracted from the sera of patients with seven types of muscle disorder (DMD, Becker muscular dystrophy [BMD], distal myopathy with rimmed vacuoles [DMRV], facioscapulohumeral muscular dystrophy [LGMD], and limb-girdle muscular dystrophy 2B [LGMD2B]) and quantified based on acetylcholinesterase (AChE) activity (G). Levels of miR-1, miR-133a, and miR-206 in the sera (H) and in EVs isolated from the sera (I) of mdx and mdxSmpd3+/− mice, with expression levels normalized to spiked-in cel-39. Levels of miR-1 (J), miR-206 (K), miR-133a (L), and miR-31 (M) in the sera of mdx and mdxSmpd3−/− mice, normalized to U6. The number of animals used is indicated in parentheses.

Additional file 18: Fig. S11. miR-1, miR-133a, and miR-206 levels in the GAS of wt, mdx, and mdxSmpd3+/− mice at 12 weeks of age were analyzed using real-time qPCR.

Additional file 19: Fig. S12. (A) H2K myotubes differentiated for three days were incubated with or without GW4869 (5, 10, 15, 20, 25, and 30 μM) in serum-depleted medium for 24 h. (B) H2K myotubes differentiated for six days and transfected with miR-1, miR-133a, and miR-206 were incubated in serum-depleted medium with or without GW4869 (25 μM) for four days. (C) Extracellular vesicles (EVs) were extracted and quantified based on acetylcholinesterase (AChE) activity from H2K and C2C12 cells. Data presented are the mean ± standard error (SE) of absorbance at 450 nm of CCK-B. Each independent experiment was repeated at least three times. (D) EV content of the sera of 12-week-old wt, mdx, and mdxSmpd3−/− mice. (E) EVs were extracted from the sera of the mice (n = 6), mdx (n = 6), and mdxSmpd3−/− mice (n = 12) and quantified based on AChE activity. *p < 0.05, **p < 0.01.

Additional file 20: Fig. S13. Expression analysis in the hippocampus of mdxSmpd3−/− mice. (A) Representative western blot analysis (top) of whole hippocampal protein homogenates from wt, mdx, and mdxSmpd3−/− mice at 5 weeks (A), 9 weeks (B), 12 weeks (C), and 19 weeks (D) of age using anti-Bdnf and anti-Gapdh antibodies. The ratio of mBdnf/Gapdh is expressed as arbitrary units [AU] (bottom). (E–G) Expression of Bdnf isoforms in the hippocampi of wt, mdx, and mdxSmpd3−/− mice at 12 weeks of age based on real-time RT-PCR. (E) Exon/intron structure and alternative transcripts of the mouse Bdnf gene. Exons are indicated as boxes and introns are indicated as lines. Filled black regions in the boxes indicate the translated regions, in which ATG represents the translated start codon. The expression levels of each Bdnf isoform from exon 1 to exon IX are shown as log2 (fold-changes) in mdx mice relative to wt mice (F) and in mdxSmpd3−/− mice relative to mdx mice (G). The levels of four myomiRs, precursor-microRNA-1 (H), primary-microRNA-1 (I), precursor-microRNA-133a (J), and precursor-microRNA-206 (K), were analyzed in the hippocampi of Smpd3+/− (#11 and #238) and mdx mice at 12 weeks of age. (L) Expression of genes induced in the hippocampi of wt, mdx, and mdxSmpd3−/− mice at 12 weeks of age was measured via real-time RT-PCR (n = 3 per genotype). *p < 0.05, **p < 0.01. (M) Expression levels of the Nfatc1, Gabar2, Egr1, Arc, Ddx2, and Git1 genes in the hippocampi of mdxSmpd3−/− and mdx mice. *p < 0.05.

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Authors’ contributions

Conception and design of the work: YM, MS, YA, HK, TI, ST, and KH; methodology: YM, JT, YO, DY, MS, SM, MT, RY, YA, YUI, TI, and KH; acquisition, analysis, and/or interpretation of the data: YM, JT, YO, DY, MS, SM, MT, RY, YA, YUI, TI, and KH; patient recruitment: YA, HK, AJ, YO, ST, and KH; original draft: YM and KH; review and editing: YM and KH. All authors participated in the critical review of the manuscript and approved the submitted version.

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Availability of data and materials

Datasets generated for this study are included in the article and Supplementary Material, and all experimental data are available upon reasonable request.

Ethics approval and consent to participate

This study was conducted in accordance with the guidelines set out in the Declaration of Helsinki and was approved by the Research Ethics Committee of the NCNP (approval ID: A2011-113). Informed consent was obtained from all participants before they were enrolled in the trial. All mice lines used in this study were treated in accordance with the guidelines provided by the Ethics Committee for the Treatment of Laboratory Animals of the National Center of Neurology and Psychiatry (approval ID: 2015006), which has adopted the three fundamental principles of replacement, reduction, and refinement.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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