RESEARCH ARTICLE

Blocking skeletal muscle DHPRs/Ryr1 prevents neuromuscular synapse loss in mutant mice deficient in type III Neuregulin 1 (CRD-Nrg1)

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Abstract

Schwann cells are integral components of vertebrate neuromuscular synapses; in their absence, pre-synaptic nerve terminals withdraw from post-synaptic muscles, leading to muscle denervation and synapse loss at the developing neuromuscular junction (NMJ). Here, we report a rescue of muscle denervation and neuromuscular synapse loss in type III Neuregulin 1 mutant mice (CRD-Nrg1¹⁻/⁻), which lack Schwann cells. We found that muscle denervation and neuromuscular synapse loss were prevented in CRD-Nrg1¹⁻/⁻ mice when presynaptic activity was blocked by ablating a specific gene, such as Snap25 (synaptoosomal-associated 25 kDa protein) or Chat (choline acetyltransferase). Further, these effects were mediated by a pathway that requires postsynaptic acetylcholine receptors (AChRs), because ablating Chma1 (acetylcholine receptor a1 subunit), which encodes muscle-specific AChRs in CRD-Nrg1¹⁻/⁻ mice also rescued muscle denervation. Moreover, genetically ablating muscle dihydropyridine receptor (DHPR) β1 subunit (Cacnb1) or ryanodine receptor 1 (Ryr1) also rescued muscle denervation and neuromuscular synapse loss in CRD-Nrg1¹⁻/⁻ mice. Thus, these genetic manipulations follow a pathway—from presynaptic to postsynaptic, and, ultimately to muscle activity mediated by DHPRs and Ryr1. Importantly, electrophysiological analyses reveal robust synaptic activity in the rescued, Schwann-cell deficient NMJs in CRD-Nrg1¹⁻/⁻ Cacnb1¹⁻/⁻ or CRD-Nrg1¹⁻/⁻ Ryr1¹⁻/⁻ mutant mice. Thus, a blockade of synaptic activity, although sufficient, is not necessary to preserve NMJs that lack Schwann cells. Instead, a blockade of muscle activity mediated by DHPRs and Ryr1 is both necessary and sufficient for preserving NMJs that lack Schwann cells. These findings suggest that muscle activity mediated by DHPRs/Ryr1 may destabilize developing NMJs and that Schwann cells play crucial roles in counteracting such a destabilizing activity to preserve neuromuscular synapses during development.
Author summary

Nerve cells (neurons) communicate with each other through specialized focal connections called synapses. Synapses are commonly assembled into a tripartite structure composed of pre- and post-synaptic neurons, and a glial cell that is closely associated with both pre- and post-synaptic components. At the vertebrate neuromuscular synapse (the neuromuscular junction, NMJ), the tripartite structure is composed of a nerve terminal, a muscle fiber and a glial cell called the terminal Schwann cell. Previous studies have shown that Schwann cells are vital to the NMJ—without Schwann cells, the NMJ fails to form. Here, we report unexpected findings that bipartite NMJs can be established in vivo, in the absence of Schwann cells. These results were accomplished by ablating specific genes in mice, ultimately resulting in a total blockade of muscle activity. Thus, Schwann cells become dispensable for the NMJ if muscle activity is blocked. These findings suggest that muscle activity normally destabilizes the NMJ during synapse formation and that Schwann cells play crucial roles in NMJ formation by opposing this muscle-derived destabilizing activity in order to preserve the developing neuromuscular synapses.

Introduction

Like all chemical synapses in the brain, the NMJ—the synaptic connections between motor neurons and skeletal muscles—is assembled as a tripartite structure that includes a presynaptic motor nerve terminal, a postsynaptic muscle cell and a terminal Schwann cell, which caps the motor nerve terminal. These three cellular components interact to support the normal physiological function of the NMJ [1–10]. Importantly, Schwann cells are required for the maintenance of the NMJ [11–15] and play crucial roles in the re-establishment of the NMJs during nerve regeneration after injury [16–18]. Motor neurons in turn produce the glycoprotein neuregulin 1 (NRG1), predominantly as the cysteine-rich domain isoform of neuregulin 1 (type III NRG1, or CRD-NRG1) [19–21]. NRG1 interacts with receptor tyrosine kinase erbB receptors and play crucial roles in synapse formation and function [19, 22–35].

Emerging evidence suggests that NRG1/erbB expression is correlated with the state of skeletal muscle innervation/denervation [36]. Deficiencies in NRG1/erbB signaling—as shown in CRD-Nrg1−/−, erbB2−/− and erbB3−/− mutant mice—lead to a loss of Schwann cells and, consequently, a retraction of motor nerve terminals from diaphragm muscle, resulting in muscle denervation and neuromuscular synapse loss [37–43]. These defects are likely due to the loss of Schwann cells, rather than a loss of NRG1/erbB signaling from motor neurons to muscles, since deleting erbBs specifically in muscles does not affect NMJ formation and function [44]. How an absence of Schwann cells may cause muscle denervation and neuromuscular synapse loss, however, remains unclear.

Synaptic activity plays crucial roles in sculpting neural circuits [45, 46]. Terminal Schwann cells are known to play important roles in regulating synaptic activity at the NMJ [5, 47–49], suggesting possible relationships among Schwann cells, activity and synapse formation. In this study, we ask the question if an absence of specific activity at pre-synaptic nerve terminals, post-synaptic acetylcholine receptors (AChRs), or muscle fibers, may affect NMJ formation in the absence of Schwann cells. To address this question, we blocked pre-synaptic activity in CRD-Nrg1−/− mice, which lack Schwann cells, by ablating specific genes known to be required for transmitter release from the nerve terminal. Surprisingly, blocking neurotransmitter release results in a rescue of muscle denervation and prevents the neuromuscular synapse loss that normally occurred in CRD-Nrg1−/− mice. We further show that these effects were mediated...
by postsynaptic acetylcholine receptors (AChRs), because genetic elimination of muscle-specific AChRs in CRD-Nrg1−/− mice also rescued muscle denervation. And finally, we show that these effects were mediated through muscle activity because genetically ablating either dihydropyridine receptors (DHPRs) or ryanodine receptor 1 (Ryr1) also rescues muscle denervation and neuromuscular synapse loss in CRD-Nrg1−/− mice. Together, these results demonstrate that bipartite NMJs lacking Schwann cells can be established if muscle activity is blocked, suggesting that muscle activity mediated by DHPRs/Ryr1 plays a key role in preserving Schwann cell-deficient NMJs.

**Results**

**Blocking pre-synaptic activity in CRD-Nrg1−/− mice prevents neuromuscular synapse loss**

To investigate how synaptic activity might impact the development of neuromuscular synapses in CRD-Nrg1−/− mice, we took several genetic approaches. First, we took advantage of previously characterized mutant mice that lack pre-synaptic activity. For example, evoked synaptic transmission is completely blocked in mutant mice deficient in synaptosomal-associated 25 kDa protein (SNAP25) [50], a key protein component of Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) complexes required for regulated exocytosis in all chemical synapses [51–55]. We therefore bred CRD-Nrg1 mutant mice with Snap25 mutant mice, and generated double mutant mice deficient in both CRD-Nrg1 and Snap25 (CRD-Nrg1−/− Snap25−/−).

Surprisingly, in contrast to a loss of phrenic innervation in the diaphragm muscle in CRD-Nrg1−/− mice [38] (see also Fig 1A, second left panel), the diaphragm muscles in CRD-Nrg1−/− Snap25−/− mice were fully innervated, despite defasciculation (Fig 1A, first right panel). Immunostaining of the diaphragm muscles (E18.5) using a mixture of antibodies (anti-NF150 and anti-Syt2 antibodies) revealed the presence of phrenic innervation in wild-type (CRD-Nrg1+/+ Snap25+/+) mice (Fig 1A, second panel). In contrast, the diaphragm muscle in CRD-Nrg1−/− Snap25−/− mice was fully innervated, although the nerves appeared highly defasciculated (Fig 1A, right panel). More important, post-synaptic endplates labeled by Texas Red conjugated α-bgt were juxtaposed with presynaptic nerve terminals, indicating that the NMJs were established in the diaphragm muscles in CRD-Nrg1−/− Snap25−/− mice (Fig 1B). Furthermore, the average size of postsynaptic endplates in CRD-Nrg1−/− Snap25−/− mice was significantly larger than those in control or CRD-Nrg1−/− mice (Fig 1C).

These results indicated that muscle denervation and NMJ loss were rescued in CRD-Nrg1 mutant mice that were also deficient in Snap25 (CRD-Nrg1−/− Snap25−/−). At the NMJ, motor nerve terminals are known to co-release both acetylcholine (ACh) and adenosine 5′-triphosphate (ATP), which has been shown to regulate the expression and stability of post-synaptic AChRs [56–59]. Ablating Snap25 blocks vesicular exocytosis and thus blocks the release of both ACh and ATP. This raises the question whether the effects seen in CRD-Nrg1−/− Snap25−/− mice were due to a blockade of the release of ACh, ATP, or both.

To address this question, we turned to mutant mice deficient in ChAT—the enzyme required for synthesizing ACh [60, 61]. We blocked cholinergic synaptic transmission in CRD-Nrg1−/− mice by generating double mutant mice deficient in both CRD-Nrg1 and Chat (CRD-Nrg1−/− Chat−/−). As shown in Fig 2, the diaphragm muscles in CRD-Nrg1−/− mice were transiently innervated at E14.5 (Fig 2B) but completely denervated by E15.5 (Fig 2B). In contrast, diaphragm muscles in the CRD-Nrg1−/− mice that also lacked ChAT (CRD-Nrg1−/− Chat−/−) were fully innervated at E15.5 (Fig 2C), E16.5 (S1 Fig) and E18.5 (Fig 2D). Furthermore, the
Fig 1. Blocking pre-synaptic activity prevents synapse loss in CRD-Nrg1−/− mice. Embryonic diaphragm muscles (E18.5) were immunostained with anti-NF150 and anti-Syt2 antibodies to reveal pre-synaptic nerves, and labelled with Texas Red conjugated α-bgt to mark post-synaptic endplates. A: Low power views of hemi-diaphragm muscles, showing the phrenic nerves trunk and nerve terminals. Arrowheads in wild-type muscle (CRD-Nrg1+/+ Snap25+/+) point to the nerve terminals. In CRD-Nrg1−/− Snap25+/+, the phrenic nerve is absent (∗ indicates the sensory nerves projecting from the edge towards the center). In CRD-Nrg1−/− Snap25−/− muscle, the phrenic nerve innervation is rescued, and the nerves are highly defasciculated (arrows). B: High-power images showing individual neuromuscular synapses (arrowheads). Neuromuscular synapses are absent in CRD-Nrg1−/− Snap25+/− but are present in CRD-Nrg1−/− Snap25−/− muscle (arrowheads). C: The average size of postsynaptic endplates in the diaphragm muscles is significantly increased in CRD-Nrg1−/− Snap25−/− mice (138.3 ± 11.4 μm², n = 177, N = 3), compare with control (72.7 ± 7.0 μm²).
that Schwann cells were present in control mice but absent in CRD-Nrg1−/− mice (80.5 ± 2.2 μm²; n = 287, N = 3, P = 0.0036), but not significantly different from those in Snap25−/− mice (133.6 ± 13.9 μm²; n = 198, N = 3, P = 0.7670). Scale bars: A: 400 μm; B: 20 μm. n: number of postsynaptic endplates; N: number of mice.

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Intramuscular nerves in CRD-Nrg1−/− (Fig 2B) and CRD-Nrg1−/−Chat−/− (Fig 2C and 2D) were highly defasciculated. Higher-power images further revealed that presynaptic nerve terminals were juxtaposed to postsynaptic AChR-enriched endplates in CRD-Nrg1−/−Chat−/− mice (Fig 2E). Similar to control mice, the apposition of pre-synaptic nerve terminals and post-synaptic end-plates in CRD-Nrg1−/−Chat−/− mice progressed throughout neuromuscular synaptogenesis (E15.5, E16.5 and E18.5) (Fig 2). We counted 526 postsynaptic endplates from the diaphragm muscles of 3 CRD-Nrg1−/−Chat−/− mice (E18.5); 100% of these 526 endplates were juxtaposed by presynaptic nerve terminals (Fig 2E). Furthermore, the endplates in CRD-Nrg1−/−Chat−/− or Chat−/− mice were noticeably larger than those in control or CRD-Nrg1−/− mice. For example, the average endplate sizes were 120.4 ± 14.3 μm² in CRD-Nrg1−/−Chat−/− mice (n = 526, N = 3) and 115.1 ± 7.7 μm² in Chat−/− (n = 576, N = 3), which represented an increase of more than 50% compared to the end-plates in either control (77.2 ± 5.7 μm², n = 450, N = 3) or CRD-Nrg1−/− mice (79.7 ± 3.6 μm², n = 313, N = 3) (Fig 2F).

In CRD-Nrg1−/− mice, motor neuron numbers were markedly reduced in the spinal cord [38]. To determine if motor neurons were also rescued in CRD-Nrg1−/−Chat−/− mice, we examined cervical segments of spinal cords using anti-choline transporter (CHT) antibodies, which label motor neurons [62]. We found that the somata of motor neurons in the ventral horn of the spinal cord were labeled CHT-positive in both control (S2A Fig, left panels) and CRD-Nrg1−/−Chat−/− mice (S2A Fig, right panels). Furthermore, we counted the numbers of motor axons from phrenic nerves (S2B Fig) and found that the average motor axon numbers per phrenic nerve were similar between control (248 ± 8, N = 3 mice) and CRD-Nrg1−/−Chat−/− (244 ± 11, N = 3 mice) (S2C Fig). Together, these results demonstrated that the loss of phrenic nerve innervation and neuromuscular synapses in single mutant mice deficient in CRD-NRG1 (CRD-Nrg1−/−) were rescued in double mutant mice deficient in both CRD-NRG1 and ChAT (CRD-Nrg1−/−Chat−/−).

Absence of Schwann cells in CRD-Nrg1−/−Chat−/− mice

Using anti-S100β antibodies, which recognize developing Schwann cells [39], we confirmed that Schwann cells were present in control mice but absent in CRD-Nrg1−/−Chat−/− mice (Fig 3E). The absence of Schwann cells was further demonstrated by electron microscopy (EM). In control and Chat single mutant mice (i.e., CRD-Nrg1−/−Chat−/− mice; Fig 3I and 3J), individual axons were regularly interspersed, and each axon was wrapped by Schwann cell processes. In contrast, phrenic nerves were completely devoid of Schwann cells in CRD-Nrg1−/−Chat−/− mice (Fig 3I and 3J, right panels), and the axons were tightly packed together with very little extracellular space in between.

Ultrastructural analyses of the NMJs also showed that terminal Schwann cells were absent at the NMJs in CRD-Nrg1−/−Chat−/− mice. Instead, the flanks of presynaptic nerve terminals were directly exposed to the interstitial space in double mutant mice (Fig 4B). No other cell type was found as a substitute for Schwann cells. Occasionally, thin processes of fibroblast-like cells were seen in the interstitial space around the NMJs, but this feature was readily observed in both control and CRD-Nrg1−/−Chat−/− double mutant mice. Although these processes could be seen near the nerve terminal membrane in a few instances, in no case did they wrap, cap or flank the nerve terminals. Consistent with our results in light microscopy (Fig 2D and 2E), no NMJs were observed under EM in E18.5 diaphragm muscles in CRD-Nrg1−/− mice. In contrast,
CRD-Nrg1 in α conjugated diaphragm muscles (D, low power; E, high power) labeled by anti-syntaxin1 antibodies (green) and Texas Red-conjugate immunostaining using a mixture of anti-NF150 and anti-Syt2 antibodies, and postsynaptic end-plates (red) by Texas Red–bungarotoxin. At E14.5, the diaphragm muscle in CRD-Nrg1−/− mice is denervated, leaving the end-plates vacant (bordered by dashed lines). Interestingly, muscle denervation is fully rescued in CRD-Nrg1+/− mice. Similarly, the nerves are highly defasciculated (arrows in C). High-power images show formation of nascent neuromuscular synapses (arrowheads in C) on the right two panels in C. D-E: Confocal images of E18.5 whole-mount diaphragm muscles (D, low power; E, high power) labeled by anti-syntaxin1 antibodies (green) and Texas Red-conjugated α-bungarotoxin. At E14.5, the diaphragm muscle in CRD-Nrg1−/− mice is innervated, but the nerves are highly defasciculated (arrows in B). However, at E15.5, the diaphragm muscle in CRD-Nrg1−/− mice is denervated, leaving the end-plates vacant (bordered by dashed lines). Interestingly, muscle denervation is fully rescued in CRD-Nrg1+/− mice.

Fig 2. Blockade of cholinergic neurotransmission (Chat−/−) prevents synapse loss caused by CRD-Nrg1 deficiency. A-G: Initial NMJ formation in CRD-Nrg1+/+ and CRD-Nrg1−/− Chat−/− mice. Confocal images (low power, left panels; high power, right panels) of embryonic diaphragm muscles at E14.5 and E15.5. Presynaptic nerves (green) are revealed by immunostaining using a mixture of anti-NF150 and anti-Syt2 antibodies, and postsynaptic end-plates (red) by Texas Red-conjugated α-bungarotoxin. At E14.5, the diaphragm muscle in CRD-Nrg1−/− mice is innervated, but the nerves are highly defasciculated (arrows in B). However, at E15.5, the diaphragm muscle in CRD-Nrg1−/− mice is denervated, leaving the end-plates vacant (bordered by dashed lines). Interestingly, muscle denervation is fully rescued in CRD-Nrg1+/− mice. Similarly, the nerves are highly defasciculated (arrows in C). High-power images show formation of nascent neuromuscular synapses (arrowheads in C) on the right two panels in C. D-E: Confocal images of E18.5 whole-mount diaphragm muscles (D, low power; E, high power) labeled by anti-syntaxin1 antibodies (green) and Texas Red-conjugated α-bungarotoxin (red). In control mice (CRD-Nrg1+/+ Chat−/−), the nerve extends nerve branches (arrowheads in D) targeted to the central region of the muscle. The nerve is absent in CRD-Nrg1−/− Chat−/− mice. The lack of innervation is rescued in CRD-Nrg1+/− Chat−/− mice. The nerve branches are defasciculated (arrow in D, CRD-Nrg1+/− Chat−/−), but the nerve terminals are intensely labeled by anti-syntaxin1 antibodies (arrowheads in D, CRD-Nrg1+/− Chat−/−) and are localized to the central region of the muscle. High power images in E show nerve terminals juxtaposing with AChR clusters and form NMJs (arrowheads in E) in control (CRD-Nrg1+/+ Chat−/−), Chat−/− and CRD-Nrg1+/− Chat−/− muscles. However, nerve terminals are absent in CRD-Nrg1−/− Chat−/− muscle, leaving AChRs vacant. F: Quantitative analyses of postsynaptic endplate size in E18.5 diaphragm muscles. The average endplate size is 77.2 ± 5.7 μm² in control (n = 450, N = 3), 79.7 ± 3.6 μm² in CRD-Nrg1+/+ (n = 313, N = 3), 115.1 ± 7.7 μm² in Chat−/− (n = 576, N = 3) and 120.4 ± 14.3 μm² in CRD-Nrg1+/− Chat−/− mice (n = 526, N = 3). The average endplate size in CRD-Nrg1+/− Chat−/− mice is significantly increased (P < 0.05) compared with control or CRD-Nrg1−/− mice. The endplate size is not statistically significant (n. s.) between CRD-Nrg1−/− Chat−/− and Chat−/− mice (one-way ANOVA, followed by Tukey test). N: number of mice; n: number of end-plates.

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numerous NMJs were identified under EM in E18.5 diaphragm muscles in control (n = 26), Chat−/− (n = 14) and CRD-Nrg1+/− Chat−/− (n = 18) mice. Among these NMJs, a striking feature was that the numbers of nerve terminal profiles per NMJ were markedly increased in both Chat−/− and CRD-Nrg1+/− Chat−/− mice compared with control mice. Specifically, the numbers of nerve terminal profiles per NMJ were significantly increased in Chat−/− (10.57 ± 1.75, n = 14, P = 0.004) and CRD-Nrg1+/− Chat−/− (15.28 ± 1.86, n = 18, P = 4.707 x 10^-5), respectively, compared with the control (6.17 ± 0.53, n = 26). The difference in presynaptic nerve terminal numbers per NMJ between Chat−/− and CRD-Nrg1+/− Chat−/− mice, however, was not statistically significant (P = 0.083).

Strikingly, despite the absence of terminal Schwann cells, the ultrastructure of presynaptic nerve terminals in CRD-Nrg1+/− Chat−/− mice appeared similar to that of control mice: multiple nerve terminals, each with abundant synaptic vesicles, mitochondria, glycogen granules, and other membranous structures, were observed. The postsynaptic compartment also displayed typical ultrastructural features: electro-dense postsynaptic membranes and abundant sub-synaptic organelles, such as mitochondria and ribosomes (compare Fig 4A to Fig 4B). Thus, bipartite neuromuscular synapses composed of only presynaptic nerve terminals and postsynaptic muscle membrane were established in the absence of Schwann cells in CRD-Nrg1+/− Chat−/− mice.

Ablating post-synaptic AChRs preserves NMJs in CRD-Nrg1−/− mice

AChRs are expressed at multiple sites within the NMJ, including presynaptic nerve terminals, terminal Schwann cells and postsynaptic muscles [10, 63]. Although Schwann cells were absent in CRD-Nrg1−/− mice, it remained possible that a blockade of cholinergic transmission (as in CRD-Nrg1+/− Chat−/− mice) may affect synaptic transmission at either pre-synaptic or post-synaptic sites, or both. To determine whether the effects of blocking cholinergic synaptic transmission were mediated through postsynaptic AChRs, we examined the NMJs of mice selectively lacking postsynaptic AChRs–mice lacking the gene encoding the AChR α1 subunit (Chrna1−/− mutants), which is selectively expressed in muscle but not in motor neurons or Schwann cells [64].
We found that muscle innervation was also completely rescued in double mutant mice lacking both AChRα1 and CRD-Nrg1 (CRD-Nrg1+/− Chrna1−/−) (Fig 5A), similar to the results displayed in CRD-Nrg1+/− Chat−/− (Fig 2D) and CRD-Nrg1−/− Snap25−/− mice (Fig 1A). Furthermore, the rescued motor nerve terminals in CRD-Nrg1+/− Chrna1−/− mice were intensely labeled by anti-syntaxin1 antibodies (Fig 5B), indicating that pre-synaptic terminal differentiation proceeded in the absence of AChRs, which is consistent with previous results shown in Chrna1−/− mice [64].

Deleting muscle DHPRs preserves NMJs in the absence of Schwann cells

Muscle AChRs are nonselective cation channels, and their activation leads to an influx of cations, including Na+ and Ca2+, and triggers muscle action potentials (muscle electrical activity), mediated by the voltage-gated sodium channels. Muscle action potentials depolarize the sarcolemma and activate voltage-sensitive dihydropyridine receptors (DHPRs), the L-type Ca2+...
channels localized to the T tubules, and ultimately activate ryanodine receptors (RyR) in the sarcoplasmic reticulum [65, 66]. It has been previously shown that genetic deletion of Cacnb1, the gene encoding the β1 subunit of DHPRs, blocks DHPR function [67] without affecting muscle electrical activity [68].

To determine the role of muscle activity mediated by DHPRs in NMJ formation in the absence of Schwann cells (i.e., in CRD-Nrg1−/− mice), we examined double mutant mice deficient in both CRD-Nrg1 and Cacnb1. Remarkably, ablating skeletal muscle DHPRs (Cacnb1−/−) in CRD-Nrg1−/− mice (i.e., in CRD-Nrg1−/−/Cacnb1−/−) prevented muscle denervation (Fig 6A). And, similar to those observed in CRD-Nrg1−/−/Snap25−/− (Fig 1A), CRD-Nrg1−/−/Chat−/− (Fig 2C and 2D, S1 Fig), and CRD-Nrg1−/−/Chrna1−/− (Fig 5A) mice, intramuscular nerves in CRD-Nrg1−/−/Cacnb1−/− were highly defasciculated (Fig 6A). Furthermore, neuromuscular synapses were established in CRD-Nrg1−/−/Cacnb1−/− mice. We counted 233 end-plates from E18.5 diaphragm muscles in CRD-Nrg1−/−/Cacnb1−/− mice (N = 3 mice), and found that 100% of AChR clusters were juxtaposed to the pre-synaptic nerve terminals (Fig 6B). Furthermore, the average size of the postsynaptic endplate increased more than 2-fold in CRD-Nrg1−/−/Cacnb1−/− mice (161.3 ± 3.6 μm², N = 3, n = 233), compared with control (74.9 ± 4.1 μm², N = 3, n = 287) or CRD-Nrg1−/− mice (78.0 ± 1.8 μm², N = 3, n = 239) (Fig 6C). Importantly, re-introducing
Genetic rescue of neuromuscular synapse loss

Cacnb1 back into skeletal muscle by transgenic expression of Cacnb1 using a muscle-specific promoter–human skeletal muscle actin (HSA-Cacnb1) in CRD-Nrg1−/−Cacnb1−/− mice–abolished the rescue of muscle innervation and synapse loss (Fig 6D). Therefore, ablating Cacnb1 in skeletal muscles is specifically required for the rescue of muscle innervation and synapse loss in CRD-Nrg1−/−Cacnb1−/− mice.

Ultrastructural analysis confirmed that Schwann cells were indeed absent in the nerves (Fig 7C) and the NMJs in CRD-Nrg1−/−Cacnb1−/− mice (Fig 7E). Similar to the results obtained with the NMJs in CRD-Nrg1−/−Chat−/− mice, the numbers of pre-synaptic nerve terminals per NMJ

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Fig 5. Ablating postsynaptic AChRs (Chrna1−/−) preserves innervation in CRD-Nrg1−/−Chrna1−/− mice. A: Low power views of hemi diaphragm muscle (E18.5) from wild-type (CRD-Nrg1+/+Chrna1+/+), Chrna1−/−, CRD-Nrg1−/− and CRD-Nrg1+/+Chrna1−/− mice, labeled with anti-syntaxin1 antibodies (green) and Texas Red conjugated α-bgt (red). AChRs are absent in CRD-Nrg1−/−Chrna1−/− and CRD-Nrg1−/−Chrna1−/− muscles due to Chrna1 deficiency. CRD-Nrg1 deficiency led to an absence of innervation in CRD-Nrg1−/−Chrna1−/− muscles. Deleting both CRD-Nrg1 and Chrna1 (CRD-Nrg1−/−Chrna1−/−) restored innervation, and the nerve terminals in CRD-Nrg1−/−Chrna1−/− muscles are intensely labeled by anti-syntaxin1 antibodies (arrowheads). Scale bars: A: 400 μm; B: 20 μm.

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Fig 6. Removal of skeletal muscle dihydropyridine receptors (Cacnb1−/−) prevents synapse loss in CRD-Nrg1−/− mice. Immunofluorescence staining of embryonic diaphragm muscles (E18.5) using anti-NF150 and anti-syt2 antibodies (green), and Texas Red conjugated α-bgt (red). A: Innervation patterns in the indicated genotypes. Muscles from CRD-Nrg1−/−Cacnb1−/− mice lack innervation, and this phenotype is rescued in CRD-Nrg1−/−Cacnb1+/− muscles, despite the nerve being highly defasciculated (arrow). B: High-power images of embryonic diaphragm muscles (E18.5) were labeled by anti-syntaxin1 antibodies (green) and Texas Red conjugate d defici ency led to an absence of innervation in CRD-Nrg1−/−Chrna1−/− muscles. Deleting both CRD-Nrg1 and Chrna1 (CRD-Nrg1−/−Chrna1−/−) restored innervation, and the nerve terminals in CRD-Nrg1−/−Chrna1−/− muscles are intensely labeled by anti-syntaxin1 antibodies (arrowheads). Scale bars: A: 400 μm; B: 20 μm.
were markedly increased in CRD-Nrg1−/−Cacnb1−/− mice, compared with controls. On average, there were 9.44 ± 1.03 (n = 26) nerve terminals at the NMJs in CRD-Nrg1−/−Cacnb1−/−, 8.68 ± 0.93 (n = 19) in Cacnb1−/−, and 6.29 ± 0.70 (n = 21) in control mice. While the nerve terminal numbers were not statistically different between CRD-Nrg1−/−Cacnb1−/− and Cacnb1−/− mice, the nerve terminal numbers were significantly (P = 0.019) increased in CRD-Nrg1−/−Cacnb1−/− mice, compared with those observed in control mice. However, the ultrastructure of the presynaptic nerve terminal, basal lamina and postsynaptic membrane appeared remarkably similar to those in control NMJs (compare Fig 7D to Fig 7E), except that terminal Schwann cells were absent in CRD-Nrg1−/−Cacnb1−/− mice (Fig 7E).
Deleting muscle RyR1 preserves NMJs in the absence of schwann cells

The activation of muscle DHPRs leads to the activation of muscle ryanodine receptors (RyRs), triggering the release of Ca\(^{2+}\) from the sarcoplasmic reticulum into the muscle cytosol, leading to muscle contractions, a process generally referred to as excitation-contraction coupling (E-C coupling) [65, 69–72]. There are three Ry genes in rodents—Ryr1, Ryr2 and Ryr3, each with distinct expression patterns: Ryr1 is predominantly expressed in skeletal muscle, Ryr2 in cardiac muscle and brain, and Ryr3 in brain [73–80]. To determine if muscle activity mediated by ryanodine receptors plays a role in NMJ formation in CRD-Nrg1−/− mice, we examined double mutant mice deficient in both CRD-Nrg1 and Ryr1 (CRD-Nrg1−/−Ryr1−/−). We found that, similar to CRD-Nrg1−/−Cacnb1−/− mice, neuromuscular synapse loss and muscle denervation were rescued in CRD-Nrg1−/−Ryr1−/− mice (Fig 8A and 8B). Specifically, the diaphragm muscles in CRD-Nrg1−/−Ryr1−/− mice were robustly innervated by highly defasciculated nerves (Fig 8A), similar to those observed in CRD-Nrg1−/−Snap25−/− (Fig 1A), CRD-Nrg1−/−Chat−/− (Fig 2C and 2D, S1 Fig), CRD-Nrg1−/−Chrna1−/− (Fig 5A and 5B) and CRD-Nrg1−/−Cacnb1−/− (Fig 6A) mice. Pre-synaptic nerve terminals were juxtaposed with post-synaptic AChR clusters in CRD-Nrg1−/−Ryr1−/− mice (Fig 8B). Furthermore, the average size of postsynaptic endplates was significantly increased in CRD-Nrg1−/−Ryr1−/− mice, compared with those of control mice (Fig 8C). Together, these results demonstrate that blocking E-C coupling, similar to blocking muscle excitation (as in CRD-Nrg1−/−Cacnb1−/− mice), is sufficient to reverse the loss of neuromuscular synapses in CRD-Nrg1−/− mice.

Schwann cell-deficient neuromuscular synapses exhibited increased spontaneous synaptic activity

Presynaptic nerve terminals at the NMJ release neurotransmitters spontaneously, leading to miniature end plate potentials (mEPPs) in post-synaptic muscle fibers [81, 82]. To determine the levels of spontaneous synaptic activity at the NMJs lacking Schwann cells, we carried out electrophysiological analyses in acutely isolated diaphragm muscle/phrenic nerve preparations. No spontaneous synaptic activity was detected in CRD-Nrg1−/− muscles (N = 6 mice, n = 36 cells), since the diaphragm muscles in CRD-Nrg1−/− muscles were completely denervated due to withdrawal of the nerve terminals. However, spontaneous muscle action potentials were readily observed in CRD-Nrg1−/− muscles—similar to spontaneous muscle action potentials recorded in control mice—and muscle contraction was observed following spontaneous muscle action potentials (Fig 9F). Spontaneous muscle action potentials were also observed in CRD-Nrg1−/−Cacnb1−/− and CRD-Nrg1−/−Ryr1−/− muscles, but these mutant muscles failed to contract after firing action potentials (Fig 9F).

Notably, significantly increased spontaneous synaptic activity was detected in both CRD-Nrg1−/−Cacnb1−/− and CRD-Nrg1−/−Ryr1−/− double mutant mice, compared with that in control, Cacnb1−/−, or Ryr1−/− single mutant mice (Fig 9A). In control mice, mEPP frequencies were approximately 1 event per minute (1.0 ± 0.1, n = 65 cells, N = 8 mice) (Fig 9B). Strikingly, mEPP frequencies increased 7200% over control in CRD-Nrg1−/−Cacnb1−/− mice (72.8 ± 9.4, n = 42 cells, N = 3 mice). Furthermore, mEPP frequencies in CRD-Nrg1−/−Cacnb1−/− mice were 260% higher than that in Cacnb1−/− mice (20.1 ± 4.1, n = 39 cells, N = 3 mice) (Fig 9B). Similarly, mEPP frequencies in CRD-Nrg1−/−Ryr1−/− mice (57.3 ± 8.3, n = 41 cells, N = 3 mice) were also significantly increased, compared with those in Ryr1−/− (21.6 ± 3.5, n = 75 cells, N = 7 mice) (Fig 9B).

MEPP amplitudes (Fig 9C), rise time (10–90%) (Fig 9D) and half-decay time (Fig 9E) were significantly increased in both CRD-Nrg1−/−Cacnb1−/− (amplitude: 3.29 ± 0.19 mV, rise time: 9.61 ± 0.41 ms; half-decay time: 20.35 ± 1.00 ms) and CRD-Nrg1−/−Ryr1−/− (amplitude:
3.35 ± 0.16 mV, rise time: 10.68 ± 0.54 ms; half-decay time: 23.94 ± 0.65 ms) mice, compared with control (amplitude: 2.38 ± 0.11 mV, rise time: 5.23 ± 0.60 ms; half-decay time: 13.16 ± 0.71 ms). However, MEPP amplitudes, rise time (10–90%) and half-decay time were not significantly different between CRD-Nrg1−/−Cacnb1−/− and Cacnb1−/− mice (amplitude: 3.28 ± 0.26 mV, rise time: 10.99 ± 0.63 ms; half-decay time: 18.12 ± 1.64 ms), or between CRD-Nrg1−/−Ryr1−/− and Ryr1−/− mice (amplitude: 3.70 ± 0.17 mV, rise time: 11.33 ± 0.43 ms; half-decay time: 23.41 ± 0.71 ms) (Fig 9C–9E). Thus, the increase in mEPP frequencies in CRD-Nrg1−/−
Cacnb1<sup>−/−</sup> and CRD-Nrg1<sup>−/−</sup>Ryr1<sup>−/−</sup> mice appeared to be the common, and, significant change between NMJs without Schwann cells (i.e., in CRD-Nrg1<sup>−/−</sup>Cacnb1<sup>−/−</sup> and CRD-Nrg1<sup>−/−</sup>Ryr1<sup>−/−</sup> mice) and those with Schwann cells (i.e., in Cacnb1<sup>−/−</sup> and Ryr1<sup>−/−</sup> mice). This suggests that the increase in spontaneous synaptic activity in both CRD-Nrg1<sup>−/−</sup>Cacnb1<sup>−/−</sup> and CRD-Nrg1<sup>−/−</sup>Ryr1<sup>−/−</sup> NMJs is attributable to the pre-synaptic elements. Together, these data demonstrated that neuromuscular synapses lacking Schwann cells exhibit increased pre-synaptic activity.
Discussion

Terminal Schwann cells are normally required for the assembly of the classic tripartite structure of the NMJs. Mutant mice deficient in type III Neuregulin 1, as well as mutant mice deficient in erbB2 or erbB3, lack Schwann cells, and consequently, lose the NMJs in the diaphragm muscle, after a transient nerve-muscle contact during development [37–41]. In this study, we found that NMJs can be established in CRD-Nrg1\(^{-/-}\) mice, in the absence of Schwann cells, if either synaptic or muscle activity is blocked. These findings are surprising because it shows that bipartite NMJs can be established \emph{in vivo}, in the absence of Schwann cells. Specifically, neuromuscular synapses are established in the absence of Schwann cells in CRD-Nrg1\(^{-/-}\) mice if one of the following genes is also ablated: Snap25 (neurotransmitter release), Chat (cholinergic transmission), Chrna1 (postsynaptic AChR), Cacnb1 (skeletal muscle DHPRs, the voltage sensor and L-type Ca\(^{2+}\) channel on the muscle membrane) or Ryr1 (skeletal muscle ryanodine receptors). These genetic manipulations follow a pathway that ultimately leads to muscle activity mediated by (DHPR/RyR) (Fig 10). Importantly, electrophysiological analyses revealed robust synaptic activity in the rescued, Schwann-cell deficient NMJs in CRD-Nrg1\(^{-/-}\)-Cacnb1\(^{-/-}\) or CRD-Nrg1\(^{-/-}\)-Ryr1\(^{-/-}\) mutant mice. Thus, a blockade of synaptic activity, although sufficient, is not necessary to preserve NMJs that lack Schwann cells. Instead, a blockade of muscle activity mediated by DHPRs and RyR1 is both necessary and sufficient for preserving NMJs that lack Schwann cells.

How might muscle inactivity contribute to the formation of neuromuscular synapses in the absence of Schwann cells? We can envision several distinct mechanisms. The first possibility is that muscle inactivity (i.e., a blockade of muscle activity) leads to increases in nerve branching, as shown previously [60, 61, 64, 83–85], which might accordingly increase the access of Schwann cell-deficient synapses to trophic factors that may preserve synapses [see review in

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**Fig 10.** The main findings are illustrated (A). Neuromuscular synapses (NMJs) are normally formed as a tripartite structure including a presynaptic nerve terminal (green), a postsynaptic muscle (orange) and a Schwann cell (glia, pink). Schwann cells are essential for the formation of the NMJ; in the absence of Schwann cells, nerve terminals withdraw from muscles, resulting in synapse loss (synapse degeneration) and muscle denervation (as shown in CRD-Nrg1\(^{-/-}\) mice). These defects are rescued by (1) a blockade of evoked neurotransmitter release (CRD-Nrg1\(^{-/-}\)-Snap25\(^{-/-}\)); (2) a blockade of neurotransmitter synthesis (CRD-Nrg1\(^{-/-}\)-Chat\(^{-/-}\)); (3) a blockade of post-synaptic AChRs (CRD-Nrg1\(^{-/-}\)-Chrna1\(^{-/-}\)); (4) a blockade of muscle dihydropyridine receptors (CRD-Nrg1\(^{-/-}\)-Cacnb1\(^{-/-}\)); and (5) a blockade of muscle ryanodine receptor 1 (CRD-Nrg1\(^{-/-}\)-Ryr1\(^{-/-}\)). These genetic rescues reveal a common pathway (B) that ultimately leads to muscle activity mediated by DHPR/RyR1. Therefore, a blockade of muscle activity is the key to rescuing muscle denervation/synapse loss in the absence of Schwann cells. Together, these genetic manipulations indicate that the blockade of muscle activity prevents muscle denervation and neuromuscular synapse loss caused by CRD-NRG1 deficiencies in mice.
ref. [86]). If this mechanism were to operative, one might expect to observe an increase in nerve branching and hence trophic access in mutants lacking both activity and Schwann cells. However, our data contradict this scenario because, despite marked increases in nerve defasciculation, no increases in nerve branching were detected in Schwann cell-deficient mutants, compared with mutant mice that retain Schwann cells (i.e., compare CRD-Nrg1−/−Snap25−/− versus Snap25−/−; CRD-Nrg1−/−Chat−/− versus Chat−/−; CRD-Nrg1−/−Chrna1−/− versus Chrna1−/−; CRD-Nrg1−/−Cacnb1−/− versus Cacnb1−/−; and CRD-Nrg1−/−Ryr1−/− versus Ryr1−/− mice).

These data further indicate that the well-documented increases in nerve branching induced by inactivity [60, 61, 64, 83–85] require the presence of Schwann cells.

A second possibility is that, muscle fibers normally destabilize presynaptic nerve terminals during neuromuscular synaptogenesis. And, this negative and dynamic destabilizing factor(s) requires active nerve and, ultimately, muscle activity. Terminal Schwann cells play crucial roles to antagonize the destabilizing activity and thus to stabilize the NMJ. In the absence of Schwann cells, the destabilizing activity of such factors on presynaptic terminals is unopposed, which leads to synapse loss at the NMJ. In this scenario, blocking either nerve or muscle activity (as in CRD-Nrg1−/−Snap25−/−, CRD-Nrg1−/−Chat−/−, CRD-Nrg1−/−Chrna1−/−, CRD-Nrg1−/−Cacnb1−/−, or CRD-Nrg1−/−Ryr1−/− mice), would lead to a blockade of the muscle-derived destabilizing factor(s), which would allow Schwann cell deficient NMJs to form. Identification of the muscle-derived, muscle activity dependent factor(s) that regulate NMJ formation will provide important new insights into the mechanisms underlying neuromuscular synapse formation, maintenance and elimination.

A third, non-mutually exclusive possibility from the second possibility described above, is that Schwann cells may regulate synaptic activity itself. In this instance, the absence of Schwann cells would be predicted to lead to dysregulated synaptic activity. We have previously shown that spontaneous neuromuscular synaptic activity is markedly increased in Cacnb1−/− and Ryr1−/− mice, compared with control, likely due to precocious maturation of the NMJs in Cacnb1−/− and Ryr1−/− mice [68]. Remarkably, spontaneous synaptic activity is significantly further increased in Schwann cell deficient NMJs (i.e., in CRD-Nrg1−/−Cacnb1−/− and CRD-Nrg1−/−Ryr1−/− mice), compared with NMJs in Cacnb1−/− or Ryr1−/− mice, respectively. These further increases in spontaneous synaptic activity are likely due to the lack of Schwann cells at the NMJ in CRD-Nrg1−/−Cacnb1−/− and CRD-Nrg1−/−Ryr1−/− mice, suggesting that Schwann cells may regulate spontaneous synaptic activity during NMJ synaptogenesis. We do not know how evoked synaptic transmission is affected at the NMJs in CRD-Nrg1−/−Cacnb1−/− and CRD-Nrg1−/−Ryr1−/− mice since we were unable to obtain recordings of evoked end-plate potentials from Schwann cell deficient mutant mice due to technical difficulties. During our electrophysiological recordings, we have made numerous attempts to apply suction electrodes to the phrenic nerves in order to deliver electrical stimulation to the nerves in CRD-Nrg1−/−Cacnb1−/− or CRD-Nrg1−/−Ryr1−/− mice. However, we were unable to visualize live, unstained phrenic nerves under the microscope in double mutant mice, due to the fact that the phrenic nerves appeared transparent in the absence of Schwann cells. Nevertheless, the marked increases in spontaneous synaptic activity in NMJs lacking Schwann cells (i.e., CRD-Nrg1−/−Cacnb1−/− versus Cacnb1−/−; and CRD-Nrg1−/−Ryr1−/− versus Ryr1−/−) support the possibility, namely that Schwann cells may protect developing neuromuscular synapses by negatively regulating synaptic activity. Thus, the regulation of synaptic activity by Schwann cells appears to be critical for NMJ formation during development. Consistent with this idea, a recent Schwann cell ablation study demonstrates that these cells continue to regulate synaptic activity postnatally (Barik et al., 2016). These studies of synapses lacking Schwann cells give added support to the studies of intact synapses that demonstrate that the activation of Schwann cells by neural activity regulates presynaptic as well as postsynaptic function [48]. Previous studies
have shown that Schwann cells are required for the maintenance of presynaptic structure in developing NMJs [11, 14] and play important roles in synapse elimination [15, 87]. It is possible that Schwann cells may preserve the NMJs, at least in part, by regulating the levels of synaptic activity (and therefore regulate muscle activity).

The results of the current study show that the level of muscle activity is a key regulator of neuromuscular synapse formation–NMJs can be established even in the absence of Schwann cells if muscle activity mediated by DHPR/Ryr1 is blocked. Our data are consistent with in vitro studies by O’Brien et al., [88, 89], which show that excessive activity, either by the topical application of ACh and high [Ca\(^{2+}\)] to immature neuromuscular synapses, or by continuous stimulation of the nerve or the muscle, leads to muscle denervation. Importantly, our findings demonstrate that a blockade of muscle activity, instead of neuronal activity, is the key to preserving the developing neuromuscular synapses. We show that neuromuscular synapses are established in the absence of Schwann cells when muscle activity is eliminated. These findings further suggest that skeletal muscle activity might destabilize developing presynaptic nerves and that Schwann cells play crucial roles in counteracting such a destabilizing activity to preserve neuromuscular synapses during development.

Materials & methods

Ethics statement

All experimental protocols followed National Institutes of Health Guidelines and were approved by the University of Texas Southwestern Institutional Animal Care and Use Committee. The APN approval number is 2015–101081.

Mice

Single mutant mice used in this study were previously generated; these mutant mice include CRD-Nrg1\(^{tm1Lwr}\), MGI:1928831) [38], Snap25\(^{tm1Mcw}\), MGI: 2180178) [50], Chat\(^{tm1Fhg}\), MGI:2450310) [60], Chrna1\(^{tm1Klee}\), MGI:4462390) [64], Cacnb1\(^{tm1Rgg}\), MGI:2181804) [67, 68], and Ryr1\(^{tm1Alle}\), MGI:4887253) [90–92]. We generated double mutant mice by crossing heterozygote mice to generate compound heterozygote mice, and then bred compound heterozygote mice together to generate homozygous mutant mice. As with single mutants, all homozygous double mutant mice died perinatally, due to NMJ defects. Thus, analyses were performed on mouse embryos between E14.5–E18.5, staged by timed-mating. Embryonic day 0.5 (E0.5) marked the day when a vaginal plug was detected. Mouse embryos were collected by cesarean section from anesthetized pregnant mice. Table 1 summarizes the numbers of mouse embryos analyzed in this study.

Light microscopy

Morphological analyses of the NMJs. Analyses were performed using procedures described previously [93, 94]. Briefly, skeletal muscles were fixed in 2% paraformaldehyde, rinsed in sodium phosphate buffer saline (PBS) for 90 minutes and incubated in 100 mM glycine in PBS for 30 minutes. Muscle samples were then incubated in a blocking buffer containing 500 mM NaCl, 0.01 M phosphate buffer, 1% BSA and 0.01% thimerosal for 45 minutes. AChRs were detected using either Texas red- or Alexa Fluor 647- conjugated α-bungarotoxin (α-bgt) (2 nM, Molecular Probes). For double-labeling experiments, we used Texas Red-conjugated α-bungarotoxin (α-bgt) (2 nM, Molecular Probes) for AChRs and the following primary antibodies for pre-synaptic nerve/nerve terminals: anti-NF150 (neurofilament protein (1:1000, Chemicon, Temecula, CA), anti-synaptotagmin 2 (Syt2) (1:1000) [95], anti-syntaxin 1 (1:1000,
generous gifts from Dr. Thomas Südhof, Stanford University School of Medicine, Palo Alto, CA), followed by fluorescein (FITC)-conjugated goat anti-rabbit IgG secondary antibodies (code # 111-095-144, Jackson ImmunoResearch). For triple-labeling experiments, we used rabbit anti-S100β (1:500, Dako, Carpinteria, CA) to label Schwann cells and mouse anti-neurofilament H non-phosphorylated (1:500, SMI-32, abcam, Cambridge, MA) to label nerves, and Alexa Fluor 647-conjugated α-bungarotoxin (α-bgt) (2 nM, Molecular Probes) to label AChRs, followed by fluorescein (FITC)-conjugated goat anti-rabbit IgG (Code # 111-095-144, Jackson ImmunoResearch) and Texas red-conjugated goat anti-mouse IgG (Code # 115-075-146, Jackson ImmunoResearch). After secondary antibody incubation, muscle samples were washed extensively and mounted in anti-fade medium (Fluoro-Gel with Tris Buffer, diluted 1:1 with 50% glycerol, Electron Microscopy Sciences, Hatfield, PA). Images were acquired using a Zeiss LSM 510 confocal microscope.

**Immunostaining of motor neuron.** Cryostat sections (transverse section, 14 μm) were collected from cervical spinal segments of mouse embryos (E18.5). The sections were incubated with anti-choline transporter (CHT) antibodies [62] (polyclonal, 1:1000, generous gifts from Dr. Randy Blakely, Vanderbilt University, Nashville, Tennessee), followed by fluorescein (FITC)-conjugated goat anti-rabbit IgG secondary antibodies (#111-095-144, Jackson ImmunoResearch) and Texas red-conjugated goat anti-mouse IgG (Code # 115-075-146, Jackson ImmunoResearch). After secondary antibody incubation, muscle samples were washed extensively and mounted in anti-fade medium (Fluoro-Gel with Tris Buffer, diluted 1:1 with 50% glycerol, Electron Microscopy Sciences, Hatfield, PA). Images were acquired using a Zeiss LSM 510 confocal microscope.

**Electrophysiology**

E18.5 diaphragm muscles from mutant mice and their littermate controls were used for intracellular recording as previously described [94, 96]. Diaphragm muscles with phrenic nerves attached were acutely isolated in oxygenated (95% O₂, 5% CO₂) Ringer’s solution (136.8 mM NaCl, 5 mM KCl, 12 mM NaHCO₃, 1 mM NaH₂PO₄, 1 mM MgCl₂, 2 mM CaCl₂, and 11 mM
d-glucose, pH 7.3) [97]. End-plate regions were identified under a water-immersion objective (Olympus BX51 WI) and impaled with glass micropipettes filled with 2 M potassium citrate and 10 mM potassium chloride (resistance 20–40 MΩ). Miniature end-plate potentials (mEPPs) were acquired using an intracellular amplifier (AxoClamp-2B) and digitized with a Digidata 1332 (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed with Mini Analysis Program (Synaptosoft, Inc., Decatur, GA).

Electron microscopy
Diaphragm muscles with phrenic nerves attached were dissected in Ringer’s solution from mouse embryos at E18.5. Samples were then fixed with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and kept in the same fixative overnight at 4°C. After a rinse in 0.1 M phosphate buffer, diaphragm muscles were trimmed into small pieces. Tissues were then post-fixed with 1% osmium tetroxide for 3 hr. on ice, dehydrated through a graded series of ethanol, infiltrated, and polymerized in Epon 812 (Polysciences, Warrington, PA, USA). Prior to embedding in Epon, the phrenic nerve trunks were cut half-way from the muscles, and the nerve trunks were embedded separately. These phrenic nerves were later cross sectioned for EM. Ultrathin sections (70 nm) were prepared and mounted on Formvar-coated grids, then stained with uranyl acetate and lead citrate. Electron micrographs were acquired using a Tecnai electron microscope (Netherlands) operated at 120 kV.

Statistical analysis
End-plate sizes were measured using ImageJ (NIH), with the measurements made blind with respect to the genotypes (at least 3 mice were analyzed in each group). Raw data were pooled and calculated as the mean ± standard error of the mean (SEM). Sigma Plot (version 11.0) was used to analyze statistics. Statistical differences among multiple groups were determined using one-way analysis of variance (ANOVA), followed by a Tukey post hoc test for pairwise multiple comparison between groups.

Supporting information
S1 Fig. Rescue of diaphragm muscle denervation in CRD-Nrg1−/− Chat−/− mice (E16.5). Embryonic diaphragm muscles (E16.5) from the control (CRD-Nrg1+/+ Chat+/−) (A), CRD-Nrg1−/− Chat+/− (B), CRD-Nrg1+/− Chat−/− (C) and CRD-Nrg1−/− Chat−/− (D) mice were immuno-stained by a mixture of antibodies (anti-NF150 and anti-synaptotagmin 2) to reveal innervation pattern (green). Low power images show the innervation pattern of the entire diaphragm muscles. The phrenic nerves innervate the diaphragm muscles bilaterally. The phrenic nerves are absent in CRD-Nrg1−/− Chat−/− muscle (B); this lack of innervation is rescued in CRD-Nrg1−/− Chat−/− muscle (D). Note that the nerves are highly defasciculated in CRD-Nrg1−/− Chat−/− muscle. Scale bar: A-D: 500 μm.

(TIF)

S2 Fig. Motor neuron staining and motor axon counts in CRD-Nrg1−/− Chat−/− mice (E18.5). A: Cross sections of cervical spinal cords were immune-stained by anti-CHT antibodies, which label motor neurons (arrow) in the ventral horn of the spinal cord (upper panels show low-power images of entire spinal cords, and lower panels show high-power views of ventral horn). B: Low-power EM images of the phrenic nerve trunk (cross section) showing individual axons within the nerve trunk. C: Quantification of motor axons. The average motor axon numbers per phrenic nerve are similar between control (248 ± 8, N = 3 mice) and CRD-
Nrg1−/−Chat−/− (244 ± 11, N = 3 mice).

S3 Fig. Amplitude distribution of mEPPs. The amplitudes were plotted against either event numbers (upper panels) or percentage of total events (lower panels). In both control and mutant mice, the amplitude distribution patterns exhibited a single peak, with a right-skewed tail. The mEPP frequencies were massively increased in mutants [Cacnb1−/−, 3 mice, 39 cells, 1919 events (B); CRD-Nrg1−/−Cacnb1−/−, 3 mice, 42 cells, 8621 events (C); Ryr1−/−, 4 mice, 36 cells, 1726 events (D) and CRD-Nrg1−/−Ryr1−/− 3 mice, 41 cells, 4707 events (E)], compared with the control [8 mice, 92 cells, 329 events (A)].

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