The Onecut Transcription Factor HNF-6 Regulates in Motor Neurons the Formation of the Neuromuscular Junctions

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Abstract

The neuromuscular junctions are the specialized synapses whereby spinal motor neurons control the contraction of skeletal muscles. The formation of the neuromuscular junctions is controlled by a complex interplay of multiple mechanisms coordinately activated in motor nerve terminals and in their target myotubes. However, the transcriptional regulators that control in motor neurons the genetic programs involved in neuromuscular junction development remain unknown. Here, we provide evidence that the Onecut transcription factor HNF-6 regulates in motor neurons the formation of the neuromuscular junctions. Indeed, adult Hnf6 mutant mice exhibit hindlimb muscle weakness and abnormal locomotion. This results from defects of hindlimb neuromuscular junctions characterized by an abnormal morphology and defective localization of the synaptic vesicle protein synaptophysin at the motor nerve terminals. These defects are consequences of altered and delayed formation of the neuromuscular junctions in newborn mutant animals. Furthermore, we show that the expression level of numerous regulators of neuromuscular junction formation, namely agrin, neuregulin-2 and TGF-β receptor II, is downregulated in the spinal motor neurons of Hnf6 mutant newborn animals. Finally, altered formation of neuromuscular junction-like structures in a co-culture model of wildtype myotubes with mutant embryonic spinal cord slices is rescued by recombinant agrin and neuregulin, indicating that depletion in these factors contributes to defective neuromuscular junction development in the absence of HNF-6. Thus, HNF-6 controls in spinal motor neurons a genetic program that coordinates the formation of hindlimb neuromuscular junctions.

Introduction

The neuromuscular junctions (NMJ) are the synapses whereby spinal motor neurons (MN) control the contraction of skeletal muscles. The development of the NMJ is initiated by interaction of the growing terminal ends of MN, guided by pre-existing acetylcholine receptor (AChR) clusters at the muscle surface, with the plasma membrane of the myotubes. This initial interaction favors local clustering of AChR and stimulates in the myotubes the expression of genes encoding post-synaptic components. Organization of these components into a post-synaptic apparatus results in a concomitant remodeling of the motor terminal ends that ensures perfect apposition of pre-synaptic and post-synaptic complements [1,2,3]. Terminal Schwann cells surrounding the NMJ also contribute to support their formation and maintenance [4]. In limb muscles, NMJ formation starts around embryonic day (e) 16.5 and is completed around postnatal day (P) 14. NMJ formation is controlled by a complex interplay of multiple mechanisms coordinatedly activated in motor nerve terminals and in their target myotubes [3]. Agrin, a heparan sulfate proteoglycan, is released from nerve terminals and, through interaction with the low-density lipoprotein receptor-related protein 4, activates the Muscle-Specific Kinase complex. This stimulates the organization of the postsynaptic apparatus and, through rapsyn, clustering of the AChR. Hence, agrin counteracts the dispersal of AChR induced by ACh, contributing to stabilize the NMJ [5]. Neuregulins also contribute to stabilize the post-synaptic apparatus [6] and are required for development of the terminal Schwann cells that in turn promote NMJ maintenance [7,8]. Indeed, recent data suggest that terminal Schwann cells secrete...
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TGF-β that could increase agrin expression in MN [9]. Additional MN-secreted factors including members of the Wnt family and muscle derived signals like FGFs, GDNF and extracellular matrix proteins also contribute to NMJ maturation, stabilization and maintenance [10,11,12].

In MN, the transcriptional regulators that control the genetic programs involved in NMJ formation or maintenance remain unknown. Here, we provide evidence that the transcription factor Hepatocyte Nuclear Factor-6 (HNF-6) acts in MN to regulate the formation of the NMJ. HNF-6 belongs to the O-2 family of transcriptional activators [13,14,15,16]. These factors control cell differentiation and tissue morphogenesis in developing liver and pancreas [17,18,19,20,21,22]. Furthermore, they regulate neuronal identity, migration, maintenance or projections in different regions of the CNS [23,24,25,26,27] Onecut factors are present in MN from the onset of their differentiation until birth [28].

Here, we present evidence that HNF-6 acts in MN to regulate the formation of the NMJ. Indeed, mouse mutants for Hnf6 exhibit hindlimb locomotor defects associated with abnormal morphology of the NMJ and defective localization of the synaptic vesicle protein synaptophysin. These result from defective development and maturation delay of the hindlimb NMJ in the early post-natal period. Furthermore, the expression of factors essential for the formation or maturation of the NMJ is downregulated in Hnf6+/−/− mice aged 3 months.

The force of the hindlimb muscles was measured using an animal grip strength system (San Diego Instruments, USA), as described [30]. The Catwalk test was used to assess the gait of voluntarily walking mice (Catwalk 7, Nolus; Wageningen, The Netherlands) [31,32]. Five runs were performed per animal and gait parameters were taken into account only when the run time was inferior to 10 s [33].

**Materials and Methods**

**Animals**

All experiments were strictly performed respecting the European Community directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87-848/EEC). All mice were raised on our animal facilities and were treated according to the principles of laboratory animal care of the Animal Welfare Committee of the Université catholique de Louvain (Permit Number: UCL/MD/2009/008). Electromyography recordings, tissue perfusions and cervical dislocation were performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering. Hnf6 knockout (Hnf6−/−) mice were obtained as described [19]. As Hnf6+/− mice do not exhibit any abnormality, control animals will refer to either wildtype or Hnf6+/− individuals. Mice were genotyped by PCR (primers available upon request).

**Behavioral Tests**

All mice were handled and trained for at least two weeks before starting experiments. Animals were weighted each experiment day. Behavioral tests were applied separately for male and for female control or Hnf6−/− mice, starting when animals were aged 2 months. They were performed during five weeks. At birth, the Hnf6−/− mice were smaller than control littermates (Fig. 1A) and showed a reduced growth rate [19]. At later stages, this difference progressively normalized and around three months, the average weight of Hnf6−/− mice was similar to that of control mice (Fig. S2M).

The global neurological status of the animals was estimated based on a neurological score [29]. This neuroscore comprises 11 parameters with a maximal cumulative score of 35: general condition (maximum 4 points), paw placement onto a table top (maximum 4 points), ability to pull itself up on a horizontal bar (maximum 3 points), turnaround reflex (maximum 2 points), climbing on a inclined platform (maximum 4 points), grasp reflex (maximum 4 points), contralateral rotation when held by the base of tail (maximum 1 point), paw positioning (maximum 8 points), tail positioning (maximum 1 point), tilt head (maximum 1 point) and circling (maximum 3 points). For each parameter, the score was determined on a scale starting from 0 for severe impairment to the maximum score for healthy function. This score was applied to control or Hnf6−/− mice aged 3 months.

Tissue Preparation

Mice were anesthetized with ketamine (Ketalar 75 mg/kg) and xylazine (Rompun 5 mg/kg) solution. Mice were perfused transcardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Limbs muscles and the lumbar portion of the spinal cord were dissected, the latter was post-fixed overnight at 4°C in 4% PFA/PBS. Embryos at e18.5 were fixed in 4%PFA/PBS/0.002%NP40 at 4°C for 1 hour. Samples were rinsed three times in PBS and cryoprotected in 30% sucrose/PBS, except for limbs muscles in 20% sucrose/PBS, for 48 hours. Tissues were embedded in Tissue Tek (Prolabo, VWR) and cut (20 μm) using a Microm HM500 cryostat. Cryosections were dried at room temperature and stored at −20°C.

**Histological and Immunofluorescence Labelings, and Quantifications**

Tibialis anterior (TA), soleus, gastrocnemius and extensor digitorum longus (EDL) muscles were dissected at P70 and weighted. They were formalin-fixed, paraffin-embedded, cut (10 μm) and stained with hematoxylin/eosin. Pictures were acquired with an Olympus BX 60 fluorescence microscope (Camera Olympus XM10) with CellIF software. The muscle fiber cross-sectional area (CSA) was measured on 200 fibers for TA and 100 fibers for soleus in each animal (n = 3) using the ImageJ software. Immunofluorescence labelings on spinal cord cryosections were performed as described [28]. Muscle cryosections were treated with 0.1 M glycine in PBS for 30 min before processing. After washes in PBS, sections were permeabilized with PBS/0.3% TritonX-100 for 10 min and saturated with PBS/0.3% Triton/10% BSA for 45 min. The primary antibodies were diluted in the saturation solution and incubated O/N at 4°C. After washes in PBS/0.1% Triton, the secondary antibodies and α-bungarotoxin were diluted in PBS/0.1% Triton/10% BSA and added for 1 hour at room temperature. After washes in PBS/0.1% Triton, the slides were mounted with fluorescent mounting medium (DAKO, Via Real, Capinteria, CA, USA). Diaphragms were stained whole-mount as previously described [34]. Primary antibodies for immunofluorescence were the mouse anti Pan-neurofilament (1:2000; Covance, SMI-312), guinea pig anti vesicular acetylcholine transporter (VACHT; 1:500; Millipore, AB1588), mouse anti synaptic vesicle (SV2, 1:500; DSHB), rabbit anti synaptophysin (1:250, Life Technologies, Denver, CO) was injected i.p. 5 days before perfusion [33]. Secondary antibodies were diluted 1:2000 and were donkey anti fluorophore (1:100, Abcam, ab26128). Fluorogold (1%; Fluorochrome, Denver, CO) was injected i.p. 5 days before perfusion [33]. Secondary antibodies were diluted 1:2000 and were donkey anti fluorophore (1:100, Abcam, ab26128). Fluorogold (1%; Fluorochrome, Denver, CO) was injected i.p. 5 days before perfusion [33]. Secondary antibodies were diluted 1:2000 and were donkey anti fluorophore (1:100, Abcam, ab26128). Fluorogold (1%; Fluorochrome, Denver, CO) was injected i.p. 5 days before perfusion [33]. Secondary antibodies were diluted 1:2000 and were donkey anti fluorophore (1:100, Abcam, ab26128). Fluorogold (1%; Fluorochrome, Denver, CO) was injected i.p. 5 days before perfusion [33]. Secondary antibodies were diluted 1:2000 and were donkey anti fluorophore (1:100, Abcam, ab26128). Fluorogold (1%; Fluorochrome, Denver, CO) was injected i.p. 5 days before perfusion [33]. Secondary antibodies were diluted 1:2000 and were donkey anti fluorophore (1:100, Abcam, ab26128). Fluorogold (1%; Fluorochrome, Denver, CO) was injected i.p. 5 days before perfusion [33].
488, 594 or 647, anti goat/AlexaFluor 488 and anti rabbit/AlexaFluor 488 or 594 (Life Technologies, Carlsbad, CA, USA). Alpha-bungarotoxin-Alexa594 (1:2000; Life Technologies, B13422) was incubated with the secondary antibodies. Pictures were taken with a Zeiss Cell Observer Spinning Disk confocal microscope (Carl Zeiss, Zaventem, Belgium). For all images, brightness and contrast were adjusted with Adobe Photoshop CS3 extended version 10.0 after acquisition to match with the observation.

MN were quantified on each side of 3 spinal cord sections stained with cresyl violet or for Hb9 or Isl1/2 from a minimum of 3 mice. NMJ morphology was quantified on a minimum of 100 hindlimb junctions from 3 different animals. Synaptophysin distribution was classified into four categories: 1) perfect superposition between the BTX and synaptophysin labelings, 2) fragmentation of synaptophysin labeling compared to BTX staining, 3) weak synaptophysin labeling and 4) absence of synaptophysin. A minimum of 400 junctions was analyzed for each animal (n = 6).

Motor endplate maturation was evaluated in P14 newborn mice based on criteria previously described [36,37]. A minimum of 100 junctions was analyzed for each animal (n = 3).

**Electronic Microscopy**

Mice were anesthetized as mentioned above and perfused with a 2% PFA- 2.5% glutaraldehyde- 0.1 M phosphate buffer- 7% saccharose- 4% polyvinylpyrrolidone solution. TA muscles were dissected and were conserved in the perfusion solution at 4°C.

**Figure 1. Absence of HNF-6 results in abnormal locomotion.** A. Newborn Hnf6−/− mice (right) are smaller than their control littermates (left) and show paresis of the hindlimbs (arrows). B. Neurological score of male or female control (blue) or Hnf6−/− (red) mice. The score is reduced in both male and female Hnf6−/− mice (n=7). C, D, Hnf6−/− mice (D) exhibit a feet-clasping posture of the hindlimbs upon tail suspension, both for the legs (arrows) and for the feet (inset). E. The hindlimb muscular strength of male and of female control (blue) or Hnf6−/− (red) mice normalized to the body weight is reduced in Hnf6−/− mice (n=4). F–M, Catwalk analyses were performed for male (F,H,J,K) or female (G,I,L,M) control (F,G,J–M) or Hnf6−/− (H,J,M–M) mice. Locomotion alterations characterized by reduced overlap of fore paw (light colors) and of hind paw (dark colors) prints, reduced area of hind paw contacts, variability in the distance between the left (green) and the right (red) prints and less linear displacement, are observed in Hnf6−/− mice. Quantifications indicate that the intensity of the hind paw contacts is reduced (K,L) while the hindlimb base of support (the distance between the left and right hind paws) is increased (M). In contrast, these parameters are not affected for the forelimbs (n=4 in each group). BOS: base of support; LF: left front; LH: left hind; RF: right front; RH: right hind. Student’s t-test; * = p<0.05; ** = p<0.01; *** = p<0.001. doi:10.1371/journal.pone.0050509.g001
Fixed tissues were then rinsed in 0.1 M phosphate buffer and postfixed for 1 h with 1% osmium tetroxide in the same buffer. They were dehydrated in ethanol (30% to 100%) and embedded in Epon. Longitudinal thin sections (70 nm) were obtained with a LEICA Ultracut T and collected on 150-mesh copper grids, double stained with uranyl acetate and lead citrate, before examination with electron microscope HITACHI H7650 TEM at 80 kV. Images were collected with an Orius camera with GATAN Digital Micrograph software.

Electromyography

Electromyography recordings were made using a portable Dantec system (Dantec, Les Ulis, France) and were performed as described [38]. Briefly, mice were anesthetized as mentioned above and electrical activity in gastrocnemius and in TA muscles was monitored.

RT-qPCR

Total RNA was extracted from P0 hindlimb muscles, liver or spinal cord using the Tripure Isolation Reagent (Roche). One microgram of total RNA was transcribed into cDNA with Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies). Primers for RT-qPCR were: agrin isoform Z, which is the isoform present in neurons (forward 5'-GACGCTGTGGATCTCTCCGTT-3', reverse 5'-AGGACAGCCAGAAACACCATCT-3'), neuregulin-1 (forward 5'-CAGGAACTGAGCAGCACACA-3', reverse 5'-TGGACAGGTCTCTCCAGCTGA-3'), neuregulin-2 (forward 5'-ATGATCTATCTGCGCCAGAAC-3', reverse 5'-GAGGGTCGCTGCTGTAAG-3'), TGF-ß Receptor II [39], choline acetyltransferase (forward 5'-CGGTTTTATTCTGTCACCA-3', reverse 5'-GGTGGGCGTCCTAGCTCTTT-3'), hepatocyte nuclear factor-6 (forward 5'-TTCCACGCGCATGTCGGGCGCTC-3', reverse 5'-GGTACTAGTCCGGTGTTTCTTC-3') and RPL 32 (forward 5'-GGGACCAGTCAGACCGATAT-3', reverse 5'-CAGGATCTGGGCGCTTGAACC-3') as internal standards. The amplification protocol for all the primers included a hot start (95°C for 1 min), 40 amplification cycles (95°C for 15 s, 60°C for 1 min) and a melting point analysis. Technical triplicates were analyzed using the iCycler software with the delta/delta CT method and normalized to RPL32.

In situ Hybridization

Choline acetyltransferase and Vesicular ACh Eterase riboprobes were provided by Dr. Ersnerberger [40], Neuregulin-1 and Neuregulin-2 by Dr. C. Lai [41] and TGF-ß Receptor II by Dr. F. Lemaigre [42]. The Agrin riboprobe corresponded to nucleotides 6327–6840 of the NM_021604.3 NCBI sequence. In situ hybridizations on sections were performed as described [43] and followed by immunofluorescence detection of Isl1/2 as described above. Pictures were taken with an AMG Evos fluorescence microscope. The length and the surface area of the NMJ-like structures were measured using the ImageJ software.

Statistics

Data are presented as mean values ± SEM. Unpaired Student’s t-test, $\chi^2$ Pearson’s test, Dunnett’s or Newman-Keuls multiple comparison tests were used to determine statistical significance using GraphPad Prism 4 software (San Diego, CA). Differences at p<0.05 were considered significant.

Results

Absence of Hnf-6 Results in Abnormal Locomotion

At birth, mice carrying a null mutation of Hnf6 exhibited a paresis of the hindlimbs (Fig. 1A). To determine the outcomes of this early abnormality, a neurological score based on 11 criteria (see Material & Methods) that enabled a global assessment of the neurological status of these mice was evaluated. Male and female Hnf6−/− mice aged 70 days showed a significant reduction in this score compared to control littermates (Fig. 1B). In particular, foot-clasping posture of the hindlimbs upon tail suspension was observed for mutant mice (Fig. 1C,D), suggestive of hindlimb muscle weakness. Therefore, limb muscular strength was evaluated using grip strength measurements. A strong reduction in hindlimb muscular strength, but not in forelimb strength (data not shown), was observed in male and in female Hnf6−/− mice. To ascertain that the observed strength reduction was not due to size differences, grip strength measurements were normalized for body weight. Nevertheless, the normalized values showed a 30 or 40% reduction in hindlimb muscular strength for Hnf6−/− male or female mice, respectively (Fig. 1E).

To determine whether this muscle weakness altered the locomotion of the mutant mice, gait parameters were evaluated by catwalk imaging. Compared to control mice, mutant individuals exhibited little overlap of fore paw and hind paw prints,
reduced area of hind paw contacts, variability in the distance between the left and the right prints and less linear displacement (Fig. 1F–I). Quantitative analyses of gait parameters confirmed a reduction in the intensity of hind paw contacts and an increase in the base of support (BOS, i.e. the distance between the left and right paws) of the hindlimbs, both for males and for females (Fig. 1J–M), indicative of hindlimb muscle weakness and posture defect. Other hindlimb parameters were normal, and forelimb parameters were not affected. Taken together, these observations suggest that the lack of HNF-6 causes locomotor alterations that result from muscle weakness and abnormal positioning of the hindlimbs.

Absence of HNF-6 Results in Muscle Hypotrophy or Hypertrophy

Hindlimb muscle weakness in Hnf6<sup>–/–</sup> animals may result from muscle degeneration and wasting. To address this question, four hindlimb muscles were weighted and the muscle fiber cross-sectional area (CSA) was measured. The TA and gastrocnemius were smaller in Hnf6<sup>–/–</sup> individuals. In contrast, the soleus was slightly bigger, whereas EDL weight was normal (Fig. 2A). In Hnf6<sup>–/–</sup> animals, the mean CSA in TA was reduced without modification in the number of fibers (Fig. 2B–D) whereas CSA in soleus was increased without change in fiber number (Fig. 2E–G). Absence of central nuclei suggested that Hnf6<sup>–/–</sup> muscles did not undergo degeneration/regeneration (Fig. 2C,F). Taken together, these data indicated that the absence of HNF-6 resulted in hypertrophy of the soleus and in hypotrophy of the TA and of the gastrocnemius. Marked hypertrophy of these two large muscles likely contributes to the observed hindlimb muscle weakness.

HNF-6 is Required for Proper Morphology of the NMJ

However, HNF-6 is not detectable in muscles [44], either by RT-PCR or by immunofluorescence (Fig. S1). This also indicates that it is not present in cells that engulf the NMJ, including terminal Schwann cells [45] and kranocytes [46]. In contrast, HNF-6 is detected in MN at each level along the antero-posterior axis of the spinal cord during development [28] and in the early post-natal period (Fig. S1). In the limb-innervating lateral motor columns, HNF-6 is restricted to subsets of MN that do not correspond to motor pools, which innervate specific limb muscle [28]. This suggested that MN defects may constitute the primary cause of the locomotion defects in Hnf6<sup>–/–</sup> mice. The amount, the molecular phenotype and the projections of lumbar MN were preserved in Hnf6<sup>–/–</sup> mice (Fig. S2). Therefore, we investigated the morphology of the NMJ in all the hindlimb muscles. Labeling of the AChR by α-bungarotoxin (BTX) unveiled that 56% of the NMJ exhibited morphological alterations in Hnf6<sup>–/–</sup> mice (Fig. 3A–E) including disorganized topology (Fig. 3C), fragmentation (Fig. 3C) or absence of gutters (Fig. 3D). These abnormalities were observed in the thigh, TA, EDL, and gastrocnemius muscles (Fig. S3). In contrast, all NMJ were normal in hindlimb soleus (Fig. S2), in forelimb muscles and in the diaphragm (Fig. S4). This indicated that HNF-6 is required for proper organization of a large proportion of the hindlimb NMJ, and suggested that NMJ alterations likely participate in the locomotion defects of Hnf6<sup>–/–</sup> mice.

HNF-6 is Required for Proper Localization of Synaptophysin at Motor Nerve Terminals

To further investigate the organization of the NMJ in the absence of HNF-6, sections in all the hindlimb muscles were

**Figure 2. Absence of HNF-6 results in muscle hypotrophy or hypertrophy.** A, Tibialis anterior, gastrocnemius, extensor digitorium longus and soleus muscles from control (blue) or Hnf6<sup>–/–</sup> (red) mice were weighted. The tibialis anterior and the gastrocnemius are hypertrophied in Hnf6<sup>–/–</sup> mice, whereas the soleus is hypotrophied. B, C, Hematoxylin-eosin stainings of transverse sections in tibialis
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HNF-6 is Required for Timely Formation and Maturation of the NMJ

Half of the hindlimb NMJ were abnormal in adult Hnf6−/− mice. To assess whether this was due to defective formation or to abnormal stabilization and maintenance of the NMJ, the development of the NMJ in Hnf6−/− hindlimb muscles was studied between birth and P14. In control newborns at P0, AChR were arranged in plaques overlaid with sparse dotted synaptophysin aggregates likely corresponding to presynaptic vesicles (Fig. 4A). At P7, motor endplates reorganized and started to display perforations while matching between motor nerve terminals and endplates increased (Fig. 4B). At P14, the most mature junctions displayed a “pretzel-like” shape while synaptophysin was superposed to the endplate gutters, indicative of a precise apposition of the nerve terminals to the motor endplates (Fig. 4C). In contrast, the formation of the NMJ in hindlimb muscles was severely affected and delayed in Hnf6−/− newborns. At P0, AChR were normally organized in plaques but synaptophysin was clustered and was not superposed to the motor endplates (Fig. 4D). At P7, motor endplates presented very few perforations, with sparse superposition of the synaptophysin labeling (Fig. 4E). At P14, a majority of NMJ displayed a round morphology lacking any “pretzel-like” organization, while synaptophysin labeling was fragmented (Fig. 4F). These observations indicated that, in the absence of Hnf6, synaptophysin does not properly localize at the motor nerve terminals and that the maturation of the NMJ seems to be delayed. To confirm this, maturation of the NMJ was evaluated at P14 based on four morphological stages [47]. This quantification showed an excessive amount of NMJ in an immature configuration (M1–M2) and fewer mature NMJ in Hnf6−/− mice (Fig. 4G), confirming the maturation delay of the junctions in these animals. Maturation delay of the NMJ was previously associated with abnormal clustering of presynaptic vesicle proteins, including VACHT and synaptophysin, in the terminal MN axonal compartment [10]. Accordingly, synaptophysin and VACHT were ectopically detected within MN axons at P14 in Hnf6−/− newborns, whereas they were excluded from the terminal axonal compartment in control animals (Fig. 4H–K). Taken together, these observations suggested that NMJ formation is altered and delayed in hindlimb muscles of Hnf6 mutant mice (Fig. 4L). Given that a large proportion of the hindlimb NMJ was abnormal in Hnf6−/− adult mice, it is likely that these early defects hamper final maturation of the junctions.

HNF-6 Regulates in Motor Neurons the Expression of Several Actors Involved in NMJ Formation

As HNF-6 is present at birth in spinal MN, we hypothesized that it could regulate the expression of some of the MN actors involved in NMJ formation. Global expression of agrin and of choline acetyltransferase (ChAT) significantly decreased in Hnf6−/− spinal cord at P0, while expression of neuroginin-2 (nrg-2) and of TßRII trended to decrease (p = 0.17, respectively) at P5. To determine whether these perturbations specifically affected MN, in situ hybridization for these factors was combined to immunofluorescence for Isl1/2, which enabled to identify spinal MN, on lumbar spinal cord sections of P0 newborns. Agrin, nrg-2 and TßRII expression was downregulated in Hnf6−/− MN and in surrounding ventral interneurons (Fig. 5B–M). These data indicate that HNF-6 stimulates in MN the expression of several factors involved in NMJ formation.

To determine whether these perturbations contributed to the defective NMJ formation observed in Hnf6−/− mice, we relied on an in vitro co-culture system combining myotubes and...
Figure 3. HNF-6 is required for proper morphology of the NMJ. A–D'', Labeling of acetylcholine receptors by α-bungarotoxin (red) and immunofluorescence detection of synaptophysin (green) and of neurofilaments (blue) on hindlimb sections of control (A–A'') or Hnf6−/− (B–D'')
HNF-6 is required for the formation of NMJ. A–F, Labeling of acetylcholine receptors by α-bungarotoxin (red) and immunofluorescence detection of synaptophysin (green) and of neurofilaments (blue) on hindlimb sections of control (A–C) or Hnf6−/− (D–F) mice at postnatal day 0 (A,D), 7 (B,E) or 14 (C,F). The progressive increase in apposition of the motor nerve terminals to the motor endplates is disrupted in the absence of HNF-6, and the maturation of the motor endplate is defective. G, Quantification of NMJ maturation in control (blue) or Hnf6−/− (red) mice based on 4 morphological stages (M1: oval plaque; M2: single perforation; M3: multiple perforations; M4: mature junction). The maturation of the junctions is delayed in the absence of HNF-6 (n = 3). H–K, Labeling of VACHT (H,J, red) or synaptophysin (I,K, green) and of neurofilaments (blue) in control (H,J) or Hnf6−/− (I,K) mice at postnatal day 14. VACHT and synaptophysin were present in the terminal portion of motor axons in Hnf6−/− mice (I,K) whereas they were restricted to nerve terminals in control mice (H,J). L, Schematic representation of NMJ formation in control and in Hnf6−/− mice. BTX: α-bungarotoxin; PanNF: Pan-neurofilament; P: postnatal day; SYN: synaptophysin; VACHT: vesicular acetylcholine transporter. Student’s t-test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001. Scale bar = 5 μm.

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(A) mRNA expression (relative levels)

- **Control**
- *Hnf6-/-*

| Gene   | Control | Hnf6-/- |
|--------|---------|---------|
| Agrin  | ![Control Agrin](image) | ![Hnf6-/- Agrin](image) |
| ChAT   | ![Control ChAT](image) | ![Hnf6-/- ChAT](image) |
| Nrg1   | ![Control Nrg1](image) | ![Hnf6-/- Nrg1](image) **|** |
| Nrg2   | ![Control Nrg2](image) | ![Hnf6-/- Nrg2](image) |
| TβRII  | ![Control TβRII](image) | ![Hnf6-/- TβRII](image) |

(B) Immunohistochemistry

- **Control**
- *Hnf6-/-*

| Gene   | Control | Hnf6-/- |
|--------|---------|---------|
| Agrin  | ![Control Agrin](image) | ![Hnf6-/- Agrin](image) |
| Nrg1   | ![Control Nrg1](image) | ![Hnf6-/- Nrg1](image) |
| VChAT  | ![Control VChAT](image) | ![Hnf6-/- VChAT](image) |

| Gene   | Control | Hnf6-/- |
|--------|---------|---------|
| Agrin  | ![Control Agrin](image) | ![Hnf6-/- Agrin](image) |
| Nrg2   | ![Control Nrg2](image) | ![Hnf6-/- Nrg2](image) |
| TβRII  | ![Control TβRII](image) | ![Hnf6-/- TβRII](image) |
embryonic spinal cord slices [48]. In this system, NMJ-like structures developed 2–3 weeks after plating slices of e12.5 control or Hnf6−/− spinal cord on differentiated control myotubes (Fig. 6). These structures are not detected when muscle cells are cultured alone, suggesting that their formation requires interactions of axon terminals with myotubes as observed in vivo for NMJ (data not shown). First, we assessed whether in vitro formation of these NMJ-like structures was affected by the absence of HNF-6 from the neuronal compartment. BTX labeling at the end of the culture period suggested that the formation of these structures was less efficient with Hnf6−/− than with control spinal cord slices (Fig. 6A,B). As the morphology of these structures was less well-established than that of their in vivo counterparts, their size and the surface covered by AChR were measured to evaluate their development [49]. The length and the surface of the NMJ-like structures after co-culture with Hnf6−/− spinal cord slices was significantly smaller than that obtained with control tissues (Fig. 6I,J). Thus, the growth of the NMJ-like structures obtained in vitro was decreased when HNF-6 was absent from the neuronal compartment. This was consistent with the hypothesis that HNF-6 acts in MN to control the development of the NMJ. It also validated our co-culture system to study the mechanisms that cause defective NMJ development in Hnf6 mutant animals. To address this question, we assessed whether activation of the agrin, neuregulin or TGF-ß signaling pathway was able to rescue the growth deficit of NMJ-like structures observed with Hnf6−/− neuronal tissue. In co-cultures with control spinal cord slices, recombinant agrin, neuregulin or TGF-ß had no significant effect on the growth of NMJ-like structures (Fig. 6C–J). In co-cultures with Hnf6−/− tissues, recombinant agrin (Fig. 6C,D,I,J) and neuregulin (Fig. 6E,F,I,J) completely restored the growth defect of these structures (Fig. 6C–F,I,J), while TGF-ß was rather deleterious (Fig. 6G–J). These data indicate that activation of the agrin or neuregulin signaling pathways rescues the mutant phenotype in this in vitro coculture system, and suggest that the downregulation of agrin and of nrg-2 expression in Hnf6−/− newborn MN contributes to defective NMJ formation.

Discussion

Oncut transcriptional activators control different aspects of CNS development including generation or maintenance of specific neuronal populations [24,25,26,27], neuronal migration [24] and central projections of the trigeminal neurons [23]. Here, we present evidence that HNF-6 controls in MN part of the genetic program necessary for the development of hindlimb NMJ. Indeed, the formation of hindlimb junctions was altered and the expression of multiple factors described to participate in NMJ development, including agrin, neuregulin-2 and TßRII, was downregulated in the lumbar MN of Hnf6 mutant newborns. Furthermore, recombinant agrin or neuregulin did rescue defective in vitro formation of NMJ-like structures due to the lack of HNF-6 from the neuronal compartment.

Only part of the hindlimb NMJ was altered in the Hnf6 mutants, probably because HNF-6 is present in only part of the MN [28]. In particular, the NMJ of the soleus muscle were not affected.

However, this could not be attributed to the absence of HNF-6 from the soleus MN pool in wild-type animals (data not shown). Similarly, the reason why forelimb NMJ were not affected remains unclear. Indeed, the distribution of HNF-6 in MN is similar at brachial or lumbar levels of the spinal cord [28]. Noteworthy, the distribution of other transcription factors including PEA3 [50], ERß1 [51] and Hox factors [52] is different in brachial or lumbar limb-innervating MN, suggesting that the genetic programs that control forelimb and hindlimb innervation are not identical. Whether another factor compensates for the absence of HNF-6 in brachial MN remains to be determined. As the NMJ of the soleus were normal in Hnf6−/− mice, the hypertrophy of the soleus observed in these animals is probably secondary to the hypotrophy of the TA and gastrocnemius muscles.

Downregulation of agrin, neuregulin-2 and TßRII in Hnf6−/− MN suggests that these genes are, either directly or indirectly, regulated by HNF-6. Although RT-qPCR experiments showed a significant decrease in the expression levels of agrin and only a trend to the reduction for neuregulin-2 and TßRII, in situ hybridization experiments indicated a strong reduction of these three factors in Hnf6−/− MN as well as in surrounding ventral interneurons, consistent with the expression of Hnf6 in these neuronal populations [28]. However, in situ hybridization is only a semi-quantitative method with a relatively high detection threshold. Therefore, it is likely that the expression levels of agrin, neuregulin-2 and TßRII are downregulated below this threshold in the Hnf6−/− MN, as indicated by the RT-qPCR experiment, but are not completely abrogated as suggested by the in situ hybridization data. This is consistent with the requirement for agrin in NMJ formation [5]. Although regulation of agrin or neuregulin genes by HNF-6 has not been described yet, TßRII is negatively regulated by HNF-6 in liver, probably indirectly [17,39]. This regulation, along with that of TGF-ß antagonists, enables Onecut factors to maintain low TGF-ß signaling in the liver parenchyma and thereby creates an environment permissive for hepatocyte differentiation [17]. Our observations suggest that HNF-6 acts oppositely in MN and rather supports activation of the TGF-ß pathway.

In Drosophila, numerous studies have shown that TGF-ß signaling in MN regulates NMJ development. Mutations in genes that encode components of this pathway lead to presynaptic defects resulting in reduced numbers of NMJ, disrupted T-bars and impaired neuromuscular transmitter release [53,54,55,56]. Recently, it has been shown in Xenopus that TGFß-1 acts as a Schwann cell-derived signal that promotes NMJ formation, probably by stimulating agrin expression in MN [9]. Therefore, support of TßRII expression by HNF-6 may contribute to maintain MN responsive to this signal, and thereby to indirectly stimulate agrin production.

Agrin plays a pivotal role in AChR clustering and NMJ maintenance. Lack of agrin results in activity-dependant dispersal of AChR clusters, impaired postsynaptic differentiation and progressive loss of NMJ [57,58]. Therefore, downregulation of agrin expression in Hnf6 mutant mice may contribute to NMJ fragmentation. However, absence of severe NMJ depletion and of muscle denervation in these mutant animals suggests that agrin
Figure 6. Depletion of agrin and of neuregulin contributes to defective NMJ formation in Hnf6 mutant mice. A–H, NMJ-like structures obtained in vitro by co-culture of control myotubes with control (A,C,E,G) or Hnf6−/− (B,D,F,H) embryonic spinal cord slices in medium supplemented with vehicle only (A,B) or with recombinant agrin (C,D), neuregulin (E,F) or TGFβ (G,H). I, J, Quantification of the length (I) and of the area (J) of the NMJ-like structures obtained with control (blue) or Hnf6−/− (red) spinal cord slices. The size and area deficits observed with Hnf6−/−
expressed levels were only partly reduced in Hnf6−/− MN. Furthermore, agrin is also produced by the muscles and by terminal Schwann cells [59], which may partly compensate for the reduction in agrin signaling from MN in Hnf6 mutant animals. Nevertheless, the growth defect of the NMJ-like structures observed in vivo with Hnf6−/− spinal cord slices was completely rescued by recombinant agrin, suggesting that decreased agrin levels contribute to the NMJ abnormalities in Hnf6 mutant animals.

Expression level of nrg-2 was also decreased in Hnf6−/− MN, and activation of neuregulin signaling rescued the mutant phenotype in co-culture experiments. Hnf6−/− mice showed a reduced localization of AChR at the post-synaptic membrane [61]. Similarly, α-dystrobrevin-dependent AChR anchoring was severely altered in mice carrying inactivation of the Nrg receptors ErbB2 and ErbB4 in skeletal muscles, resulting in progressive NMJ wasting [6]. These data suggest that Nrg-1 and Nrg-2 may provide redundant signals that use the same intracellular signaling pathway to stabilize the post-synaptic apparatus. Therefore, downregulation of Nrg-2 in Hnf6−/− mice may contribute to morphological alterations of adult NMJ but is unlikely to account for the early maturation defects of these junctions. Taken together, these data suggest that combined reduction in agrin, in neuregulin and in TGF-β signaling causes the NMJ defects observed in the absence of HNF-6. This indicates that HNF-6 may act in lumbar MN to coordinate the genetic program that participates in hindlimb NMJ formation and maintenance.

Abnormal distribution of synaptophysin was observed in Hnf6−/− MN. Synaptophysin is one of the major integral membrane proteins of the small (30-50 nm diameter) electron-translucent transmitter-containing vesicles in neurons and accounts for ±10% of total synaptic vesicle proteins. The exact function of synaptophysin remains unclear. Mice lacking synaptophysin are healthy and show no apparent defect, suggesting that their NMJ are intact and functional although their organization was not studied [62,63,64,65,66]. In the absence of HNF-6, synaptophysin at the motor nerve terminals was either fragmented, reduced or absent, although the levels of synaptophysin mRNA in the spinal cord were similar in control and in Hnf6−/− newborn mice (data not shown). Abnormal synaptophysin distribution was not due to defective localization of the synaptic vesicles at the nerve terminals because other synaptic vesicle proteins, including VACHT and SV2, were properly distributed and a normal amount of synaptic vesicles was observed beneath the presynaptic membrane using electron microscopy. Therefore, abnormal synaptophysin distribution in Hnf6−/− mice seems to result from deficient translation or from defective targeting of synaptophysin toward the synaptic vesicles. How HNF-6 may contribute to these processes remains to be investigated.

Finally, the absence of HNF-6 caused abnormal locomotion and positioning of the hindlimbs. Some of the observed defects, including reduced muscle strength and posture defects, probably partly result from abnormal NMJ formation. However, other neuronal populations of the CNS are defective in Hnf6−/− animals [24,25] (spinal interneurons in this study; data not shown) and likely contribute to the locomotion defects.

In conclusion, we have shown that the transcription factor HNF-6 coordinates in lumbar MN a genetic programs involved in hindlimb NMJ formation. Perturbations of this program caused defective formation of the NMJ that resulted in a reduction in the hindlimb muscle strength. Why this defect is restricted to hindlimb muscles, how HNF-6 controls in MN the expression of regulators of NMJ development and whether HNF-6 additionally modulates the expression of other regulators of NMJ formation remain to be investigated.

Supporting Information

Figure S1 HNF-6 is expressed in MN but not expressed in muscle. A,B, Expression levels of Hnf6 in liver (positive control), spinal cord or hindlimb muscles of P0 control animals (n = 3) evaluated on gel (A) and by quantitative real-time PCR (B). C–E, Detection of HNF-6 in e18.5 liver (positive control), C) or lumbar spinal cord (D) and in P0 hindlimb muscle (E) of control animals. HNF-6 is present in the liver and in the spinal cord. In contrast, it is not detected in the hindlimb muscles. Student’s t-test; * = p<0.05, ** = p<0.01. Scale bar = 100 μm. (TIF)

Figure S2 Spinal motor neurons are not affected by the absence of HNF-6. A–D, Transverse sections in the lumbar spinal cord of e18.5 control (A,C) or Hnf6−/− (B,D) embryos labeled for the motor neuron markers Hb9 (A,B) and Is11/2 (C,D). MN are present in the ventral horn of control (A,B) and of Hnf6−/− (C,D) mice. E, Quantification of motor neurons in hemissections in the lumbar spinal cord of control (blue) or Hnf6−/− (red) mice at e18.5. The amount of motor neurons was similar in control and in Hnf6−/− mice (n = 3). F,G, Transverse sections in the lumbar spinal cord of adult (F) or Hnf6−/− (G) mice stained with cresyl violet. The dashed lines delineate the border between the gray and the white matter. MN (arrows) are present in the ventral horn of control and of Hnf6−/− mice. H, I, VACHT is detected in these cells, both in control (H) and in Hnf6−/− (I) mice, indicating that these are cholinergic neurons, as expected. J–K, Fluorogold was injected i.p. in control (J) or Hnf6−/− (K) mice. In both cases, 5 days after injection, the cell bodies of MN contain Fluorogold, indicating that these neurons project toward the periphery. L, The amount of motor neurons was similar in control and in Hnf6−/− mice (n = 5). M, Table showing the weight of P70 control or Hnf6−/− mice. At that stage, the weight of Hnf6−/− mice is similar to that of control animals. VACHT: vesicular acetylcholine transporter. Scale bars = 100 μm. (TIF)

Figure S3 NMJ are altered in Hnf6−/− tibialis anterior, gastrocnemius and EDL muscles, but not in Hnf6−/− soleus muscle. A–H’, Labeling of acetylcholine receptors by α-bungarotoxin (red) and immunofluorescence detection of synaptophysin (green) and of neurofilaments (blue) on tibialis anterior (A–B’), gastrocnemius (C–D’), EDL (E–F’) and soleus (G–H’) muscles of control (A–G’) or Hnf6−/− (B–H’) mice. A–G’, In control mice, NMJ display the expected “pretzel-like” shape and show perfect apposition of the synaptophysin labeling to the motor endplate. B–H’, In Hnf6−/− mice, junctions show disorganized topology and defective localization of synaptophysin (arrowhead)
in tibialis anterior, gastrocnemius and EDL muscle. In contrast, the apposition of nerve terminals to the motor endplates and the endplate morphology are normal in Hsf6−/− soleus muscle. BTX: α-bungarotoxin; SYN: synaptophysin. PanNF: Pan Neurofilament; EDL: extensor digitorum longus. Scale bar = 5 μm. (THF)

**Figure S4** HNF-6 is not required for the formation of forelimb and diaphragm NMJ. A–D’, Labeling of acetylcholine receptors by α-bungarotoxin (red) and immunofluorescence detection of synaptophysin (green) on forelimb muscle sections (A–B’) or on diaphragm (C–D’) of control (A–A’,C–C’) or Hsf6−/− (B–B’,D–D’) mice at postnatal days 14. The apposition of the nerve terminals to the motor endplates and the endplate maturation are normal in forelimb and in diaphragm NMJ of Hsf6−/− mice. BTX: α-bungarotoxin; SYN: synaptophysin. Scale bar = 5 μm. (THF)

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