# Rescuing $Z^+$ agrin splicing in *Nova* null mice restores synapse formation and unmasks a physiologic defect in motor neuron firing

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**Synapse formation at the neuromuscular junction (NMJ) requires an alternatively spliced variant of agrin ($Z^+$ agrin) that is produced only by neurons. Here, we show that Nova1 and Nova2, neuron-specific splicing factors identified as targets in autoimmune motor disease, are essential regulators of $Z^+$ agrin. *Nova1*/*Nova2* double knockout mice are paralyzed and fail to cluster AChRs at the NMJ, and breeding them with transgenic mice constitutively expressing $Z^+$ agrin in motor neurons rescued AChR clustering. Surprisingly, however, these rescued mice remained paralyzed, while electrophysiologic studies demonstrated that the motor axon and synapse were functional-spontaneous and evoked recordings revealed synaptic transmission and muscle contraction. These results point to a proximal defect in motor neuron firing in the absence of Nova and reveal a previously unsuspected role for RNA regulation in the physiologic activation of motor neurons.**

alternative splicing | physiology | neuromuscular junction | neuron activity

The regulation of RNA biology plays critical roles in synaptic transmission and human neurologic disease (1, 2). We have developed assays to assess the function of Nova, a neuron-specific RNA binding protein (3, 4) targeted in patients with a paraneoplastic neurologic degeneration manifest by abnormal motor control in the brainstem and spinal cord (5, 6). Nova harbors 3 KH-type RNA binding domains, and in vitro RNA selection (4, 7, 8) together with X-ray crystallography (9) revealed that Nova binds to RNA elements harboring clusters of YCAY motifs. A combination of biochemical (10–12), cross-linking and immunoprecipitation (CLIP) (13, 14), microarray (15), and bioinformatic (16) studies have led to the conclusion that Nova regulates alternative splicing of neuronal transcripts encoding synaptic proteins. Moreover, identification of Nova targets has begun to predict specific defects in the synaptic physiology of *Nova* KO mice (16, 17).

Studies of the neuromuscular junction (NMJ), arguably the best-understood mammalian synapse, provided an early example of the role of RNA regulation in neurons. Motor neurons synthesize a 200-kDa protein termed agrin, named for its ability to promote aggregation of AChR clusters on the muscle surface directly beneath the nerve terminal (18, 19). Most cell types synthesize agrin, but only neurons produce $Z^+$ agrin, an alternatively spliced isoform. The $Z^+$ exons encode a domain of 8–19 amino acids that confers up to a 1,000-fold increase in AChR clustering activity relative to $Z^-$ agrin (20). *Agrin* KO mice, and mice in which the $Z^+$ exons have been deleted ($agrin^{Z-/Z-}$), are paralyzed and die at birth from diaphragmatic paralysis (19, 21).

$Z^+$ agrin induces AChR clusters through interaction with the agrin receptor, Lrp4, leading to phosphorylation of the muscle-specific receptor tyrosine kinase MuSK (refs. 19 and 22–24 and reviewed in refs. 25 and 26). AChR clusters are prepatterned in the central, prospective synaptic region of the muscle independent of innervation (27–29), and this muscle prepattern dictates where motor axons grow and form synapses (30, 31). Motor axons modify this prepattern by releasing both $Z^+$ agrin and ACh. ACh acts to disperse AChR, even in *agrin* null mice (32, 33), and agrin/choline acetyl transferase double KOs (DKOs) show persistent AChR prepatterned clusters, although functional NMJs do not form (33). These observations have led to a model of NMJ formation (26) in which the initial role of $Z^+$ agrin is to inhibit the local dispersion of AChR clusters mediated by ACh release (33).

Despite the central role for $Z^+$ agrin in synapse biology, the mechanisms regulating its neuronal alternative splicing are unknown. Here, we observe that $Nova1^{-/-}/Nova2^{-/-}$ (*Nova* DKO) mice are born alive but are paralyzed, which led us to examine $Z^+$ agrin splicing in these mice. We find that Nova is a critical regulator of $Z^+$ agrin splicing, but surprisingly, rescuing $Z^+$ agrin expression in motor neurons rescues AChR clustering, but leaves mice paralyzed. Electrophysiologic recording from motor axons in these mice revealed that spontaneous and evoked ACh release was intact. These findings reveal that AChR clustering can be dissociated from motor nerve function and suggest that Nova-regulated RNAs encoding proteins functioning proximal to the nerve axon are critical for physiologic motor neuron activity.

## Results

**Nova Regulates $Z^+$ Agrin Splicing.** More than 70 Nova RNA targets have been validated in *Nova* KO mice, revealing that Nova regulates alternative splicing of neuronal transcripts encoding synaptic proteins (13–16, 34). Screens for alternatively spliced exons in the spinal cord of *Nova1* null mice failed to show changes in $Z^+$ agrin splicing (15). These studies focused on Nova1, which is highly expressed in the ventral spinal cord, in contrast to Nova2, which is expressed in a graded manner, with greatest expression in the dorsal spinal cord (4). However, when we generated *Nova* DKO mice, we observed that they were born alive but paralyzed (see below), prompting us to reexplore whether a redundant action of *Nova2* might have masked a role for Nova proteins in $Z^+$ agrin splicing.

We developed a more sensitive agrin splicing assay than used

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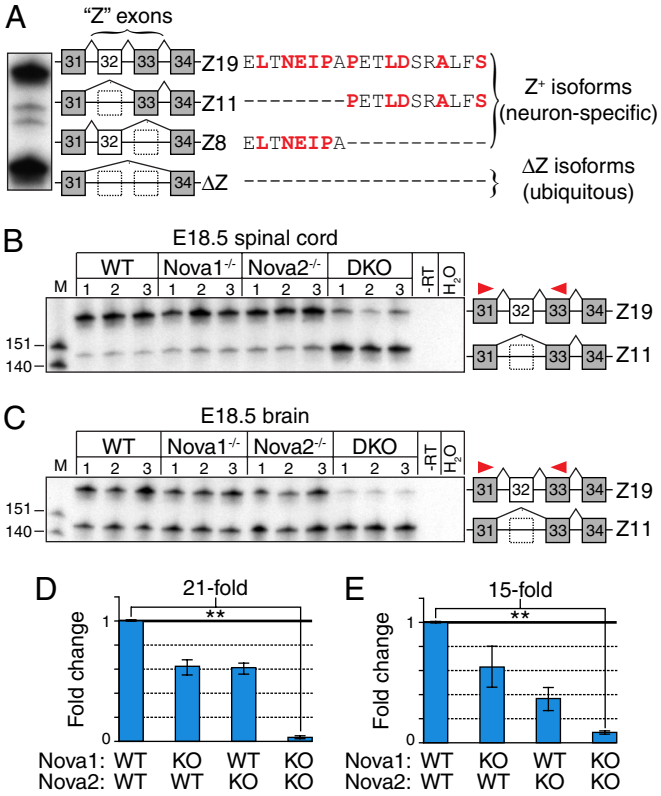


**Fig. 1.** Nova regulates alternative splicing of *Agrin*. (*A*) Agrin $Z^+$ isoforms detectable by RT-PCR and shown schematically, with conserved residues between human and chick shown in red. (*B* and *C*) The presence or absence of agrin $Z^+$ exon 32-containing isoforms measured by RT-PCR of total RNA from E18.5 WT or *Nova*-null spinal cord (*B*) and total brain (*C*). Results of 3 biological replicates per genotype are shown. Primers (red arrowheads) amplify exon 32-containing isoforms. (*D* and *E*) Quantitation of data in *B* and *C*, normalized to WT, showing a 15- to 21-fold reduction in utilization of *agrin* exon 32 in *Nova* DKO mice. Error bars represent SD.

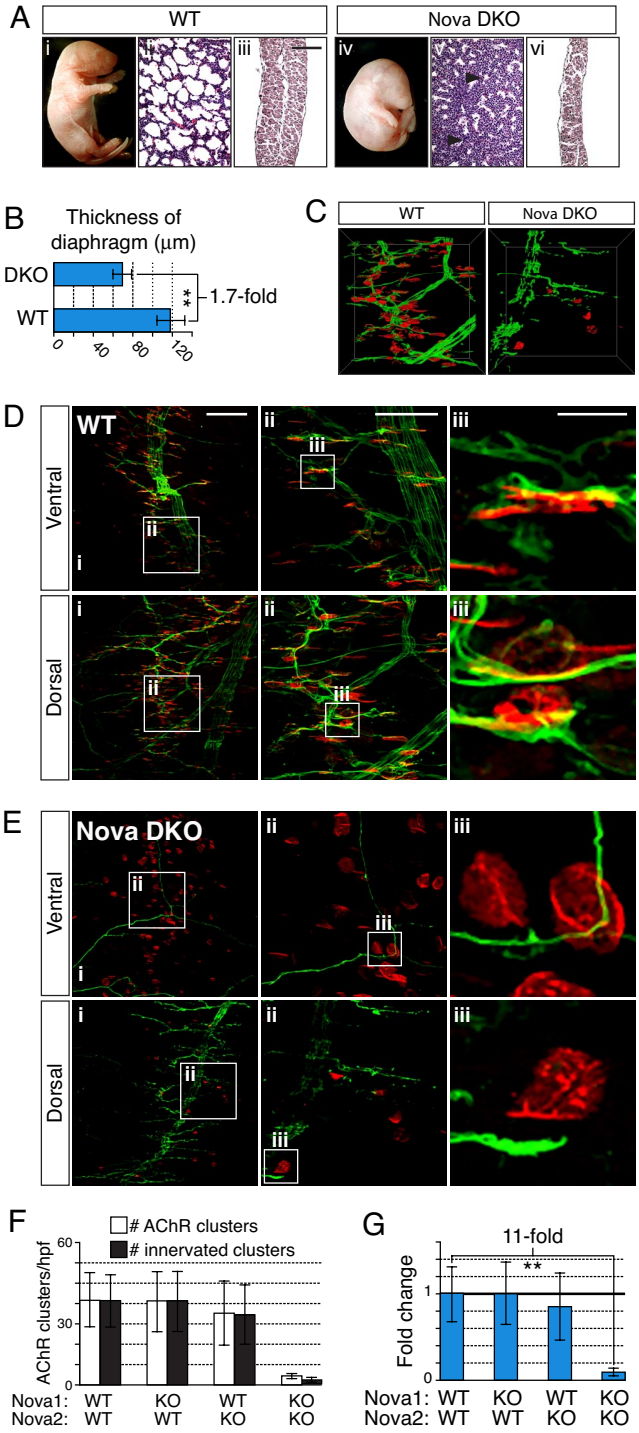


**Fig. 2.** *Nova* DKO mice are paralyzed with defective neuromuscular synapses. (*A*) Paraffin-embedded sections of E18.5 *Nova* WT (*Left*) and DKO (*Right*) animals stained with haematoxylin and eosin reveals lung atelectasis and collapsed alveolar air spaces (arrowheads) and diaphragmatic atrophy (*vi*) in DKO animals. (Scale bar: 100 μm.) (*B*) Quantitation of diaphragmatic muscle thickness reveals a 1.7-fold reduction in DKO mice. (*C*) 3D reconstructions of high-magnification images (63×) of NMJ staining of the diaphragm of E18.5 WT and *Nova* DKO mice. Nerve and nerve terminals are shown in green (synaptophysin and neurofilament), AChRs are shown in red (*a*-bungarotoxin). (*D* and *E*) NMJ staining (as in *C*) from WT (*D*) or Nova DKO (*E*) mice. White rectangles indicate areas shown at higher magnification in *ii* and *iii*. (Scale bars: *i*, 100 μm; *ii*, 50 μm; *iii*, 10 μm.) (*F*) Quantitation of total number of AChR clusters per high power filed (63×, empty bars) and number of clusters apposed by a neurite (filled bars) in mid to ventral areas of the left hemidiaphragm where phrenic nerve is present. (*G*) Fold change in the number of AChR clusters normalized to WT. Error bars indicate SD. **, $P < 0.001$ by *t* test.

previously (Fig. S1 *A–D*). The functional $Z^+$ agrin isoforms include sequences encoded by alternate exons 32 and 33 (nomenclature of ref. 21; Fig. 1*A*). The Z8 (with 8 additional amino acids encoded by exon 32) and Z19 (with 19 amino acids encoded by exons 32 and 33) isoforms are 150-fold more potent in promoting AChR clustering than isoforms lacking exon 32 and 45-fold more potent than the Z11 isoform [including exon 33 alone (20, 35–37)]. We found a small effect (<2-fold) of either Nova1 or Nova2 on $Z^+$ agrin splicing in the spinal cord; interestingly, Nova preferentially regulated splicing of the most potent $Z^+$ agrin (exon 32-containing) isoforms (Fig. 1 *B* and *D*). This effect was much more evident when we compared WT and *Nova* DKO spinal cord RNA, indicating functional redundancy of the 2 proteins in regulating $Z^+$ agrin splicing; *Nova* DKO spinal cord produced only ≈5% of WT levels of exon 32-containing $Z^+$ agrin isoforms. Nova's action was also evident in the brain (Fig. 1 *C* and *E*) and was specific, because there were no changes in usage of the alternatively spliced *agrin* Y exon (Fig. S1 *E–H*). We independently examined Nova-dependent splicing of all exon variants between exons 31 and 34, yielding similar results, although this RT-PCR assay was less sensitive (it could not be detected in *Nova1*$^{-/-}$ mice as reported (15) (Fig. S1 *A–D* and Table S1). Taken together, these results indicate that Nova proteins function together as critical splicing factors responsible for regulating production of the potent $Z^+$ agrin isoforms in motor neurons.

**Analysis of the NMJ in *Nova*$^{-/-}$ Mice.** *Nova* DKO mice were born alive, but had no motor movement, even after noxious sensory

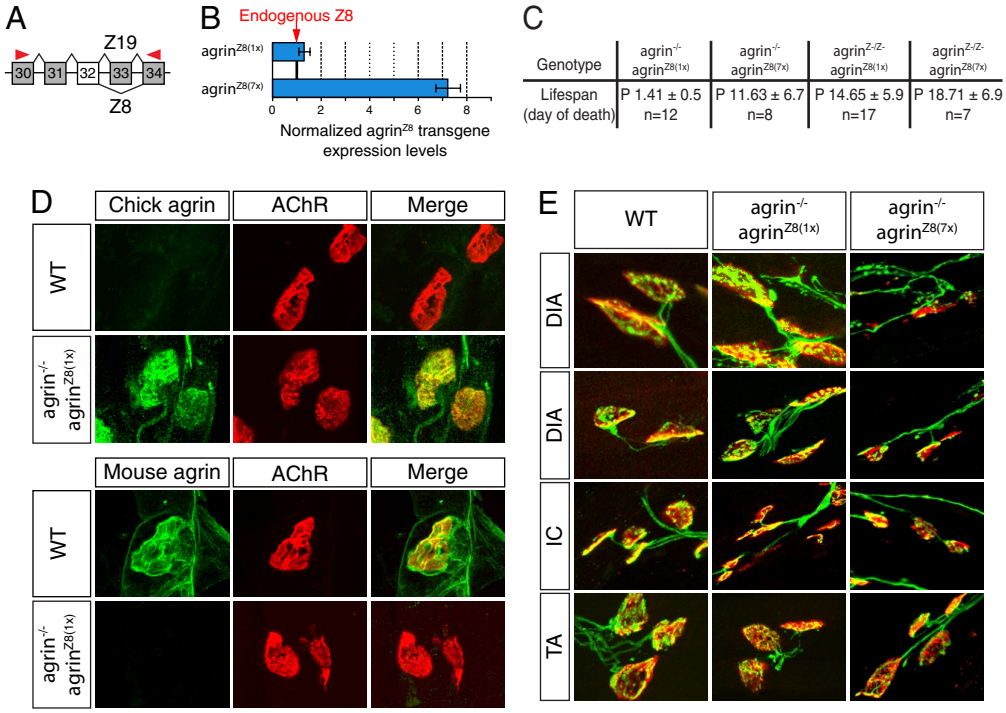


**Fig. 3.** Transgenic expression of $Z^+$ *Agrin* in motor neurons restores neuromuscular synapse formation and rescues neonatal lethality in *Agrin* mutant mice. (*A*) Schematic representation of the relative positions of mouse *agrin* primers used for qPCR quantitation. (*B*) qPCR quantitation of chick *agrin* transgene expression in the mouse spinal cord, shown as fold change compared with endogenous mouse Z8 *agrin* mRNA (see also Table S1). Error bars indicate SD. (*C*) The $agrin^{Z8(1x)}$ and $agrin^{Z8(7x)}$ transgenes rescue the lethality of $agrin^{-/-}$ and $agrin^{Z-/Z-}$ mice. (*D*) Endogenous mouse agrin is expressed at synaptic sites (stained as in Fig. 2) in WT mice but is not detectable at synapses in $agrin^{-/-}$ mice expressing $agrin^{Z8(1x)}$. In contrast, in $agrin^{-/-}/agrin^{Z8(1x)}$ mice, mouse agrin is absent but chick Y4Z8 agrin is concentrated at the NMJ, demonstrating that chick Y4Z8 agrin is correctly targeted to synapses. (*E*) Each *agrin* transgene rescues the formation of NMJ in P0 mice. These neuromuscular synapses continue to mature and become more complex by P7 in diaphragm (DIA), intercostal (IC) and tibialis anterior (TA) muscles. (Magnifications: *D* and *E*, 200×.)

stimuli (tail pinch), and died immediately after birth. These mice were stiff but otherwise had normal gross morphology (Fig. 2*Aiv*) with a beating heart. Histological analysis indicated that these animals never inhaled, because lung alveoli had failed to expand (Fig. 2*Av*), and diaphragmatic muscle atrophy (Fig. 2 *Avi* and *B*), indicating lack of functional motor innervation. Similar observations have been made in mice with severe defects in the development of the NMJ (19, 21, 22) and were consistent with the possibility that Nova's action on $Z^+$ agrin splicing had a functional consequence on NMJ development.

We analyzed the histology of the phrenic nerve muscle junction by labeling AChRs with α-bungarotoxin and nerve axons and terminals with antibodies against neurofilament and synaptophysin, respectively (29). In contrast to WT embryonic day (E) 18.5 embryos (Fig. 2 *C* and *D*), the NMJ in *Nova* DKO mice had very few AChR clusters, and only rarely were they in contact with motor axon terminals (Fig. 2 *C* and *E*). Quantitation (Fig. 2 *F* and *G* and Table S2) confirmed these observations, demonstrating that <5% of AChR clusters were apposed by nerve terminals in *Nova* DKO mice. Similar results have been seen in E18.5 $agrin^{Z-/Z-}$ mice (21), suggesting that the defect in Nova-mediated agrin $Z^+$ exon inclusion might be responsible for the defective morphology and physiologic dysfunction of the NMJ.

**Rescue of *Agrin* $Z^-$ Mice with *Agrin* $Z^+$ Transgenes.** To test whether the loss of $Z^+$ agrin underlay the NMJ defects in *Nova* KO mice, we generated transgenic mice that express $Z^+$ agrin from a chick *agrin* cDNA driven by a motor neuron-specific promoter (HB9; refs. 38–40 and Fig. 3*A*). Two independent transgenic lines were generated, one expressing nearly normal levels of Z8 *agrin* mRNA (1.25 ± 0.21-fold, $P < 0.001$; $agrin^{Z8(1x)}$ mice), and a second line overexpressing Z8 *agrin* by ≈7-fold (7.2 ± 0.49, $P < 0.001$; $agrin^{Z8(7x)}$ mice; Fig. 3*B*). This fortuitous event gave us the opportunity to assess whether the defect in NMJ formation in *Nova* DKO mice could be rescued by normal or overexpressed $Z^+$ agrin.

We first evaluated the integrity of the $agrin^{Z8(1x)}$ and $agrin^{Z8(7x)}$ transgenes by crossing them into agrin null mice ($agrin^{-/-}$). $Agrin^{-/-}$ mice bred to either $agrin^{Z8(1x)}$ or $agrin^{Z8(7x)}$ mice survived for several days or up to 2 weeks, respectively (Fig. 3*C*), and $agrin^{-/-}$ mice that carry 2 copies of $agrin^{Z8(1x)}$ survived for nearly 1 week, indicating that these transgenes rescued survival in $agrin^{-/-}$ mice in a dose-dependent manner. The lifespan of these mice is shorter than reported in a third set of rescued *agrin* null mice [in which 50% of animals lived to 50 days (41)], which may relate to different levels of transgene expression. Crossing these transgenes into $Z^-$ agrin mice ($agrin^{Z-/Z-}$) promoted survival for ≈3 weeks (Fig. 3*C*), suggesting that $Z^-$ agrin isoforms have a role in extending survival during the first few postnatal (P) weeks. Whole-mount stains from P0 and P7 mice demonstrated that both transgenes were expressed in a normal pattern and restored all of the hallmark features of neuromuscular synapses in diaphragm and limb muscles (Fig. 3 *D* and *E*).

**Rescue of Nova DKO with *Agrin* $Z^+$ Transgenes.** We crossed the $agrin^{Z8(1x)}$ and $agrin^{Z8(7x)}$ transgenes into *Nova* DKO mutant mice and evaluated their NMJ. Both *agrin* transgenes restored AChRs clusters in regions of the diaphragm muscle where the nerve was present, and these clusters were contacted by motor axons (Fig. 4*A–E* and Fig. S2). Quantitation revealed that AChR clusters were rescued in *Nova* DKO/$agrin^{Z8(7x)}$ mice to near normal levels, and between 59 and 83% were apposed by nerve terminals (Fig. 4 *D* and *E*, Fig. S2, and Table S2). These changes were also seen in additional muscles in *Nova* DKO/$agrin^{Z8(7x)}$ mice (soleus, gastrocnemius, and vastus muscles; Fig. S3). Thus, $Z^+$ *agrin* rescues AChR

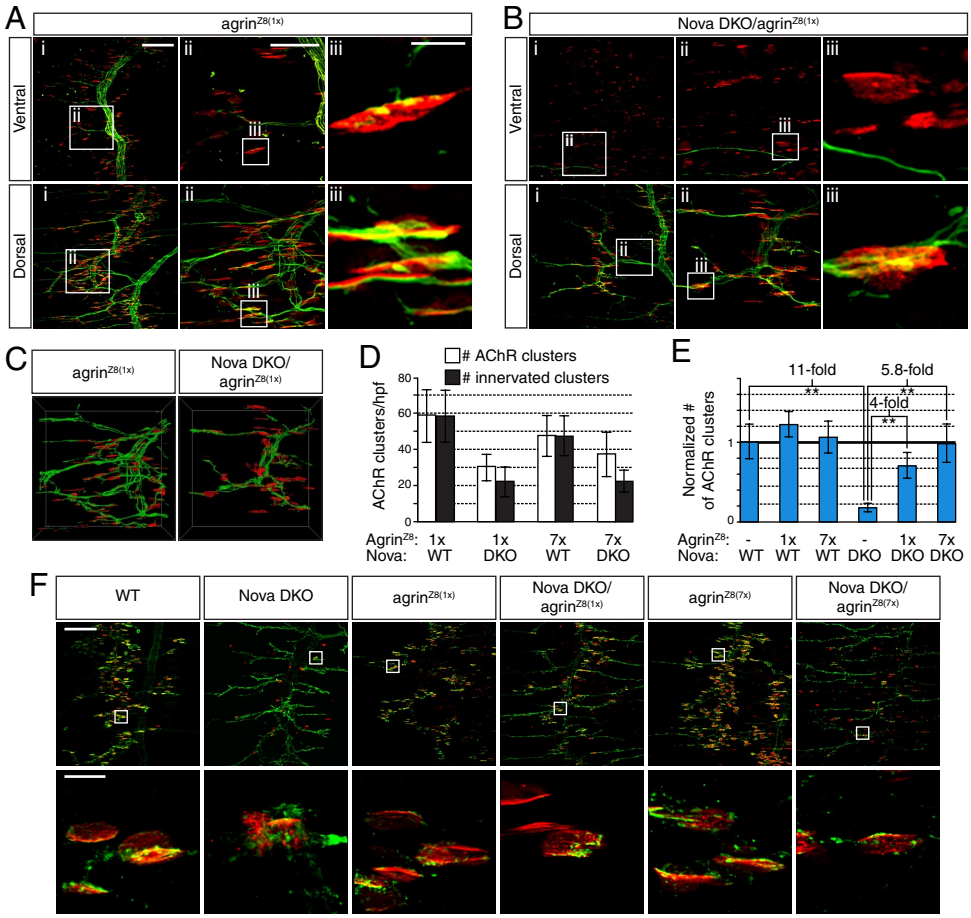


**Fig. 4.** Transgenic expression of $Z^+$ agrin in motor neurons rescues AChR clustering in the diaphragm of *Nova* DKO mice. (*A* and *B*) NMJ staining of the diaphragm of E18.5 *agrin*$^{Z8(1x)}$ (*A*) or *Nova* DKO/*agrin*$^{Z8(7x)}$ (*B*) mice, stained as in Fig. 2, showing that transgenic expression of $Z^+$ agrin in *Nova* DKO animals restores formation of AChR clusters in regions where the phrenic nerve has grown into the muscle, and these clusters are apposed by nerve terminals. Areas in white rectangles are shown at higher magnification (200×) in *ii* (63×) and *iii* (200×). (Scale bars: *i*, 100 μm; *ii*, 50 μm; *iii*, 10 μm.) (*C*) 3D reconstructions of motor endplates from *agrin*$^{Z8(1x)}$ (*Left*) and *Nova* DKO/*agrin*$^{Z8(1x)}$ (*Right*) E18.5 animals. (Magnification: 63×.) (*D* and *E*) *agrin*$^{Z8}$ transgenes rescue the number of AChR clusters in *Nova* DKO animals. The number of AChR clusters is shown as number per high power field (63×; *D*) and as fold change normalized to WT (*E*). Error bars indicate SD. **, $P < 0.001$. (*F*) Synaptophysin staining at NMJs in the diaphragm in WT, *Nova* DKO, *agrin*$^{Z8(1x)}$, and *Nova* DKO/*agrin*$^{Z8(1x)}$ E18.5 embryos. Preterminal axons containing synaptophysin-positive synaptic vesicles are shown in green; postsynaptic sites (AChR clusters) are shown in red. Areas in white rectangles are shown at higher magnification below. (Scale bars: *Upper*, 100 μm; *Lower*, 10 μm.)

clustering and nerve terminal apposition in *Nova* DKO mice, indicating that *agrin* pre-mRNA is a critical Nova target and its failure to be spliced properly at the Z site is responsible for the histologic NMJ defect.

We also noted a general increase (1.2- to 2.5-fold) in α-bungarotoxin staining throughout the diaphragm of *Nova* DKO/*agrin*$^{Z8}$ mice [Fig. 4*B*, Fig. S2, and Table S3; seen also in other muscles (Fig. S3)]. These observations suggest that ACh release may be sufficient to disperse prepatterned AChRs in *Nova* DKO/*agrin*$^{Z8}$ mice, but insufficient to elicit activity-dependent mechanisms that suppress nonsynaptic AChR expression (42, 43), or perhaps they unmask a neuronal signal necessary for proper AChR endocytosis. We also assessed the morphology of nerve terminals. In *Nova* DKO mice, nerve terminals stained with synaptophysin had an unusual shape resembling immature growth cones (Fig. 4*F*), and these defects were restored to normal appearance in *Nova* DKO/*agrin*$^{Z8}$ mice (Fig. 4*F*). We noted an abnormal presence of synaptophysin in axons in *Nova* DKO/*agrin*$^{Z8}$ mice, suggesting a defect in the distribution of synaptic proteins from the cell body to the axon terminus, but this finding was also present in *Nova* DKO mice (Fig. 4*F*).

**Motor Neuron Physiology in Nova DKO Mice.** Unexpectedly, neither the *agrin*$^{Z8(1x)}$ nor *agrin*$^{Z8(7x)}$ transgene rescued the physiologic defect in *Nova* DKO mice; both sets of mice were born paralyzed and died shortly after birth (Fig. 5*A*). This observation, together with the finding that Nova regulates RNAs encoding synaptic proteins (13–16), led us to assess the electrophysiology of the NMJ in these mice. Intracellular recordings were made at the motor endplate from acute preparations of intact phrenic nerve diaphragms from E18.5 mice. When phrenic nerve motor axons from *Nova* DKO or *Nova* DKO/*agrin*$^{Z8}$ mice were stimulated at 1 Hz, reproducible contraction of the diaphragm muscle was evident specifically in areas that were innervated in 5/6 mice (2 of 2 *Nova* DKO and 3 of 4 *Nova* DKO/*agrin*$^{Z8}$ mice). Muscle contractions could be blocked by α-bungarotoxin, a specific nAChR antagonist. Spontaneous miniature endplate potentials (MEPPs) could also be detected, and evoked responses were consistently large enough to trigger muscle action potentials (Fig. 5 *B* and *C* and Fig. S4): evoked postsynaptic potentials with a latency of 12–50 ms were recorded from 17 of 26 fibers from 3 *Nova* DKO/*agrin*$^{Z8}$ diaphragms. These results indicate that the machinery for conducting an action potential along the axon and for neurotransmission at the nerve terminal is intact in *Nova* DKO and *Nova* DKO/*agrin*$^{Z8}$ mice.

## Discussion

We provide evidence for a link between the function of a tissue-restricted splicing regulator and vertebrate development, defining the pathway (neuromuscular synapse formation), the factor (Nova), and a specific target (agrin). Although understanding of NMJ development has evolved since the discovery of agrin (18, 44), the alternatively spliced $Z^+$ agrin isoforms have retained an undisputed role in key aspects of the process. The observation that these $Z^+$ isoforms are generated only in neurons has long suggested that motor neuron-specific RNA regulation plays a crucial role in formation of the NMJ. Here, we identify Nova1 and Nova2 as the splicing factors responsible for generating $Z^+$ agrin alternatively spliced isoforms in the spinal cord and establish Nova as necessary (≈95%; Fig. 1 *A* and *B* and Table S1) for $Z^+$ agrin-mediated AChR cluster formation at the NMJ.

**Nova Regulates $Z^+$ Agrin Alternative Splicing.** Nova RNA binding proteins are expressed exclusively in neurons of the central nervous

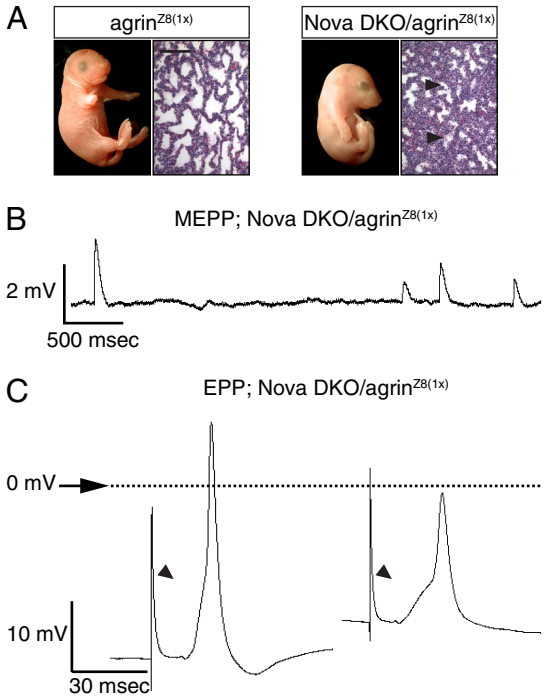


**Fig. 5.** Axons in *Nova* DKO and Nova DKO/*agrin*$^{Z8}$ mice are able to trigger action potentials. (*A*) *Nova* DKO/*agrin*$^{Z8(1x)}$ are stiff and fail to breath, as assessed by lung histology (see Fig. 2*A*). (Scale bar: 100 $\mu$m.) (*B* and *C*) Electrophysiologic recordings. Stimulus of spontaneous (*B*) and evoked (*C*) motor endplate potentials. Acute nerve-diaphragm preparations were made from E18.5 *Nova* DKO/*agrin*$^{Z8(1x)}$ mice. MEPPS were recorded (*B*), and the phrenic nerve was stimulated at 1 Hz and action potentials were recorded at the motor endplate (*C*). Arrowheads indicate action potential threshold. Dashed line = 0 mV.

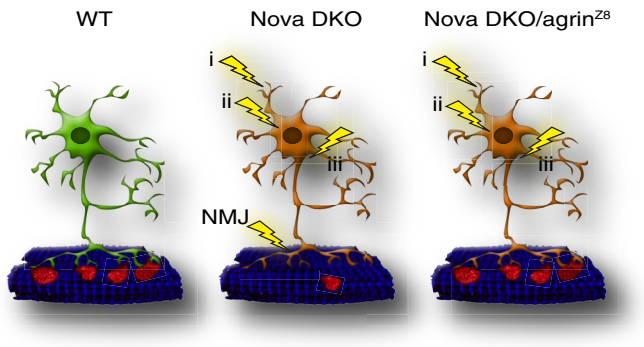


**Fig. 6.** Model of Nova action in motor neuron function. WT neuron with AChR clusters (red) at NMJ. *Nova* DKO neurons fail to induce AChR clusters at the NMJ caused by defect in *agrin* $Z^+$ splicing, which is rescued in *Nova* DKO/*agrin*$^{Z8}$ transgenic rescue. Electrophysiologic recording in *Nova* DKO mice or *Nova* DKO/*agrin*$^{Z8}$ mice indicates that stimulation of the phrenic nerve (motor axon) allows AChR release and evokes action potentials in the diaphragm (muscle). Nonetheless the mice are paralyzed, suggesting a defect remains that is proximal to the motor axon. Thus, in addition to affecting the NMJ, Nova-regulated RNAs may encode proteins that mediate motor neuron inputs (*i*), transduction of synaptic signals within the motor neuron (*ii*), or the firing of the motor neuron at the axon hillock (*iii*).

system, where they act to regulate a subset of alternatively spliced transcripts. Three unbiased approaches, CLIP (13, 14), a splicing microarray (15), and bioinformatic analysis (16), have determined that the great majority of Nova-regulated targets validated in *Nova* KO brain encode proteins that function at the neuronal synapse (13–16). Synaptic physiology in *Nova* KO mice was first examined by analyzing inhibition of long-term potentiation of slow inhibitory postsynaptic potentials in the hippocampus, a process that depends on the Nova CLIP targets *GIRK2*, *GABA$_B$*, and *CAMKII*, and that was found to be absent in *Nova2*$^{-/-}$ mice (17). Here, we examine another aspect of synaptic function implicated by analysis of Nova splicing targets. Based on our observation of neuromuscular paralysis in *Nova* DKO mice (Fig. 2*A*), we found that *Nova1* and *Nova2* synergize to regulate $Z^+$ agrin exon splicing in the spinal cord (Fig. 1 *B* and *C*). Unexpectedly however, restoring $Z^+$ agrin expression in motor neurons of *Nova* DKO mice is not sufficient to rescue motor paralysis, despite the fact that NMJ synapses appear morphologically normal in *Nova* DKO/*agrin*$^{Z8}$ mice (Fig. 4 and Fig. S3). Thus, Nova-dependent RNA regulatory events in motor neurons, in addition to agrin regulation, are likely to be critical for the proper formation and function of the NMJ.

**Defining the Role Of $Z^+$ Agrin in NMJ Formation.** In cultured myotubes, agrin organizes AChRs into aggregates without inducing AChR production (48). Early models of $Z^+$ agrin as a neural inducer of postsynaptic differentiation have been challenged by recent studies showing that AChR clusters form in the prospective synaptic region of the muscle even in the absence of agrin or innervation. In normal development, AChR clusters that become apposed to nerve terminals are stabilized and maintained by $Z^+$ agrin, whereas nonsynaptic AChR clusters are actively dispersed by neuronal ACh release (26).

Our observations are consistent with this view, but suggest additional layers of complexity. In the dorsal region of the diaphragm muscle, few AChR clusters remain in the diaphragm where the nerve has invaded, and of those that do remain, only approximately one-third appear to be in perfect apposition to nerve terminals (Fig. 2 *D–F* and Fig. S2). This finding suggests that there was sufficient ACh release in this region of the muscle to extinguish the AChR prepattern. At the same time, *Nova* DKO mice show AChR clusters that are prepatterned in the ventral region of the diaphragm not innervated by the phrenic nerve (Fig. 2*C*), in accordance with previous reports showing that AChR prepatterning occurs in the absence of motor innervation (29). Despite the apparent release of ACh, and the $Z^+$ agrin-mediated rescue of AChR clusters, the NMJs that formed in the dorsal region of the muscle appeared morphologically normal but were functionally incompetent, given that *Nova* DKO/*agrin*$^{Z8}$ mice are paralyzed. Because *Nova* is expressed in a neuronal-cell autonomous fashion, these observations support the conclusion that there must be additional Nova-dependent, agrin-independent neuronal factors required for motor neuron function.

**A Role for Nova in Motor Neuron Firing.** Nova RNA regulation appears to coordinate 2 different aspects of motor function, one at the NMJ, and a second, revealed by our electrophysiology results, proximal to the motor axon (Fig. 6). Given that *Nova* DKO mice remain paralyzed after rescue of $Z^+$ agrin splicing and AChR clustering, and despite having axons that are able to trigger motor action potentials (Fig. 6), the nature of this presumed proximal defect is of great interest. We hypothesize that an integrated action of Nova-regulated neuron-specific protein variants is necessary for motor neurons to fire. Prior studies have linked *Nova* to the balance of neuronal excitation and inhibition, both at hippocampal synapses (17) and in the paraneoplastic neurologic disease paraneoplastic opsoclonus-myclonus ataxia (POMA), where targeting of Nova leads to a failure of brainstem and spinal motor inhibition (2, 5). Nova-regulated RNAs could encode protein variants that function in motor neuron inputs (e.g., balancing inhibition and excitation), dendrites (at the level of neurotransmitter receptors, scaffolding, or

signaling pathways), and/or the proximal motor axon (e.g., inward rectifying channels).

## Conclusions

The relationship between RNA regulation and motor neuron function discovered here is reflected not only in the link to POMA, but in a number of other motor system diseases. Accumulated evidence suggests that the dysregulation of RNA splicing or splicing machinery underlies disorders such as myotonic dystrophy, spinal cerebellar ataxias, spinal muscular atrophy, Rett syndrome, and perhaps amyotrophic lateral sclerosis (1, 45). The finding that *Nova* is necessary for physiologic motor neuron firing may reflect a more general role for RNA–protein regulation in motor neurons than previously appreciated; moreover, because Nova is also expressed in the central nervous system (3–5), these studies may be relevant to neurophysiology throughout the brain.

## Materials and Methods

Methods are described in detail in *SI Text*.

**Histology.** Staining of the NMJ was performed with a Zeiss Axiovert 200 inverted microscope by using Alexa Fluor 594 $\alpha$-bungarotoxin and methods described in *SI Text*.

**Mice.** Chick *agrin* transgenic mice were made with full-length chick *agrin* including the Z8 exon (46) fused to the 5′ regulatory region ($\approx$9 kb) from the *Hb9* gene (38). These mice were mated to chick *agrin* transgenic, and triple heterozygotes were backcrossed for 10 generations.

**RNA Expression.** RNA was amplified with primers to *agrin* exons 32 and 33 [also known as exons 31a and 31b (15)]. Quantitative PCR (qPCR) data were collected from 4 biological replicates with a MyiQ single-color real-time PCR detection system, using mouse $\beta$-actin as internal control, and $\Delta\Delta$Ct calculations.

**Quantitative Immunofluorescence.** AChR clusters and $\alpha$-bungarotoxin signals were quantitated with ImageJ software (http://rsb.info.nih.gov/ij).

**Electrophysiology.** Standard electrophysiological methods (47) were used to record from E18.5 acutely isolated phrenic nerve-diaphragm muscles. Synaptic events were recorded by using sharp electrodes (20–30 megaohms) with pClamp10 (Axon Instruments) and analyzed by using MiniAnalysis (Synaptosoft). To test for cholinergic transmission, muscle contraction was blocked with a saturating dose (5 $\mu$g/mL) $\alpha$-bungarotoxin.

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