Cell

# SMN Is Required for Sensory-Motor Circuit Function in *Drosophila*

Wendy L. Imlach,[1,2] Erin S. Beck,[1,3] Ben Jiwon Choi,[1,2] Francesco Lotti,[1,2] Livio Pellizzoni,[1,2] and Brian D. McCabe[1,2,3,*]
[1]Center for Motor Neuron Biology and Disease
[2]Department of Pathology and Cell Biology
[3]Department of Neuroscience
Columbia University Medical Center, 630 West 168th Street, New York, NY 10032, USA
*Correspondence: brian@mccabelab.org
http://dx.doi.org/10.1016/j.cell.2012.09.011

## SUMMARY

Spinal muscular atrophy (SMA) is a lethal human disease characterized by motor neuron dysfunction and muscle deterioration due to depletion of the ubiquitous survival motor neuron (SMN) protein. *Drosophila* SMN mutants have reduced muscle size and defective locomotion, motor rhythm, and motor neuron neurotransmission. Unexpectedly, restoration of SMN in either muscles or motor neurons did not alter these phenotypes. Instead, SMN must be expressed in proprioceptive neurons and interneurons in the motor circuit to nonautonomously correct defects in motor neurons and muscles. SMN depletion disrupts the motor system subsequent to circuit development and can be mimicked by the inhibition of motor network function. Furthermore, increasing motor circuit excitability by genetic or pharmacological inhibition of $K^+$ channels can correct SMN-dependent phenotypes. These results establish sensory-motor circuit dysfunction as the origin of motor system deficits in this SMA model and suggest that enhancement of motor neural network activity could ameliorate the disease.

## INTRODUCTION

Animal behaviors such as locomotion depend upon the coordinated activity of neuronal networks. Disruption of individual components of neural circuits by injury or disease can produce a cascade of deleterious secondary effects upon other networked neurons. It has been hypothesized that the chronic dysfunction of neuronal circuits may ultimately lead to degeneration of neurons within the network, both exacerbating the damage and masking the primary cause of the disorder (Palop and Mucke, 2010). Identifying the molecular, cellular, and physiological basis of disease is central to understanding the adult motor neuron disorder amyotrophic lateral sclerosis (ALS) (Rothstein, 2009) and the juvenile disease spinal muscular atrophy (SMA) (Burghes and Beattie, 2009). The genetics of ALS are complex, and only a minority of cases are due to the inheritance of mutations in a diverse range of genes. By contrast, SMA, the most common inherited cause of infant mortality (Pearn, 1978), is both recessive and monogenic. Both ALS and SMA are characterized by motor neuron functional alterations and degeneration, which has focused research on cell-autonomous changes in motor neurons themselves. However, recent studies of ALS mouse models have identified contributions of other spinal cord cells such as astrocytes to disease pathology, suggesting that interactions between motor neurons and other partner cells may be an important contributing factor to motor neuron disease (Ilieva et al., 2009).

SMA is caused by recessive mutations in the survival motor neuron 1 (*SMN1*) gene (Lefebvre et al., 1995) that are not compensated for by *SMN2*, a human-specific gene (Burghes and Beattie, 2009). Although *SMN2* is almost identical to *SMN1*, nucleotide differences alter its splicing pattern, resulting in an ~90% reduction of full-length SMN messenger RNA (mRNA) expression (Lorson et al., 1999; Monani et al., 1999). Therefore, SMA is caused by low levels of SMN as opposed to the complete loss of SMN (Burghes and Beattie, 2009). SMN is a multifunctional protein that has been implicated in a variety of cellular processes linked to RNA metabolism (Pellizzoni, 2007). SMN is ubiquitously expressed and has been highly conserved during evolution (Schmid and DiDonato, 2007). In genetic model organisms, complete removal of SMN results in loss of cell viability. In contrast, the reduced level of SMN found in SMA patients does not appear to significantly perturb the majority of organ systems (Crawford and Pardo, 1996). However, SMA patients develop motor problems and muscle weakness—with the proximal limb and trunk muscles stereotypically being the most severely affected—progressing eventually to respiratory insufficiency and death (Swoboda et al., 2005). Postmortem studies show that SMA patients have pathologically abnormal motor neurons and evidence of motor neuron loss (Simic, 2008); however, it is currently unclear whether this is the primary origin of motor system dysfunction or a terminal consequence.

Reduction of SMN to low levels—similar to the situation in human SMA—has been studied using several animal models (Schmid and DiDonato, 2007). In the SMA mouse model SMN-Δ7, death occurs 2 weeks after birth (Le et al., 2005). This is accompanied by a modest loss of motor neurons; however, these mice have a profound early impairment of motor behavior

well before this loss occurs (Park et al., 2010a). Examination of the neuromuscular junctions (NMJ) of SMN-Δ7 mice reveals that most terminals are innervated, though some have structural abnormalities (Kariya et al., 2008; Kong et al., 2009; Ling et al., 2012; McGovern et al., 2008). NMJ neurotransmission is aberrant in these mutants, with an ~50% reduction in quantal content (Kariya et al., 2008; Kong et al., 2009); however, the high safety factor of the neuromuscular junction means that these terminals still produce normal muscle twitch tension (Ling et al., 2010). Therefore, given the early severe defects observed in the motor behavior of SMA mice, the disruption of motor neuron function is surprisingly modest.

SMN mutant mice have been used to grossly assess the tissue requirements for SMN by both selective SMN rescue and depletion experiments. Expression of transgenic SMN throughout the nervous system and some muscles (Gavrilina et al., 2008) or through central nervous system (CNS) virus delivery (Passini et al., 2010) gives a robust rescue of SMN-Δ7 motor behavior and survival, whereas muscle-specific SMN expression does not (Gavrilina et al., 2008). In contrast, selective genetic reduction of SMN in the motor neurons of mice is not lethal, though NMJ structural and electrophysiological abnormalities are observed (Park et al., 2010b), and genetic restoration of SMN in motor neurons alone does not rescue mutant lethality (Gogliotti et al., 2012; Martinez et al., 2012). In addition to the motor neuron defects, pronounced early deficits of spinal reflexes and reduced numbers of proprioceptive synaptic inputs onto motor neurons have recently been described in SMN-Δ7 mice, although functional contribution of these changes to the SMA phenotype is not yet known (Ling et al., 2010; Mentis et al., 2011). Collectively, these findings suggest the possibility that neurons other than motor neurons could contribute to the motor deficits in SMA.

Here, we have exploited *Drosophila* mutants with low levels of SMN to determine the essential cellular site and requirement for SMN in the motor system of this model. Using previously described loss-of-function *smn* mutants (Chan et al., 2003; Chang et al., 2008; Rajendra et al., 2007), we confirmed that depletion of SMN in *Drosophila* results in reduced muscle growth and defective locomotion similar to SMA phenotypes and showed that this was accompanied by aberrant rhythmic motor output and neuromuscular junction neurotransmission. Surprisingly, we found that none of these defects could be rescued by transgenic restoration of SMN in either the muscles or motor neurons of *Drosophila smn* mutants. Rather, we discovered that SMN must be restored in both proprioceptive neurons and cholinergic interneurons in order to rescue *smn* mutant phenotypes. Our results reveal that the disruption of motor neurons and muscles is a secondary consequence of a primary dysfunction of sensory-motor network activity in this SMA model, and we demonstrate that genetic or pharmacological strategies that increase motor circuit excitability can positively benefit *smn* mutant phenotypes. Furthermore, in a companion manuscript (Lotti et al., 2012 [this issue of *Cell*]), we demonstrate that an SMN-dependent gene required for normal motor circuit function in *Drosophila* is also disrupted in the motor circuits of SMN mutant mice. These results suggest that disruption of motor circuit function may be critical to SMA and that strategies designed to manipulate the activity of motor networks might be employed to ameliorate SMA patient symptoms.

## RESULTS

### *Drosophila smn* Mutants Have Muscle and Motor System Defects

To model the low level of SMN found in SMA patients in *Drosophila*, we utilized the zygotic protein null *smn* allele, $smn^{X7}$, which is a small deficiency that removes the entire *smn* coding region without disrupting nearby loci (Chang et al., 2008). The remaining SMN in these animals is contributed by maternal protein, which provided <6% of the level of SMN compared to controls at the third instar larval stage (Figure S1A available online). $smn^{X7}$ mutants never initiated pupation but instead persisted as third instar larvae, often surviving several days at this stage, which is consistent with other *smn* mutant alleles (Chan et al., 2003). To confirm that this phenotype was dependent on SMN, we ubiquitously expressed a transgenic upstream activation sequence (UAS) flag-tagged SMN construct (Chang et al., 2008) in $smn^{X7}$ mutants using Actin-Gal4. This restored normal pupation of $smn^{X7}$ mutants, with 100% of larvae initiating pupation and >80% subsequently eclose to produce adults (data not shown). Thus, $smn^{X7}$ mutants have low levels of SMN at late larval stages and can be rescued by transgenic SMN. We used this mutant allele for all subsequent experiments except where noted.

In humans, depletion of SMN affects both muscles and motor function. We noted that *Drosophila smn* mutant larvae were smaller than control animals and examined whether this was associated with a reduction in muscle size. We labeled *smn* mutant and control larval muscles with phalloidin and found that *smn* mutants had a 46% ($p < 0.001$) reduction in muscle surface area compared to controls (Figures 1A–1C and Table S1). This defect was fully rescued by ubiquitous expression of transgenic SMN. *smn* mutant larvae were also sluggish and moved less frequently than controls. To quantify this defect, we used video capture and tracing software to measure their locomotion. We found that *smn* mutants showed a 63% ($p < 0.001$) decrease in locomotion velocity compared to control animals, which was restored to control levels by ubiquitous expression of transgenic SMN (Figures 1D–1F). Thus, similar to SMA patients, *Drosophila* larvae with low levels of SMN have muscle and locomotion defects.

Locomotion of *Drosophila* larvae has been linked to the rhythmic activity of segmental central pattern-generating networks (CPGs) in the ventral nerve cord (VNC) (Fox et al., 2006), which receive inputs from both the brain hemispheres (Cattaert and Birman, 2001) and proprioceptive sensory neurons (Cheng et al., 2010; Hughes and Thomas, 2007; Song et al., 2007) and output activity to motor neurons. To measure the activity of these pattern-generating neurons, we recorded the spontaneous activity of motor neurons in preparations where we left the brain and VNC in situ (Fox et al., 2006). In control animals, we observed periodic bursting of motor activity at regular intervals, which was consistent with previous studies (Cattaert and Birman, 2001; Fox et al., 2006) (Figure 1G). In contrast, this activity was disrupted in *smn* mutants, which had short, irregular bursts that varied in

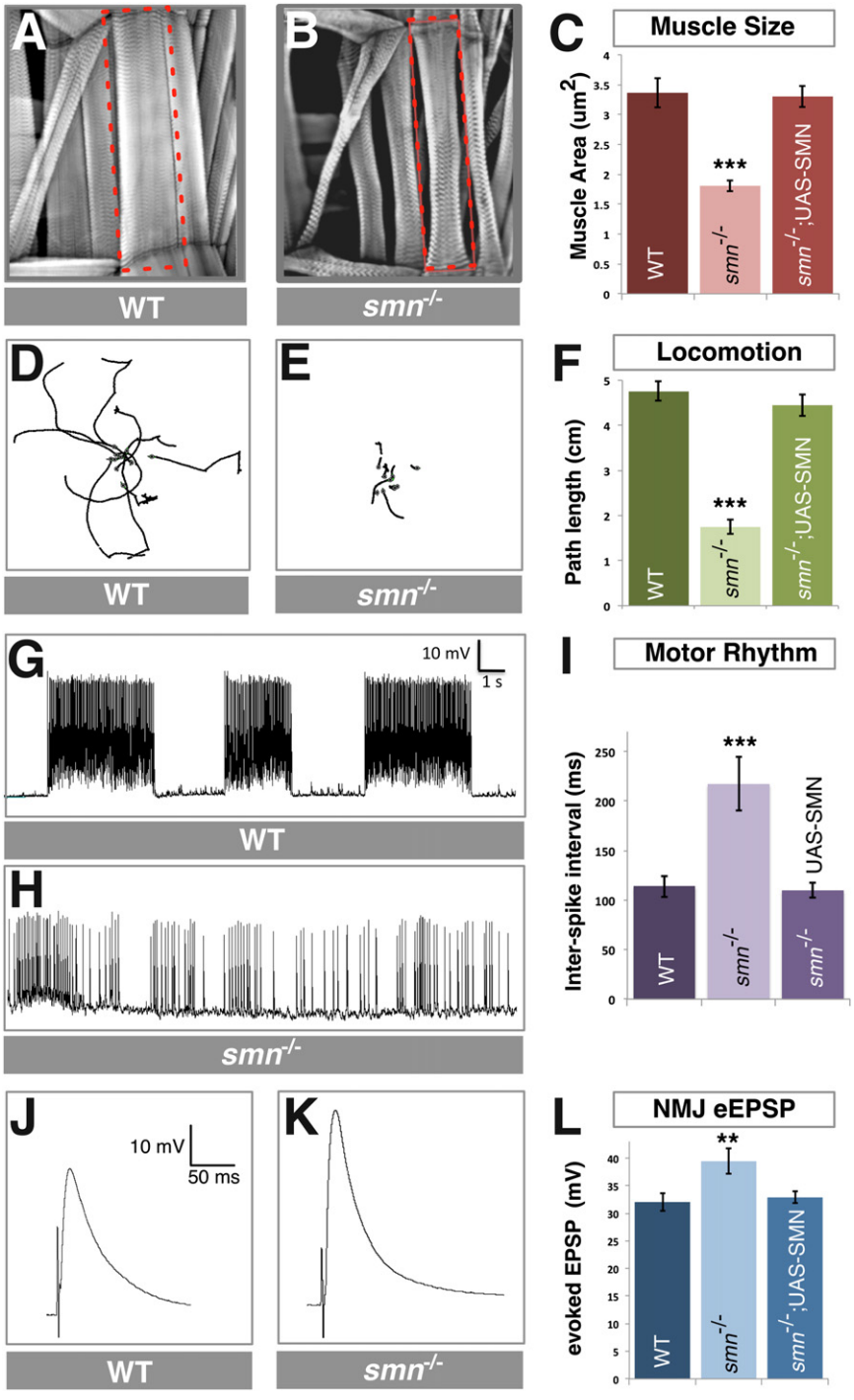


**Figure 1. *smn* Mutants Have Reduced Muscle Size, Decreased Locomotion, Defective Motor Rhythm, and Aberrant NMJ Neurotransmitter Release**

(A–C) Sample images of muscles from segment A3 of control (A) and $smn^{X7}$ mutant (B) third instar larvae labeled with TRITC-phalloidin show a reduction of muscle surface area (C) that is fully rescued by ubiquitous expression of UAS-flag-SMN driven by Da-Gal4 (genotype: Da-Gal4/UAS::flagSMN; $smn^{X7}/smn^{X7}$).

(D–F) Ten sample superimposed 60 s larval locomotion path traces from control (D) and $smn^{X7}$ mutants (E). *smn* mutant larvae have reduced velocity compared to controls that was corrected by ubiquitous expression of transgenic SMN (F).

(G–I) Recordings from muscle 6 in segment A1 of semi-intact larval preparations where the brain, ventral nerve cord, and motor neurons are intact. Control larvae produce a regular motor rhythm with periodic bursting activity corresponding to peristaltic muscle contractions (G). *smn* mutant larvae have an irregular motor pattern with short and uncoordinated bursts as shown by an increase in the average interspike interval (I) that is rescued by ubiquitous expression of SMN.

(J–L) Representative traces recorded from muscle 6 of segment A3 in control (J) and $smn^{X7}$ mutant (K) larvae. $smn^{X7}$ mutants have increased eEPSP amplitude as compared to controls (K). This is corrected by ubiquitous expression of SMN (L).

Data are represented as mean ±SEM; $^{**}p < 0.01$ and $^{***}p < 0.001$. See also Figure S1 and Table S1.

duration (Figure 1H). We quantified this defect by measuring the average interspike interval between all spontaneous spike events in *smn* mutants and controls over a fixed time period. Compared to controls, *smn* mutants showed a 90% ($p < 0.001$) increase of interspike interval (Figure 1I). As with locomotion, normal rhythmic motor activity was fully restored by ubiquitous expression of transgenic SMN. Thus, the output of motor circuits is defective in *Drosophila smn* mutants.

To investigate the neurotransmitter release properties of individual motor neurons, we removed the brain and stimulated motor neurons directly by using a suction electrode (Imlach and McCabe, 2009). Compared to controls, we found a 23% ($p < 0.005$) increase of evoked excitatory postsynaptic potential (eEPSP) amplitude at the NMJs of *smn* mutants (Figures 1J–1L). The increase of NMJ eEPSP amplitude in $smn^{X7}$ mutants was restored to control levels by ubiquitous expression of transgenic SMN (Figure 1L). We also observed a 60% ($p < 0.05$) increase in miniature excitatory postsynaptic potential (mEPSP) frequency. In contrast, mEPSP amplitude at *smn* mutant NMJ terminals was not different than controls (Figures S2A and S2B), leading to a 64% ($p < 0.001$) increase in quantal content (Figure S2C). These findings are consistent with a presynaptic change in the neurotransmitter release properties of motor neurons in *smn* mutants. As our electrophysiology results differed from a previous report (Chan et al., 2003), we also examined transheterozygous combinations of $smn^{X7}$ with other *smn* mutant alleles and confirmed similar changes in eEPSP amplitudes in these mutants that were not

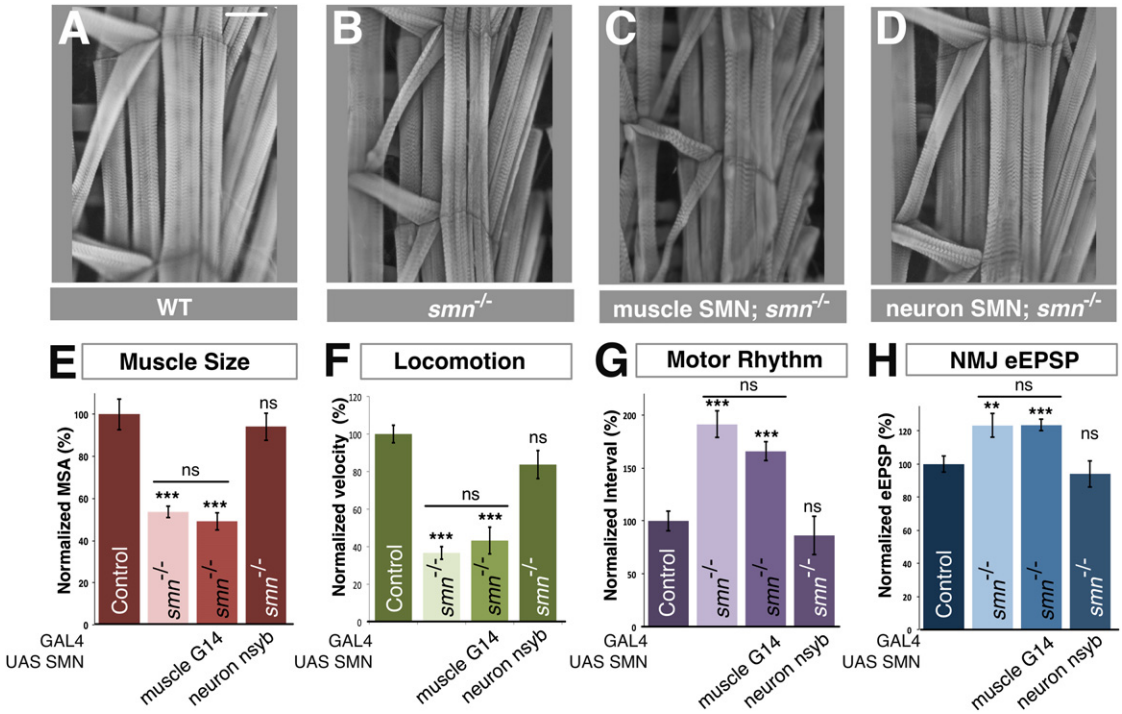


**Figure 2. SMN Expression Is Required in Neurons, but Not in Muscles, to Rescue *smn* Mutants**

(A–D) Sample images of muscles from segment A3 of control (A), $smn^{X7}$ mutant (B), $smn^{X7}$ mutants with transgenic SMN expression only in muscles (G14-Gal4/UAS::flagSMN; $smn^{X7}$/ $smn^{X7}$) (C), or neurons (nsyb-Gal4/UAS::flagSMN; $smn^{X7}$/$smn^{X7}$) (D). Restoration of SMN expression in muscles has no effect on muscle size; however, restoration in neurons fully rescues muscle surface area. Scale bar, 50 μm.

(E–H) Quantification of muscle surface area (E), locomotion (F), motor rhythm (G), and NMJ eEPSP amplitude (H) normalized to controls. Expression of transgenic SMN in neurons rescues all of *smn* mutant phenotypes, whereas expression in muscles does not.

Data are represented as mean ±SEM; **p < 0.01 and ***p < 0.001. See also Figure S2.

found in heterozygous $smn^{X7}$ animals (Figure S2D). When we examined the morphological features of $smn^{X7}$ mutant NMJs, we observed no significant difference in the number of synaptic boutons compared to controls (Figure S2E). In summary, we find that *Drosophila smn* mutants have increased NMJ-evoked neurotransmitter release that is accompanied by defects in muscle growth, locomotion, and motor rhythm.

## Restoration of SMN in the Nervous System Rescues *smn* Mutant Phenotypes

In order to identify the cell-autonomous requirement for normal SMN levels, we used iteratively more tissue-restricted Gal4 drivers to assess rescue of *smn* mutants. We began by expressing transgenic SMN only in the muscles of $smn^{X7}$ mutants using G14-Gal4, a larval muscle-specific driver. This produced no significant increase in muscle surface area (Figures 2C and 2E) or effects upon the locomotion, rhythmic motor output, and NMJ eEPSP amplitude of *smn* mutants (Figures 2F–2H). We therefore tested SMN restoration only in the nervous system of *smn* mutants using the neuron-specific *nsyb*-Gal4 driver. In contrast to muscle restoration of SMN, panneuronal restoration of SMN fully rescued the muscle surface area of *smn* mutants to control levels (Figures 2B, 2D, and 2E) and also completely restored their locomotor velocity, rhythmic motor output, and NMJ eEPSP amplitudes (Figures 2F–2H). Neuron-only rescue of *smn* mutants was not sufficient to produce viable *Drosophila* adults (data not shown), presumably due to the SMN level in tissues that are not rescued, becoming depleted to the point where cellular viability was compromised. Our results established that the defects of muscle growth in *smn* mutant larvae are due to a nonautonomous requirement for normal SMN levels in the nervous system rather than in muscle fibers themselves.

## SMN Is Required in Cholinergic Neurons and Not in Motor Neurons

The *Drosophila* VNC, like the human spinal cord, is populated by neurons with diverse neurotransmitter expression. All *Drosophila* motor neurons, in addition to a subset of central interneurons, are glutamatergic (Daniels et al., 2008). Because we observed presynaptic defects in neurotransmitter release at the NMJ in *smn* mutants, we first tested the ability of transgenic SMN expression in motor neurons to rescue *smn* mutants. We used OK371-Gal4, an enhancer trap inserted in the vesicular glutamate transporter promoter, to express transgenic SMN only in the glutamatergic neurons of *smn* mutants. This produced no difference in muscle surface area, locomotion velocity, or rhythmic motor output compared to *smn* mutants alone (Figures 3E–3G). Surprisingly, we also observed no reduction of the aberrant increase of eEPSP amplitude at the NMJs of these animals (Figures 3B, 3C, and 3H). We confirmed this unexpected result

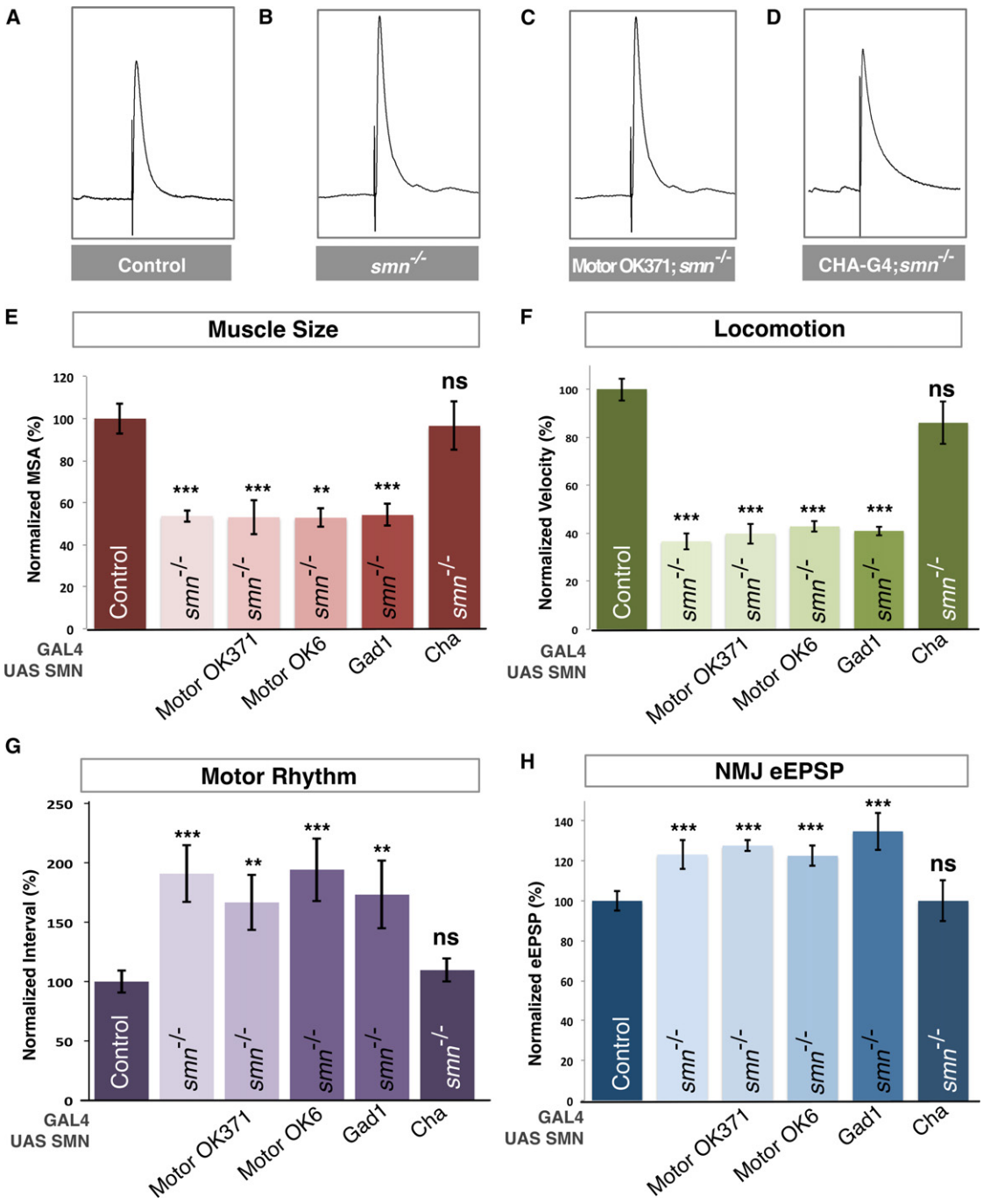


**Figure 3. SMN Expression Is Required in Cholinergic Neurons and Not in Motor Neurons**

(A–D) Representative traces of control (A), *smn*$^{X7}$ mutant (B), transgenic SMN expressed in the motor neurons of *smn* mutants (OK371-Gal4/UAS::SMN; *smn*$^{X7}$/*smn*$^{X7}$) (C), and transgenic SMN expressed in the cholinergic neurons of *smn* mutants (Cha-Gal4/UAS::SMN; *smn*$^{X7}$/*smn*$^{X7}$) (D). Expression of transgenic SMN in motor neurons does not restore NMJ eEPSP amplitudes in *smn* mutants; however, expression of SMN in cholinergic neurons does.

(E and F) Quantification of muscle surface area (E), locomotion (F), motor rhythm (G), and NMJ eEPSP amplitude (H) normalized to controls. Expression of transgenic SMN in the motor neurons of *smn* mutants with OK371-Gal4 or OK6-Gal4 or in GABAergic neurons with GAD1-Gal4 does not rescue any phenotype. Expression of transgenic SMN in cholinergic neurons rescues all phenotypes.

Data are represented as mean ±SEM; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Significance was calculated versus controls.

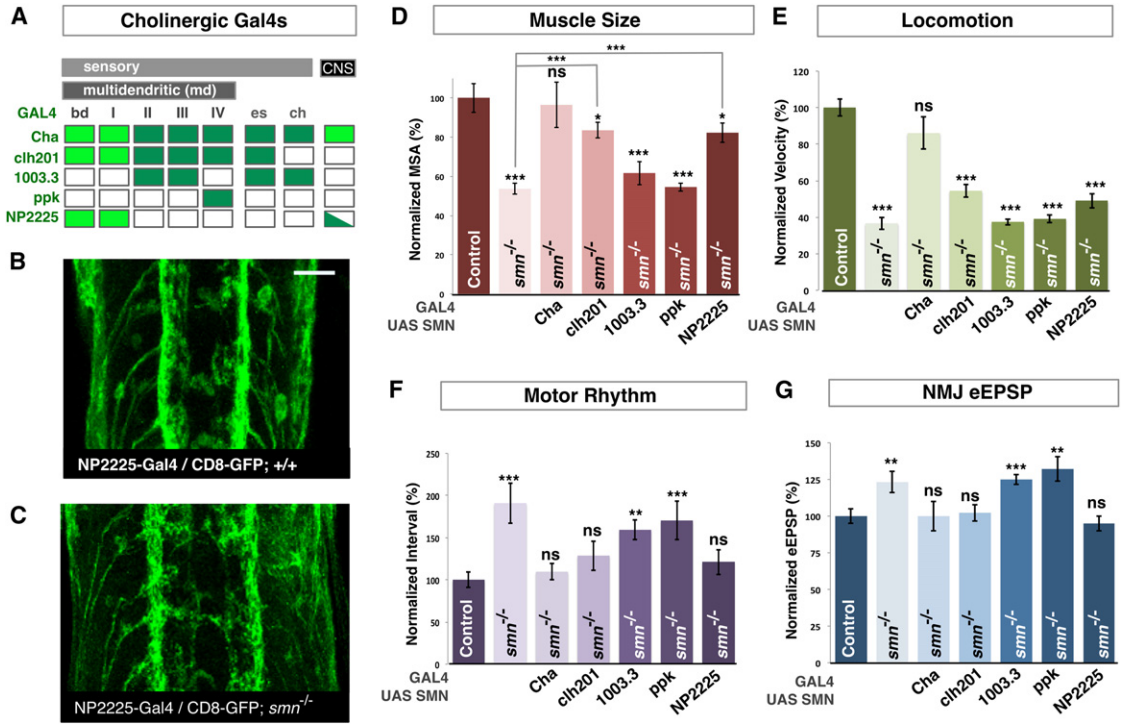


**Figure 4. SMN Is Required in Both Proprioceptive and Central Cholinergic Neurons**

(A) Expression pattern of cholinergic neuron Gal4 lines (dark green). Cha-Gal4 is expressed in both central and sensory cholinergic neurons. Clh201-Gal4 is only expressed in md and es sensory neurons. 1003.3-Gal4, ppk-Gal4, and NP2225-Gal4 are expressed in subsets of md, es, or ch sensory neurons. Bright green indicates the ability to rescue *smn* mutant phenotypes.

(B and C) UAS::CD8-GFP labeling the axons of bd and type I md sensory neurons with NP2225-Gal4 in the ventral nerve cord of wild-type (B) or $smn^{X7}$ mutants (C). Sensory axons project normally into the CNS in *smn* mutants. Scale bar, 10 μm.

(D–G) Quantification of muscle surface area (D), locomotion (E), motor rhythm (F), and NMJ eEPSP amplitude (G) normalized to controls (genotype: Gal4/UAS::flagSMN; $smn^{X7}/smn^{X7}$). Expression of transgenic SMN in both central and sensory cholinergic neurons in *smn* mutants with Cha-Gal4 fully rescuing all phenotypes. Restoration of SMN in all sensory neurons or only proprioceptive type I md and bd neurons with NP2225-Gal4 increases muscle size and fully rescues motor rhythm and NMJ eEPSP amplitude but does not rescue locomotion. In contrast, restoration of SMN with 1003.3-Gal4 or ppk-Gal4 does not rescue any *smn* mutant phenotype.

Data are represented as mean ±SEM; *p < 0.05, **p < 0.01, and ***p < 0.001. Significance was calculated versus controls except where otherwise indicated.

by using a second independent motor-neuron-specific driver, OK6-Gal4 (Figures 3E–3H). Therefore, similar to the requirement for SMN in muscle growth, the aberrant neurotransmitter release at the NMJ of *smn* mutants is not the result of the cell-autonomous loss of SMN in motor neurons. This result prompted us to investigate whether SMN was required in other neuron types in the *Drosophila* motor circuit.

Inhibitory inputs are important regulators of motor circuit function (Featherstone et al., 2000), so we next used glutamic acid decarboxylase 1 promoter Gal4 to restore SMN in GABAergic neurons; however, we observed no significant rescue of any *smn* mutant phenotype (Figures 3E–3H). The majority of excitatory neurons in the *Drosophila* nervous system are cholinergic (Salvaterra and Kitamoto, 2001), and motor neurons receive synaptic input from cholinergic neurons (Baines, 2006). We therefore restored transgenic SMN in *smn* mutants by using choline acetyltransferase (Cha) promoter-driven Gal4. In contrast to glutamatergic and GABAergic drivers, expression of transgenic SMN in cholinergic neurons completely rescued the muscle growth, locomotion, and rhythmic activity defects of *smn* mutants (Figures 3E–3G). Moreover, expression of SMN in cholinergic neurons also fully rescued eEPSP amplitudes at the NMJ terminals of *smn* mutants to control levels (Figures 3D and 3H). Thus, expression of SMN only in cholinergic neurons is sufficient to fully rescue *smn* mutant phenotypes and can nonautonomously rescue the SMN-dependent defects of both motor neurons and muscles.

## SMN Is Required in Both Proprioceptive and Central Cholinergic Neurons

All *Drosophila* larval sensory neurons, in addition to the majority of excitatory central neurons, are cholinergic (Salvaterra and Kitamoto, 2001). To dissect the requirement for normal SMN levels between these two populations, we examined the ability of transgenic SMN expression in sensory neurons alone to rescue *smn* mutant phenotypes. *Drosophila* sensory neurons are categorized into three major types: multiple dendrite neurons (md), of which there are five subclasses (bd, I, II, III, and IV); external sense organ neurons (es); and chordotonal neurons (ch). We first used a panel of sensory neuron Gal4 drivers (Figure 4A) to

restore SMN only in major types of sensory neurons. We found that when we reinstituted SMN in all md neurons and es sensory neurons, but not ch neurons or central neurons, both the rhythmic motor output and evoked NMJ eEPSP amplitudes of *smn* mutants were restored to control levels, and muscle surface area was increased to 83.5% of controls ($p < 0.05$) (Figures 4D, 4F, and 4G). However, expression of transgenic SMN with this driver did not significantly change the locomotion of *smn* mutants (Figure 4E). In contrast, expression of SMN in ch neurons did not rescue any *smn* mutant phenotype (Figures 4D–4G). Using additional Gal4 drivers that are expressed in smaller subsets of md or es sensory neurons (Figure 4A), we found that it was sufficient to restore SMN only in bd and type I md neurons to rescue defects of rhythmic motor output and NMJ neurotransmission and to increase the muscle growth of *smn* mutants (Figures 4D, 4F, and 4G). Expression of SMN in both the CNS and peripheral cholinergic neurons with Cha-Gal4 fully rescues all phenotypes, including locomotion and muscle size (Figures 4D–4G). This suggests that, in addition to bd and type I md sensory neurons, SMN must be restored in at least one other additional population of cholinergic neurons that resides within the CNS to completely correct *smn* mutant locomotion and to fully restore muscle size.

Both bd and type I md sensory neurons are required for proprioceptive feedback to the motor circuit of *Drosophila* larvae (Cheng et al., 2010; Hughes and Thomas, 2007). To determine whether these neurons were morphologically disrupted by SMN depletion, we examined the sensory or axonal processes of the bd and type I md neurons labeled in *smn* mutants; however, we found no obvious defects in sensory processes (data not shown), and the axons of these neurons projected into the CNS similarly to controls (Figures 4B and 4C). Our data therefore suggested that reduced SMN in proprioceptive neurons might disrupt their function rather than their development or connectivity.

## *smn* Mutant Phenotypes Can Be Rescued after Embryogenesis

*Drosophila* larval neurons develop, connect, and become functional during the 21 hr of embryonic development prior to hatching (Baines, 2006). To determine whether SMN depletion could have disrupted nervous system assembly during this period, we used the "GeneSwitch" RU486-drug-inducible Gal4 system to control the temporal restoration of transgenic SMN. We first asked whether *smn* mutant phenotypes could be rescued by expression of transgenic SMN subsequent to the completion of embryogenesis by exposing *smn* mutant larvae and controls carrying the neuron-specific elav-GeneSwitch driver to RU486-containing media immediately after hatching and throughout the subsequent larval period (Figure 5A). When transgenic SMN expression was not induced, we found no difference compared to *smn* mutants alone (Figures 5C–5F). In contrast, when SMN expression was induced immediately after embryogenesis, third instar larval muscle size, locomotion, rhythmic motor output, and motor neuron eEPSP amplitudes were indistinguishable from control animals (Figures 5B–5F). This result established that restoration of SMN expression after embryogenesis can rescue *smn* mutants, suggesting that they do not have persistent defects in motor circuit assembly.

We next delayed SMN expression in the nervous system of *smn* mutants until progressively later larval stages. When we induced transgenic SMN in *smn* mutants at 48 or 96 hr after embryo hatching, we found intermediate phenotypes where muscle volume, motor rhythm defects, and locomotion were only partially restored compared to controls (Figures 5C and 5D). In contrast, NMJ eEPSP amplitudes were completely restored in smn mutants to control levels by only 48 hr of SMN expression (Figures 5B and 5F). These results revealed a differential phenotypic sensitivity to the timing of SMN restoration with NMJ neurotransmitter fully corrected by elevating SMN levels at even late stages, whereas locomotion, motor rhythm, and muscle growth required an earlier or longer duration of exposure to increased SMN levels.

## Inhibiting Cholinergic Neuron Activity Mimics Aspects of SMN Depletion

During *Drosophila* embryonic development, complete removal of cholinergic input onto motor neurons results in motor neuron hyperexcitability and increased neurotransmission (Baines et al., 2001). We hypothesized, therefore, that the nonautonomous changes of motor neuron properties in *smn* mutants might be explained by defective excitatory input from cholinergic neurons in the motor circuit. Complete loss or inhibition of all cholinergic neuron activity results in embryonic lethality (Kitamoto et al., 2000), so in order to test this hypothesis, we employed transgenes designed to partially inhibit neurotransmission in cholinergic neurons. We used lines that either express moderate levels of the human inward-rectifying channel Kir2.1, which inhibits membrane depolarization (Paradis et al., 2001), or express membrane-tethered Plectreurys Toxin II (PLTXII), which inhibits synaptic N-type voltage-gated calcium channels (Wu et al., 2008). To determine the effectiveness of this approach, we first expressed these transgenes in motor neurons alone using OK6-Gal4. We found that Kir2.1 reduced eEPSP amplitudes by 32% ($p < 0.001$), whereas expression of PLTXII reduced eEPSP amplitudes by 96% ($p < 0.001$), indicating that both transgenes were capable of partially inhibiting neurotransmission.

To examine the effects on the motor system of inhibiting cholinergic neuron function, we then expressed each of these Kir2.1 or PLTXII in the cholinergic neurons of wild-type animals using Cha-Gal4. Expression of either transgene in cholinergic neurons had no effect on muscle surface area (Figure 6C); however, expression of these transgenes significantly inhibited locomotion by 41% ($p < 0.001$) and 42% ($p < 0.001$), respectively (Figure 6D). They also disrupted spontaneous rhythmic motor activity, inducing a 54% ($p < 0.05$) or 59% ($p < 0.05$) increase in average interspike intervals (Figures 6B and 6E). Importantly, inhibition of cholinergic neuron function also resulted in increased eEPSP amplitudes at the NMJs of glutamatergic motor neurons (Figures 6A and 6F). Expression of Kir2.1 in cholinergic neurons produced a 50% increase ($p < 0.001$) in NMJ eEPSP amplitudes, whereas expression of PLTXII induced a 45% increase ($p < 0.001$) (Figure 6F). Therefore, inhibition of cholinergic neuron activity replicated a number of the features

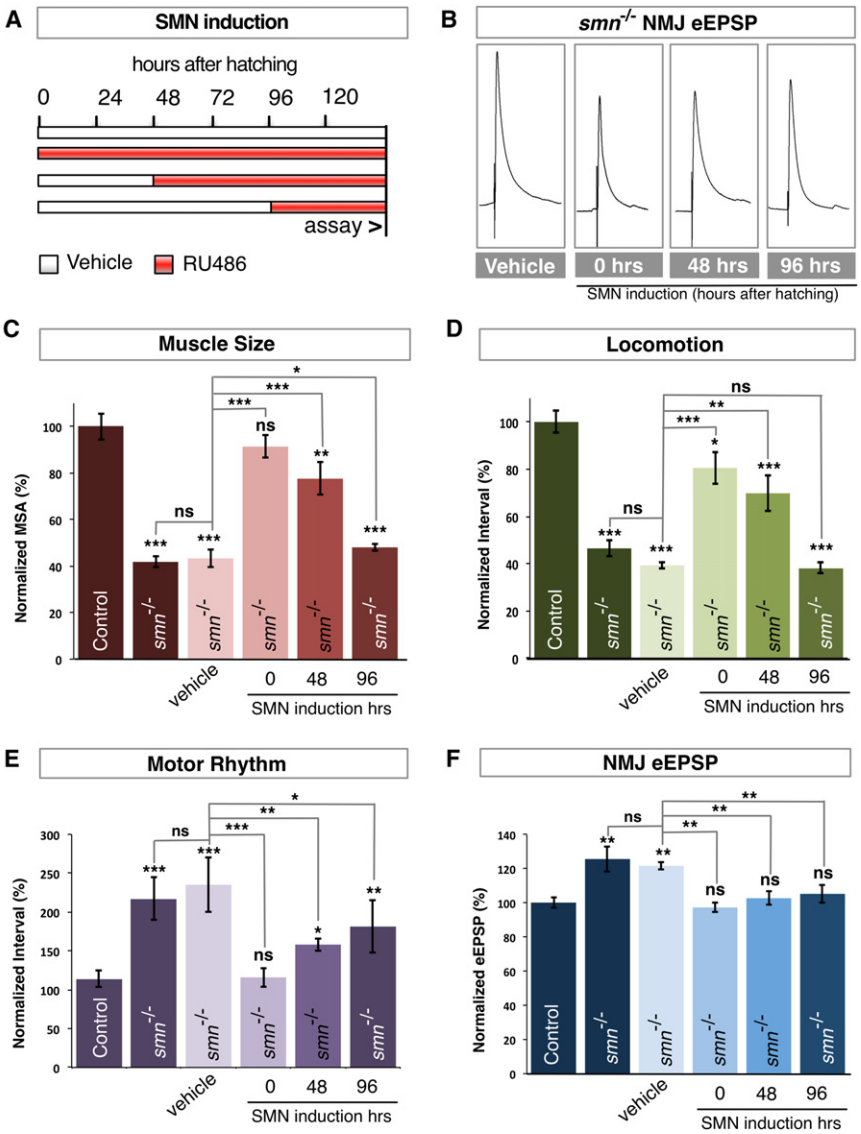


**Figure 5. Restoration of SMN after Embryogenesis Rescues *smn* Mutants**

(A) Schematic of transgenic SMN induction in the nervous system. RU486 is required for the activation of transgene induction by geneswitch Gal4. Elav::geneswitch/UAS::flagSMN; $smn^{X7}/smn^{X7}$ larva were transferred to either vehicle media or RU486-containing media immediately, 48 hr, or 96 hr after hatching.

(B) Representative traces recorded from *smn* mutants that were cultured on either vehicle media or RU486 media 0, 48, or 96 hr after hatching. Induction of SMN at each time point fully restored normal eEPSP amplitude.

(C–F) Quantification of muscle surface area (C), locomotion (D), motor rhythm (E), and NMJ eEPSP amplitude (F) normalized to controls. Muscle size, locomotion, and motor rhythm are fully rescued if transgenic SMN is induced immediately after hatching, but if SMN induction is delayed, rescue is incomplete. Induction of SMN for only 48 hr is, however, sufficient to completely restore normal NMJ eEPSP amplitude.

Data are represented as mean ±SEM; *p < 0.05, **p < 0.01, and ***p < 0.001. Significance was calculated versus controls except where otherwise indicated.

of *smn* mutants, including non-cell-autonomous effects on the neurotransmitter release properties of motor neurons, which is consistent with cholinergic neurons in the motor circuit having reduced function in *smn* mutants.

## Increasing Neuronal Excitability Rescues *smn* Mutant Phenotypes

Building upon the hypothesis that motor circuits have functional deficits in *smn* mutants, we next asked whether increasing the excitability of cholinergic neurons in these animals could increase motor network activity and alter *smn* mutant phenotypes. Inhibition of the Shaker (Sh) type $I_A$ $K^+$ current by a dominant negative (SDN) transgene enhances membrane excitability and increases both the amplitude and duration of eEPSPs at synaptic terminals (Mosca et al., 2005). We expressed the SDN transgene in the cholinergic neurons of smn mutants and examined the effect upon mutant phenotypes (Figures 7A–7G). Expression of SDN completely restored the muscle surface area, locomotor velocity, rhythmic motor activity, and eEPSP amplitudes of *smn* mutants to levels indistinguishable from control animals (Figures 7D–7G). This striking result established that increasing the activity of cholinergic neurons in the motor circuit of *smn* mutants can result in robust phenotypic rescue.

As genetic methods to inhibit $K^+$ channel activity benefited *smn* mutant phenotypes, we next asked whether pharmacological antagonists of $K^+$ channels could also be effective. 4-aminopyridine (4-AP) is an FDA-approved small-molecule inhibitor of voltage-activated vertebrate (Hayes, 2007) and *Drosophila* $K^+$ channels (Wicher et al., 2001). We added 4-AP to larval media and titrated the compound to identify the maximum dosage at which the drug could be tolerated without lethality in wild-type larvae (2 mM). We then examined the effects of exposure of 4-AP throughout the larval period in both control and *smn* mutants. In control animals, 4-AP had no effect on muscle size but reduced larval locomotion by 35% ($p < 0.01$), decreased rhythmic motor activity by 40% ($p < 0.01$), and reduced NMJ eEPSP amplitudes by 21% ($p < 0.001$), indicating mild systemic toxicity at this dose (Figures 7D–7G). Despite this, when we grew *smn* mutants on 4-AP-containing media throughout the larval period, muscle surface area was increased by 66% ($p < 0.001$) compared to untreated *smn* mutants (Figure 7D). Locomotion was also increased by 55% ($p < 0.05$) and was not significantly different to controls

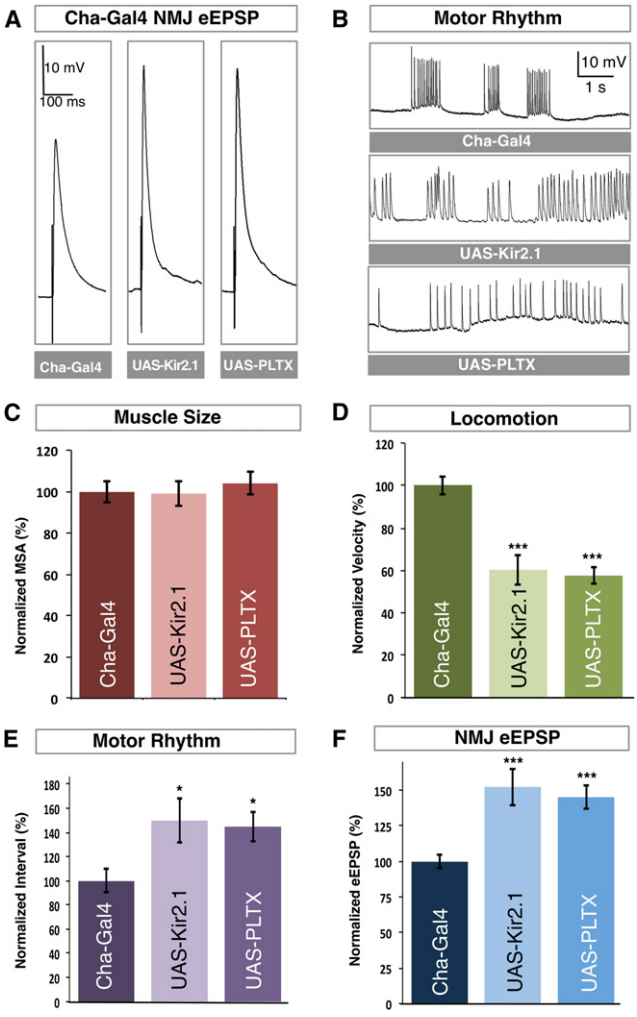


**Figure 6. Inhibiting Cholinergic Neuron Activity Mimics *smn* Mutant Phenotypes**

(A) Representative traces recorded from the NMJ of control or UAS-human Kir2.1 or UAS-PLTXII expressed in cholinergic neurons with Cha-Gal4. Inhibiting cholinergic neuron excitability with Kir2.1 or neurotransmitter release with PLTXII increases motor neuron NMJ eEPSP amplitude.

(B) Expression of Kir2.1 or PLTX in cholinergic neurons disrupts rhythmic motor activity.

(C–F) Quantification of muscle surface area (C), locomotion (D), motor rhythm (E), and NMJ eEPSP amplitude (F) normalized to controls. Expression of Kir2.1 or PLTXII in cholinergic neurons does not alter muscle size but does reduce locomotor speed, disrupt motor rhythm, and increases NMJ eEPSP amplitude. Data are represented as mean ±SEM; *$p < 0.05$ and ***$p < 0.001$.

treated with 4-AP (Figure 7E). Defects in rhythmic motor activity in *smn* mutants were substantially improved, with the aberrant increase in interspike interval reduced to 31% ($p < 0.001$) above controls treated with 4-AP (Figure 7F). Finally, the increased NMJ EPSP amplitude of *smn* mutants treated with 4-AP was reduced by 27% ($p < 0.001$), which is not significantly different than that of 4-AP-treated controls (Figure 7G). Thus, pharmacological inhibition of $K^+$ channels, similar to genetic inhibition, can benefit *smn* mutant phenotypes, which is consistent with the defective excitability of motor circuits being a critical consequence of SMN depletion.

## DISCUSSION

Across organisms, the function of the motor system seems uniquely sensitive to low levels of the ubiquitous protein SMN, the molecular defect responsible for SMA (Burghes and Beattie, 2009; Schmid and DiDonato, 2007). This is also true in *Drosophila*, in which *smn* mutants have reduced muscle size and locomotion, which we find is accompanied by defects in rhythmic motor output and motor neuron neurotransmitter release. Surprisingly, restoration of SMN in the motor neurons or muscles of these mutants provided no phenotypic rescue. Instead, SMN must be reinstated in both cholinergic proprioceptive and central neurons to rescue *smn* mutant phenotypes, including non-cell-autonomous defects in both motor neurons and muscles. Proprioceptive neurons provide essential inputs to motor circuits (Hughes and Thomas, 2007), and cholinergic interneurons are critical for *Drosophila* CNS function (Kitamoto et al., 2000), including synaptic output onto motor neurons (Baines et al., 2001). Restoration of SMN after the completion of nervous system development is sufficient to rescue SMN-dependent phenotypes, arguing that it is not the connectivity but rather the function of motor circuits that is disrupted by depletion of SMN. Two lines of evidence further support this conclusion. First, inhibiting the activity of cholinergic neurons can mimic a number of *smn* mutant phenotypes, including nonautonomous effects on motor neurons. Second, increasing the excitability of motor circuits through $K^+$ channel inhibition can rescue *smn* mutant defects. Our results therefore demonstrate that depletion of SMN in *Drosophila* causes the dysfunction of a select subset of neurons in the motor circuit, which consequently disrupts the activity of other networked components of the motor system such as motor neurons and muscles. These findings establish this model of SMA as a paradigm for a neurological disease induced by neuronal circuit dysfunction.

### The Contribution of Cholinergic Neurons to *Drosophila* Motor Circuits

Although our results exclude a cell-autonomous requirement for normal SMN levels in *Drosophila* motor neurons to rescue *smn* mutants, our data do establish that SMN has to be restored in at least two groups of motor circuit neurons for full rescue of larval phenotypes. One of these groups is bd and type I md sensory neurons, which are essential components of a proprioceptive sensory feedback circuit necessary for coordinated contractile locomotion of *Drosophila* larvae (Hughes and Thomas, 2007). Both bd and type I md subsets of sensory neurons express the mechanosensitive NompC transcript receptor potential (TRP) channel, which is essential for proprioception (Cheng et al., 2010). Sensory feedback does not seem to be necessary for *Drosophila* larval central pattern generator assembly or basic embryonic and larval movement (Crisp et al., 2008); however, without sensory input, both rhythmic motor circuit activity (Fox et al., 2006) and coordinated locomotion behavior are severely disrupted (Hughes and Thomas, 2007; Song et al., 2007). Rescue of SMN in bd and type I md sensory

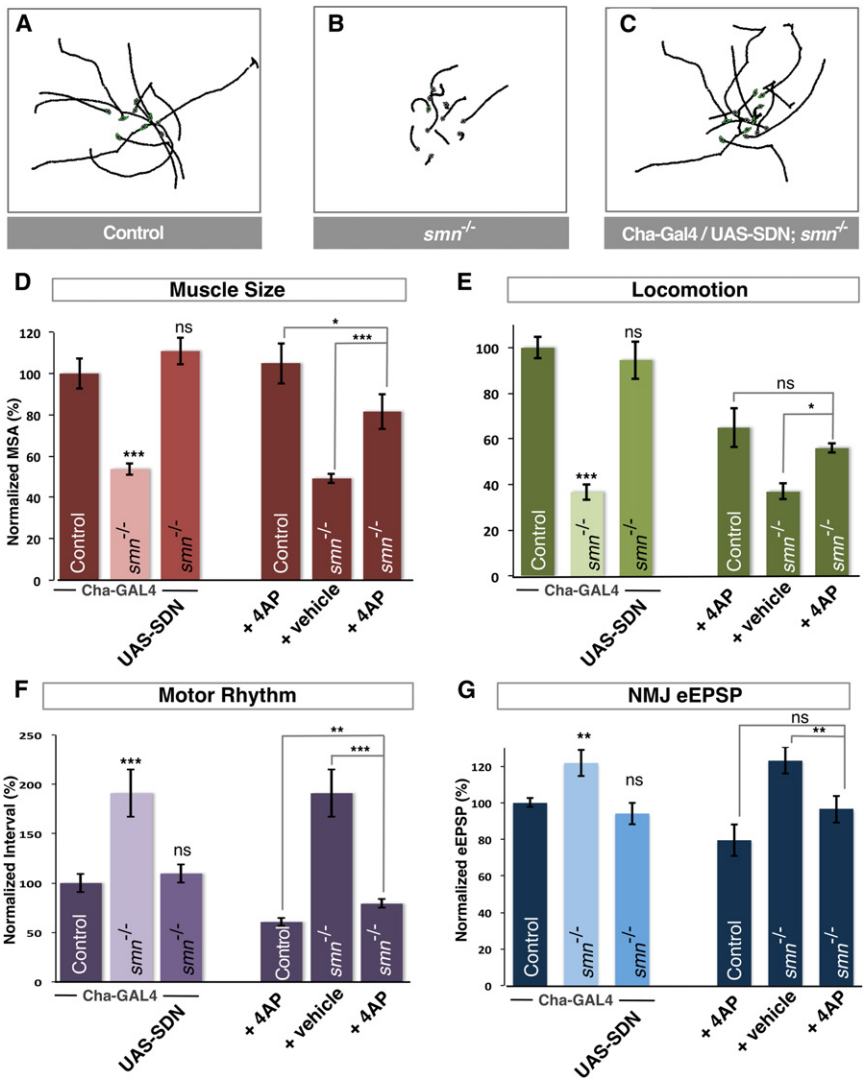


**Figure 7. Genetic or Pharmacological Inhibition of $K^+$ Channels Ameliorates *smn* Mutant Phenotypes**

(A–C) Locomotion path traces from control (A), *smn* mutants (B), and *smn* mutants expressing a UAS dominant negative Shaker $K^+$ channel (UAS-SDN) in cholinergic neurons with Cha-Gal4 (C). Expressing SDN rescues the locomotion of *smn* mutants.

(D–G) Quantification of muscle surface area (D), locomotion (E), motor rhythm (F), and NMJ eEPSP amplitude (G) normalized to controls. Expression of SDN in cholinergic neurons with Cha-Gal4 restores muscle size (D), locomotion (E), motor rhythm (F), and NMJ eEPSP (G) of *smn* mutants to control levels. Addition of 2 mM 4-AP to culture media throughout larval development does not alter muscle size in control animals but increases the muscle size of *smn* mutants (D). 4-AP administration inhibits locomotion, motor rhythm, and eEPSP size in control animals. Administration of 4-AP to *smn* mutants corrects locomotion (E) and NMJ eEPSP (G) to levels not significantly different from control 4-AP-treated animals and substantially corrects defects in motor rhythm (F).

Data are represented as mean ±SEM; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Significance was calculated versus controls except where otherwise indicated.

neurons can restore the rhythmic motor output of *smn* mutants, which is consistent with an important role for sensory input in regulating this activity (Fox et al., 2006). However, restoration of SMN in proprioceptive neurons alone is not sufficient to correct the locomotion velocity of *smn* mutants, indicating that additional neurons require wild-type levels of SMN in order to restore full mobility.

SMN expression in all cholinergic neurons can completely rescue all *smn* mutant larval phenotypes, including locomotion. Our results therefore implicate an additional cell-autonomous requirement for SMN in one or more groups of central cholinergic neurons. Establishing the identity of these central neurons will be a challenge, given our limited understanding of central motor circuitry in *Drosophila*. It is tempting to speculate that these neurons could be descending inputs from the brain (Cattaert and Birman, 2001) or other connections between segmental central pattern generators that promote the coordination necessary for effective locomotion. However, although rescue analysis demonstrates that individual components of the motor circuit can make significant contributions to some *smn* mutant phenotypes, other phenotypes such as muscle growth additively require SMN in both central and peripheral cholinergic neurons. Therefore, our data suggest that the effect of SMN depletion on the motor network is an amalgam of specific defects in distinct neurons that sum to produce a generalized disruption of the motor system.

Why are cholinergic motor circuit neurons selectively susceptible to SMN depletion? In a companion manuscript (Lotti et al., 2012), we describe a sequence of molecular events that link reduction of SMN to selective motor circuit dysfunction. We show that loss of *Drosophila* SMN disrupts minor splicing, which is required for the expression of genes with rare U12-type introns (Patel and Steitz, 2003). Through a genome-wide analysis of *Drosophila* U12 intron-containing genes, we identified a transmembrane protein, Stasimon, that has both reduced expression in *smn* mutants and increased NMJ eEPSP amplitudes when mutated, which is similar to the *smn* mutant phenotype. We found that, like SMN, Stasimon is required in cholinergic neurons, but not in motor neurons, to affect NMJ electrophysiology. Furthermore, we demonstrate that transgenic expression of Stasimon can fully restore normal NMJ eEPSP amplitudes in *smn* mutants in addition to increasing muscle size. These data establish that reduction of SMN decreases expression of a subset of genes that are particularly sensitive to SMN-dependent splicing disruption. Some of these genes,

such as *stasimon*, are critically required for the normal function of cholinergic motor circuit neurons in *Drosophila*. These results establish a mechanistic chain linking the role of SMN in RNA splicing to the selective vulnerability of motor circuit function when SMN is depleted.

## Parallels between *Drosophila* and Mammalian Motor Circuits

Although the basic elements of motor circuits—proprioceptive neurons, interneurons, and motor neurons—are conserved between *Drosophila* and humans, the neuronal constituents and connections that make up *Drosophila* central motor circuitry are at present unknown, limiting comparisons with mammalian circuits. However, it is known that the neurotransmitters employed in each system are different (Marder and Rehm, 2005). For example, human and mouse motor neurons are cholinergic, whereas proprioceptive neurons are glutamatergic, the inverse of the neurotransmitters employed in *Drosophila* motor circuits. Therefore, one possible interpretation of our results is that cholinergic neurons have a particular and conserved sensitivity to the reduced levels of SMN. Neurotransmitter release is defective from the cholinergic motor neurons of SMN-Δ7 mice (Kong et al., 2009; Park et al., 2010a), and this defect does appear to require the cell-autonomous presence of normal SMN levels in these neurons (Park et al., 2010b). Nonetheless, SMN-Δ7 mutants have normal muscle twitch tension (Ling et al., 2010), targeted depletion of SMN in motor neurons does not cause lethality (Park et al., 2010b), and selective restoration of SMN in motor neurons alone or cholinergic neurons alone produces only a few days of survival benefit to mutant animals (Gogliotti et al., 2012; Martinez et al., 2012). These results imply that, if indeed cholinergic neurons are selectively affected by reduction of SMN, additional neurons in the mammalian motor circuit must also be involved.

An alternative, though not necessarily exclusive, interpretation is that conserved network elements of motor circuits are vulnerable to low levels of SMN. In support of this, it has recently been shown that SMN-Δ7 mice have early reduced responses to afferent fiber activation (Mentis et al., 2011), which are accompanied by a later decrease in glutamatergic proprioceptive synapses from sensory afferents onto motor neurons (Ling et al., 2010; Mentis et al., 2011). SMA patients have also been reported to have reduced or absent H-reflexes (Renault et al., 1983), which could be consistent with decreased activity of motor reflex circuits. Interestingly, in a companion manuscript (Lotti et al., 2012), we show that the splicing and expression of the SMN-dependent gene Stasimon is preferentially disrupted in the proprioceptive neurons of SMN-Δ7 motor circuits, though motor neurons are also affected. The concordant evidence for defective sensory-motor function in both mammalian and *Drosophila* SMN mutants is striking but also unexpected, even with our limited understanding of the central circuitry of both systems. For example, both mouse and human motor neurons receive direct synaptic input onto both somata and dendrites from sensory afferents (Chen et al., 2003), whereas *Drosophila* motor neuron dendrites do not appear to contact proprioceptive axon processes (Zlatic et al., 2009). Restoration of SMN in the proprioceptive neurons of *Drosophila smn* mutants is sufficient to restore normal NMJ neurotransmitter release properties in motor neurons. This suggests that, even without direct synaptic contact, increasing SMN in these neurons can influence motor neuron electrophysiological properties, presumably through intermediate interneuron connections. Therefore, it is possible that, although the specific details of motor circuit wiring differ between *Drosophila* and vertebrates, the essential relationships and function of motor networks are conserved and selectively susceptible to depletion of SMN.

## Manipulating Motor Circuit Excitability in *smn* Mutants

*Drosophila smn* mutants have increased NMJ eEPSP amplitude and mEPSP frequency, which is consistent with an increased excitability of motor neurons. Hyperexcitability of motor neurons has also been described in the SMA-Δ7 mouse model (Mentis et al., 2011). In *Drosophila*, this increase in neurotransmitter release properties is not corrected by restoring SMN in motor neurons themselves but is rescued by expressing SMN in cholinergic neurons. Hyperexcitability of *Drosophila* motor neurons has previously been reported in embryos in which cholinergic neurotransmission is completely inhibited (Baines et al., 2001). Congruent with this, we could replicate the increased evoked neurotransmitter release from *smn* mutant motor neurons by inhibiting cholinergic neurotransmission in larvae, which is consistent with a homeostatic compensatory increase in the excitability of motor neurons when synaptic inputs are reduced. A similar phenomenon has recently been described in chicken magnocellular neurons, which, when deafferentated by removal of the cochlea, increase in excitability (Kuba et al., 2010). Increasing neuronal excitability by inhibiting $K^+$ channels in *smn* mutants gave a remarkably robust rescue of muscle size, locomotion, rhythmic motor output, and NMJ neurotransmission. The Shaker type $I_A$ $K^+$ current plays a critical role in the regulation of membrane excitability in *Drosophila* neurons, and expression of a dominant negative construct inhibiting the Sh current (Mosca et al., 2005) in cholinergic neurons of *smn* mutants fully rescues all the larval phenotypes we examined. Together, these results strongly argue that decreased excitability of motor circuit neurons is a key physiological outcome of reduced levels of SMN.

Treatment with the small-molecule $K^+$ channel antagonist 4-AP also showed benefit to *Drosophila smn* mutant phenotypes. In wild-type animals, 4-AP treatment did not affect muscle size but did reduce locomotion and inhibited NMJ neurotransmitter release as might be anticipated by systemic inhibition of $K^+$ channels, which are present throughout the nervous system and in muscles (Wicher et al., 2001). Nonetheless, administration of 4-AP significantly increased both the muscle area and locomotion of *smn* mutants and fully corrected defects in rhythmic motor output and NMJ neurotransmission. Treatment with 4-AP has been linked to functional improvement of patients with spinal cord injury, myasthenia gravis, and Lambert-Eaton syndrome (Hayes, 2007) and can improve muscle twitch tension in a canine hereditary motor neuron disease (Pinter et al., 1997). A sustained release preparation of 4-AP was recently approved by the FDA for human clinical use in multiple sclerosis (Chwieduk and Keating, 2010). Our data suggest that the efficacy of 4-AP in

the *Drosophila smn* mutant model is likely via its activity upon cholinergic neurotransmission in the sensory-motor circuit. Extrapolating this finding to humans, investigation of compounds like 4-AP that can act within the spinal cord to modify the excitability of motor neural networks could be a fruitful therapeutic strategy to ameliorate the symptoms of SMA.

## EXPERIMENTAL PROCEDURES

### *Drosophila* Stocks

The mutants $smn^{X7}$ (Chang et al., 2008), $smn^{73Ao}$ (Chan et al., 2003), $smn^{E33}$ (Rajendra et al., 2007) were utilized. Gal4 and UAS lines are described in Supplemental Information.

### Muscle Measurement

Muscle area measurements were carried out at muscle 6, segment A3 of phalloidin-stained muscle fillet preparations (Brent et al., 2009).

### Locomotion

Larval locomotion assays were essentially performed as previously described (Suster and Bate, 2002).

### Motor Rhythm

Spontaneous motor rhythm was recorded as previously described (Fox et al., 2006). To measure the average interspike interval, the peak detection feature of MiniAnalysis (Synaptosoft, Inc.) was used to detect all spontaneous eEPSPs events that occurred during a 3 min period.

### NMJ Electrophysiology

Intracellular recordings from muscle 6, segment A3 were performed as previously described (Imlach and McCabe, 2009).

### Drug Treatment

Gene-switch GAL4 SMN expression was induced by culturing larvae with RU486 at 10 μg/ml for 148, 96, 72, or 48 hr prior to phenotypic measurement in *smn* mutants (controls were all assayed at wandering L3 stage). SMN induction was confirmed by western blot. For 4-AP treatment, 2 mM 4-AP (Sigma) was added to the yeast paste on which larvae were cultured immediately after hatching and throughout the subsequent larval period.

### Statistical Methods

Significance was tested by analysis of variance (ANOVA).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.09.011.

## ACKNOWLEDGMENTS

We are very grateful to Spyros Artavanis-Tsakonas and Greg Matera for *smn* stocks and reagents. We also thank Haig Keshishian, James Hodge, Cynthia Hughes, John Thomas, Michael Nitabach, Andrea Pizzo, Jonathan Javitch, Graeme Davis, Julie Simpson, and Wesley Grueber for additional stocks and advice. We thank George Mentis and especially Chris Henderson for critical reading of this manuscript. L.P. was supported by NIH-NINDS R01NS069601 and with B.D.M. by the Columbia University Motor Neuron Center. B.D.M. was supported by DoD-W81XWH-08-1-0009, DoD-W81XWH-11-1-0753, and the Gatsby Initiative in Brain Circuitry.

Received: November 21, 2011
Revised: June 15, 2012
Accepted: September 10, 2012
Published: October 11, 2012

## REFERENCES

Baines, R.A. (2006). Development of motoneuron electrical properties and motor output. Int. Rev. Neurobiol. *75*, 91–103.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J. Neurosci. *21*, 1523–1531.

Brent, J.R., Werner, K.M., and McCabe, B.D. (2009). Drosophila larval NMJ dissection. J. Vis. Exp. *24*, 1107.

Burghes, A.H., and Beattie, C.E. (2009). Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? Nat. Rev. Neurosci. *10*, 597–609.

Cattaert, D., and Birman, S. (2001). Blockade of the central generator of locomotor rhythm by noncompetitive NMDA receptor antagonists in Drosophila larvae. J. Neurobiol. *48*, 58–73.

Chan, Y.B., Miguel-Aliaga, I., Franks, C., Thomas, N., Trülzsch, B., Sattelle, D.B., Davies, K.E., and van den Heuvel, M. (2003). Neuromuscular defects in a Drosophila survival motor neuron gene mutant. Hum. Mol. Genet. *12*, 1367–1376.

Chang, H.C., Dimlich, D.N., Yokokura, T., Mukherjee, A., Kankel, M.W., Sen, A., Sridhar, V., Fulga, T.A., Hart, A.C., Van Vactor, D., and Artavanis-Tsakonas, S. (2008). Modeling spinal muscular atrophy in Drosophila. PLoS ONE *3*, e3209.

Chen, H.H., Hippenmeyer, S., Arber, S., and Frank, E. (2003). Development of the monosynaptic stretch reflex circuit. Curr. Opin. Neurobiol. *13*, 96–102.

Cheng, L.E., Song, W., Looger, L.L., Jan, L.Y., and Jan, Y.N. (2010). The role of the TRP channel NompC in Drosophila larval and adult locomotion. Neuron *67*, 373–380.

Chwieduk, C.M., and Keating, G.M. (2010). Dalfampridine extended release: in multiple sclerosis. CNS Drugs *24*, 883–891.

Crawford, T.O., and Pardo, C.A. (1996). The neurobiology of childhood spinal muscular atrophy. Neurobiol. Dis. *3*, 97–110.

Crisp, S., Evers, J.F., Fiala, A., and Bate, M. (2008). The development of motor coordination in Drosophila embryos. Development *135*, 3707–3717.

Daniels, R.W., Gelfand, M.V., Collins, C.A., and DiAntonio, A. (2008). Visualizing glutamatergic cell bodies and synapses in Drosophila larval and adult CNS. J. Comp. Neurol. *508*, 131–152.

Featherstone, D.E., Rushton, E.M., Hilderbrand-Chae, M., Phillips, A.M., Jackson, F.R., and Broadie, K. (2000). Presynaptic glutamic acid decarboxylase is required for induction of the postsynaptic receptor field at a glutamatergic synapse. Neuron *27*, 71–84.

Fox, L.E., Soll, D.R., and Wu, C.F. (2006). Coordination and modulation of locomotion pattern generators in Drosophila larvae: effects of altered biogenic amine levels by the tyramine beta hydroxlyase mutation. J. Neurosci. *26*, 1486–1498.

Gavrilina, T.O., McGovern, V.L., Workman, E., Crawford, T.O., Gogliotti, R.G., DiDonato, C.J., Monani, U.R., Morris, G.E., and Burghes, A.H. (2008). Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. Hum. Mol. Genet. *17*, 1063–1075.

Gogliotti, R.G., Quinlan, K.A., Barlow, C.B., Heier, C.R., Heckman, C.J., and Didonato, C.J. (2012). Motor neuron rescue in spinal muscular atrophy mice demonstrates that sensory-motor defects are a consequence, not a cause, of motor neuron dysfunction. J. Neurosci. *32*, 3818–3829.

Hayes, K.C. (2007). Fampridine-SR for multiple sclerosis and spinal cord injury. Expert Rev. Neurother. *7*, 453–461.

Hughes, C.L., and Thomas, J.B. (2007). A sensory feedback circuit coordinates muscle activity in Drosophila. Mol. Cell. Neurosci. *35*, 383–396.

Ilieva, H., Polymenidou, M., and Cleveland, D.W. (2009). Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J. Cell Biol. *187*, 761–772.

Imlach, W., and McCabe, B.D. (2009). Electrophysiological methods for recording synaptic potentials from the NMJ of Drosophila larvae. J. Vis. Exp. *24*, 1109.

Kariya, S., Park, G.H., Maeno-Hikichi, Y., Leykekhman, O., Lutz, C., Arkovitz, M.S., Landmesser, L.T., and Monani, U.R. (2008). Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. Hum. Mol. Genet. *17*, 2552–2569.

Kitamoto, T., Xie, X., Wu, C.F., and Salvaterra, P.M. (2000). Isolation and characterization of mutants for the vesicular acetylcholine transporter gene in Drosophila melanogaster. J. Neurobiol. *42*, 161–171.

Kong, L., Wang, X., Choe, D.W., Polley, M., Burnett, B.G., Bosch-Marcé, M., Griffin, J.W., Rich, M.M., and Sumner, C.J. (2009). Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. J. Neurosci. *29*, 842–851.

Kuba, H., Oichi, Y., and Ohmori, H. (2010). Presynaptic activity regulates Na(+) channel distribution at the axon initial segment. Nature *465*, 1075–1078.

Le, T.T., Pham, L.T., Butchbach, M.E., Zhang, H.L., Monani, U.R., Coovert, D.D., Gavrilina, T.O., Xing, L., Bassell, G.J., and Burghes, A.H. (2005). SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. Hum. Mol. Genet. *14*, 845–857.

Lefebvre, S., Bürglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M., et al. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. Cell *80*, 155–165.

Ling, K.K., Lin, M.Y., Zingg, B., Feng, Z., and Ko, C.P. (2010). Synaptic defects in the spinal and neuromuscular circuitry in a mouse model of spinal muscular atrophy. PLoS ONE *5*, e15457.

Ling, K.K., Gibbs, R.M., Feng, Z., and Ko, C.P. (2012). Severe neuromuscular denervation of clinically relevant muscles in a mouse model of spinal muscular atrophy. Hum. Mol. Genet. *21*, 185–195.

Lorson, C.L., Hahnen, E., Androphy, E.J., and Wirth, B. (1999). A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc. Natl. Acad. Sci. USA *96*, 6307–6311.

Lotti, F., Imlach, W.L., Saieva, L., Beck, E.S., Le, H.T., Li, D.K., Jiao, W., Mentis, G.Z., Beattie, C.E., McCabe, B.D., et al. (2012). An SMN-dependent U12 splicing event essential for motor circuit function. Cell *151*, this issue, 440–454.

Marder, E., and Rehm, K.J. (2005). Development of central pattern generating circuits. Curr. Opin. Neurobiol. *15*, 86–93.

Martinez, T.L., Kong, L., Wang, X., Osborne, M.A., Crowder, M.E., Van Meerbeke, J.P., Xu, X., Davis, C., Wooley, J., Goldhamer, D.J., et al. (2012). Survival motor neuron protein in motor neurons determines synaptic integrity in spinal muscular atrophy. J. Neurosci. *32*, 8703–8715.

McGovern, V.L., Gavrilina, T.O., Beattie, C.E., and Burghes, A.H. (2008). Embryonic motor axon development in the severe SMA mouse. Hum. Mol. Genet. *17*, 2900–2909.

Mentis, G.Z., Blivis, D., Liu, W., Drobac, E., Crowder, M.E., Kong, L., Alvarez, F.J., Sumner, C.J., and O'Donovan, M.J. (2011). Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy. Neuron *69*, 453–467.

Monani, U.R., Lorson, C.L., Parsons, D.W., Prior, T.W., Androphy, E.J., Burghes, A.H., and McPherson, J.D. (1999). A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum. Mol. Genet. *8*, 1177–1183.

Mosca, T.J., Carrillo, R.A., White, B.H., and Keshishian, H. (2005). Dissection of synaptic excitability phenotypes by using a dominant-negative Shaker K+ channel subunit. Proc. Natl. Acad. Sci. USA *102*, 3477–3482.

Palop, J.J., and Mucke, L. (2010). Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. Nat. Neurosci. *13*, 812–818.

Paradis, S., Sweeney, S.T., and Davis, G.W. (2001). Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. Neuron *30*, 737–749.

Park, G.H., Kariya, S., and Monani, U.R. (2010a). Spinal muscular atrophy: new and emerging insights from model mice. Curr. Neurol. Neurosci. Rep. *10*, 108–117.

Park, G.H., Maeno-Hikichi, Y., Awano, T., Landmesser, L.T., and Monani, U.R. (2010b). Reduced survival of motor neuron (SMN) protein in motor neuronal progenitors functions cell autonomously to cause spinal muscular atrophy in model mice expressing the human centromeric (SMN2) gene. J. Neurosci. *30*, 12005–12019.

Passini, M.A., Bu, J., Roskelley, E.M., Richards, A.M., Sardi, S.P., O'Riordan, C.R., Klinger, K.W., Shihabuddin, L.S., and Cheng, S.H. (2010). CNS-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy. J. Clin. Invest. *120*, 1253–1264.

Patel, A.A., and Steitz, J.A. (2003). Splicing double: insights from the second spliceosome. Nat. Rev. Mol. Cell Biol. *4*, 960–970.

Pearn, J. (1978). Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. J. Med. Genet. *15*, 409–413.

Pellizzoni, L. (2007). Chaperoning ribonucleoprotein biogenesis in health and disease. EMBO Rep. *8*, 340–345.

Pinter, M.J., Waldeck, R.F., Cope, T.C., and Cork, L.C. (1997). Effects of 4-aminopyridine on muscle and motor unit force in canine motor neuron disease. J. Neurosci. *17*, 4500–4507.

Rajendra, T.K., Gonsalvez, G.B., Walker, M.P., Shpargel, K.B., Salz, H.K., and Matera, A.G. (2007). A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. J. Cell Biol. *176*, 831–841.

Renault, F., Raimbault, J., Praud, J.P., and Laget, P. (1983). [Electromyographic study of 50 cases of Werdnig-Hoffmann disease]. Rev. Electroencephalogr. Neurophysiol. Clin. *13*, 301–305.

Rothstein, J.D. (2009). Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Ann. Neurol. *65* (*Suppl 1*), S3–S9.

Salvaterra, P.M., and Kitamoto, T. (2001). Drosophila cholinergic neurons and processes visualized with Gal4/UAS-GFP. Brain Res. Gene Expr. Patterns *1*, 73–82.

Schmid, A., and DiDonato, C.J. (2007). Animal models of spinal muscular atrophy. J. Child Neurol. *22*, 1004–1012.

Simic, G. (2008). Pathogenesis of proximal autosomal recessive spinal muscular atrophy. Acta Neuropathol. *116*, 223–234.

Song, W., Onishi, M., Jan, L.Y., and Jan, Y.N. (2007). Peripheral multidendritic sensory neurons are necessary for rhythmic locomotion behavior in Drosophila larvae. Proc. Natl. Acad. Sci. USA *104*, 5199–5204.

Suster, M.L., and Bate, M. (2002). Embryonic assembly of a central pattern generator without sensory input. Nature *416*, 174–178.

Swoboda, K.J., Prior, T.W., Scott, C.B., McNaught, T.P., Wride, M.C., Reyna, S.P., and Bromberg, M.B. (2005). Natural history of denervation in SMA: relation to age, SMN2 copy number, and function. Ann. Neurol. *57*, 704–712.

Wicher, D., Walther, C., and Wicher, C. (2001). Non-synaptic ion channels in insects—basic properties of currents and their modulation in neurons and skeletal muscles. Prog. Neurobiol. *64*, 431–525.

Wu, Y., Cao, G., Pavlicek, B., Luo, X., and Nitabach, M.N. (2008). Phase coupling of a circadian neuropeptide with rest/activity rhythms detected using a membrane-tethered spider toxin. PLoS Biol. *6*, e273.

Zlatic, M., Li, F., Strigini, M., Grueber, W., and Bate, M. (2009). Positional cues in the Drosophila nerve cord: semaphorins pattern the dorso-ventral axis. PLoS Biol. *7*, e1000135.