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Neuromuscular defects in a *Drosophila* survival motor neuron gene mutant

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Autosomal recessive spinal muscular atrophy (SMA) is linked to mutations in the survival motor neuron (*SMN*) gene. The SMN protein has been implicated at several levels of mRNA biogenesis and is expressed ubiquitously. Studies in various model organisms have shown that the loss of function of the *SMN* gene leads to embryonic lethality. The human contains two genes encoding for SMN protein and in patients one of these is disrupted. It is thought the remaining low levels of protein produced by the second *SMN* gene do not suffice and result in the observed specific loss of lower motor neurons and muscle wasting. The early lethality in the animal mutants has made it difficult to understand why primarily these tissues are affected. We have isolated a *Drosophila smn* mutant. The fly alleles contain point mutations in *smn* similar to those found in SMA patients. We find that zygotic *smn* mutant animals show abnormal motor behavior and that *smn* gene activity is required in both neurons and muscle to alleviate this phenotype. Physiological experiments on the fly *smn* mutants show that excitatory post-synaptic currents are reduced while synaptic motor neuron boutons are disorganized, indicating defects at the neuromuscular junction. Clustering of a neurotransmitter receptor subunit in the muscle at the neuromuscular junction is severely reduced. This new *Drosophila* model for SMA thus proposes a functional role for SMN at the neuromuscular junction in the generation of neuromuscular defects.

INTRODUCTION

Autosomal recessive spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality and is characterized by a loss of lower motor neurons in patients, associated with muscle paralysis and atrophy. Patients are classified into four types on the basis of the age of onset of the disease as well as range of mobility. The genetics indicate that all phenotypes are caused by the overall decrease in activity of SMN, the Survival Motor Neuron protein (1). The human genome contains two genes that encode SMN protein both localized at 5q11.2–13.3. The presence of the two genes encoding the same protein located close together is most likely the result of an evolutionary recent genomic duplication (1,2). In patients, the *SMN*1 gene is affected by mis-sense, nonsense or splice site mutations (3). The second gene, *SMN*2, only differs from the *SMN*1 gene by a few base pairs, none affecting the amino acid sequence. However the transcripts produced by the second gene mostly encode for a truncated, unstable SMN protein due to one of the mutations leading to alternative splicing of the *SMN*2 transcript (4,5). As a result, only low levels of full-length SMN protein are produced from *SMN*2. The severity of the SMA phenotypes appears to be closely linked to the level of active SMN protein produced (6,7).

The *SMN* gene is highly conserved across species and, except for in human and closely related species, is present in a single copy only and expressed in most tissues investigated (2,8–13). The SMN protein has been found associated with numerous other proteins (14–22) and with itself, both in cytoplasm and in the nucleus (23–25). The association with nuclear riboproteins (nRNPs, small and heterogeneous) and other RNA binding proteins and its presence in a complex that functions in the formation and transport of spliceosomal snRNPs, indicates a role for SMN in mRNA biogenesis (26,27). Indeed SMN has been found to stimulate splicing (28). The SMN protein has, however, also been found associated with proteins not

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necessarily involved in RNA biogenesis (29,30). Despite the array of proteins known to interact with SMN, a clear function that is specific to the neuromuscular phenotypes described in patients has not been found (for reviews see 31,32).

To investigate SMN function in SMA, several animal models have been generated. Loss of the single *SMN* gene in the mouse leads to early embryonic death (10). In fact, complete loss of *SMN* is lethal in cell culture, yeast and *C. elegans* (9,13,33,34). These observations are consistent with SMN performing an essential role in the cell. To generate an SMA animal model in mouse, several genetic backgrounds have been created. One approach taken was to generate a mouse mutant where in a *Smn* null mutant background, a human genomic region containing *SMN*2 and other genes supplemented activity (35). The resulting mice could be typed into two groups on basis of the severity of their phenotypes. However amongst all progeny, only some of the mice with milder phenotypes displayed SMA-like defects. In a similar set-up, a construct containing just the human *SMN*2 gene was used to supplement the *Smn* mutant mice (36). High copy numbers of the *SMN*2 transgene completely rescued. When the *SMN*2 transgene was present in low copy number, surviving progeny showed abnormal motor behavior and loss of motor neurons. Recently these $Smn^{-/-}$; *SMN*2 animals have also been used in combination with transgenes encoding forms of SMN found in patients (37). It has also been found that the lower levels of SMN in the heterozygous *Smn* mutant mouse lead to mild SMA phenotypes (38). From these results it seems that reduced overall levels of SMN activity can lead to SMA phenotypes, while human *SMN*2 can rescue the null mutant mice. To investigate the neuromuscular specificity of the disease, conditional mutants have been generated. Using the Cre–Lox recombination system in mice, it has been shown that motor neurons require SMN function (39). However, confusingly, using similar techniques to disrupt *Smn* in muscle only, a dystrophic phenotype was observed (40). Both investigations depended on full removal of *Smn* activity in the target tissues and thus generated a background that is not strictly identical to the situation in SMA patients where low overall *SMN*2 based activity is always present. In general, the mouse models can replicate the human disease situation but the transgenic combinations required are complex. It has therefore been difficult to address the questions relating the role of SMN and the SMA phenotype. For instance, it is not clear how to relate a reduction in activity of this snRNP-associated protein with the neuromuscular phenotypes observed in SMA. Similarly, it is not clear if the reduction in SMN activity primarily affects muscle, neurons or both tissues. Furthermore, no clear cellular or molecular defects have been described in patients or the available models that could explain the observed lower motor neuron loss.

Invertebrate models have been shown to be useful in mirroring human neurogenetic disorders while offering advanced genetics and physiological tools (41). We present here the isolation and characterization of an *smn* mutant in *Drosophila melanogaster*. We show that, due to maternal wild-type *smn* activity, *smn* zygotic mutant embryos survive and mutant larvae show severe motor abnormalities and finally succumb to defects that involve paralysis. The delayed lethality has allowed us to study the effects of decreased activity of *smn* in the neuromuscular system of a model organism. We show that the motor defects can only be rescued by providing *smn* activity in both muscles and neurons. More importantly, the phenotypes are associated with defects at the neuromuscular junction (NMJ). We have determined a decrease in efficiency of synaptic transmission in the larval neuromuscular system and a disruption of clustering of postsynaptic neurotransmitter receptor. We have created and provide an initial characterization of a new model for SMA and our results point to primary defects associated with the NMJ.

RESULTS

The *Drosophila* genome contains a single homologue of the *smn* gene (12). To obtain a mutant in the *smn* gene, we reasoned that this mutant should be lethal at some stage in the fly life cycle. We analysed candidate recessive-lethal mutants in the region where the *smn* gene is located based on the genome annotation (73A7-9). Sequence analysis showed that one of the many lethal mutations in this region, the mutant l(3)*73Ao*, contained a point mutation in the coding region of *smn*, resulting in a mis-sense mutation (Fig. 1A). We recombined this mutant with an isogenized stock to remove any unwanted lethal mutations in the genetic background. The resulting smn^{73Ao} mutant was homozygous lethal at late larval stages. In a small-scale mutagenesis screen, we isolated a second allele (smn^{B}) in which a mis-sense mutation was found in the same region of the Smn protein as in smn^{73Ao} (Fig. 1A). The smn^{73Ao} and smn^{B} alleles do not complement each other. In addition, the homozygous mutant phenotype displayed by these alleles (see below) is similar to the phenotype when these point mutations are placed in *trans* over the genomic deletion covering the *smn* gene. When the smn^{73Ao} and smn^{B} alleles are combined, a marginally weaker phenotype is observed. We confirmed that both alleles are mutants in *smn* by fully rescuing the phenotypes and associated lethality with a ubiquitously expressed wild-type *smn* construct (see Fig. 3A). The amino acid substitutions in Smn protein in these alleles lie within the conserved carboxy-terminal domain of the protein (containing the YXXG motif). Similar mutations have been identified in *SMN*1 in a number of SMA patients and these are thought to impair SMN oligomerization and/or binding of associated proteins (1,23,42). We have previously reported strong self-interaction for wild-type *Drosophila* Smn protein using yeast two-hybrid analysis (12). In a similar experiment, self-oligomerization for the protein encoded by smn^{73Ao} and binding of the protein encoded by smn^{73Ao} to wild-type Smn protein were significantly reduced, compared with wild-type Smn with itself (Fig. 1B). This suggests that the protein that generated *in vivo* from the mutant smn^{73Ao} is defective in self-binding.

Homozygous smn^{73Ao} animals die as late larvae; the second allele smn^{B} is lethal within the same period. This observation is in contrast with the early embryonic lethality observed in different animal models lacking the single *SMN* gene or where activity of this gene has been considerably reduced (10). Northern blot analysis shows that *smn* is expressed at high levels at stages during early embryogenesis where no zygotic transcription takes place as well as at slightly lower levels in females, while at low levels in all other developmental stages (Fig. 2A). This shows that *smn* in flies is contributed to the

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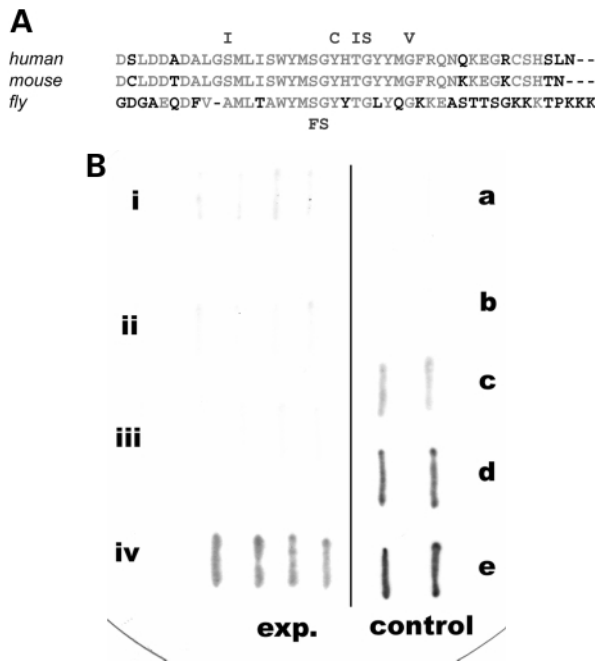

Figure 1. *Drosophila smn* mutants missense mutations in the YxxG domain of the *smn* gene leading to loss of self-binding. (**A**) Amino acid sequences of *Homo sapiens* (human) (starting at amino acid 252), *Mus musculus* (mouse; starting at amino acid 247) and *Drosophila melanogaster* (fly; starting at amino acid 184) carboxy-terminal domains of SMN protein. Conserved residues (and conservative changes) are shown in grey. The amino acid residues above the human sequence are the result of mis-sense point mutations found in SMA patients; below the lines are those found in *Drosophila* mutants smn^{73Ao} and smn^{B} (G202S and S201F respectively). (**B**) Yeast-two-hybrid interaction studies between wild-type and mutant proteins. On the left-hand side, experimental analysis is shown of various constructs of prey and bait (exp.). The coloration in the X-gal assay suggests a strong self-interaction in the wild-type *smn* positive control (both bait and prey are wild-type *Drosophila smn*) (iv) (all plated in quadruplicate). There is no or little interaction between mutant smn^{73Ao} with itself (both bait and prey SMN encoded by smn^{73Ao} gene) (i), and wild-type *smn* bait (prey is smn^{73Ao}) (ii) or prey (bait is smn^{73Ao}) (iii). On the right-hand side, for comparison (control), yeast control strains (a–e), ranging from no protein interaction in (a) to strongest interaction in (e) were plated. When control interactions and coloration (on right) were quantified and compared with experimental data (on left), interactions in (i) were 8% of maximum, (ii) 6%, (iii) 3% and (iv) 68%.

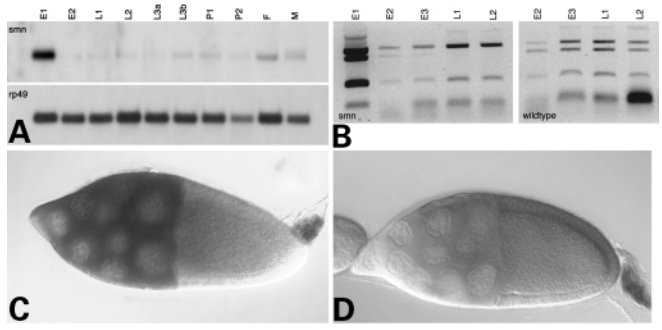

Figure 2. *smn* mRNA is maternally transmitted. (**A**) Northern blot analysis of *smn*. The gene is expressed at all developmental stages (in stages beyond early embryogenesis, the expression seen represents zygotic plus maternal expression), with strong expression in early embryo stages (<2 h after egg laying, AEL). No significant variation in expression level was found in the ribosomal protein control (*rp49*) (except stage P2). E1, embryo (0–2 h AEL); E2, embryo (14–18 h AEL); L1, first instar larva; L2, second instar larva; L3a, third instar larva; L3b, third instar larva (wandering larva); P1, pupa (white); P2, pupa (brown, 2 day); F, adult female; M, adult male. (**B**) Restriction enzyme digests of purified PCR fragments of *smn*. In the left panel, samples derived from homozygous smn^{73Ao} animals; in the right panel, samples were isolated from heterozygous smn^{73Ao}/*TM3 Kruppel*-GFP. The largest clearly visible band represents the mutant transcripts (at 488 bp), while the fragment just below (409 bp) is representative of the control, wild-type *smn* transcripts. Stages are as in (A), except E2, embryo (9–11 h AEL), and E3, embryo (19–21 h AEL). (**C**) *In situ* hybridization with *smn* probe on egg chambers. High expression of *smn* is found in the nurse cells. Areas of low hybridization represent the nuclei of the nurse cells. (**D**) No hybridization above background is seen when probed with *smn* sense RNA.

embryo by the mother, as has been reported for *SMN* in *Caenorhabditis elegans* (11). We analysed the extent of maternal *smn* RNA contribution in the early life stages of the fly by making use of the polymorphism introduced into the smn^{73A0} mutant gene, to distinguish mutant from wild-type transcripts. In Figure 2B, digests of polymerase chain products (PCR) are shown. The polymorphism removes a restriction enzyme site in the mutant DNA and, as is seen in the mutant samples, the wild-type digested products are no longer clearly detectable from early larval stages, while all products remain visible in the wild-type control. *In situ* hybridization on female egg chambers confirmed strong expression of *smn* in the nurse cells, the cells that contribute maternal RNAs to the oocyte (Fig. 2C and D). To test if this maternal activity enables embryonic survival, we generated female flies that contain an smn^{73Ao} mutant germline (see Materials and Methods). Oocytes from these females only contain *smn* mRNA that encodes for the mutant protein. Only a few eggs were laid, which died at very early developmental stages (between 0 and 4 h after egg laying at 25°C), showing little morphology (data not shown). Therefore many oocytes do not appear to reach maturity and if they do and are fertilized, the embryonic lethality is not rescued by the paternal wild-type chromosome. These observations show that a large quantity of maternal transcript is deposited in the egg while removal leads to oocyte or embryonic death. We conclude that the survival of the zygotic mutant animals is due to the presence of maternal wild-type *smn* activity.

Interestingly, we found that the zygotic mutant larvae displayed a progressive loss of mobility and increased uncoordinated movement before death occurred. We quantified the loss of mobility by studying the rate of locomotory body wall contractions. A $53 \pm 5\%$ decrease in contraction rate was seen for smn^{73Ao} homozygous mutant versus either heterozygous third instar larvae or *smn* mutant larvae where *smn* activity was supplied by a transgene. This phenotype indicates a requirement for *smn* activity in the muscles or their controlling neurons, which is not met by the decreasing levels of maternal activity but can be rescued by providing wild-type *smn* activity in the mutant backgrounds. This creates a situation where low levels of *smn* activity are provided in an *smn* mutant background. Thus, we believe this new fly model can be used to study aspects of the SMA aetiology.

First, we used these larvae to study the requirement of *smn* for continued normal movement by supplementing wild-type *smn* to either muscle or neurons, or both tissues. Expression of *smn* ubiquitously, starting in embryogenesis, leads to a normal lifespan and no mobility defects (Fig. 3A). When the gene was provided in embryonic and larval muscles, partial rescue of the

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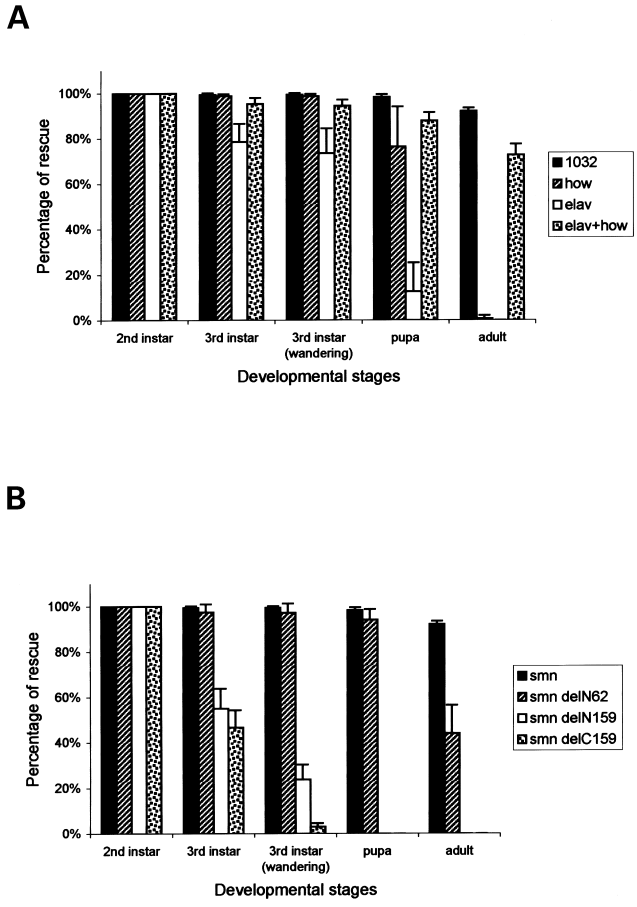

Figure 3. Expression of *smn* in muscle and neuron can rescue the loss of mobility in smn^{73Ao} zygotic mutant larvae. (**A**) Full-length wild-type *Drosophila smn* (UAS-*smn*) was expressed using different tissue-specific GAL4 drivers: *1032*-GAL4 (ubiquitous), how^{24B}-GAL4 (mesoderm), and *elav*-GAL4 (pan-neural). Various degrees of rescue were achieved depending on the GAL4 driver that was used (each bar represents the mean value ± standard deviation of four independent measurements $n > 200$ per each measurement). The results for homozygous mutant animals without transgenic rescue are identical to the situation as described in (B) for *smn*ΔC157. (**B**) Rescue by expression of constructs encoding truncated Smn. UAS-*smn*, UAS-*smn*ΔN62, UAS-*smn*ΔN159 and UAS-*smn*ΔC157 (see Materials and Methods) were expressed using *1032*-GAL4 (ubiquitous expression). Rescue to adult stages was only observed using UAS-*smn*ΔN62 (each bar represents the mean value ± standard deviation of four independent measurements; $n > 200$ per each measurement).

phenotypes was observed, while pan-neural expression showed weaker rescue (Fig. 3A). However, expression in both muscle and neurons allowed many flies to emerge normally (Fig. 3A). Although it is not clear if these drivers are strictly limited to the tissues where they are supposed to be expressed, this result indicates that neuromuscular tissues are more sensitive to the levels of *smn* activity than the rest of the animal. In addition, it appears that, when *smn* is expressed at high levels in these tissues additional to the existing low levels, for many animals a normal lifespan ensues.

To study the importance of the Smn protein domains, we expressed constructs encoding truncated forms in the zygotic mutant background using a ubiquitous driver. Expression of a construct encoding Smn protein without the carboxy-terminus, or only this domain, did not rescue the mutants that normally die in larval stages (Fig. 3B). However, expression of Smn lacking the amino-terminus rescued over 40% of homozygous mutant animals to adult stages (Fig. 3B). These results suggest that the amino-terminus of Smn is, at least partially, dispensable for rescue of the neuromuscular phenotypes and lethality.

Despite the *smn* mutant larvae displaying significant loss of mobility, no obvious muscular or neuronal defects are observed using markers for muscle (phalloidin) and neurons (anti-HRP antibody, data not shown) when mobility defects are first observed. The mutant animals survive as larvae without entering pupation for up to 10 days. Mild muscular dystrophy is observed in dead larvae but no obvious cell death is observed in the animals presented below. The observed abnormal motor behavior indicates a neuromuscular defect and, together with our finding that *smn* activity is required in both neurons and muscle, we focused our efforts on studying the NMJ in more detail.

To begin to assess NMJ synaptic function in mutant and control larvae, two-electrode voltage-clamp recordings were made from identified single muscle cells. Excitatory post-synaptic currents (EPSCs) were recorded following electrical stimulation of pre-synaptic motorneurons from wild-type and *smn* mutant muscle cells. The amplitude of EPSC recorded in mutant larvae was significantly reduced in comparison to control animals (Fig. 4A and B). These observations are consistent with the hypothesis that *smn* zygotic mutant larvae have a dysfunctional NMJ.

We used markers that label the NMJ post- and/or pre-synaptically to analyse its architecture. We used several available antibodies that label various structures within the NMJ or total muscle or neurons [including Elav-GFP, HRP, anti-Cysteine String Protein (csp), anti-Sap47 as well as other GAL4 lines that label neurons and/or muscle when used to express GFP]. The results all indicate a mild architectural defect at the NMJ that is presented here using two antibodies that label structures within the NMJ. The Discs-large protein is found mostly in post-synaptic membrane (43) while Synapsin is exclusively located pre-synaptically (44). In wild-type NMJs (or in rescued mutant larvae, data not shown), the pre-synaptic area falls almost entirely within the post-synaptic domain (Fig. 5A and C). In the *smn* mutant boutons, the pre-synaptic and post-synaptic labeling overlap to varying degrees (Fig. 5B and D). We conclude that the architecture of the bouton is not normal in the mutant NMJ. No clear difference in branching or number of boutons is observed. However a statistically significant increase in the number of enlarged boutons is observed in *smn* mutants compared with heterozygous larvae, or to mutant larvae that are rescued using a *smn* transgene (Fig. 5E). Although the observed changes in the *smn* mutant NMJ are variable and thus difficult to clearly define, it is possibly that some component of the NMJ would be consistently affected. We continued looking using available antibodies against NMJ markers (see for instance Fig. 6). The function of the NMJ is crucially dependent on the presence and correct localization of the neurotransmitter and its receptor; in *Drosophila* L-glutamate serves as an excitatory neurotransmitter at the NMJ. We assayed the localization of the glutamate receptor (GluR subunit IIA) (45). Interestingly, the receptor is expressed in the muscle but the post-synaptic clustering of

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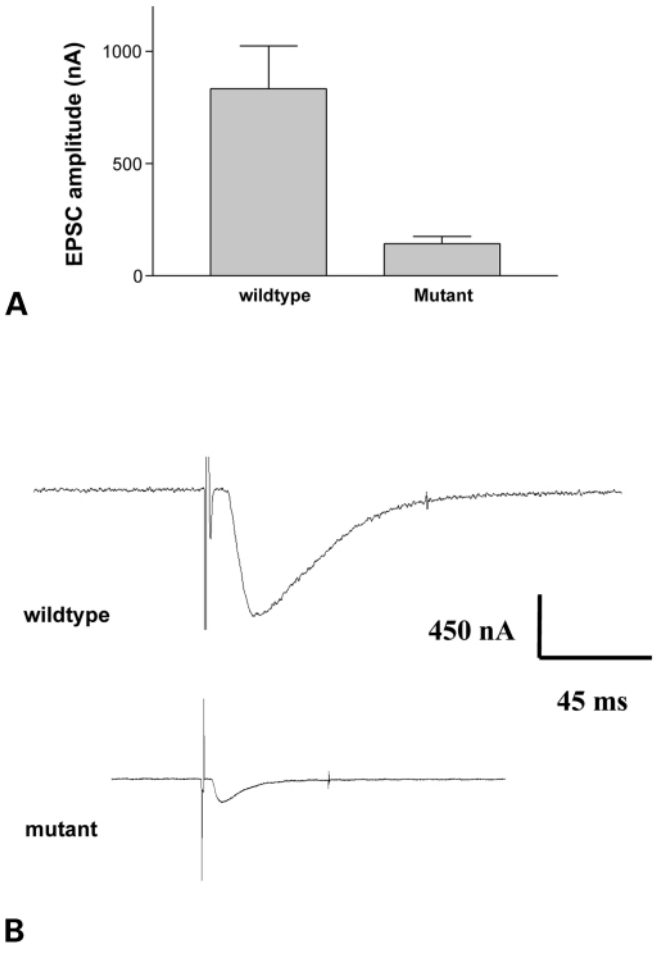

Figure 4. Post-synaptic currents in muscle 6 of third instar larvae. (**A**) Experimentally evoked excitatory post-synaptic currents (EPSCs) were significantly reduced in homozygous smn^{73Ao} mutant larvae compared to control wild-type larvae. EPSCs results combined for $n = 6$, wild-type (*st p e*/*st p e*) and $n = 8$, mutant. Shown are EPSC amplitude + SEM. An unpaired *t*-test gives a value of 0.0014 ($t = 4.109$ and d.f. = 12). An *F*-test to compare variances indicates significant difference ($F = 26.05$, Dfn = 5 and Dfd = 7) It was possible to reversibly block EPSCs by adding cobalt chloride (10 mm) to the bathing medium, indicating that the currents recorded were not passive electronic events but evoked active synaptic events (not shown). Because full rescue of the motility phenotype was only observed using drivers that lead to expression in both muscle and neuron while some but significant rescue was found expressing the wild-type gene in either tissue, we did not study the EPSCs in such rescued animals. (**B**) Example traces of wild-type and mutant recordings. A scale bar for amplitude and length of time is shown.

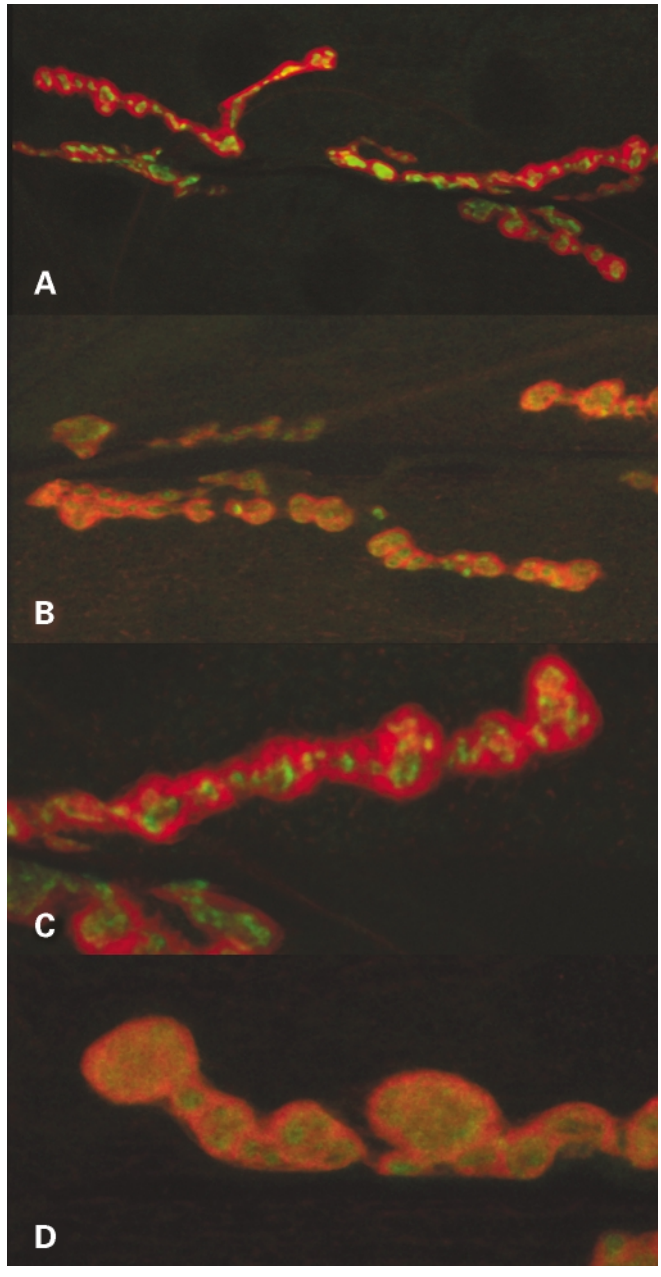

Figure 5. *smn* mutant larvae show defects at the neuromuscular junction. (**A**–**D**) Immuno-staining of NMJ of muscles 6–7 of control (A and C; for genotypes see Materials and Methods) and mutant (B and D) were stained with anti-Synapsin (presynaptic) (44) (in green) and anti-Discs large (pre- and postsynaptic) (65) (in red). (A) and (B) were imaged using 100× objectives without digital zoom; (C) and (D) were zoomed 2× digitally. The mutant (smn^{73Ao}) shows disorganized boutons (type I) compared with the wild-type control; the organization of the pre- and post-synaptic areas is abnormal in the mutant. In (B) and (D), the green staining is less obvious and more yellow staining is observed (indicating overlap between green and red staining but no clear loss of green staining is observed in the mutant). The pictures shown are representative examples of the mutant phenotypes; a range of severity of the observed changes is seen. (**E**) smn^{73Ao} mutant shows an increased number of enlarged boutons. NMJs of muscles 6 and 7 in abdominal segments 2 and 3 were analysed. *Smn* mutant shows an increased number of enlarged boutons (type I, middle column) compared with the control (heterozygous mutant *smn, TM3* is balancer chromosome, left column) or rescued *smn* mutant (using ubiquitous expression of *smn*, right column). Each bar represents the mean value ± SEM; $P < 0.01$; $n \geq 8$.

receptors is disrupted in the *smn* mutant; the discrete large clusters localized at the boutons are lacking (Fig. 6). The observed defect in clustering is completely rescued by ubiquitous expression of wild-type *smn* (Fig. 6G–I).

DISCUSSION

We have found a mutant in the *Drosophila smn* gene. We isolated two alleles that both lead to mis-sense mutations in the encoded Smn protein. These mutations are located in a region of the protein which is highly conserved and where several of the human point mutations have been found (3). Based upon

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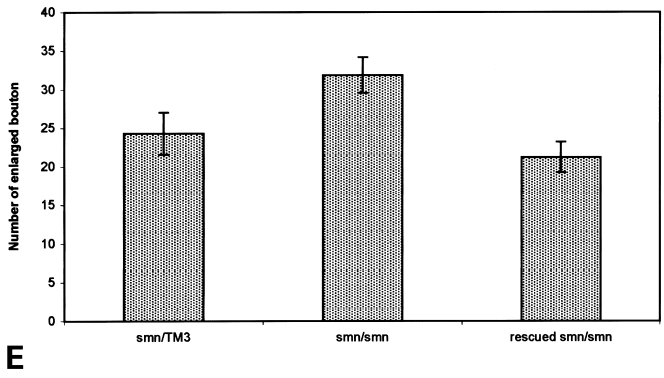

Figure 5 *continued*.

genetic behaviour and the very early lethality when the germ line in females is mutant, we suggest that the smn^{73Ao} allele is strong if not non-functional for *smn* activity. This would agree with the severely reduced self-binding ability that the encoded protein displays. Mis-sense mutations found in patients in this region of *SMN*1 (42) indicate that the loss of protein oligomerization can be correlated to disease severity (23). Our results further confirm the functional importance of SMN oligomerization in SMA as suggested for the human protein.

We wanted to investigate if *Drosophila* could be used as a model to study SMA and, upon characterization of the *smn* mutant, we found that mutant larvae develop severe motor abnormalities. The survival of these larvae past embryogenesis can be attributed to the presence of maternal mRNA coding for wild-type Smn protein, a situation not found in mammalian systems. Because the mutations in our *smn* alleles are similar to mutations found in SMN1 in patients, any mutant Smn protein produced in these larvae would be similar to the mutant proteins produced in such SMA patients (defective in oligomerization). In addition, low levels of wild-type protein will be present in the larvae due to translation of maternal transcripts and persistent protein. We have thus created a *Drosophila* background with disrupted Smn activity in later life and, interestingly, these animals display a motor phenotype. However some aspects of our model do not necessarily recapitulate the situation in SMA patients. First, the presence of the maternal *smn* activity in *Drosophila* will obscure any embryonic developmental defects that low levels of SMN activity might result in. Second, the *Drosophila* larvae mutant for *smn* never reach the adult fly life stage; this is most likely due to the degeneration of the imaginal structures (Y.B.C. and M.v.d.H., unpublished data). These are the structures that develop into the adult fly body and are actively growing in the larvae. Thus the low levels of *smn* activity in these mutant larvae do not support the growth and survival of these tissues but allow the polyploidal larval tissues to function, except the neuromuscular system. These animals have allowed us to examine the defects that possibly underlie SMA without the cell lethality associated with total loss of *SMN* function.

In this background, ubiquitous expression of an amino-terminal truncated version of the Smn protein partially rescues phenotypes. A similar observation in a cell model was published recently (46). The region deleted in the construct is thought to be required for SIP-1/Gemin2 binding (21). This

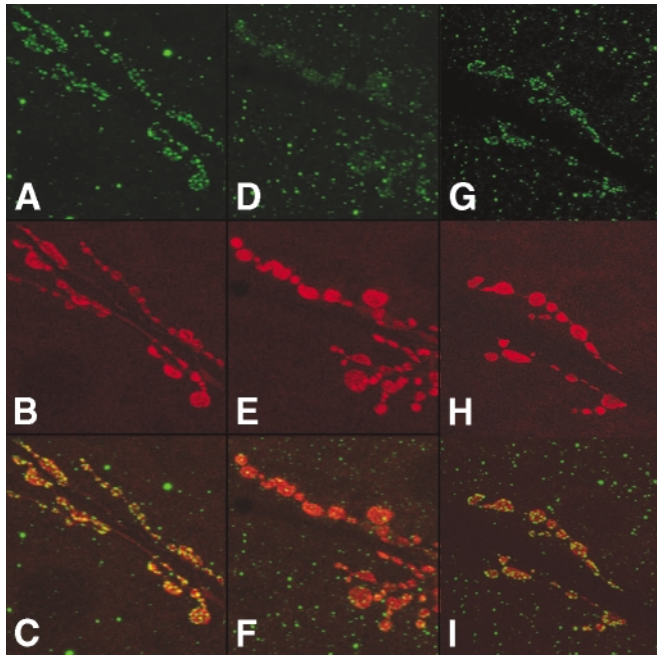

Figure 6. *smn* mutant shows loss of large glutamate receptor clusters at the NMJ. (**A**, **D** and **G**) Immunostaining of NMJ of muscles 6–7 with antibodies against D-GluRIIA (45) concentrating on type I boutons; (**B**, **E** and **H**) localization of Sap-47 (66); (**C**, **F** and **I**) merges of the images above. The homozygous smn^{73Ao} mutant larvae clearly show loss of large GluR clusters underlying the pre-synaptic area (compare A–C with D–F); this defect can be restored by ubiquitous expression of *smn* (driven by ubiquitous driver; *1032*-GAL4) (G–I). The localization of the synaptic terminal associated protein SAP-47 is not altered in the NMJ of mutant versus wild-type or rescued larvae.

would infer that an interaction between these two proteins supported by the amino-terminus is not required for rescue of the neuromuscular *smn* phenotype in flies. Interestingly, a point mutation found in the amino-terminal region of SMN1 in patients can when present in a transgene construct in the $Smn^{-/-}$; SMN2 mice rescue SMA type I phenotypes to less severe types II or III (37). This result and ours are consistent with such an interpretation, indicating that possibly an amino-terminal truncated or mutated SMN protein can partially function as a scaffold to support oligomerization and/or protein binding. More importantly, by utilizing localized expression of wild-type *smn*, it is clear that in our model activity needs to be provided in both muscles and neurons to rescue the larval motility phenotype. This is consistent with results obtained in mouse models where, using recombination, *Smn* function was removed from either muscle or neuron, thus uncovering a requirement in both. However in these experiments, the genetic defect created led to complete loss of the wild-type *Smn* allele in these tissues and therefore possible cell death. In our model, the defect is based upon levels of *smn* activity that allow survival of most other tissues in the animals and should thus resemble the SMA aetiology better. Our results indicate that any attempt to rescue SMA in patients should aim for up-regulating SMN activity in both muscle as well as motor neurons.

To begin to understand the molecular defect that leads to the specific neuromuscular phenotypes in this model, we analysed

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the *smn* mutant larvae in more detail. We see no gross abnormalities in the muscles or motor neurons of animals that show motor abnormality. However upon closer inspection using antibodies that label NMJ structures, we see disorganization and an increased number of enlarged boutons. Although it is possible that the observed defects in localization of components at the NMJ are primary, the severity of these phenotypes is variable amongst mutant animals. Physiological and morphological defects at the NMJ have only recently been observed in a mouse model (37); such studies could have been hampered by the lack of a mild SMA-like genetic and phenotypic background or patient material. Disruption of the neuromuscular junction has been implicated in neuromuscular atrophic phenotypes (47), but a clear hint at a possible defect was lacking. In addition, recently, in the somatic *Smn* mutant motor neurons in the mouse, aberrant organization of the cytoskeleton at the NMJ has been observed (48). We observe a reduction in the amplitude of EPSCs, indicating that a pre- and/or post-synaptic decrease in efficiency at the NMJ may contribute to the phenotypes. This reduction can be rescued by providing *smn* activity, but it is not clear if it is a primary defect. Interestingly, a decrease in amplitudes of muscle action potentials in type III SMA mice has recently been reported (37). Further experiments on these animals including pharmacology and measuring spontaneous amplitudes would possibly lead to further insights.

Perhaps more telling is the consistent reduction of large GluR clusters in the post-synaptic domain in the smn^{73Ao} mutant muscle, while this defect is completely rescued by supplying wild-type *smn* activity. For clustering to occur and be maintained, signalling between the pre- and post-synaptic sides has to take place. This signalling involves chemical and/or electrical activities (49,50). It is possible that high levels of *smn* activity would be required to generate one such signal. However the proposed role for SMN in RNA metabolism does not immediately point to a clear target.

Control of translation seems to play a role at both sides of the synapse; on the pre-synaptic side, translation of an orthologue of the microtubule-associated protein 1B (MAP1B), *futsch*, is controlled by a fly homologue of the Fragile X mental retardation protein (51,52). We have not found any genetic interaction between *futsch* and *smn* (N.T. and M.v.d.H., unpublished data) and a role in this pathway is therefore not immediately obvious. The concentration of L-glutamate in the pre-synaptic terminal is tightly controlled and has a role in the regulation of the post-synaptic receptor field (53). SMN could be required for the regulation of neurotransmitter levels or its non-vesicular release to regulate expression of post-synaptic receptor. It is interesting to note here that in a canine model of motor neuron disease (hereditary canine spinal muscular atrophy, HCSMA) reduced levels of neurotransmitter release have been reported to underlie the motor dysfunction (54,55).

A relation between glutamate receptor activation and increased levels of SMN in primary cultures of rat motor neurons at the dendritic site of the neuron has been described recently (56). In addition, amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by loss of lower and upper motor neurons, has been reported to be associated with aberrant RNA processing of a glutamate transporter (57).

On the post-synaptic side of the *Drosophila* NMJ, translation of GluR is controlled locally by translation initiation factors (58). It is possible that *smn* influences one such factor; elF4E can undergo alternatively splicing and SMN is thought to influence splicing (58,59). At vertebrate NMJs, clustering of the nicotinic acetylcholine receptors (nAChRs) is an intensely studied process and proper clustering is required for function (60). Thus receptor clustering driven by signalling across the synapse is a conserved process necessary for function of the synapse, despite differences in neurotransmitter.

In studies of SMA, it has been difficult to recreate the situation in patients where it is clear that global differences in levels of SMN activity lead to large differences in effects on the patient's motor neurons and muscles. Similarly in our *Drosophila* model, variability of phenotypes is observed, obstructing molecular definitions. However it is clear that our results indicate defects at the NMJ. As an alternative route to molecular studies, *Drosophila* genetics could be employed using this mutant; a random genetic screen without any preconception to its outcome should allow the isolation of suppressor loci that could indicate how *smn* function influences the motor system.

MATERIALS AND METHODS

Fly stocks

All fly stocks were maintained at 25°C on standard medium. *l(3)73Ao e/TM3* from the Szeged stock centre was recombined with an isogenic line *st p e*. The resulting chromosomes *l(3)73Ao p e* were balanced over *TM8* and *TM6B* to generate the smn^{73Ao} mutant stock used. The deficiency that covers the *smn* region was (Df(3L)*st-g24*). how^{24B}-GAL4 was recombined with smn^{73Ao} to generate *w*; smn^{73Ao}*p* how^{24B}-GAL4/*TM6B*. Virgin females of *1032*-GAL4; smn^{73Ao}*p e*/*TM6B*, *elav*-GAL4; smn^{73Ao}*p e*/*TM6B*, *w*; smn^{73Ao}*p* how^{24B}-GAL4/*TM6B* or *elav*-GAL4; smn^{73Ao}*p* how^{24B}-GAL4/*TM6B* were crossed with *w*; UAS-*smn*; smn^{73Ao}*p e*/*TM6B*. Non-*TM6B* early second instar larvae were picked for assays. UAS-*smn* has been described previously (11). *1032*-GAL4; smn^{73Ao}*p e*/*TM6B* was crossed with *w*; UAS-*smn*; smn^{73Ao}*p e*/*TM6B*, *w*; UAS-*smn*ΔN62 (construct misses the amino acids up until 63); smn^{73Ao}*p e*/*TM6B*, *w*; UAS-*smn*ΔN159 (construct misses amino acids up until 160); smn^{73Ao}*p e*/*TM6B* and *w*; UAS-*smn*ΔC157 (construct misses all amino acids after 157); smn^{73Ao}*p e*/*TM6B*. Non-*TM6B* early second instar larvae were picked for rescue assay. The UAS-GAL4 system has been described (61); how^{24B}-GAL4 ($P\{GawB\}^{how\text{-}24B}$) leads to expression in muscles starting in all mesodermal tissue in the embryo (61), *elav*-GAL4 ($P\{GawB\}elav^{c155}$) leads to expression in all embryonic and larval neural tissue (62); *1032*-GAL4 ($P\{GawB\}^{1032.hx}$) generates ubiquitous expression of any UAS construct present (63). Several other GAL4 lines were investigated (localized expression in muscle, NMJ or in motor neurons) but none of these showed rescue. We assessed the levels of expression of all our GAL4 lines by driving GFP and the lines shown here had very high levels of expression while the lines we do not use show lower levels. For the generation of germ line clone containing females for *smn*, the FRT2A yeast

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recombination site was recombined onto the *smn*^{73Ao} chromosome. Females with germ line clones were generated according to standard procedures using *ovoD1* FRT2A males (64).

Mutagenesis

A total of 2500 isogenic *st p e* males were treated with 25 mM EMS according to standard protocols. Mutagenized males were crossed and balanced and with Df(3L)*st-g24 Ki roe p*/*TM6B*, *Tb*. Mutants that did not complement the deficiency were isolated and crossed with the *smn*^{73Ao} stock to verify complementation.

Northern blot analysis

RNA of a *yw* stock at different developmental stages was extracted using the RNeasy kit (QIAGEN). Sixteen micrograms of total RNA of each sample were used to make the northern blot using the NorthernMax^{TM} kit (Ambion). A full length *smn* probe was labelled with [^{32}P]dCTP and hybridized to the blot following the manufacturer's protocol.

Reverse transcriptase and polymerase chain reaction

RNA of *smn*^{73Ao} homozygous animals and from *smn*^{73Ao}/marked balancer chromosome (providing both the means to select these animals as well as wild-type SMN activity) was isolated (see above). First-strand cDNA was synthesized according to standard procedures using oligo-dT. A fragment of 690 bp of *smn* was generated using primers with PCR. The resulting DNA was purified and digested with the enzyme *Msp*I. In the wild-type (heterozygous) sample this analysis should result in three fragments of respectively 79, 202 and 409 bp. In the mutant samples, this would be 202 and 488 bp.

In situ hybridization

Ovaries of *Oregon R* (wild-type) females were fixed and hybridized with DIG-labelled RNA probes, sense and antisense *smn*, according to standard protocols.

Protein interaction

Yeast two-hybrid assays were carried out using the ProQuest^{TM} Two-hybrid System (GibcoBRL). Full-length wild-type *smn* and mutant *smn*^{73Ao} were cloned into pDBLeu bait and pPC86 prey vectors. Both bait and prey vectors were co-transformed into yeast strain MaV203. Protein interactions were assessed by X-gal assay according to the manufacturer's manual.

Immuno-histochemistry

Third instar larvae of homozygous *smn* mutant (*w*; *smn*^{73Ao}*p e*), wild-type (w; *st p e*/*TM3 Ser act*-GFP and/or *st p e*/*st p e*) controls and *1032*-GAL4; *smn*^{73Ao}/UAS-D-*SMN smn*^{73Ao} were dissected and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS); all animals were carefully staged. Fixed, dissected larvae were washed in PBS + 0.1% Triton-X and incubated with anti-Discs large, anti-Synapsin, anti-SAP47 and anti-GluRIIA antibodies overnight at 4°C. Samples were incubated with secondary antibodies (Jackson Laboratories) after washing away primary antibodies. Neuromuscular junctions of muscle 6–7 (A2–A6) were imaged using a Leica TCS NT system and bouton sizes (type I boutons) were measured using AxioVision3.1 software. Numbers of enlarged boutons (over 15 μm^2) in both mutant and control were counted.

Electrophysiology

Third instar larvae were pinned onto a Sylgard® lined recording chamber (nominal volume 2 ml) using 100 μm diameter, stainless steel pins. Preparations were perfused with a modified standard physiological saline cooled to 5°C (in mM): NaCl, 128; KCl, 2; $CaCl_2$, 0.4; $MgCl_2$, 4; sucrose, 70 mM; HEPES, 5; pH 7.2, with NaOH. The larvae were opened along the dorsal mid-line and internal organs removed to reveal the ventral muscle field and ventral ganglion. Segmental nerves were sectioned from the ventral ganglion to eliminate excitatory potentials evoked by CNS activity. Two electrode voltage-clamp recordings were made from muscle 6–7 at a holding potential of −60 mV, using a GeneClamp 500 (Axon Instruments, USA). Borosilicate glass micro-electrodes were filled with 4 M K^+ acetate: 10 mM KCl and had resistances of between 7 and 15 MΩ. EJCs were evoked by stimulating severed nerves (0.8–1 ms; 0.5–5 V; a saline-filled class suction electrode connected to a Grass S48 stimulator, via an SIU5 stimulus isolator; Astro-Med Inc., USA). Currents can be blocked using cobalt and are reduced by repeated stimulation. Clamp conditions were stable and sufficient for the experiments from which data were used.

Currents were filtered (1 kHz), digitized for storage (6.6 kHz) with a Digidata 1322A acquisition system and analysed using pClamp 8.0 software (Axon Instruments, USA). Readings from five to 10 consecutive EJCs were averaged for each datum. Statistical analysis and graphing was performed using GraphPad Prism 3.0 (GraphPad Software Inc., USA).

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