Absence of UCHL 1 function leads to selective motor neuropathy

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Abstract

Objective: The aim of this study was to investigate the role of ubiquitin C-terminal hydrolase-L1 (UCHL1) for motor neuron circuitry and especially in spinal motor neuron (SMN) health, function, and connectivity. Methods: Since mutations in UCHL1 gene leads to motor dysfunction in patients, we investigated the role of UCHL1 on SMN survival, axon health, and connectivity with the muscle, by employing molecular and cellular marker expression analysis and electrophysiological recordings, in healthy wild-type and Uchl1nm3419 (UCHL1−/−) mice, which lack all UCHL1 function. Results: There is pure motor neuropathy with selective degeneration of the motor, but not sensory axons in the absence of UCHL1 function. Neuromuscular junctions (NMJ) are impaired in muscle groups that are innervated by slow-twitch or fast-twitch SMN. However, unlike corticospinal motor neurons, SMN cell bodies remain intact with no signs of elevated endoplasmic reticulum (ER) stress. Interpretation: Presence of NMJ defects and progressive retrograde axonal degeneration in the absence of major SMN soma loss suggest that defining pathology as a function of neuron number is misleading and that upper and lower motor neurons utilize UCHL1 function in different cellular events. In line with findings in patients with mutations in UCHL1 gene, our results suggest a unique role of UCHL1, especially for motor neuron circuitry. SMN require UCHL1 to maintain NMJ and motor axon health, and that observed motor dysfunction in the absence of UCHL1 is not due to SMN loss, but mostly due to disintegrated circuitry.

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Introduction

To date five different patients are identified with mutations in their ubiquitin C-terminal hydrolase-L1 (UCHL1) gene. The first two patients displayed Parkinson-like symptoms, with defects in their motor function and spasticity in the legs.1 Recently, three siblings with very early neurodegeneration associated with upper motor neuron dysfunction were identified to have Glu7Ala mutation in their UCHL1 gene.2 These clinical reports sparked interest in exploring the role of UCHL1 function for motor neuron circuitry and motor neuron diseases, especially the ones in which voluntary movement is impaired.

UCHL1 is critically important for protein homeostasis due to its dual hydrolase and ligase functions, by adding or removing ubiquitin to poly-ubiquitin chains.1,3,5 This is a unique ability of UCHL1 in maintaining free ubiquitin levels and a central role within the ubiquitin proteasome system (UPS) in neurons.3,5 Defects in protein turnover and UPS has been closely associated with motor neuron diseases, and it is suggested that motor neurons heavily depend on the proper function of the UPS.7–11 Therefore, investigating the role of UCHL1, especially for motor neurons, and within the context of motor neuron diseases is important.

To date numerous mouse models are developed and they revealed important information about the involvement of UCHL1 in both neurodegeneration and cellular proliferation.12–15 For example, the UCHL1 knockout mouse with a targeted deletion of the exons 6 through 8 of the UCHL1 gene showed its requirement for the normal structure and function of the neuromuscular junctions (NMJ).12 In line with reports that demonstrate motor dysfunction in Uchl1 nm3419 (UCHL1+/-) mice, which lack all UCHL1 function,15 we recently defined a unique importance of UCHL1 for motor neuron circuitry.16 Mice that lacked UCHL1 developed motor dysfunction as early as postnatal day (P) 40, and corticospinal motor neurons (CSMN) displayed an early and profound degeneration with spine loss and disintegration of apical dendrites. However, the role of UCHL1 on the spinal component of motor neuron circuitry remained elusive.

To reveal the potential link between UCHL1 function and motor circuitry, we investigated spinal motor neuron (SMN) health and connectivity using electrophysiology, histology and molecular marker expression analysis in healthy as well as diseased mice that lacked all UCHL1 function. Our findings revealed defects in the motor, but not the sensory branch of the femoral nerve and disintegration of NMJ. Interestingly, despite early and profound CSMN degeneration, SMN soma remained intact in the spinal cord. NMJ in soleus, and extensor digitorum longus (EDL) muscles, which are mainly innervated by slow-twitch and fast-twitch SMN, respectively, displayed profound disintegration in the absence of UCHL1 function. In vivo recordings further delineate the early functional defects even when neurons are intact and display no signs of increased endoplasmic reticulum (ER) stress or neuron loss. Altogether, our studies suggest that UCHL1 plays different roles in CSMN and SMN and that circuitry defects are more relevant to developing disease pathologies in the absence of UCHL1.

Materials and Methods

Mice

All animal procedures were approved by Northwestern University Animal Care and Use Committee, and conformed to the standards of the National Institutes of Health. Uchl1 nm3419 (UCHL1+/-) mice identified by G. Cox at The Jackson Laboratory carry a spontaneous 795 base-pair intragenic deletion that results in the removal of the final 24 base-pair of exon 6 and the first 771 base-pairs of intron 6 (Fig. 1C). Heterozygous mice (UCHL1+/-) were viable, fertile, and were bred together to generate UCHL1 deficient (UCHL1-/-) mice. All mice were on the C57BL/6J background. Survival times and motor function defects were comparable between males and females with 100% penetrance. Primers used to determine genotype are UCHL1 forward: ttgacggctgtgtgtgctaatg, WT reverse: ctaagggaaggg

Tissue collection and histology

Mice were deeply anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) and perfused with 4% PFA in...
Spinal cords were isolated from WT and UCHL1/C0 in dissociation solution. Tissue was homogenized in mice at P100. Lumbar spinal cords were quickly dissected in cold PBS. Tissue was homogenized in sodium cacodylate buffer, and 1-μm-thick serial sections were collected in a cryostat parallel to the muscle fiber. Gastrocnemius muscle was dehydrated in alcohol series, embedded in paraffin, and 4-μm-thick cross sections were collected at the Northwestern University Mouse Histology and Phenotyping Laboratory (n = 3 for P40, P65, P80, and P100). Nissl, hematoxylin–eosin (H&E), and nonspecific esterase staining (NSE) staining were performed as previously described.17 Motor and sensory branches of the femoral nerve and the ventral roots were isolated at P200 (n = 3), postfixed in 4% PFA in sodium citrate, pH 9.0, at 80°C. Spinal cords were sectioned = 3), postfixed in 4% PFA at 4°C. Total protein lysate (10 μg; determined by BCA Kit; Pierce, Rockford, IL) were resolved by 7.5% SDS-PAGE, transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, Marlborough, MA), and immunoblotted with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-UCHL1 (1:1000; ProteinTech, Rosemont, IL, cat # 14730-1-AP, lot # 00024921), anti-jNK (1:250; Cell-Clicking, Danvers, MA, cat # 9252S, lot # 15), anti-p-jNK (1:1,000, Cell Signaling, Danvers, MA, cat # 4668S, lot # 11), and anti-ac- tin (1:1,000; Millipore, Temecula, CA, cat # MAB1501R, lot # 2521458). The membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Grand Island, NY) for 30 min at 37°C and washed with PBS-T. Blots were developed by enhanced chemiluminescence (Thermo Scientific, Grand Island, NY) using HRP-conjugated secondary antibodies.

**Immunocytochemistry**

Immunocytochemistry was performed on every 6th section of mouse spinal cords, and on continuous serial sections for soleus, and EDL muscle. Primary antibodies were purchased from Millipore unless otherwise stated. Antibodies: anti-ChAT (choline acetyltransferase) (1:500, cat # AB144, lot # 2234133); anti-UCHL1 (1:1000; ProteinTech, Rosemont, IL, cat # 14730-1-AP, lot # 00024921), anti-NCAM (1:1000, cat # AB2503, lot # LV1487828); anti-NF-H (1:1000, cat # AB5539, lot # 2519335); anti-PERK (1:1000; Cell Signaling Technology, Danvers, MA, cat # C33E10, lot # 5); anti-PDI (1:1000; Cell Signaling Technology, Danvers, MA, cat # C81H6, lot # 2); and anti-synaptophysin (1:200; Chemicon, Temecula, CA, cat # MAB368, lot # 3388841), anti-calretinin (1:500, cat # AB5054, lot # 2465124), anti-p-jNK (1:50, Cell Signaling, Danvers, MA, cat # 4668S, lot # 11), anti-S100 (1:500, DAKO, Carpinteria, CA, cat # Z031129-2, lot # 00084964). α-bungarotoxin ([α-BTX], 1:500; Invitrogen, Grand Island, NY, cat # B35451, lot # 1724058) was applied together with anti-NF-H primary antibody. Antigen retrieval was performed for PERK and PDI immunocytochemistry; sections were treated with 0.01 mol/L sodium citrate, pH 9.0, at 80°C water bath for 3 hr prior to incubation with primary antibody. After PBS washes, either fluorescent conjugated (goat AlexaFlour 488, Cy3) or biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) were used. When using biotinylated secondary antibodies, ABC Kit (Vector Labs Inc., Burlingame, CA), and DAB substrate (Vector Labs Inc., Burlingame, CA) were used for detection, as directed by the manufacturer. For p-jNK immunocytochemistry was performed as previously described.19 For UCHL1/S/100 co-immunohistochemistry, a sequential protocol was
used; sections were first incubated with rabbit anti-UCHL1 primary antibody, followed by goat anti-rabbit FAB fragments (Jackson ImmunoResearch, West Grove, PA) to change the antibody species, and donkey anti-goat Alexa Fluor 647. Sections were then incubated with rabbit anti-S100 (DAKO, Carpinteria, CA, cat # Z031129-2, lot # 00084964) and α-BTX conjugated with AlexaFluor 568 (1:500; Invitrogen, Grand Island, NY, cat # B35451, lot # 1724058), and S100 was detected using donkey anti-rabbit AlexaFluor 488 conjugated secondary antibody. In another set of experiments S100 primary antibody was omitted to control for cross-reactivity of secondary antibodies.

Electron microscopy

Mice were perfused with EM grade 4% PFA. Motor and sensory branch of femoral nerve were dissected out and postfixed in 2% PFA and 0.5% glutaraldehyde overnight. Tissue was then postfixed in buffered 2% OsO4 rinsed with distilled water and stained in 1% uranyl acetate, again rinsed with distilled water, dehydrated in ascending grades of ethanol with transition fluid propylene oxide and embedded in resin mixture with Embed 812 and cured in a 60°C oven for 3 days. Tissue was mounted on resin block, and was sectioned on a Leica Ultracut UC6 ultramicrotome. 70-nm-thin sections were collected on resin block, and was sectioned on a Leica Ultracut UC6 ultramicrotome. 70-nm-thin sections were collected on 200 mesh copper-palladium grids. Grids were counter stained with 8% radioactive depleted uranyl acetate for 20 min. Grids were examined using FEI Tecnai Spirit G2 TEM, and digital images were captured on an FEI Eagle camera.

In vivo electrophysiological recording from motor neuron units

WT and UCHL1−/− mice (n = 3 for postnatal day (P) 40 and P80) were anesthetized with an IP injection of pentobarbital sodium (70 mg/kg), and thereafter maintained by IV infusion of supplemental doses of pentobarbital (6 mg/kg) as needed. A tracheotomy was performed, and mice were artificially ventilated with pure oxygen (SAR-830/P ventilator; CWE, Ardmore, PA). The end tidal PCO2 was maintained around 4% (MicroCapstar; CWE, Ardmore, PA). The heart rate was monitored, and the central temperature was kept at 38°C using an infrared heating lamp. A catheter was introduced in the external jugular vein for anesthesia supplementation and fluid infusion. All the nerves of the hindlimb were dissected and cut, except for the branches innervating the triceps surae muscle (gastrocnemius medialis and lateralis, and soleus), which were left intact, and mounted on a monopolar- electrode. The distal tenden of the triceps surae was dissected free and sutured to an isometric force transducer (BG-100, Kulite Instruments, Leonia, NJ). The length of the muscle was adjusted so that the twitch response was maximal. A pair of fine silver wires was inserted under the fascia of the triceps surae to record electromyography activity. The force and the compound muscle action potential produced by stimulation of the nerve at low frequency (0.5 Hz) and at varying intensity were recorded for offline analysis, as previously described.

Imaging and quantification

Nikon SMZ1500 and Nikon Eclipse TE2000-E fluorescence microscopes equipped with Intensilight C-HGFI (Nikon Inc., Melville, NY) were used. Epifluorescence images were acquired using a Digital Sight (DS)-QHMC CCD camera (Nikon Inc.), and light images were acquired using a Ds-Fi1 camera (Nikon Inc.). Confocal images were collected using a Zeiss 510 Meta confocal microscope (Carl Zeiss Inc., Thornwood, NY).

The total number of ChAT-stained neurons were counted and averaged from at least five cervical and lumbar spinal cord sections, blinded to the genotype (n = 4). Statistical differences were determined by one-way analysis of variance (ANOVA) with post hoc Tukey’s multiple comparison parametric test.

Number of total and degenerating axons in the toluidine blue stained nerve cross sections were quantified by two independent observers, blinded to the genotype (n = 3). Degenerating axons were defined based on their dark cytoplasmic inclusions, disintegration of their myelin sheet, and engulfment by phagocytes. Thin sections containing two entire femoral nerve branches (n = 3) were imaged using a 63× objective. Images were analyzed in Image J [http://imagej.nih.gov/ij/] using G-ratio calculator 1.0 plugin to calculate G ratios and axon diameters. Statistical differences were determined by unpaired t-test.

Soleus and EDL muscles were sectioned serially at 30 μm. Quantifications were done on at least 100 NMJ per subject by two independent observers, blinded to the genotype (n = 3) at p40 and p100. Reported is percent of innervation considered as a full merge of α-BTX and anti-NF-H and percent healthy considered as the NMJ ability to maintain its pretzel-like secondary structure. Statistical differences were determined by one-way ANOVA with post hoc Tukey’s multiple comparison parametric test.

Statistical analysis

All statistical analyses were performed using Prism software (version 6; Graphpad Software Inc., La Jolla, CA). Statistically significant differences were determined after
either one-way ANOVA with post hoc Tukey’s multiple comparison tests or t-test considered. Statistically significant differences were considered at least $P < 0.05$, and values were expressed as the mean ± SEM.

**Results**

**Spinal motor neuron soma remain intact in the absence of UCHL1 function**

UCHL1 is a neuronal protein expressed in neurons (Fig. 1A), but not the Schwann cells and myelin sheaths surrounding the motor axons (Fig. S1) in the mouse spinal cord. UCHL1 protein is detected in both the cytoplasm and the nucleus of WT spinal motor neurons (SMN) that are located in the ventral horn of the spinal cord and express ChAT (Fig. 1A). In addition, UCHL1 is present in SMN axons (Fig. 1B), which extend toward muscle targets and innervate muscle fibers at the site of neuromuscular junctions (NMJ). Motor end-plates, visualized by α-BTX staining, are innervated by SMN axons, co-localized with neurofilament-H (NF-H) and UCHL1 immunocytochemistry (Fig. 1B), further confirming presence of UCHL1 even at the site of NMJ. SMN numbers remained comparable between WT and UCHL1−/− mice at P200 (WT cervical: 54 ± 3; lumbar: 51 ± 4; UCHL1−/− cervical: 44 ± 5; lumbar: 47 ± 6; n = 4; one-way ANOVA followed by Tukey’s multiple comparisons test; Fig. 2D), revealing a lack of prominent SMN degeneration in the absence of UCHL1 function.

These striking differences between CSMN and SMN, and lack of prominent SMN loss suggested SMN might not be as vulnerable as CSMN are in the absence of UCHL1 function. Since mice displayed hindlimb paralysis, we focused our attention to the lumbar section of the spinal cord, and because CSMN displayed apical dendrite degeneration and spine loss in the absence of UCHL1,16 we decided to investigate whether neurons in spinal cord suffered a similar loss of synaptic input. Synaptophysin is used to mark locations of synapses on ChAT+ SMN (Fig. 2C) and calretinin+ interneurons (Fig. S2) in WT and UCHL1−/− mice at P200. The numbers of synaptophysin+ puncta per 10 μm of SMN soma length were comparable between WT (lumbar: 1.3 ± 0.3; n = 4) and UCHL1−/− mice (lumbar: 1.4 ± 0.1; n = 3, two-tailed unpaired t-test; Fig. 2E). Similar to SMN, there was no significant difference in the numbers of synaptophysin+ puncta per 10 μm of interneuron (calretinin+) soma length in WT (lumbar: 1.4 ± 0.1 n = 2) and UCHL1−/− mice (lumbar: 1.3 ± 0.2: n = 3, two-tailed unpaired t-test). In addition, unlike CSMN,16 SMN did not display any signs of increased ER stress. The expression of PERK, and PDI, two prominent markers of increased ER stress were comparable between WT and UCHL1−/− SMN (Fig. 2F; Fig. S3). Our results show that the average number of synapses on SMN soma was not altered in UCHL1−/− mice suggesting that SMN and CSMN were affected differently in the absence of UCHL1 function. Since SMN did not display major cell loss in UCHL1−/− mice, which develop motor dysfunction, we reasoned presence of potential defects in sensory input.

**Axonal degeneration is restricted to motor axons**

Sensory input is one of the major modulators of motor function in the spinal cord. Some of the phenotype that
is observed as “motor dysfunction” in mice could indeed stem from sensory input defects. To investigate the potential contribution of the sensory system to the observed motor function deficits, motor and sensory branches of the femoral nerve and the ventral root were examined (Fig. 3A), and the extent of axonal degeneration was quantified. The total numbers of either axons in sensory branch of the femoral nerve (WT: 865.7 ± 11.8, n = 3; UCHL1−/−: 897 ± 17.2, n = 3; Fig. 3B and E) or proximal motor axons at the level of ventral root (WT: 951 ± 89, n = 3; UCHL1−/−: 1004 ± 114, n = 5; Fig. 3C and G) were comparable between WT and UCHL1−/− mice even at P200. In addition, no signs of degeneration were detected in the femoral nerve sensory branch (WT: 9 ± 3%, n = 3; UCHL1−/−: 9.4 ± 0.1%, n = 4; Fig. 3F) and in the ventral root (WT: 13.5 ± 1.3, n = 3; UCHL1−/−: 16 ± 0.7%, n = 5; Fig. 3H). Even though the total numbers of distal motor axons at the level of femoral nerve (Fig. 3D) were comparable between WT and UCHL1−/− mice (WT: 459 ± 13, n = 3; UCHL1−/−: 448 ± 14, n = 3, Fig. 3I), there was significant axonal degeneration only in the motor branch of the femoral nerve in UCHL1−/− mice (WT: 6 ± 2%, n = 3; UCHL1−/−: 22 ± 3%, n = 3; P = 0.0146, two-tailed unpaired t-test; Fig. 3J). To further assess selective degeneration in the motor branch of the femoral nerve, we measured the G-ratio and axon diameters. G-ratio is a measure of myelin thickness that is proportional to the size of the fiber based on the inner area/total area of the axon fiber. G-ratio is significantly reduced in the motor branch of the femoral nerve (WT, 0.674 ± 0.009, n = 3; UCHL1−/−, 0.607 ± 0.008, n = 3; P = 0.0048, two-tailed unpaired t-test; Fig. 3K). As hypermyelination (decreased G-ratio) could result either due to increased thickness of myelin layer or pathological shrinkage of the axonal caliber,23 we next evaluated the axon size distribution of the femoral motor axons. Analysis of axonal diameter also revealed a decline especially in the percentage of the large axons with a diameter of >7.5 μm (3–3.5 μm range: WT, 7.7 ± 0.8 μm; UCHL1−/−, 12.7 ± 1.3 μm; P = 0.0271,
Figure 3. There is selective, progressive and directional motor axon degeneration in the absence of UCHL1−/− function. (A) A schematic diagram showing a cross section of the spinal cord with both sensory and motor axons. SMN axons are present in the motor branch of the femoral nerve and in the ventral root, whereas the sensory axons of the DRG are present in the sensory branch of the femoral nerve. Toluidine blue staining of the motor branch of the femoral nerve (B), ventral root (C), and the sensory branch of the femoral nerve (D) at P200 in WT and UCHL1−/− mice. Boxed areas are enlarged to the side. (E-J) Average number of total axons and the average percentage of degenerating axons in the motor branch of the femoral nerve (E-F), ventral root (G-H), and the sensory branch of the femoral nerve (I-J). (K) G-ratio quantification of the femoral nerve motor branch axons. (L) Distribution of femoral nerve motor branch axons based on axon diameter. Scale bars: B, C, and D = 100 μm; B and C inset = 20 μm; D inset = 10 μm. *P < 0.05, **P < 0.01, ***P < 0.001.
two-tailed unpaired t-test; 7.5–8 μm range: WT, 6.9 ± 0.4 μm; UCHL1−/−, 1.9 ± 0.2 μm; P = 0.0004, two-tailed unpaired t-test; 8–8.5 μm range: WT, 5.9 ± 1.2 μm; UCHL1−/−, 1.2 ± 0.2 μm; P = 0.0186, two-tailed unpaired t-test; 8.5–9 μm range: WT, 4 ± 1.2 μm; UCHL1−/−, 0.4 ± 0.2 μm; P = 0.0484, two-tailed unpaired t-test; n = 3, WT: 1908 axons, n = 3, UCHL1−/−: 1160 axons, Fig. 3L).

EM analysis revealed that cytoplasmic composition of both motor and sensory branch of the femoral nerve were comparable between WT and UCHL1−/− mice (Fig. 4A and G). The orientation of neurofilaments and microtubules were normal, and mitochondria were present (Fig. 4B-D, F and H). However, axons in the motor branch (Fig. 4B-F) showed severe shrinkage (atrophy) of the cytoplasmic area. In some axons the atrophy of the cytoplasmic area was mild (Fig. 4C), whereas in others, especially in larger caliber nerves, it was massive, leading to the collapse of myelin sheath and a lobular appearance (Fig. 4B, D and E). In few cases, the axons were totally absent from the myelin sheath (Fig. 4E) which folds onto itself. In final stage the axon was scavenged by macrophages (Fig. 4F). Altogether, these results demonstrate selective axonal defects only in the motor, but not in the sensory axon, which rule out the possibility that the observed motor dysfunction is primarily related to sensory abnormalities. Moreover, axonopathy is observed only in the distal motor axons at the level of femoral nerve, but not proximal to SMN cell bodies at the level of the ventral root, suggesting the presence of a progressive and directional axonal degeneration from periphery toward soma in the absence of UCHL1 function. This striking selective motor, but not sensory, axonopathy suggested potential defects at NMJ.

**UCHL1 is required for maintaining the structure of the NMJ**

Even though UCHL1−/− mice grow with comparable size and rate with their littermates, there is progressive muscle atrophy only at later ages (Fig. S4A). Because UCHL1 is not expressed in the WT muscle (Fig. 1F), and axonal degeneration is detected only in the motor axons, muscle atrophy is thought to be a consequence of NMJ defects. Visualization of the motor end-plates with α-BTX and the presynaptic innervation by NF-H immunocytochemistry revealed profound structural defects in two major muscle groups only in UCHL1−/− mice (Fig. 5A-B). The EDL muscle, which is mainly innervated by fast-twitch SMN revealed profound NMJ defects in the absence of UCHL1 function (P40 WT: 82 ± 4.5% innervated, 97.7 ± 1.3% healthy, n = 3; P100 WT: 65.3 ± 10.5% innervated, 97 ± 2.1% healthy, n = 3; P40 UCHL1−/− mice: 84 ± 3.1% innervated, 94.7 ± 0.9% healthy, n = 3; P100 UCHL1−/− mice: 30.3 ± 4.2% innervated, 54.3 ± 14.8% healthy, n = 3; EDL innervation: P40 UCHL1−/− versus P100 UCHL1−/− adjusted P value = 0.0014, P100 WT versus P100 UCHL1−/− adjusted P value = 0.018; E.D.L. health: P40 UCHL1−/− versus P100 UCHL1−/− adjusted P value = 0.022, P100 WT versus UCHL1−/− adjusted P value = 0.016; one-way ANOVA followed by Tukey’s multiple comparisons test; Fig. 5C,E and F). Likewise, soleus muscle, which is mainly innervated by slow-twitch SMN displayed major NMJ defects (P40 WT: 97 ± 2.5% innervated, 89.3 ± 5.2% healthy, n = 3; P100 WT: 81 ± 6.6% innervated, 93 ± 1% healthy, n = 3; P40 UCHL1−/− mice: 79.7 ± 7.9% innervated, 86.7 ± 2.9% healthy, n = 3; P100 UCHL1−/− mice: 39.3 ± 6.8% innervated, 48.3 ± 6.8% healthy, n = 3; soleus innervation: P40 UCHL1−/− versus P100 UCHL1−/− adjusted P value = 0.0085, P100 WT versus P100 UCHL1−/− adjusted P value = 0.007; soleus health: P40 UCHL1−/− versus P100 UCHL1−/− adjusted P value = 0.0016, P100 WT versus P100 UCHL1−/− adjusted P value = 0.0006, one-way ANOVA followed by Tukey’s multiple comparisons test; Fig. 5D,G and H). In addition, denervated muscle fibers were observed in the gastrocnemius muscle from UCHL1−/− mice by nonspecific esterase (NSE) staining and neural cell adhesion molecule (NCAM) immunocytochemistry (Fig. 5B and D). Similar NMJ defects were reported in ubiquitin-specific protease 14 (Usp14) deficient ataxia (αα) mice, due to an increase in c-Jun N-terminal kinase (JNK) phosphorylation. Therefore, we decided to investigate JNK and phospho JNK (pJNK) levels in UCHL1−/− mice. There was an increase in pJNK levels in the UCHL1−/− spinal cord lysates compared to WT at P100 (an increase between 25 and 62% was observed in three independent experiments; Fig. S5A-B). pJNK was also detected at the NMJ of WT mouse EDL muscle at P200 (Fig. S5C) suggesting their possible involvement, although more experiments are needed to investigate this further. These qualitative and quantitative findings in different muscle groups confirmed that UCHL1 was not primarily important for a subset of SMN population, but rather was a key molecule required by a broad range of SMN to maintain a functional NMJ, potentially through a mechanism involving JNK pathway.

**Transmission of excitation to the muscle fibers is less efficient in UCHL1−/− mice**

To investigate how these expressionanalyses correlate with in vivo motor function defects, we studied the electrical activity of muscle fibers in intact WT and UCHL1−/− mice at P40 and P80, using a novel in vivo...
Axon fibers in the motor branch of femoral nerve display selective axonal degeneration. (A) A representative cross section image of axons within the motor branch of the femoral nerve in WT mice. Axons with different sizes, and the intact myelin sheath around them is observed with electron microscope. (B) A representative cross-sectional image of axons within the motor branch of the femoral nerve in UCHL1−/− mice. Axons fibers with various degrees of degeneration are present. (C-E) Representative images displaying stages of axonal defects. (C) Initial stage of axon fiber collapse, (D) intermediary stage showing invagination of myelin sheath, and (E) a completely collapsed axon fiber. (F) An example of completely degenerated axon fiber being scavenged by macrophage. (G-H) Representative cross-sectional electron micrographs of sensory branch of the femoral nerve in WT (G) and UCHL1−/− (H) mice appear comparable, and both are devoid of major axonal defects. Scale bar: 2 μm.

Figure 4.
The nerves innervating the triceps surae (TS) muscle of WT and UCHL1−/− mice were stimulated and the electrical activity of muscle fibers (i.e., compound muscle action potential [CMAP]), as well as the force generated by the muscle in response to repeated low-frequency nerve stimulation were recorded (Fig. 6A). There was no difference between the delay of the CMAPs and the onset of force. The maximum force produced by the TS muscle in response to a single stimulus in the WT and UCHL1−/− mice was detected with high precision and indicated a lower force for UCHL1−/− than WT mice at P40 and P80 (Fig. 6B-C). To account for the muscle atrophy observed in UCHL1−/− mice (Fig. S4), we normalized the force by the weight of the TS muscle. The normalized force values were comparable between WT and UCHL1−/− mice at P40, but significantly lower in UCHL1−/− mice at P80 (Fig. 6B-C). In WT mice, the electrical activity of muscle was very consistent from stimulation to stimulation, and lasted for only a short period of time, displaying only a few peaks at P40 and P80. However, the profile of CMAPs was very different in UCHL1−/− mice, with the appearance of a high latency activity that could last for several tens of milliseconds (Fig. 6C). Furthermore, CMAPs showed
numerous jitter from stimulation to stimulation only in UCHL1−/− mice at P80 (n = 3). These in vivo recordings further revealed the importance of UCHL1 function not only for the formation, but also for the maintenance of NMJ function in a broad range of muscle groups. In the absence of UCHL1, there is gradual defect at the site of SMN and muscle interaction, which progressively results into directional motor axon degeneration that extends from NMJ to SMN soma. However, unlike CSMN, SMN cell bodies not vastly affected and maintain their integrity even at later stages.

**Discussion**

To date many different aspects of UCHL1 function and its involvement in a broad perspective of cellular events have been studied,6,13 and yet its distinct role and importance is not fully understood. Even though UCHL1 is a unique deubiquitinating enzyme (DUB) with abilities to add and remove ubiquitin from a protein, and is involved in many different cellular events, patients with mutations in their *UCHL1* gene display early neurodegeneration and motor dysfunction.1,2,25 Motor neuron diseases are one of the most complex diseases of the nervous system, not only because the motor neuron circuitry requires proper function and connectivity of many different neuron populations, but also because maintaining homeostasis of the system is a demanding act.

Patients with defective UCHL1 and that display spasticity in the legs and problems with locomotion and voluntary movement.1,2,25 Similarly, the UCHL1−/− mice have poor rotarod performance, decreased grip strength and abnormal gait, with complete hindlimb paralysis by P100, which is relatively early when compared with other mouse models for motor neuron diseases. We previously found that in the absence of UCHL1, CSMN display very early and profound degeneration with spine loss and disintegration of apical dendrites,16 suggesting a potential role for maintenance of spines and neuronal connectivity. Selective axonal degeneration in the same mouse is remarkable. In many diseases the motor and the sensory systems are affected either to a different degree or pattern. Axonopathy of distal ends of long central nervous system axons, especially upper motor neurons defines hereditary spastic paraplegia (HSP) syndrome. However, peripheral neuropathy has been described as either a rare or
common feature in more than a dozen genetic types of HSP. In our study, we find distal motor neuropathy but not motor-sensory neuropathy, in support of our observations, some HSP subjects exhibit strictly distal motor neuropathy. Axon length-dependent motor neuropathy is also a common feature of distal hereditary motor neuropathies (dHMN), and many forms of dHMN also have a significant upper motor neuron component. Delineating the sensory versus motor system defects in the same model system is rather complicated and challenging, and yet we found that in the absence of UCHL1 function, it is mainly the motor neuron circuitry that is affected, and the sensory axons remain healthy.

NMJ is a specialized synapse and UCHL1 was previously reported to be important for the maintenance of the structure and function of not only the NMJ, but also hippocampal neuron synapses. For example, inhibition of UCHL1 activity increases hippocampal neurons spine size, while decreasing spine density. Reduced hippocampal CA1 LTP was reported in another mouse model of UCHL1, accompanied by reduction in memory in passive avoidance learning and exploratory behavior. Inhibition of UCHL1 function by LDN reduced basal synaptic transmission and LTP in hippocampal slices. Moreover, exogenous UCHL1 rescues β-amyloid-induced decreases in synaptic function (LTP). These studies suggest that UCHL1 plays a broad role in maintaining synaptic structure and functional connectivity in many different neuron types in the cortex.

Interestingly, lack of UCHL1 function also resulted in increased ER stress mainly in CSMN as they failed to maintain their homeostasis and became vulnerable to degeneration. Even though ER stress was also increased in other cortical neurons, they were able to cope with the stress, whereas CSMN failed. However, Guanabenz, which other cortical neurons, they were able to cope with the stress, whereas CSMN failed. However, Guanabenz, which also hippocampal neuron synapses. Reduced hippocampal CA1 LTP was reported in another mouse model of UCHL1, accompanied by reduction in memory in passive avoidance learning and exploratory behavior. Inhibition of UCHL1 function by LDN reduced basal synaptic transmission and LTP in hippocampal slices. Moreover, exogenous UCHL1 rescues β-amyloid-induced decreases in synaptic function (LTP). These studies suggest that UCHL1 plays a broad role in maintaining synaptic structure and functional connectivity in many different neuron types in the cortex.

Interestingly, lack of UCHL1 function also resulted in increased ER stress mainly in CSMN as they failed to maintain their homeostasis and became vulnerable to degeneration. Even though ER stress was also increased in other cortical neurons, they were able to cope with the stress, whereas CSMN failed. However, Guanabenz, which alleviates ER stress, improved CSMN survival in vitro. So even though a broad spectrum of neurons required UCHL1 function for spine health and connectivity, it seem to play a unique role for CSMN, especially within the context of ER stress. This is no surprise as there is a direct relationship between problems with the UPS and increased ER stress.

In contrast to the profound and early CSMN degeneration, SMN remained intact in the spinal cord with no obvious cell loss in the same mice that lacked all UCHL1 function. This is not only interesting, but also is very informative. UCHL1 seem to play distinct roles in upper and lower motor neuron populations. CSMN could be more dependent on UCHL1 function for retaining its cellular cytoarchitecture, maintaining the integrity of its apical dendrite and the spine health, as well as controlling or maintaining ER stress at manageable levels, whereas, SMN may require UCHL1 function for other cellular events.

Unlike CSMN, SMN display no increase in ER stress in the absence of UCHL1 function, suggesting that UCHL1 is not as critically important for SMN, as it is for CSMN for managing ER stress. Different from CSMN, however, SMN display major axonal degeneration phenotype in the UCHL1−/− mice. AAV-mediated anterograde transduction of CSMN revealed lack of corticospinal tract axon defects in the absence of UCHL1 function and ruled out the possibility of a “dieback” mechanism for progressive CSMN degeneration. However, in the absence of UCHL1, there was indeed a “dieback” phenomenon for SMN axon fibers, which showed defects in the NMJ, in the axon bundles of motor branch of the femoral nerve, but not at the site of ventral root. Interestingly, CST axons did not degenerate before CSMN, and the presence of directional and progressive axonal degeneration was specific to the motor, and not to the sensory axons in the femoral nerve.

Previous studies documented NMJ defects in the absence of UCHL1 function, however, role of UCHL1 function on the health and stability of SMN remained elusive. In this study we began to elucidate a unique role for UCHL1 function in the lower motor neuron circuitry as revealed by denervated and unhealthy NMJ in the EDL and soleus muscles, which are two important muscle groups in the leg that are the site of disease initiation in some patients, and that are primarily innervated by different types of SMN. By investigating these two different muscle groups, we studied whether NMJ defects were more restricted to a subgroup of SMN population, but found a rather broad defect in both muscle groups. In vivo electrophysiological recordings from intact and alive mice further revealed motor unit defects especially in the hindlimb of UCHL1−/− mice. NMJ defects are the primary pathogenic event in ALS patients as well as the hSOD1G93A ALS mouse model. We now show, UCHL1−/− mice display severe HLP, muscular atrophy, accompanied by partial/complete denervation of the NMJ, and postsynaptic fragmentation.

UCHL1 is important for regulating free ubiquitin levels in neurons, and our data demonstrates its absence leads to NMJ denervation. Interestingly, another DUB, Usp14, which removes ubiquitin from proteosomal substrates to maintain intracellular free ubiquitin levels seems to have a very similar function. axO ataxia mice, which have a spontaneous deletion of the Usp14 gene have neuromuscular problems where NMJ undergo structural degeneration. Usp14 deficiency leads to an increase in K63-linked ubiquitination of mixed lineage kinase 3 (MLK3), which in turn leads to an increase in JNK phosphorylation. Our data demonstrates a similar increase in pJNK levels, suggesting a common mechanism for the importance of UPS system and ubiquitin homeostasis for
maintenance of proper NMJ structure and function. Moreover, in a mouse model of Huntington’s Disease, mutant huntingtin protein contributed to selective vulnerability of affected neuron populations by inhibiting fast axonal transport through increased JNK activity. Since CSMN and SMN are affected differently in the absence of UCHL1, it is potentially possible that alterations in the regulation of different JNK substrates in the two cell types might explain the different pathologies observed.

Despite severe NMJ defects and progressive axonal degeneration, there is very limited cellular pathology and SMN remain intact in the spinal cord, even by P200. Since we are accustomed to define pathology with cell loss, this is rather interesting. Building evidence suggests, however, that pathology is most correlated with loss of function, and even in the absence of cell loss there could be severe pathology.

In an attempt to study and understand complex diseases, we suggest to shift our attention from genes, but focus more on circuits as well as functional defects within different components of affected circuits in diseases. Here, we find that UCHL1 function is primarily important for the motor neuron circuitry. Even though CSMN and SMN need UCHL1 function for different cellular events, in its absence they both are affected and the whole motor circuitry becomes dysfunctional. This may in part explain why patients with mutations in their UCHL1 gene display early degeneration and motor defects.

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Author Contributions

B.G., J.H.J., M.M., C.J.D., C.J.H., and P.H.O. designed this study; B.G., J.H.J., M.C.S., M.J.S., M. M., M. G., J. L. K., G. S., C. J. D., and P.H.O. conducted experiments; G.A.C. shared UCHL1+/− mice; all authors collected and analyzed data; and B.G., J.H.J., M. C. S., and P.H.O. wrote the manuscript.

Conflict of Interest

The sponsor had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. UCHL1 is expressed in the motor axons at the ventral root (A,B) and femoral nerve (C,D) level, but not the myelin sheets (S100, green) wrapping around them. Figure S2. There is no difference in the synaptic input on to calretinin+ interneurons in the spinal cord. Figure S3. SMN of UCHL1—/— mice do not display increased ER stress. PERK (A) and PDI (B) expression levels are comparable between WT and UCHL1—/— SMN at P40 and P100. Figure S4. There is progressive muscle atrophy in the absence of UCHL1 function. Figure S5. pJNK levels are increased in spinal cord of UCHL1—/— mice.