Behavioral and electrophysiological outcomes of tissue-specific *Smn* knockdown in *Drosophila melanogaster*

Christina Timmerman[a] and **Subhabrata Sanyal**[a,b,*]

[a]Department of Cell Biology Emory University School of Medicine, Atlanta, GA 30322, USA

[b]Department of Neurology Emory University School of Medicine, Atlanta, GA 30322, USA

Abstract

Severe reduction in Survival Motor Neuron 1 (SMN1) protein in humans causes Spinal Muscular Atrophy (SMA), a debilitating childhood disease that leads to progressive impairment of the neuro-muscular system. Although previous studies have attempted to identify the tissue(s) in which SMN1 loss most critically leads to disease, tissue-specific functions for this widely expressed protein still remain unclear. Here, we have leveraged RNA interference methods to manipulate SMN function selectively in *Drosophila* neurons or muscles followed by behavioral and electrophysiological analysis. High resolution measurement of motor performance shows profound alterations in locomotor patterns following pan-neuronal knockdown of SMN. Further, locomotor phenotypes can be elicited by SMN knockdown in motor neurons, supporting previous demonstrations of motor neuron-specific SMN function in mice.

Electrophysiologically, SMN modulation in muscles reveals largely normal synaptic transmission, quantal release and trans-synaptic homeostatic compensation at the larval neuro-muscular junction. Neuronal SMN knockdown does not alter baseline synaptic transmission, the dynamics of synaptic depletion or acute homeostatic compensation. However, chronic glutamate receptor-dependent developmental homeostasis at the neuro-muscular junction is strongly attenuated following reduction of SMN in neurons. Together, these results support a distributed model of SMN function with distinct neuron-specific roles that are likely to be compromised following global loss of SMN in patients. While complementary to, and in broad agreement with, recent mouse studies that suggest a strong necessity for SMN in neurons, our results uncover a hitherto under-appreciated role for SMN in homeostatic regulatory mechanisms at motor synapses.

Keywords

Drosophila; motor behavior; synaptic transmission; homeostasis; Spinal Muscular Atrophy; SMN

1. Introduction

Spinal Muscular Atrophy (SMA) is a severely debilitating childhood disorder that leads to progressive muscle weakness resulting from a loss or degeneration of motor neuron

[*]Corresponding author Room No. 444, 615 Michael St., NE, Cell Biology Dept., Whitehead Building, Emory University, Atlanta, GA 30322 ssanya2@emory.edu Phone: 404-727-1250 Fax: 404-727-6256.

innervation. It is a relatively common autosomal recessive disorder and can affect up to 1 in 6000 babies (Lefebvre et al., 1995; Pearn, 1978; Sleigh et al., 2011). SMA results predominantly from mutations in the *Survival Motor Neuron 1 gene* (*SMN1*). Severity is related to the copy number and activity of a near identical homolog in humans, *SMN2* (Coovert et al., 1997; Lefebvre et al., 1997). The *SMN2* gene carries a C-to-T transition leading to aberrant splicing in 80–90% of the *SMN2* transcript and loss of exon 7 (*SMNΔ7*) resulting in a protein with reduced stability (Lorson et al., 1999; Lorson and Androphy, 2000; Monani et al., 1999). The overall reduction of the SMN protein leads to severe neuromuscular degeneration including the loss of motor neuron cell bodies over time (Balabanian et al., 2007; Jablonka et al., 2000; Monani et al., 2000). SMN is a highly conserved ubiquitously expressed protein that is required critically for normal mRNA splicing since it affects the biogenesis of U snRNP particles across species (small nuclear ribonucleoprotein) (Fischer et al., 1997; Kroiss et al., 2008; Lee et al., 2009; Liu et al., 1997; Meister et al., 2001; Pellizzoni et al., 2002). Complete loss of SMN is cell lethal, therefore, SMA is essentially a disorder that results from reduced SMN availability (Feldkotter et al., 2002; Mailman et al., 2002; McAndrew et al., 1997). Several mouse models of SMA also suggest that full-length SMN is required for cell viability, and that disease severity is closely linked to the dosage of *SMN* gene product. Thus, total loss of *Smn* leads to early lethality (Frugier et al., 2000), whereas introduction of two copies of the human *SMN2* gene results in normal birth followed by progressive decline in neuromuscular and motor function and subsequent death by post-natal day 7 (PND 7). Introduction of four copies of *SMN2*, however, leads to complete rescue (Hsieh-Li et al., 2000; Monani et al., 2000). Interestingly, introduction of *SMNΔ7* – the mutant form of human SMN missing exon 7 - in an *Smn*$^{-/-}$; *SMN2*$^{+/+}$ background provides additional rescue, such that animals now die between PND 6 and 13 (Le et al., 2005). Given that SMN is required in all cells, why its reduction leads to specific neuromuscular symptoms or whether SMA symptoms arise from SMN reduction in motor neurons or muscle (or even other tissues), still remain outstanding questions, though recent experiments indicate prominent neuronal functions (Gogliotti et al., 2012; Martinez et al., 2012; Park et al., 2010; Imlach et al., 2012; Lotti et al., 2012).

Since SMN is a highly conserved protein, invertebrate models such as *C. elegans* and *Drosophila* have also been used to understand molecular functions for SMN and to more rapidly identify networks of genes within which SMN functions (Briese et al., 2009; Chan et al., 2003; Chang et al., 2008; Dimitriadi et al., 2010; Rajendra et al., 2007; Sen et al., 2011; Sleigh et al., 2011). These studies have revealed remarkable similarities in SMN function in the regulation of U snRNP biogenesis (Cauchi, 2010; Cauchi et al., 2008; Kroiss et al., 2008; Lee et al., 2009; Shpargel et al., 2009) and NMJ development and physiology (Cauchi et al., 2008; Chan et al., 2003; Chang et al., 2008; Praveen et al., 2012; Rajendra et al., 2007; Shpargel et al., 2009). While a number of these studies have used classical loss-of-function alleles in *Drosophila Smn*, more recent work has made use of gene-targeted RNAi methodologies, that can be used to knock down, but not deplete SMN function in selected tissues in conjunction with the versatile GAL4-UAS system (Brand and Perrimon, 1993; Chang et al., 2008). Thus, RNAi based knockdown is a closer approximation to the situation in SMA patients, and also permits experiments in which tissue-specific reduction in SMN can be achieved *in vivo* in an otherwise normal animal. If it is true that different tissues have different thresholds for susceptibility to reduction in SMN function, then selective, and relatively mild, knockdown of SMN might uncover fundamentally conserved tissue-specific functions for SMN (Sleigh et al., 2011). Here, we use this strategy to test neuronal versus muscle-specific functions for SMN in *Drosophila* in the regulation of motor behavior and synaptic physiology (Figure 1). Our results highlight both neuronal and muscle functions for *Drosophila Smn*, similar to mouse studies, but with stronger neuronal outcomes. But more significantly, they also point to a very precise role for SMN in the regulation of homeostatic mechanisms that maintain parity at the NMJ. These findings are especially relevant in the

context of putative developmental roles for SMN in mammals and suggest a unique function for SMN in the homeostatically driven development of mature motor neurons (Foust et al., 2010; Gogliotti et al., 2012; Hammond et al., 2010; Martinez et al., 2012; Sleigh et al., 2011). Behavioral and synaptic phenotypes described in this report should also provide useful platforms for the functional testing of other *Smn*-interacting genes that have been discovered through genetic and biochemical screening approaches (Dimitriadi et al., 2010; Sen et al., 2011).

2. Results

2.1 *Smn* perturbation in neurons or muscle causes behavioral deficits

Gross locomotor defects in *Drosophila Smn* mutants and in mutants for the fly homolog of *Gemin3* – a DEAD-box RNA helicase complexed with SMN, have been reported previously (Cauchi et al., 2008; Chan et al., 2003; Praveen et al., 2012; Shpargel et al., 2009; Imlach et al., 2012). Since *Smn* mutants are often pupal and adult lethal, these studies revealed abnormal locomotion predominantly at larval stages and showed that systemic or cholinergic neuron-specific add-back of SMN significantly rescued these motor phenotypes. Consequently, a relationship between normal SMN function and motor physiology has been established in *Drosophila*, similar to human patients and other mammalian model systems (Cauchi et al., 2008; Chan et al., 2003; Shpargel et al., 2009; Imlach et al., 2012). However, it is currently unclear whether locomotor phenotypes in *Smn* mutants arise from a loss of SMN in neurons or muscle. In this study, we attempted to answer this question using the GAL4-UAS (Brand and Perrimon, 1993) method of tissue-targeted transgene expression and recently available reagents to either over-express the full length wild type SMN gene product or knockdown *Smn* function using RNA interference (Chang et al., 2008; Sen et al., 2011). We chose to use these RNAi reagents since their efficacy in knocking down endogenous levels of SMN in neurons and muscles, as well as their potency in generating neuro-muscular defects have been well characterized (Chang et al., 2008), and a reduction in SMN, rather than its completely removal, more closely approximates the situation in SMA (western blots in Figure 2A and C). Since RNAi mediated knockdown of *Smn* does not lead to lethality in most cases, we were also able to measure adult locomotor behavior at a resolution much higher than possible for larvae. Such measurements allow for a thorough analysis of locomotor parameters that will also be useful as screening tools in future genetic interaction experiments. A complementary strategy to rescue strong loss-of-function mutations in SMN using tissue directed rescue has recently been reported (Imlach et al., 2012).

Pan-neuronal reduction of SMN using the $elav^{C155}$-GAL4 driver results in significant changes to the normal locomotor patterns of flies in the Buridan's assay (Gotz, 1980). In this assay, 5–6 day old flies are allowed to walk between two diametrically placed landmarks on a circular arena while their movement is recorded in real time followed by off-line analysis (Freeman et al., 2012; Neuser et al., 2008). In general, our results suggest that neuronal loss of SMN leads to slower walking speed, less overall activity and a larger number of pauses between bouts of walking as compared to genetic controls containing the GAL4 line (Figure 2A). These phenotypes are seen when *Smn* is knocked down using either of the two RNAi lines directed to the C- or N-terminal portion of the *Drosophila Smn* mRNA (Chang et al., 2008) confirming the specificity of these phenotypes. Neuronal over-expression of SMN, however, does not lead to any discernible defects in locomotion. Single fly tracks and cumulative occupancy plots (heat map coded to show regions of highest occupancy during the performance of the behavior in red) also demonstrate the extent of disruption of locomotor activity in neuronal *Smn* knockdown animals (Figure 2B).

Changes in SMN levels in muscle do not produce strong locomotor phenotypes in adults (Figure 2C and D). Although muscle-specific roles for SMN have been recognized in both mammalian and *Drosophila* models (Chan et al., 2003; Chang et al., 2008; Dachs et al., 2011; Lee et al., 2012; Rajendra et al., 2007; Sen et al., 2011), RNAi mediated knockdown or over-expression of wild type SMN does not result in appreciable locomotor defects. Mild changes are seen in the number of pauses when SMN is either more or less than control levels, suggesting that these are likely to result from non-specific effects. Single representative fly tracks as well as occupancy plots further confirm that changes of SMN in muscle tissue do not affect locomotor performance in this assay. Overall, results presented in Figure 2 show the presence of profound locomotor deficits in adult animals following selective knockdown of *Smn* in neurons. These findings suggest that in *Drosophila* motor phenotypes previously observed in *Smn* mutants are likely to be due to loss of SMN in neurons but do not clarify whether SMN is required in specific neuronal subsets for normal locomotor behavior.

2.2. Knockdown of *Smn* in glutamatergic neurons, but not cholinergic neurons, leads to behavioral deficits

Since pan-neuronal reduction in SMN resulted in locomotor deficits, we asked whether SMN is preferentially required in a subset of neurons for normal motor behavior. Given that motor neurons are strongly implicated in SMA, we first tested if *Smn* knockdown in adult motor neurons produces behavioral phenotypes. *Drosophila* motor neurons are glutamatergic. Therefore, we used the VGlut-GAL4 line (OK371) to either over-express or knockdown *Smn* in all glutamatergic neurons (note that there are many non-motor glutamatergic neurons in the *Drosophila* nervous system that are also targeted by OK371-GAL4) (Mahr and Aberle, 2006). OK371-GAL4 driven *Smn* RNAi resulted in phenotypes very reminiscent of pan-neuronal *Smn* knockdown (Figure 3A and B). While the average speed of walking was not significantly altered, total distance moved and overall activity were significantly reduced, and number of pauses elevated, following *Smn* knockdown in the glutamatergic nervous system. These phenotypes were not observed following over-expression of *Smn* in glutamatergic neurons. Single fly tracks and occupancy plots also confirmed aberrant locomotor behavior in the knockdown animals, though these were not as strong as those following pan-neuronal knockdown of *Smn*. By contrast, *Smn* perturbation in cholinergic neurons did not have any effect on locomotion at all (Figure 3C and D). Thus, when *Smn* was over-expressed or knocked down with the Cha-GAL4 driver (Salvaterra and Kitamoto, 2001), none of the parameters were altered significantly and single fly tracks and occupancy plots reiterated normal locomotor behavior in all genotypes tested. Since by all accounts, Cha-GAL4 is a strong driver and has elicited phenotypes in many previous studies including those that have used RNAi mediated knockdown (for examples see Ghosh and Feany, 2004; Kitamoto, 2001; Lima and Miesenbock, 2005; Sakai et al., 2009), we do not favor the idea that the absence of phenotypes is a consequence of weak expression of the SMN-RNAi construct. Instead, taken together with results described in the previous section, these experiments suggest to us that glutamatergic motor neurons are likely to be a prominent locus for *Smn*-dependent locomotor phenotypes in *Drosophila*. We next tested whether altered synaptic transmission at motor synapses might underlie behavioral deficits following tissue-specific knockdown of SMN.

2.3. Neuronal perturbation of *Smn* does not alter baseline and high-frequency synaptic transmission

Analysis of adult behavior shows that reduced SMN in motor neurons results in behavioral deficits in a visuo-motor assay. We wondered whether we could detect a possible basis for these motor phenotypes rooted in synaptic physiology. Since the larval neuro-muscular system is simpler and experimentally more accessible than in the adult, we used this model

synapse to test our idea. Baseline synaptic transmission was measured at the muscle 6/7 motor synapse in abdominal segment A2 in 1 mM external calcium as reported previously (Freeman et al., 2011; Sen et al., 2011). *elav*[C155]-GAL4 was used to drive expression of full-length *Smn* or RNAi constructs as described in previous sections. Interestingly, we did not observe any meaningful change in synaptic transmission under these conditions (Figure 4A and B). Mean excitatory junction potential (EJP) amplitude, mini evoked junction potential (mEJP) amplitude and the quantal content of release remained unaltered in all genotypes tested, barring a modest increase in mEJP amplitude following over-expression of *Smn*. These data indicate that normal parameters of synaptic transmission are not affected by *Smn* perturbation in neurons, although a 50% reduction in the growth of this synapse and the number of synaptic boutons under these conditions of *Smn* knockdown have been reported previously (Chang et al., 2008).

We next tested whether *Smn* knockdown impairs synaptic transmission when the system is stressed during high-frequency stimulation under conditions that maximize quantal content of release. We drove the N4 RNAi line with *elav*[C155]-GAL4 since this should result in the strongest pre-synaptic knockdown of *Smn*. Motor neurons innervating muscle 6/7 were stimulated at 10 Hz for a duration of 10 minutes in an external calcium concentration of 10 mM. Under these conditions more synaptic vesicles are released than can be recovered, leading to a depletion of the readily releasable pool of synaptic vesicles and a rapid decline in the size of the EJP that reflects fewer vesicles being released per action potential. Following this rapid decline, the synapse recovers partially and reaches a stable transmission rate due to vesicle recruitment from the reserve pool of vesicles. After a transition from 10 Hz stimulation to 0.1 Hz stimulation, the synapse recovers almost immediately to pre-stimulus levels since the balance between vesicle fusion and vesicle retrieval is restored. This assay, therefore, examines mechanisms underlying rapid recruitment of distinct vesicle pools and synaptic vesicle fusion and recycling (Dickman et al., 2005; Seabrooke and Stewart, 2011). When challenged with high-frequency stimulation *Smn* knockdown synapses performed as well as control ones (Figure 4C). The rate and extent of decline, reserve pool recruitment and recovery post-stimulation are not significantly different between knockdown and control animals suggesting normal vesicle dynamics at these synapses. Taken together, results in this section imply that behavioral deficits following neuronal knockdown might not result from obvious changes in baseline synaptic transmission at the neuro-muscular junction. This is in apparent conflict with earlier reports in *Drosophila* (Chan et al., 2003) and several studies in rodents (Kong et al., 2009; Martinez et al., 2012; Ruiz et al., 2010). However, these studies did not selectively remove *Smn* in neurons, and synaptic transmission phenotypes in these situations could have arisen from developmental changes triggered by reduced SMN in both neurons and muscles and indeed in other tissues. In a situation where *Smn* was selectively reduced in rodent motor neurons, synaptic and behavioral defects were also mild and improved dramatically postnatally (Park et al., 2010).

2.4. *Smn* perturbation in muscle affects quantal size

Increasingly, a role for normal SMN function in muscle is being appreciated (Dachs et al., 2011; Lee et al.; Martinez et al., 2012). These studies suggest that SMN in muscle might contribute to the regulation of normal muscle development and synaptic physiology. In *Drosophila*, previous experiments have clearly documented post-synaptic enrichment of SMN protein, synaptic growth phenotypes at the larval neuro-muscular junction that have resulted from *Smn* knockdown (Chang et al., 2008), strong muscle atrophy in *Smn* mutants (Rajendra et al., 2007) and genetic interactions between *Smn* and *FGF* signaling pathways in muscle (Sen et al., 2011). Based on this information, we asked whether *Smn* perturbations in muscle might result in synaptic phenotypes at the larval NMJ.

how^{24B}-GAL4 driven expression of full-length *Smn* or *Smn*-RNAi resulted in reduced mean EJP and mEJP amplitude (Figure 5A and B) resulting in indistinguishably similar quantal content of release across genotypes. Although it seems surprising that both over-expression and knockdown of *Smn* lead to equivalent synaptic phenotypes, this is in line with the observation that both over-expression and knockdown of *Smn* in muscle leads to pupal/adult lethality (Chang et al., 2008) and abnormally long pupal cases (our unpublished observations). While the reasons behind the similarity of these phenotypes are unclear, it seems likely that these are indeed due to perturbations in muscle SMN function. Quantal size phenotypes prompted us to examine the staining intensity and distribution of post-synaptic glutamate receptors. A severe disorganization of post-synaptic Acetylcholine receptors at the NMJ has been widely observed in rodent models of SMA (Dachs et al., 2011; Kariya et al., 2008; Kong et al., 2009; Lee et al., 2008; Ling et al.; Murray et al., 2008; Ruiz et al., 2010) and a loss of glutamate receptor staining has also been reported in *Drosophila Smn* mutants (Chan et al., 2003). However, we did not observe any changes in overall glutamate receptor staining intensity or distribution when *Smn* was knocked down in muscles using the stronger N4 RNAi transgene (Figure 5C). Similarly, no change in glutamate receptor distribution is observed following neuronal knockdown of *Smn* either (data not shown). These results suggest that a reduction in SMN exclusively in the muscle (or in neurons) is not sufficient to cause strong synaptic transmission defects or receptor misaggregation, although it is sufficient to cause adult lethality (Chang et al., 2008). Changes in quantal size might also be simply due to small alterations in muscle size (Imlach et al., 2012) that we were not able to detect readily, and therefore, input resistance. It is possible that neuro-muscular symptoms in SMA arise from a progressive distortion in NMJ development or maintenance precipitated by a chronic loss of SMN in both compartments (Martinez et al., 2012). If this were true, compartment-specific knockdown of SMN might reveal the very initial events that trigger this collapse.

2.5. Pre-synaptic knockdown of *Smn* abolishes long-term homeostatic compensation at the NMJ

Rodent models of SMA show early synaptic transmission deficits at the NMJ that typically worsen with age (Dachs et al., 2011; Kariya et al., 2008; Kong et al., 2009; Lee et al., 2012; Ling et al., 2011; Murray et al., 2008; Ruiz et al., 2010). Additionally, recent studies suggest that homeostatic feedback mechanisms that maintain parity of synaptic transmission might be compromised when SMN levels are reduced in motor neurons. In one study, selective reduction of SMN in motor neurons resulted in reduced quantal content of transmission at post-natal day 8 (PND8) in mice (Park et al., 2010). Strikingly, these defects were sharply reduced by PND10–12, and by 3 months, NMJs showed overcompensation as they displayed higher mini-end plate potentials (mEPPs), EPPs and quantal content than control animals (Park et al., 2010). These data reveal aberrations in homeostatic mechanisms at the synapse following neuronal knockdown of *Smn*. Conversely, two other studies have addressed the tissue-specificity of SMN by rescuing SMN expression in either neurons or muscle in an *Smn* mutant background (Gogliotti et al., 2012; Martinez et al., 2012). While neuronal, but not muscle, add-back of SMN was able to rescue synaptic transmission defects at the NMJ in *Smn* mutants, surprisingly, neuronal supplementation of SMN also prevented loss of central motor neuron synaptic input. This observation suggests that under normal conditions neuronal SMN might regulate retrograde signaling mechanisms that maintain homeostatic drive at synapses.

To directly evaluate whether SMN plays a role in homeostatic signaling mechanisms in neurons, we used two assays that test either a rapid, gene expression-independent or a long-term, gene expression requiring form of homeostasis at the larval NMJ. An activity-dependent block of post-synaptic glutamate receptors at the larval NMJ using the wasp

venom Philanthotoxin (PhTox) triggers a fast homeostatic response from the pre-synapse that results in a significant increase in quantal content, while quantal size remains predictably reduced (Frank et al., 2006; Frank et al., 2009). We tested whether *Smn* knockdown in neurons or muscles affected this homeostatic response. Figure 6A shows that PhTox-dependent homeostatic responses remained robust in either case. Thus, reduction of SMN using the N4 RNAi transgene did not alter the synapse's ability to engage a fast homeostatic increase in quantal content following glutamate receptor attenuation with PhTox (reflected in the reduced mEJP amplitudes in Figure 6A). These data suggest that local signaling mechanisms that are involved in this form of homeostasis do not require SMN activity (Figure 6D).

Since aberrations in homeostasis in rodent models of SMA occur over a developmental time period, we next tested a form of homeostatic compensation at the larval NMJ that is longer term and likely requires changes in gene expression. A strong genetic reduction in one glutamate receptor subunit, *GluRIIA*, leads to severely attenuated post-synaptic currents and smaller mEJP amplitudes (Davis et al., 1998; Petersen et al., 1997). However, over developmental time, the pre-synapse compensates through an increase in quantal content (Davis et al., 1998 1999; Haghighi et al., 2003; Penney et al., 2012; Petersen et al.,1997). When we reduced SMN in neurons in a *GluRIIA* mutant background, we saw a virtual absence of homeostatic compensation (Figure 6B and C and Supplementary Table 1). By contrast, SMN reduction in muscles did not affect this form of homeostasis. These observations point to very specific neuronal functions for SMN in the control of long-term homeostatic responses. Such a function would also be consistent with a general role for SMN in the regulation of RNA metabolism and gene expression (Figure 6D).

3. Discussion

Although there are obvious differences between *Drosophila* and mammalian neuro-muscular organization, modeling SMA in flies is a productive approach given the high degree of conservation in SMN function and the ease and rapidity of genetic analysis in flies (Dimitriadi et al., 2010). Indeed, previous work in *Drosophila* has suggested that *Smn* regulates U snRNP biogenesis (Cauchi, 2010; Kroiss et al., 2008; Lee et al., 2009; Shpargel et al., 2009) and loss of SMN results in both synaptic and motor defects that are comparable to those seen in mouse models of SMA (Chan et al., 2003; Chang et al., 2008; Rajendra et al., 2007; Shpargel et al., 2009). Recent work has also made use of RNA interference to knockdown *Smn* in target tissues (Chang et al., 2008; Sen et al., 2011) to better mimic the situation in SMA patients. Experiments with these validated RNAi reagents have uncovered morphological phenotypes at the NMJ when SMN was knocked down in either neurons or muscle (Chang et al., 2008; Sen et al., 2011). In the current study we have used the same RNAi reagents in conjunction with the GAL4-UAS system to reduce SMN in neurons or muscle tissue followed by an assessment of such perturbation on motor performance and synaptic transmission at the NMJ (Figure 1). Muscle knockdown of SMN did not result in strong behavioral or synaptic transmission defects, though similar manipulations have been shown to affect synaptic morphology in larvae and lead to adult lethality (Chang et al., 2008; Sen et al., 2011). This suggests a situation in *Drosophila* that is very similar to that in mice, where SMN plays a role in muscles to ultimately impact lifespan, but is not critically required for normal synaptic function (Gogliotti et al., 2012; Martinez et al., 2012). On the other hand, we find distinct deficits in visuo-motor performance when SMN is reduced neuronally, though baseline synaptic transmission under these conditions is largely unaffected. This contrasts with earlier findings in *Drosophila* (Chan et al., 2003) and is likely due to the compartment-specific knockdown of SMN in our experiments. Interestingly, a recent report suggests that larval locomotor phenotypes in strong hypomorphic mutations in SMN arise from SMN deficiency in cholinergic neurons (Imlach

et al., 2012). While our complementary manipulations do not reveal any effect of cholinergic knock down of SMN on locomotor activity in adult flies (perhaps due to insufficient knock down with the RNAi reagent available to us), we were able to identify a very specific loss of homeostatic compensation between the pre- and post-synapse when SMN was reduced neuronally (Figure 6D). We propose that this might reflect an early and subtle event in SMA etiology that predates more dramatic SMA sequelae comprising loss of normal NMJs and neuro-muscular degeneration.

Severe mouse models of SMA (e.g. $Smn^{-/-}$; $SMN2^{+/+}$) show clearly discernible loss of neuro-muscular morphology, strong synaptic transmission defects, loss of pre-synaptic inputs to spinal motor neurons, muscle degeneration and eventual death of motor neurons (Balabanian et al., 2007; Jablonka et al., 2000; Monani et al., 2000). Several studies have also used Cre-loxP derived conditional knockout models of SMA in mice to address the question of tissue-specificity of SMN vis-à-vis the incidence of SMA-like symptoms. Complete removal of exon 7 in specific tissues led to cell lethality, a situation, though consistent with a vital role for SMN in all cells, significantly different from that found in SMA patients (Cifuentes-Diaz et al., 2001; Cifuentes-Diaz et al., 2002; Frugier et al., 2000; Nicole et al., 2003; Vitte et al., 2004). Recent studies have more successfully approximated the patient condition, while trying to distinguish tissue-specific functions for SMN *in vivo* (Gogliotti et al., 2012; Martinez et al., 2012; Park et al., 2010). In one study, *Smn* exon 7 was removed in neurons in a background where *SMN2* was introduced (Park et al., 2010). Surprisingly, these mice showed mild SMA symptoms that improved with age. However, this manipulation did uncover subtle homeostatic regulatory phenotypes at the neuro-muscular junction (NMJ) that may not have been noticed in the presence of the typically severe SMA phenotypes (Kariya et al., 2008; Michaud et al., 2010; Murray et al., 2010; Murray et al., 2008; Ruiz et al., 2010). In another study, full-length SMN protein was added back to either neurons or muscle in a mutant *SMN* background that otherwise results in viable "SMA" mice (Lutz et al., 2011; Martinez et al., 2012). This study revealed that while both neuronal and muscle add-back of SMN could significantly rescue survival and motor behavior, only neuronal expression rescued synaptic function at the NMJ and also motor neuron somal synapses, through putative homeostatic mechanisms (Martinez et al., 2012). A third study showed, by adding back SMN selectively to motor neurons using an *Hb9-Cre*, that SMN supplementation in motor neurons could rescue the vast majority of motor defects and restored normal sensory-motor synapses (Gogliotti et al., 2012). Together, these observations hint at neuron-specific roles for SMN in the homeostatic regulation of normal synaptic connectivity and transmission in mammals.

Phenotypic end-points in severe SMA mouse models closely mirror SMA pathology in patients, particularly Type1 SMA. However, the progressive nature of these phenotypes, increased fetal expression of *SMN* and the general observation that SMA phenotypes can be most effectively rescued by adding back full length SMN perinatally, suggest that defining events in SMA pathology might occur at very early stages of development (Baumer et al., 2009; Burlet et al., 1998; Butchbach et al., 2010; Foust et al., 2010; Gabanella et al., 2005; Hammond et al., 2010; Murray et al., 2010; Narver et al., 2008). Thus, obvious target tissues in SMA such as motor neurons and muscle might be exquisitely susceptible to a reduction in SMN function such that subtle defects in neuro-muscular development or physiology are the first to appear at these loci – consistent with the threshold hypothesis for SMA (Sleigh et al., 2011). Milder models of SMA in mice such as those mentioned in the previous section, seem to support this idea in some ways since they reveal aberrations in the homeostatic regulation of synaptic connectivity that may not be discernible in the presence of more severe phenotypes (Gogliotti et al., 2012; Martinez et al., 2012; Park et al., 2010). These observations point to a fundamental impairment in communication between motor neurons and their pre-synaptic input following loss of SMN. Over time, this might lead to an

asynchrony in an otherwise tightly coordinated program of synaptic development leading to eventual synaptic degeneration.

Our experiments in *Drosophila* probably represent relatively mild manipulations of *Smn*. Consistent with this idea, animals are largely viable and do not show a dramatic loss of locomotion at larval stages that is seen in *Smn* loss-of-function mutants (Chan et al., 2003; Shpargel et al., 2009). However, neuronal knockdown of *Smn* does result in adult locomotor dysfunction that can be discerned with sophisticated visuo-motor assays. Further electrophysiological analysis at the larval NMJ provides a possible underlying mechanism, with the obvious caveat that these recordings are made at larval stages, while our behavioral analysis is in adults. Although most parameters of synaptic transmission are near normal, we detect specific phenotypes when *Smn* is perturbed in neurons or muscles. Muscle knockdown of *Smn* results in altered quantal size. Although we did not detect any changes in glutamate receptor staining intensity or distribution, increased mEJP size might still result from altered post-synaptic receptor sensitivity as electrophysiological measures are typically more sensitive than immuno-histochemistry. Alternatively, altered quantal size might result from changes in synaptic vesicle size or neurotransmitter packaging, or from small changes in muscle size and input impedance. Neuronal knockdown of *Smn* leads to a defect in homeostatic compensation. A severe impairment of synaptic transmission as described previously (Chan et al., 2003), might not have allowed the detection of this phenotype. In general, this phenotype finds resonance with defects in homeostatic regulatory mechanisms that have been highlighted previously in mild SMA models in mice (Gogliotti et al., 2012; Martinez et al., 2012; Park et al., 2010) and might represent a "weak link" that is most susceptible to a loss of SMN in neurons. It is also conceivable that an SMN-dependent loss of calibration in homeostatic drive is one of the earliest events that disrupts developmental coupling between the pre- and post-synapse during a critical period in synaptogenesis. It is interesting to note that this phenotype is only uncovered when Glutamate receptor expression is reduced, suggesting that low neuronal SMN limits the capacity for a homeostatic response when the demand for such a response is high. Further experiments are required to test whether this prediction holds in mouse models of SMA.

Our results might also help in understanding the molecular function of SMN in neurons. While SMN is clearly involved in U snRNP biogenesis, it is not clear whether this function of SMN is the one most compromised in neurons (Buhler et al., 1999; Gabanella et al., 2007; Pellizzoni et al., 1999; Shpargel and Matera, 2005; Wan et al., 2005). For example, in *Drosophila*, *Smn* null mutants show normal mRNA splicing (Praveen et al., 2012; Rajendra et al., 2007). In addition, low transgene-mediated expression of wild type SMN rescues SMA-like phenotypes in flies without any improvement in snRNA levels (Praveen et al., 2012). By contrast, a recent report implicates aberrant U12 splicing events in a collection of U12 intron containing genes following SMN perturbations (Lotti et al., 2012). While snRNA levels have not been tested in our manipulations, phenotypic analysis shows clearly that a gene expression-requiring homeostatic compensation is completely abolished through a neuron-specific reduction in SMN (red arrows in Figure 6D). By contrast, a rapid gene expression-independent form of homeostasis at the NMJ is normal (grey arrows in Figure 6D), suggesting perhaps a role for SMN in the regulation of nuclear gene expression. Whether this occurs through control of mRNA metabolism, remains to be investigated. However, it is interesting to note that this form of long-term homeostasis also requires BMP/ TGF-β signaling in the pre-synapse, a signaling pathway recently shown to interact with SMN in flies (Chang et al., 2008; Haghighi et al., 2003). In this model, one might envisage SMN playing a role either in the relay of a homeostatic "signal" in the pre-synaptic compartment, or in the execution of a suitably graded response (Figure 6D). Although our studies have previously shown a role for SMN in the regulation of FGF signaling in the muscle, this function does not seem to be necessary for homeostatic regulation of synaptic

transmission (Sen et al., 2011). Finally, it is of note that motor neurons in *Drosophila* are glutamatergic. Whether GluR-dependent homeostatic signaling in flies has a bearing on cholinergic neuro-muscular synapses or glutamatergic sensory-motor synapses in mammals remains to be seen.

4. Experimental Procedure

4.1. *Drosophila* strains, genetics and husbandry

Drosophila strains were reared in standard corn meal–dextrose–yeast containing food at 25 °C in controlled humidity incubators under a constant 12 h light:dark cycle. $elav^{C155}$-GAL4 and how^{24B}-GAL4 have been described previously (Brand and Perrimon, 1993; Luo et al., 1994). UAS-SMN, UAS-SMN-RNAi[C24] and UAS-SMN-RNAi[N4] were from Spyros Artavanis-Tsakonas and have been validated and described earlier (Chang et al., 2008). *GluRIIA* mutants (*GluRIIA*^{Sp16}) and deficiency (*Df(2l)cl-h4*) were from Pejmun Haghighi (Penney et al., 2012).

4.2. Antibodies, immuno-histochemistry and western blotting

Larval dissection, staining and confocal microscopy were performed according to standard protocols (Franciscovich et al., 2008). Briefly, larvae were dissected in Ca^{2+} free HL3 ringer's solution (in mM): NaCl 70, KCl 5, MgCl2 20, NaHCO3 10, Sucrose 115, Trehalose 5, BES 5 (pH 7.2). Dissections were fixed in 4% paraformaldehyde, stained with primary antibody overnight, and followed by incubation in Alexa Fluor conjugated secondary antibody. Rabbit anti-dGluRIII (Marrus et al., 2004) was used at 1:1000 and Alexa-568 conjugated anti-HRP (Molecular Probes) was used at 1:500. Anti-rabbit Alexa 488 conjugated secondary antibody (Molecular Probes) was used at 1:400. An inverted 510 Zeiss LSM microscope was used for imaging. For quantitative fluorescence care was taken to prepare samples identically. Samples were imaged and analyzed double blind, and all imaging was interleaved such that control and experimental samples were imaged alternately on the same day. Confocal settings including black level (offset/contrast), gain, pixel dwell time and the number of iterative samplings for noise reduction were kept constant. Western blotting was carried out according to standard procedures (Freeman et al., 2012). Rabbit polyclonal anti-SMN antibodies were used at 1:1000 (Chang et al., 2008).

4.3. Electrophysiology

Baseline evoked and spontaneous junction potentials were measured as described previously (Sen et al., 2011). Briefly, wandering third instar larvae were dissected in normal HL3 ringer's solution (Stewart et al., 1994) (in mM): NaCl 70, KCl 5, MgCl2 20, CaCl2 1, NaHCO3 10, Sucrose 115, Trehalose 5, BES 5 (pH 7.2). Intracellular recording electrodes with tip resistances between 25–50 MΩ were filled with 3 M KCl. Only those recordings were used where the resting membrane potential was more polarized than −60 mV and the muscle input resistance was greater than 10 MΩ. Muscle 6 (VL3) in abdominal segment A2 was used for all recordings. A train of 25 supra-threshold stimuli at 0.5 Hz was delivered in each experiment to record compound EJPs from both Is and Ib terminals, from which mean peak EJP values were obtained by averaging the last 20 traces. EJP amplitudes were corrected for non-linear summation using Martin's correction factor, assuming a reversal potential of 0 mV and a membrane capacitance factor of 0.55 (Kim et al., 2009; McLachlan and Martin, 1981). 8–10 separate animals were used for each genotype. A 2 minute continuous recording was used to measure mEJP amplitude. Traces were low-pass filtered at 1 kHz and analyzed using Clampfit or Mini-Analysis programs (Synaptosoft). 8–10 separate animals were analyzed for each genotype. Quantal content was determined by dividing the mean corrected EJP amplitude for a given synapse by the mean mEJP amplitude. Statistical significance was determined using one-way ANOVA. Representative traces were plotted

using MS-Excel from episodic recordings exported from Clampfit as axon text files. High-frequency stimulation was carried out in a modified HL3 Ringer's solution containing 10 mM Ca^{2+} as described previously (Dickman et al., 2005) (in mM): NaCl 70, KCl 5, MgCl2 10, CaCl2 10, NaHCO3 10, Sucrose 115, Trehalose 5, BES 5 (pH 7.2). Rapid PhTox (Sigma Aldrich Inc.) mediated homeostasis was measured as described earlier in 0.3 mM Ca^{2+} containing HL3.1 Ringer's solution (Feng et al., 2004; Frank et al., 2006) (in mM): NaCl 70, KCl 5, MgCl2 10, CaCl2 0.3, NaHCO3 10, Sucrose 115, Trehalose 5, BES 5 (pH 7.2). Recordings in a loss of *GluRIIA* background were carried out in the same HL3.1 Ringer's with 0.3 mM Ca^{2+}.

4.4. Behavioral analysis

2–3 day old flies were collected and their wings cut close to the thorax. These flies were then allowed to recover for an additional 3 days. Flies were individually placed on a circular platform 10 cm in diameter and surrounded by a moat of water 2 cm in width. Flies normally walked back and forth between two diametrically opposite vertical black bars within a brightly illuminated cylinder. A 5 mega-pixel webcam mounted centrally above the platform recorded their movement for a period of 5 minutes from within a Buridan tracking program from Bjorn Brembs. These recordings were then analyzed using custom designed Buridan analysis software (from Bjorn Brembs) written in the statistical package *R*. Results from this analysis were plotted in MS-Excel. Statistical significance was determined using one-way ANOVA. Single representative fly tracks and occupancy plots were also generated using the Buridan analysis software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

NMJ	Neuro-muscular junction
SMA	Spinal Muscular Atrophy
SMN	Survival Motor Neuron
UAS	Upstream Activating Sequence
RNAi	RNA interference
FGF	Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
GluR	Glutamate Receptor

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Highlights

- Knock down of *dSmn* in the nervous system impairs locomotion

- Knock down of *dSmn* in muscle does not impair locomotion

- Synaptic transmission is normal following neuronal or muscle knock down of *dSmn*

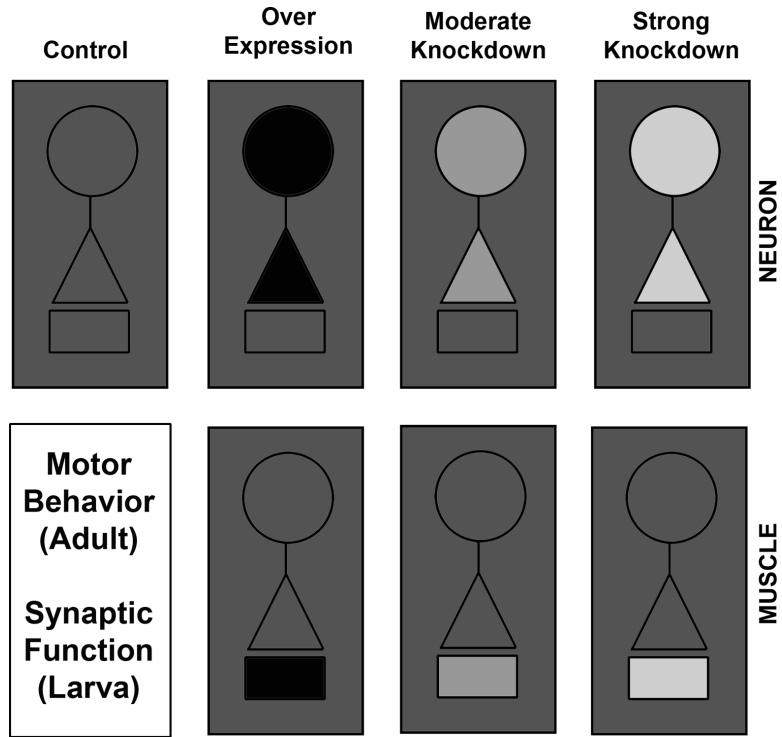- Homeostasis of synaptic transmission is impaired by neuronal *dSmn* knock down

Figure 1. Experimental paradigm to test tissue-specific requirement for SMN in the maintenance of normal motor physiology
The experimental strategy to test compartment-specific functions for SMN is outlined schematically. SMN is expressed widely in many tissues (grey outer box). In this study, SMN has either been over-expressed (black) in neurons or muscles, or has been reduced through RNAi mediated knockdown (lighter shades of grey reflected moderate to strong knockdown). SMN levels in other tissues are presumed to be intact and comparable to wild type control animals. Experimental animals are then subjected to behavioral assays to measure motor function in adults. Electrophysiological measurements are used to evaluate various aspects of synaptic function at the third instar larval NMJ. In all figures, circles represent the neuronal soma, triangles represent the pre-synaptic compartment and small rectangles represent post-synaptic muscle.
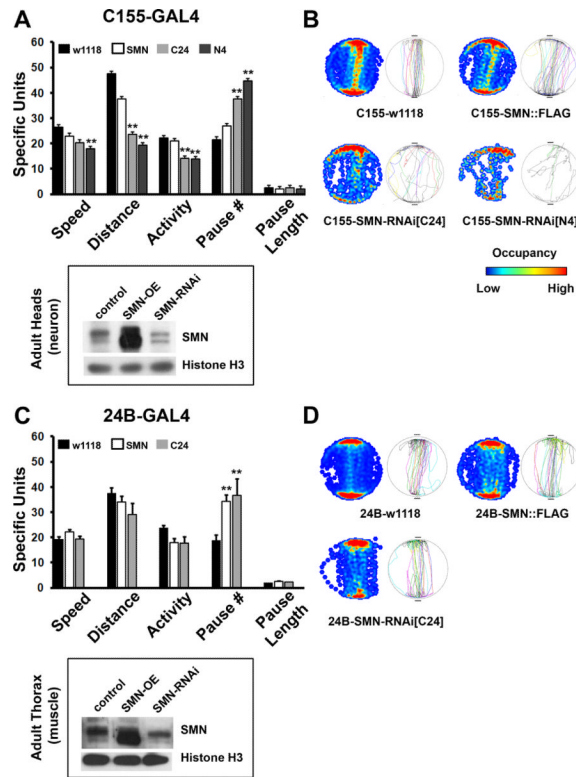
Figure 2. Perturbation of SMN in the nervous system disrupts normal motor behavior
(A) Various parameters of locomotion as measured by the Buridan's assay are shown for neuronal perturbations of SMN using the *elav*[C155]-GAL4 driver line. SMN knockdown using two RNAi lines results in decreased speed, distance covered and overall activity, while the number of pauses is increased significantly without affecting mean pause length. Western blot from adult heads shows strong over-expression (SMN-OE) and significant but not complete knock down (SMN-RNAi) of SMN protein levels in adult heads (a predominantly neuronal population). **(B)** Single representative fly traces (left circles) and cumulative occupancy plots (right circles with heat maps) show disrupted locomotor behavior following pan-neuronal knockdown of SMN. **(C)** Muscle perturbation of SMN using the *how*[24B]-GAL4 driver line. The number of pauses is significantly increased following either over-expression or knockdown of SMN in the mesoderm. Western blot of protein extract from adult thorax shows strong over-expression (SMN-OE) and significant but not complete knock down of SMN in muscle tissue (SMN-RNAi). **(D)** Single fly tracks and cumulative occupancy plots show mild impairment of locomotion. Note that muscle expression of SMN-RNAi-N4 is pupal lethal as reported previously. 10 individual animals that are 5–6 days old are tested for each genotype. Error bars are SEM, double asterisks represent $p < 0.01$ as tested by ANOVA.
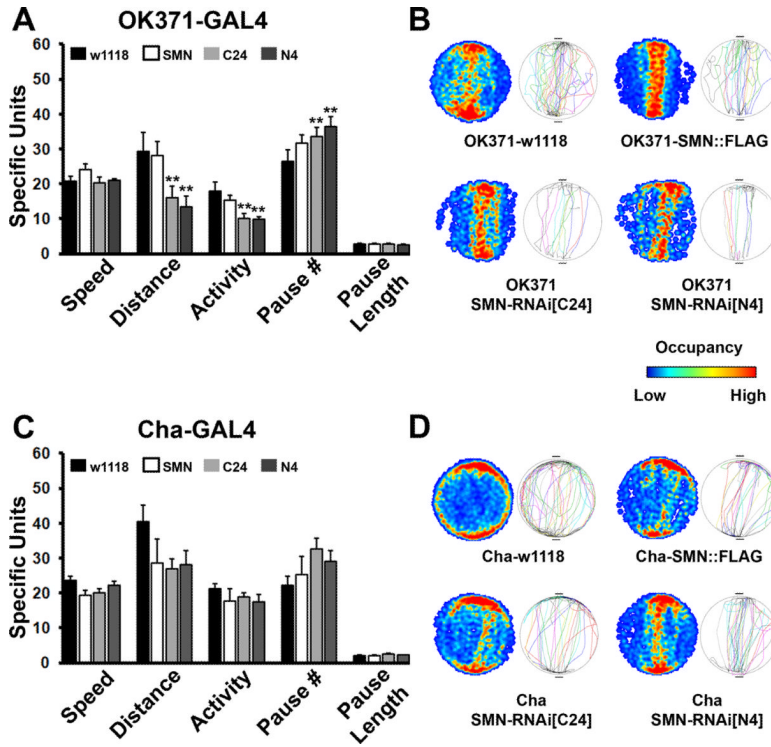
Figure 3. SMN reduction in motor neurons is sufficient to impair motor function
(A, B) Parameters of locomotion, single fly tracks and occupancy plots in animals with glutamatergic neuron specific perturbations of SMN using the VGlut-GAL4 driver (OK371). RNAi mediated knockdown of SMN in glutamatergic neurons recapitulates in large part the effect of pan-neuronal knockdown. Distance and activity are reduced, while the total number of pauses is increased. SMN over-expression does not have any discernible influence on locomotion. **(C, D)** Results from the Buridan's assay for cholinergic neuron specific perturbations in SMN. None of the parameters for locomotion are significantly affected by altering SMN function in cholinergic neurons. 10 animals are tested for each genotype, error bars are SEM and double asterisks represent $p<0.01$ as determined by ANOVA.
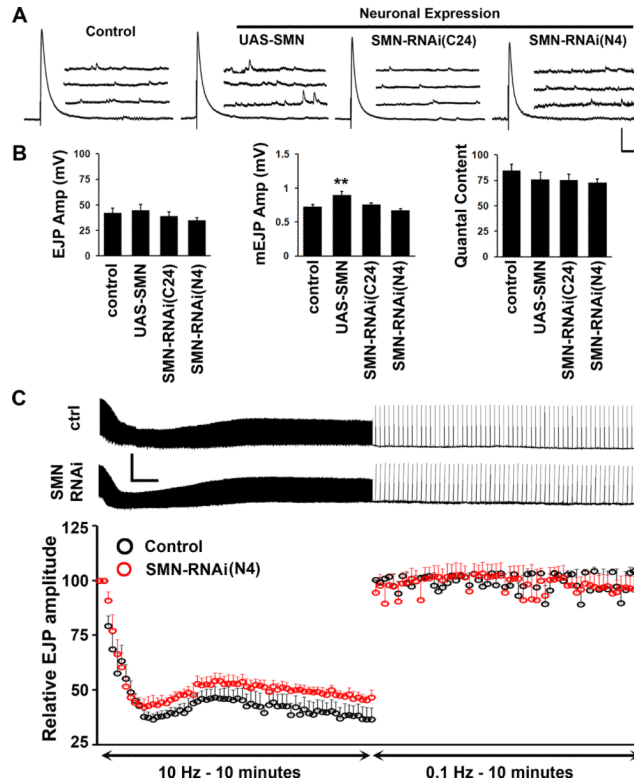
Figure 4. Baseline synaptic transmission is largely normal under conditions of neuronal SMN modulation

(A, B) Representative traces (A) and quantification of baseline synaptic transmission at the larval NMJ (muscle 6/7 synapse at abdominal segment A2) following pan-neuronal SMN perturbation (B). mEJP amplitude (quantal size) is significantly different in SMN over-expressing synapses. However, EJP amplitudes or quantal content remain unchanged. 9–10 animals are tested for each genotype. Vertical scale bar = 10 mV for EJP and 5 mV for mEJP; Horizontal scale bar = 100 ms for EJP and 200 ms for mEJP. Mean resting membrane potentials are: Control = 73mV, UAS-SMN = 74mV, SMN-RNAi-C24 = 69mV, SMN-RNAi-N4 = 68.5mV. Mean input resistance values are: Control = 12.5MΩ, UAS-SMN = 12MΩ, SMN-RNAi-C24 = 13.3MΩ, SMN-RNAi-N4 = 12.4MΩ. **(C)** High-frequency (10 Hz) stimulation in high-calcium (10 mM) containing Ringer's solution leads to gradual synaptic depression to approximately 50% of the initial EJP amplitude, followed by almost instantaneous recovery under low-frequency (0.1 Hz) stimulation. This profile is unchanged when SMN is knocked down pan-neuronally using the N4 RNAi transgene (red spheres) as compared to control animals (black spheres). Errors bars are SEM. 5 animals were tested for each genotype. Horizontal scale bar = 1min, vertical scale bar = 50mV.
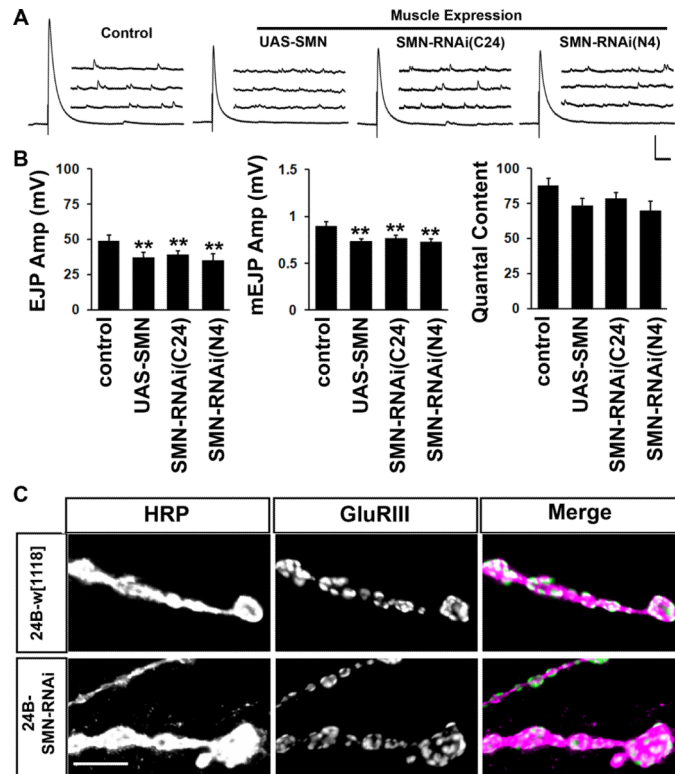
Figure 5. SMN knockdown in muscle affects the quantal size of pre-synaptic transmitter release
(A, B) Representative traces and quantification for baseline synaptic transmission at the third instar larval NMJ following muscle-specific perturbation in SMN. Both over-expression and knockdown of SMN result in smaller EJP and mEJP amplitudes. As a result quantal content is not significantly altered in experimental animals. 9–10 animals are tested for each genotype. Vertical scale bar = 10 mV for EJP and 5 mV for mEJP; Horizontal scale bar = 100 ms for EJP and 200 ms for mEJP. Mean resting membrane potentials are: Control = 74mV, UAS-SMN = 66mV, SMN-RNAi-C24 = 63mV, SMN-RNAi-N4 = 62mV. Mean muscle input resistances are: Control = 13.2MΩ, UAS-SMN = 12.4MΩ, SMN-RNAi-C24 = 12.5MΩ, SMN-RNAi-N4 = 11.8MΩ. **(C)** Staining of third instar synapses with anti-HRP and anti-GluRIII shows indistinguishable staining intensities and pattern of glutamate receptor distribution following SMN knockdown in muscles. Scale bar = 5 µm.
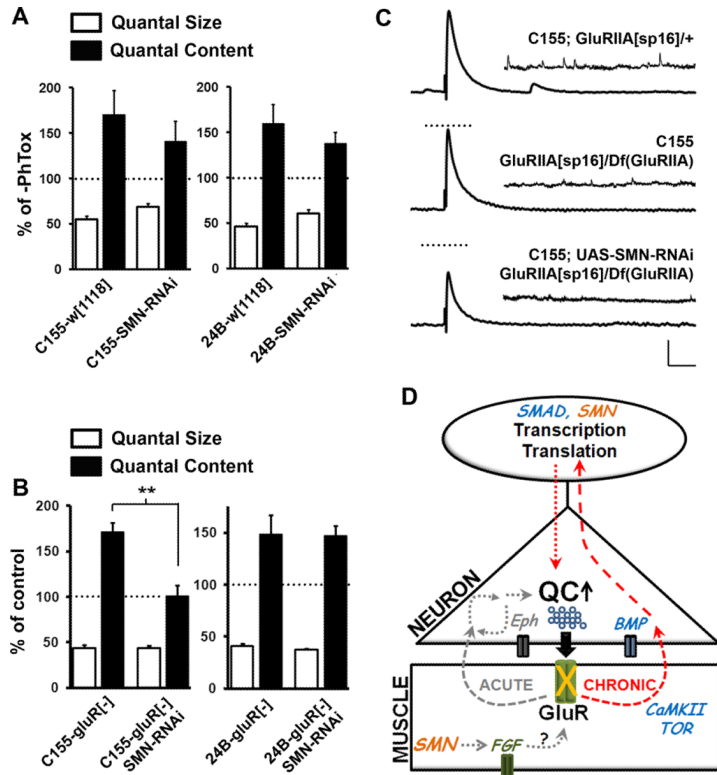
Figure 6. Loss of SMN in neurons impairs long-term homeostasis at the neuro-muscular junction
(A) PhTox induced rapid homeostatic compensation at the NMJ is normal following SMN knockdown in either neurons or muscle. Shown are the percentage reduction in mEJP amplitude (quantal size) and percentage increase in quantal content induced by a 10 minute bath application of PhTox. 10 animals were tested for each genotype. **(B)** Persistent developmental homeostasis (increased quantal content) induced by a genetic reduction in the GluRIIA subunit of glutamate receptors at the NMJ is lost when SMN is knocked down pre-synaptically in neurons. Reduction of SMN in muscles, however, does not impair this form of homeostatic compensation. **(C)** Representative traces showing the absence of homeostasis in neuronal SMN knockdown animals. 8–10 animals were tested for each genotype. Vertical scale bar = 10 mV for EJP and 2 mV for mEJP; Horizontal scale bar = 100 ms for EJP and 1 s for mEJP. **(D)** Model schematic showing a role for SMN in chronic homeostatic plasticity at the NMJ. Interestingly, this form of plasticity also requires normal BMP signaling, a pathway known to genetically interact with SMN. Chronic homeostasis is likely to rely on long-term changes in gene expression that might require normal SMN function in neurons. SMN also functions in muscles to modulate outputs of FGF signaling that control post-synaptic properties.