Shp2 Is Dispensable in the Formation and Maintenance of the Neuromuscular Junction

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Key Words

SHP2 • Neuromuscular junction • Conditional knockout • Agrin • MuSK • Synapse • Transcription • Formation

Abstract

SHP2, a protein tyrosine phosphatase with two SH2 domains, has been implicated in regulating acetylcholine receptor (AChR) gene expression and cluster formation in cultured muscle cells. To understand the role of SHP2 in neuromuscular junction (NMJ) formation in vivo, we generated muscle-specific deficient mice by using a loxP/Cre strategy since Shp2 null mutation causes embryonic lethality. Shp2^{floxed/floxed} mice were crossed with mice expressing the Cre gene under the control of the human skeletal α -actin (HSA) promoter. Expression of SHP2 was reduced or diminished specifically in skeletal muscles of the conditional knockout (CKO) mice. The mutant mice were viable and fertile, without apparent muscle defects. The mRNA of the AChR α subunit and AChR clusters in CKO mice were localized in a narrow central region surrounding the phrenic nerve primary branches, without apparent change in intensity. AChR clusters colocalized with markers of synaptic vesicles and Schwann cells, suggesting proper differentiation of

presynaptic terminals and Schwann cells. In comparison with age-matched littermates, no apparent difference was observed in the size and length of AChR clusters in CKO mice. Both the frequency and amplitude of mEPPs in CKO mice were similar to those in controls, suggesting normal neurotransmission when SHP2 was deficient. These results suggest that Shp2 is not required for NMJ formation and/or maintenance.

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Introduction

Acetylcholine receptors (AChRs) are highly concentrated at the neuromuscular junction (NMJ), essential for fast and accurate neurotransmission. Two signaling pathways are believed to contribute to the enrichment of AChRs at the NMJ. Agrin, a large extracellular matrix protein, is thought to be utilized by motoneurons to form or maintain AChR clusters in the postsynaptic membrane [1, 2]. Agrin activates MuSK, a transmembrane tyrosine kinase that is essential for agrin-induced AChR clustering [3–7]. MuSK^{-/-} mice, like agrin mutant mice, fail to form the NMJ [3, 8, 9]. Mutation analyses indicate

that the tyrosine kinase activity of MuSK is essential for its biological activity [10, 11]. Agrin induces tyrosine phosphorylation of the AChR β and δ subunits in a manner dependent on Src family kinases [12–18], which appears to be necessary for AChR cluster stability [19]. Recently, agrin was shown to activate Abl tyrosine kinases, geranylgeranyl transferase I (GGT), GTPases of the Rho family, and Pak1, a serine/threonine kinase that is activated by Rho GTPases [20–24]. Their inhibition attenuates agrin-induced AChR clustering [20, 21, 23, 24]. In addition, intracellular calcium and nitric oxide were shown to regulate AChR clustering [25–27].

Recent studies have identified several proteins that interact with MuSK including Dishevelled (Dvl), GGT, MAGI-1c and 14-3-3 γ [21, 22, 28, 29]. Dvl is a signaling molecule important for planar cell polarity [21, 22]. Disruption of the MuSK-Dvl interaction inhibits agrin- and neuron-induced AChR clustering. Expression of dominant-negative Dvl1 in postsynaptic muscle cells reduces the amplitude of spontaneous synaptic currents (SSCs) at the NMJ. Dvl1 may recruit Pak1 to the complex [21]. MAGI-1c may recruit yet unidentified signaling molecules whereas 14-3-3 γ appears to regulate synaptic gene expression via inhibition of MAPK-PI₃K signaling pathways [28, 29].

Neuregulin (NRG) is implicated in synapse-specific transcription of AChR genes [1]. NRG acts by activating its receptor – the 180-kDa ErbB receptor tyrosine kinases (ErbB2/HER2 [30, 31], ErbB3/HER3 [32, 33], and ErbB4/HER4 [34, 35]). NRG induces homo- and hetero-dimerization of ErbB receptors, thus stimulating tyrosine kinase activity [36–40]. Upon activation, tyrosine residues in the carboxyl termini become phosphorylated and serve as docking sites for cytoplasmic signaling molecules such as Shc and Grb2 to activate various downstream signaling pathways [41]. Activation of ERK kinase is required for NRG regulation of AChR gene expression in muscle cells [42–44] and for synapse-specific expression of the ϵ -transgene in vivo [45]. In addition, NRG induces expression of immediate early genes *c-jun* and *c-fos* and activates the c-JUN NH₂-terminal kinase (JNK), both of which are required for NRG-mediated expression of the ϵ -transgene [46]. Recently, NRG has been found to activate Cdk5, which is also required for AChR expression [47]. These results suggest that NRG activates multiple signaling cascades that converge to regulate AChR gene expression [48]. However, ErbB2 and ErbB4 double deletion in skeletal muscles form NMJs that are structurally and functionally normal in many aspects [49]. These data indicate that the NRG/ErbB signaling pathway to muscle

is dispensable for at least the principal aspects of postsynaptic development.

The degree of tyrosine phosphorylation results from a balance of activities of protein tyrosine kinases that phosphorylate and phosphatases which dephosphorylate substrate proteins. However, in contrast to ample evidence for the roles of ErbB tyrosine kinases in the regulation of AChR synthesis, much less is known about the relevant protein tyrosine phosphatase(s). SHP2 (previously also known as PTP1D, SH-PTP2, Syp, or PTP2C [50–54]) is a tyrosine phosphatase that contains two SH2 domains. It is up-regulated during myogenesis [55] and is upregulated in response to neuregulin (NRG) [56]. SHP2 is localized at the adult NMJ [57]. Functionally, SHP2 interacts with ErbB proteins upon NRG stimulation [57]. Expression of SHP2 in muscle cells attenuates the NRG-induced expression of the ϵ -transgene, suggesting that SHP2 negatively regulates the NRG signaling pathway and may inhibit synapse-specific transcription [57]. On the other hand, SHP2 increasingly associates with MuSK in C2C12 cells in response to agrin [Camilleri et al.: Soc Neurosci, 727.1, 2005]. Treatment with pervanadate enlarges AChR clusters induced by agrin and depletion of SHP2 by siRNA increases MuSK activation and both agrin-independent and -dependent AChR clustering in C2C12 myotubes [58]. These results from in vitro studies suggest a role of SHP2 in regulating AChR gene expression and cluster formation.

To investigate the role of SHP2 in NMJ formation and function in vivo, we generated mutant (CKO) mice whose SHP2 expression was attenuated or abolished specifically in skeletal muscle cells. The CKO mice are vital and fertile without apparent morphological defects in the muscle. AChR gene expression was analyzed by in situ hybridization. In addition to α -bungarotoxin (α -BTX), which labels AChRs, markers of presynaptic terminals and Schwann cells were analyzed for expression and localization. Moreover, neurotransmission of the NMJs in CKO mice was analyzed by electrophysiological recording. Our results suggest that SHP2 is not essential for NMJ formation or maintenance.

Materials and Methods

Generation of Shp2 Mutant Mice

HSA-Cre transgenic mice [59, 60] were mated with mice heterozygous for the Shp2 floxed allele [61]. Mice containing a HSA-Cre and a floxed allele were intercrossed to generate embryos with homozygous floxed alleles and the HSA-Cre transgene. For genotyping, DNA was isolated from tails and analyzed by PCR.

Primers for the Cre gene are: sense 5'ATG CCC AAG AAG AAG AGG AAG GT and anti-sense 5'GAA ATC AGT GCG TTC GAA CGC TAG A, which generates a 750-bp product. Primers for Shp2 floxed alleles: sense (Exo4) 5'ACG TCA TGA TCC GCT GTC AG and anti-sense (Int4AS) 5'ATG GGA GGG ACA GTG CAG TG, which generates 400 and 300 bp products from the floxed and wild-type alleles, respectively. The floxed allele was screened using primers Int3 (sense) 5' CAG TTG CAA CTT TCT TAC CTC and NullC (anti-sense) 5' GCA GGA GAC TGC AGC TCA GTG ATG, which results in 1,000 bp products. PCR cycles are (5 min, 94°C) × 1, (30 s, 94°C, 1 min, 56°C, 1 min, 72°C) × 30, and (10 min, 72°C) × 1. The use of animals was in compliance with the guidelines of the Animal Care and Use Committee of the Medical College of Georgia.

Hematoxylin-Eosin

Diaphragms were dissected in PBS (pH 7.3) at room temperature, fixed in 4% paraformaldehyde (PFA) at 4°C overnight, and embedded in paraffin. Sections (8 µm thickness) were thaw-mounted on glass slides and treated sequentially with 2 × 5 min xylene, 2 × 2 min 100% EtOH, and 2 × 2 min 95% EtOH. After rinsing in distilled water, they are placed in hematoxylin (01522, Surgipath Medical Industries) for 1 min, and rinsed first in distilled water and then in tap water for 3–5 min. Slides were rinsed in 95% EtOH for 30 s and incubated in 1% eosin (01602, Surgipath Medical Industries) for 1 min. Slides were then dipped in 95% EtOH ten times and 100% EtOH ten times, and then cleared with 3 changes of xylene for 2 min each. The slides were mounted with Permount (SP15-100, Fisher Chemicals).

Whole-Mount Immunocytochemistry

Whole-mount staining was performed as described previously [62, 63]. Briefly, dissected diaphragms were fixed in 4% PFA at 4°C overnight, rinsed with PBS (pH 7.3) at room temperature, incubated with 0.1 M glycine in PBS for 1 h, and rinsed with 0.5% Triton X-100/PBS. Muscles were blocked in the blocking buffer (3% BSA, 5% goat serum, and 0.5% Triton X-100 in PBS) for 2–4 h at room temperature or overnight at 4°C. They were then incubated with primary antibodies (neurofilament, 1:1,000, AB1983, Chemicon; synaptophysin, 1:2,000, A0010, Dako; SV2, 1:1,000, Developmental Studies Hybridoma Bank; S100, 1:1,000, Z0311, Dako; or rapsyn, 1:1,000) [45] in the blocking buffer overnight at 4°C. After washing three times for 1 h each in 0.5% Triton X-100 in PBS, the muscles were incubated with fluorescein-conjugated anti-rabbit or mouse IgG (1:500, Molecular Probes) and Texas Red conjugated α-BTX (RT α-BTX 1:2,500, Molecular Probes) 2–4 h at room temperature or overnight at 4°C. Samples were washed three times for 1 h each in 0.5% Triton X-100 in PBS, rinsed once with PBS, and flat-mounted in Vectashield mounting medium (H-1,000, Vector Laboratories). Z serial images were collected with a Zeiss confocal laser scanning microscope (LSM 510 META 3.2) and collapsed into a single image. The area and length (the longest axis) of each AChR cluster were determined with LSM 5 Image Examiner (Carl Zeiss).

Acetylcholinesterase (AChE) Histochemistry

AChE staining was performed as previously described [63]. Briefly, PFA-fixed diaphragms (see above) were rinsed 3 times in PBS and incubated in solution A (pH 5.5, containing 0.2 mM ethopropazine, 4 mM acetylthiocholine iodine, 10 mM glycine,

2 mM cupric sulfate, and 65 mM sodium acetate) for 2–4 h at 37°C. AChE activity was detected in situ by incubation in 1.25% sodium sulfide (pH 6.5) at room temperature in a hood (for usually 2–5 min). The samples were washed extensively in water and mounted in 50% glycerol in PBS.

Whole-Mount in Situ Hybridization

In situ hybridization was performed as previously described [62]. Briefly, E18.5 diaphragms were dissected out in PBS containing 2 mM EGTA and fixed in 4% PFA in PBS containing 2 mM EGTA for 1–2 h at room temperature or 4°C overnight. Samples were washed 10 min twice with PTW (PBS, 0.1% Tween-20), once with 50% MeOH/PTW, and once with 100% MeOH and then treated with 10 µg/ml proteinase K in PTW for 30 min at 37°C. After brief rinse in PTW, diaphragms were post-fixed in 4% PFA and 0.1% glutaraldehyde in PTW at room temperature for 20 min. Samples were washed once with PTW and once with PTW/hybridization buffer (1:1) containing 50% formamide, 1.3× SSC, 5 mM EDTA, 50 µg/ml yeast tRNA, 0.2% Tween-20, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 100 µg/ml heparin. Samples were pre-incubated in the hybridization buffer for 1 h at 55°C before the addition of 1 µg/ml DIG-labeled RNA probe. After overnight incubation at 55°C, diaphragms were washed in the hybridization buffer and in a 1:1 mixture of the washing solution (50% formamide/2×SSC) and TBST (150 mM NaCl, 10 mM KCl, 50 mM Tris, pH 7.5, and 0.1% Tween-20), each for 15 min at 55°C. Samples were then incubated in TBST plus 2% Boehringer Blocking Reagent (BBR) and 20% heat-treated goat serum for 1–2 h at room temperature, rinsed once with the antibody solution of TBST plus 2% BBR and 1% serum, and incubated with the antibody solution containing anti-DIG-AP antibody (1:1,000) for overnight at 4°C or 4–6 h at room temperature. After rinse with TBST, samples were incubated with a solution containing 0.1 M NaCl, 0.1 M Tris, pH 9.5, 0.05 M MgCl₂, 1% Tween-20, 0.068% NBT, and 0.035% BCIP for color development. Samples were then washed in re-fixed in PTW with 4% PFA and 0.1% glutaraldehyde at 4°C overnight.

Electrophysiology

Electrophysiological recording was performed as described previously [64]. Briefly, diaphragms with ribs were pinned on Sylgard gel in a dish and perfused with an oxygenated solution containing 145 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes-NaOH, pH 7.3, and 13 mM glucose at room temperature for at least 1 h. Microelectrodes, 20–50 MΩ when filled with 3 M KCl, were placed in the central region of muscle fibers where AChR clusters were enriched. Positions were adjusted for maximal response with mEPP rise time smaller than 1 ms. Data were digitized at 25 kHz and analyzed by PCLAMP 9.2 (Axon Instruments).

Western Analysis

Western blotting was performed as previously described [65]. Nitrocellular membranes were probed with anti-SHP2 (1:1,000, 610621, BD Transduction Laboratories, USA) and AChR α subunit (mAb35, 1:1,000). Immunoreactivity was visualized by HRP-conjugated secondary antibodies (1:2,500, Pierce) and ECL reagents (Amersham Biosciences).

Fig. 1. Muscle-specific deletion of the *Shp2* gene. **A, B** Different alleles of the *Shp2* gene. Exo4 vs. Int4AS and Int3 vs. NullC are two pairs of primers for genotyping. **C** Western blot analysis of SHP2 levels in tissues from control (*Shp2*^{loxP/loxP}) and CKO (*Shp2*^{loxP/loxP}; HSA-Cre) mice. SHP2 expression is specifically suppressed in skeletal muscles but not other tissues in CKO mice. **D** Normal morphology of skeletal muscles in CKO mice. P1 muscle sections were stained with hematoxylin-eosin (top panels) or subjected to electronic microscopic analysis (bottom panels).

Fig. 2. Localization of AChR clusters in the central region of muscle fibers in CKO diaphragms. **A** P2 diaphragms were whole-mount stained with RT α -BTX to label the AChR (red) and with rabbit anti-neurofilament antibody to label motoneurons, which were visualized by FITC-conjugated anti-rabbit antibody. Z-serial images were collected with a $\times 5$ objective using a Zeiss confocal laser scanning microscope (LSM 510 META 3.2). Images from each set of z series were collapsed to a single projected image. The phrenic nerve ran along the center of the diaphragm and small groups of axons leave the primary branches to innervate muscle fibers in both control and mutant mice. *Shp2* deficiency did not affect the distribution of endplates. **B** Quantitative analysis of endplate band width.

Fig. 3. *Shp2* deficiency did not affect the size and length of AChR clusters. **A** P15 diaphragms were whole-mount stained for AChR and nerves as in figure 2. Z-serial images were collected with a $\times 40$ oil objective using a Zeiss confocal laser scanning microscope. Shown was a single projected image collapsed from each set of z series. Nerve terminals were found in all AChR clusters. **B** Enlarged images of AChR clusters of P1 and P15 diaphragms. **C** Characterization of AChR cluster size and length. Individual AChR clusters from one $\times 40$ field were traced around their perimeters to calculate the area and length of clusters using LSM 5 Image Examiner software (Zeiss). Both the size and length were not changed in CKO mice.

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Results

Shp2 Gene Inactivation in Skeletal Muscles

To generate mice where the *Shp2* gene was silenced specifically in the skeletal muscle, mice containing the floxed *Shp2* allele were intercrossed with transgenic mice expressing Cre under the control of human skeletal α -actin (HSA) promoter. This promoter was active at E9.5 in the myotomal region of somites and drives Cre expression specifically in striated muscles [59, 60]. Wild-type and floxed mice generate 300- and 400-bp bands, respectively, in genotyping PCR reactions using the Exo4 and Int4AS primers (fig. 1A). In contrast, conditional knockout (CKO, i.e. *Shp2*^{floxed/floxed}; HSA-Cre^{+/?}) mice generated a 1,000-bp band in genotyping PCR reactions using the Int3 and NullC primers, which was absent in wild type (fig. 1B). Western blot analysis revealed a reduction of SHP2 specifically in muscles in CKO mice, in comparison with control littermates that include wild-type, *Shp2*^{floxed/+}, and *Shp2*^{floxed/floxed} mice. In contrast, SHP2 expression in other tissues was similar between control and CKO mice, including the spinal cord, brain, liver, and lung.

Normal Muscle Morphology in Shp2-Deficient Mice

CKO mice were vital and fertile and did not show apparent motility or behavior anomaly. At gross anatomy level, muscles of CKO mice are indistinguishable from control mice at postnatal day (P) 1, P15, and P60. Shown in figure 1D were representative images of HE staining of P1 diaphragm cross section. At the ultrastructural level, typical striated patterns were observed in both control and CKO mice. The sarcomeric organization indicated by Z-lines and M-lines was the same, if not identical, in control and CKO mice (fig. 1D). These results suggest that muscle development was unaffected in *Shp2*-deficient mice.

Characterization of AChR Clusters in CKO Mice

SHP2 have been implicated in regulating AChR expression and clustering, two major events in NMJ formation [57, 58]. To determine whether SHP2 is necessary for NMJ formation in vivo, we first examined the band width of endplates and the size and length of AChR clusters. Diaphragms were whole-mount stained with an anti-neurofilament antibody to label motoneuron terminals and RT α -BTX to label the AChR. Normally, AChR clusters are confined to a central region surrounding the primary phrenic nerve branches, forming the endplate band (fig. 2A, top panels) [1, 62, 63]. To analyze quantitatively

the central band width, a polygon was drawn to include most peripheral AChR clusters and the average myotube length contained in the polygon was calculated. The band width was $178.3 \pm 35.0 \mu\text{m}$ in CKO mice, which was similar to that in control mice ($167.6 \pm 24.3 \mu\text{m}$, means $\pm$ SEM, $n = 4$, $p > 0.05$) (fig. 2B), suggesting that *Shp2* deficiency may not affect overall organization of endplate banding. The notion was supported by AChE distribution. AChE is an extracellular protein that co-locates with AChR clusters at the NMJs [66]. As shown in figure 4A, AChE activity in CKO mice was localized in a central region as in control mice.

As observed in control mice, each AChR cluster co-stained with the anti-neurofilament antibody in P1 and P15 CKO mutant mice (fig. 3A, data not shown), suggesting proper innervation. We measured the size and length of AChR clusters in the right ventral portion of the diaphragms of both control and CKO mice. The size of AChR clusters was similar in control and CKO mice [$101 \pm 41.4 \mu\text{m}^2$ in control and $109 \pm 36 \mu\text{m}^2$ in CKO mice at P1 ($n = 66$, $p > 0.05$); $144 \pm 41 \mu\text{m}^2$ in control and $171 \pm 48 \mu\text{m}^2$ in CKO mice at P15 ($n = 40$, $p > 0.05$)]. Likewise, the length of AChR clusters in CKO mice was not altered [15.4 ± 3.8 in control and 15.4 ± 3.4 in CKO mice at P1 ($n = 66$, $p > 0.05$); 7.6 ± 3.4 in control and 18.4 ± 2.8 in CKO mice at P15 ($n = 40$, $p > 0.05$)] (fig. 3C). As the NMJ develops, AChR clusters become perforated into plaque-like structures [1]. Formation of such plaques was apparently not affected in CKO mice (fig. 3B). These results suggest that SHP2 may not be necessary for AChR cluster formation, maintenance, and/or maturation.

AChR Transcripts Are Concentrated at the NMJ in CKO Mice

In normal muscles, a few nuclei beneath the postsynaptic membrane are active in transcribing genes for NMJ formation and function [1]. For example, the mRNAs of AChR subunits are enriched in the synaptic region. To determine whether *Shp2* deletion affects the spatial pattern of gene expression in muscle, in situ hybridization analysis was performed using a probe specific to the AChR α subunit gene. Whole-mount in situ hybridization showed that AChR α subunit transcripts were confined in the middle region of the diaphragms in CKO mice, like those in control mice (fig. 4B). The width of the region where AChR α subunit transcripts are confined to and their intensity were similar in both control and CKO mice. These results suggested that AChR α subunit gene was transcribed properly in *Shp2*-deficient mice.

Fig. 4. AChE staining and AChR α subunit transcript distribution in CKO mice. **A** AChE activity was assayed histochemically in diaphragms of P1 mice. Images were collected with $\times 2.5$ objectives using a Zeiss microscope (Axiophot, AxioCam MRC). AChE clusters are concentrated in a center region in both control and CKO mice. **B** In situ hybridization showing AChR α subunit mRNA distribution in CKO mice. E18.5 diaphragms were incubated with digoxigenin-cRNA probes for the AChR- α subunit. Labeling was concentrated in a central band of muscle in both control and CKO mice.

Fig. 5. Normal tripartite presynaptic, postsynaptic, and glial components in CKO mice. P15 diaphragms were stained for synaptophysin, rapsyn, and S100 with respective antibodies and visualized with FITC-conjugated secondary antibodies. AChR was labeled with RT α -BTX. Z-serial images were collected with a $\times 40$ oil objective using a Zeiss confocal laser scanning microscope. Shown was a single projected image collapsed from each set of z series. Synaptophysin, rapsyn and S100 were well colocalized with AChRs in both control and CKO mice.

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Fig. 6. Normal neurotransmission at the NMJ in CKO mice. **A** Representative traces of mEPPs in control (top) and CKO (bottom) mice. **B** Statistical analysis of the frequencies and amplitudes of mEPPs in **A**.

Association of AChR Clusters with Rapsyn and Schwann Cells in CKO Mice

The NMJ forms as a result of complex interactions among motoneurons, Schwann cells, and muscle fibers. Motoneuron terminals and perisynaptic Schwann cells are closely apposed to AChR clusters in normal NMJs. To test whether SHP2 were required to maintain both pre- and postsynaptic apparatus, diaphragm sections were co-immunostained with anti-synaptophysin antibody to label nerve terminals, anti-S100 antibody to label Schwann cells, and anti-rapsyn antibody to label postsynaptic apparatus. As shown in figure 5, AChR clusters were closely apposed by synaptophysin immunoreactive nerve terminals in both control and CKO NMJs. S100 staining revealed a similar pattern as synaptophysin in both types of mice, suggesting that Schwann cells were distributed in close proximity with nerve terminals. Moreover, immunostaining of rapsyn, which is colocalized perfectly with AChR clusters and is a key molecule for AChR clustering at the NMJ, showed that rapsyn was well colocalized with

AChR in both control and CKO NMJs (fig. 5). These results demonstrate that alignment of three components (pre- and postsynaptic and Schwann cells) was not affected by Shp2 deficiency, suggesting that Shp2 is not essential for the assembly of presynaptic and postsynaptic apparatus at the NMJs.

Normal Neurotransmission at the NMJ in CKO Mice

To characterize the function of NMJs in CKO mice, we recorded miniature endplate potential (mEPP), an indicator of vesicle fusion in the presynaptic terminals and AChR density in the postsynaptic membrane. Both the frequency and amplitude of mEPPs were similar in control and in CKO mice (fig. 6). The amplitude of mEPP was 0.96 ± 0.08 mV in control (n = 11) and 0.93 ± 0.09 mV in CKO mice (n = 12, $p > 0.05$), whereas the frequency was 3.21 ± 0.52 Hz in control and 3.47 ± 0.48 Hz in CKO mice. These results were consistent with the above morphological studies, suggesting normal NMJ function in CKO mice.

Discussion

In this study, we analyzed the NMJ in mutant mice where SHP2 expression was specifically suppressed in the skeletal muscle. The CKO mice form NMJs that are morphologically indistinguishable from control mice. AChR clusters in CKO mice are distributed in the central region of the muscle fibers and are associated with both presynaptic terminals and Schwann cells. AChR transcripts were detectable in the sub-synaptic region in mutant mice. Moreover, the NMJ appears to be functional normally in CKO mice. These results demonstrate that NMJ or AChR clusters form and maintain properly when Shp2 is deficient. They argue against the notion that Shp2 may be involved in regulating synapse-specific transcription and AChR clustering [57, 58] [Camilleri et al.: *Soc Neurosci*, 727.1, 2005]. It is possible that CKO mice developed a compensatory mechanism to cope with Shp2 deficiency. Many tyrosine phosphatases have been identified and more than eleven are expressed in the skeletal

muscles [67]. The function of SHP2 may be compensated by one or more of these phosphatases. SHP2 shares high homology with SHP1, which also contains two SH2 domains. However, SHP1 is specifically expressed in hematopoietic cells [68, 69]. One other possible explanation of the lack of NMJ defects in Shp2-deficient mice is the weak activity of the HSA promoter. The Shp2 CKO mice are not null mutant, although the amount of SHP2 in CKO mice was barely detectable. However, we are unable to exclude the possibility that NMJ formation and maintenance could be sufficiently regulated by the residual level of SHP2 in the mutant mice.

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