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(54) Title: A METHOD FOR TREATING SPINAL MUSCULAR ATROPHY

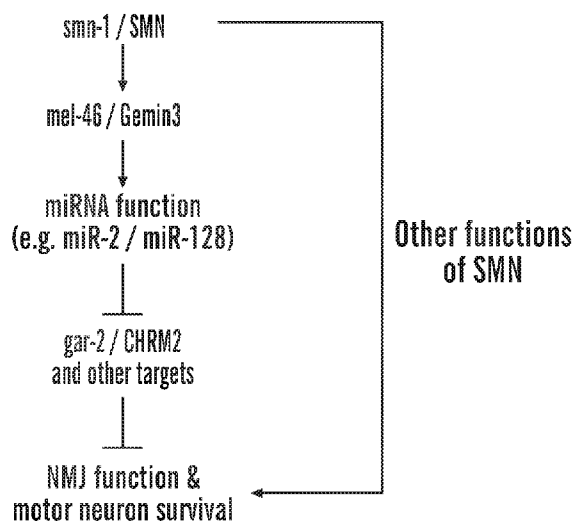


FIG. 16F

(57) Abstract: Described herein are methods and compositions for the use of treating Spinal Muscular Atrophy (SMA). Aspects of the invention relate to administering to a subject in need thereof an agent that inhibits muscarinic acetylcholine receptor. In another embodiment, administration of an agent that inhibits muscarinic acetylcholine receptor ameliorates systems and defects related to SMA. In some embodiments, the agent is methoctramine.



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A METHOD FOR TREATING SPINAL MUSCULAR ATROPHY

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This Application claims benefit under 35 U.S.C. § 119(e) of the U.S. Provisional Application No. 62/351,482 filed June 17, 2016, the contents of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

[0002] The field of the invention relates to methods for the treatment of Spinal Muscular Atrophy.

GOVERNMENT SUPPORT

[0003] This invention was made with Government support under Grant Nos. NS066888, MH020068, and NS089201 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

[0004] Spinal muscular atrophy is a genetic disorder that affects the control of muscle movement. It is caused by a loss of specialized nerve cells, called motor neurons, in the spinal cord and the part of the brain that is connected to the spinal cord (the brainstem). The loss of motor neurons leads to weakness and wasting (atrophy) of muscles used for activities such as crawling, walking, sitting up, and controlling head movement. In severe cases of spinal muscular atrophy, the muscles used for breathing and swallowing are affected.

[0005] Spinal muscular atrophy is caused by defects in the gene *SMN1*, which makes a protein that is important for the survival of motor neurons (SMN protein). In spinal muscular atrophy, insufficient levels of the SMN protein lead to degeneration of the lower motor neurons, producing weakness and wasting of the skeletal muscles. This weakness is often more severe in the trunk and upper leg and arm muscles than in muscles of the hands and feet.

[0006] There are many types of spinal muscular atrophy distinguished by the pattern of features, severity of muscle weakness, and age when the muscle problems begin. The acute infantile-onset SMA1 affects approximately 1 per 10,000 live births; the chronic forms (SMA2 and SMA3) affect 1 per 24,000 births. SMA1 and SMA3 each account for about one fourth of cases, whereas SMA2 is the largest group and accounts for one half of all cases. The age of onset for spinal muscular atrophies is as follows: SMA1(acute infantile or Werdnig Hoffman): Onset is from birth to 6 months. SMA2 (chronic infantile):

Onset is between 6 and 18 months. SMA3 (chronic juvenile): Onset is after 18 months. SMA4 (adult onset): Onset is in adulthood (mean onset, mid 30s).

[0007] Therapeutics are limited for treatment of SMA, despite being the most common cause of death in infants and a disease that can affect people at any stage of life. Thus, there is a long standing need for a treatment for spinal muscular atrophy.

SUMMARY

[0008] The methods disclosed herein are based, in part, on the discovery that defects in axon outgrowth associated with Spinal Muscular Atrophy (SMA) is reversed following treatment with the muscarinic acetylcholine receptor M2 inhibitor, methoctramine. Accordingly, aspects, disclosed herein are related to a method of treating SMA. Generally, the method comprises administering a therapeutically effective amount of an agent that inhibits a muscarinic acetylcholine receptor to a subject in need thereof. In one embodiment, the muscarinic acetylcholine receptor is muscarinic acetylcholine receptor M2. Optionally, the muscarinic acetylcholine receptor M2 is the human M2 gene, *CHRM2*.

[0009] In another embodiment, disclosed herein is a method of treating SMA, wherein the subject has been diagnosed with or is at risk of having SMA1, SMA2, SMA3, or SMA4. Optionally, SMA is attributed to a reduction in the SMN protein. Optionally, SMA is attributed to a mutation in the *SMA1* gene.

[00010] In another embodiment, the treatment for SMA comprises administering to a subject in need thereof an M2 antagonist. Optionally, SMA can be treated by administering to a subject an agent that prevents the function of the muscarinic acetylcholine receptor M2. Optionally, SMA can be treated by administering to a subject an agent that prevents muscarinic acetylcholine receptor M2 from binding neurotransmitter acetylcholine. In some embodiments of the various aspects disclosed herein, the M2 antagonist can comprise an antibody, a small molecule, a polypeptide, an oligonucleotide or an analog thereof, or an inhibitory nucleic acid molecule.

[00011] Some exemplary small molecule inhibitors of M2 include atropine, hyoscyamine, dimethindene, otenzepad, AQRA-741, AFDX-384, dicycloverine, thorazine, diphenhydramine, dimenhydrinate, tolterodine, oxybutynin, ipratropium, methoctramine, tripitramine, gallamine, and chlorpromazine. In some embodiments in all aspects described herein, the M2 inhibitor is methoctramine.

[00012] In embodiments of the various aspects described herein, the agent is administered to a mammal. In some embodiments, that subject is human.

[00013] In some embodiments of the various aspects disclosed herein, the method further comprises administering an additional anti-SMA therapeutic and/or therapy to a subject in need thereof. For example, administering a standard of care SMA therapeutic or therapy to said subject.

[00014] In some embodiments of the various aspects disclosed herein, the method further comprises administering a treatment that ameliorates neuromuscular defects and/or synaptic defects. Optionally, the subject has been diagnosed as having neuromuscular defects and/or synaptic defects.

[00015] Another aspect of the invention relates to a method for treating SMA, method comprising: administering methocarbamol to a subject in need thereof. Optionally, the method further comprising administering to the subject additional anti-SMA therapeutic and/or therapy. Optionally, administration of methocarbamol ameliorates neuromuscular defects and/or synaptic defects. Optionally, the subject has been diagnosed as having neuromuscular defects and/or synaptic defects.

[00016] Another aspect of the invention relates to a composition for the treatment of SMA, the composition comprising: methocarbamol and an agent that facilitates the passage through a blood brain barrier. In one embodiment, the agent is a pharmaceutically acceptable carrier that passes through the blood brain barrier. In another embodiment, the agent is a nanoparticle that passes through a blood brain barrier. In another embodiment described herein, the agent is a pharmaceutically acceptable compound that permeabilizes a blood brain barrier. In yet another embodiment, the agent is a peptide nucleic acid molecule that passes through a blood brain barrier.

BRIEF DESCRIPTION OF THE DRAWINGS

[00017] **FIGs. 1A-1G shows decreased MEL-46 function in *C. elegans* results in defective NMJ signaling. (FIG. 1A) *mel-46(tm1739)* animals had reduced pharyngeal pumping rates *versus* wild type (N2) control animals. Defects were fully rescued by global expression of MEL-46 behind its own promoter ([*mel-46(+)*]*#1*). Mean $\pm$ SEM; Mann-Whitney *U*-test, two-tailed. (FIG. 1B) *mel-46(tm1739)* animals paralyzed more slowly when exposed to aldicarb, an acetylcholinesterase inhibitor. Time course for paralysis on 1mM aldicarb for wild type (N2), *mel-46(tm1739)*, and *mel-46(tm1739);[mel-46(+)]#1* early larval stage L4 animals. Reintroduction of *mel-46* restored normal aldicarb sensitivity. Log-rank test. (FIG. 1C) Cholinergic neuron-specific *mel-46(RNAi)* causes resistance to aldicarb. Time course for paralysis on 1.5mM aldicarb for empty(*RNAi*), *smn-1(RNAi)*, *mel-46(RNAi)*, and *goa-1(RNAi)* young adult animals. Animals sensitive to RNAi in only cholinergic neurons (XE1581) were fed bacteria expressing double-stranded RNA (dsRNA) against *mel-46*, *smn-1*, or *goa-1* (positive control). Control animals were fed bacteria expressing an empty vector control: empty(*RNAi*). Data set previously published without *mel-46(RNAi)* (Dimitriadis et al., 2016). Log-rank test. (FIG. 1D) *mel-46(tm1739)* animals had reduced RFP::SNB-1 (synaptobrevin). Percent change from wild type (N2) control for RFP::SNB-1 in the dorsal cord of *mel-46(tm1739)* and *mel-46(tm1739);[mel-46(+)]#1* animals for ‘puncta analyzer’ parameters: puncta width (μ m), intensity (AU), and linear density (number/ μ m).**

Asterisks denote significance compared to wild type; shading indicates significant change for *mel-46(tm1739)* versus *mel-46(tm1739);[mel-46(+)]#1*. Mann-Whitney *U*-test, two-tailed. Expression of *mel-46* rescued RFP::SNB-1 puncta width defects in *mel-46(tm1739)* animals (wild type versus *mel-46(tm1739);[mel-46(+)]#1* $p = 0.82$); *mel-46(tm1739)* versus *mel-46(tm1739);[mel-46(+)]#1* $p = 0.03$), rescued SNB-1 puncta intensity defects (wild type versus *mel-46(tm1739);[mel-46(+)]#1* $p = 0.85$; *mel-46(tm1739)* versus *mel-46(tm1739);[mel-46(+)]#1* $p = 0.005$) and partially ameliorated SNB-1 puncta linear density defects (wild type versus *mel-46(tm1739);[mel-46(+)]#1* $p = 0.0004$); *mel-46(tm1739)* versus *mel-46(tm1739);[mel-46(+)]#1* $p = 0.0001$). (FIGs. 1E-1G) Representative images of RFP::SNB-1 expressed in the dorsal cord of cholinergic DA MNs for wild type, *mel-46(tm1739)*, and *mel-46(tm1739);[mel-46(+)]#1* animals. These images were taken as part of data collection. Scale bar, 5 μ m. $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00018] FIGs. 2A-2H shows MEL-46(Gemin3) is necessary for proper NMJ function. (FIG. 2A) Schematic representation of the predicted *mel-46* gene. Large arrow indicates the direction of translation. Also shown are the positions of the *yt5* G to A transition, the *tm1739* deletion and the *ok3760* complex substitution, for which the inserted sequence is indicated (Minasaki et al., 2009). (FIG. 2B) *mel-46(yt5)* animals had reduced pharyngeal pumping rates versus wild type (N2) control. Mean $\pm$ SEM; Mann-Whitney *U*-test, two tailed. (FIG. 2C) *mel-46(ok3760)* animals had reduced pharyngeal pumping rates versus wild type (N2) control. Mean $\pm$ SEM; *t*-test, two tailed. This data was collected alongside data in FIG. 1A. (FIG. 2D) Animals sensitive to RNAi in all tissues (KP3948) fed bacteria expressing double-stranded RNA (dsRNA) against *mel-46* had reduced pharyngeal pumping rates versus control animals fed bacteria expressing an empty vector control. Mean $\pm$ SEM; Mann-Whitney *U*-test, two tailed. (FIG. 2E) Animals sensitive to RNAi in only cholinergic neurons (XE1581) fed bacteria expressing double-stranded RNA (dsRNA) against *mel-46* had reduced pharyngeal pumping rates versus control animals fed bacteria expressing an empty vector control. Mean $\pm$ SEM; Mann-Whitney *U*-test, two tailed. (FIG. 2F) *mel-46(yt5)* animals were resistant to the acetylcholinesterase inhibitor, aldicarb. Broad expression of MEL-46 with *[mel-46(+)]#3*, which uses the *mel-46* promoter, did not significantly restore *mel-46(yt5)* aldicarb resistance. Time course for paralysis on 1mM aldicarb for wild type (N2), *mel-46(yt5)*, and *mel-46(yt5);[mel-46(+)]#3* early larval stage L4 animals. Log-rank test. (FIG. 2G) *mel-46(ok3760)* animals were resistant to the acetylcholinesterase inhibitor, aldicarb. Time course for paralysis on 1mM aldicarb for wild type (N2) and *mel-46(ok3760)* young adult animals. Log-rank test. This data was collected alongside data in FIG. 1B. (FIG. 2H) GABA neuron-specific *mel-46* RNAi or *smn-1* RNAi results in aldicarb hypersensitivity. Time course for paralysis on 1mM aldicarb for empty(RNAi), *smn-1(RNAi)*, *mel-46(RNAi)*, and *unc-25(RNAi)* animals. Animals sensitive to RNAi in only GABAergic neurons (XE1375) were fed bacteria expressing double-stranded RNA (dsRNA) against

mel-46, *smn-1*, or *unc-25* (positive control). Control animals were fed bacteria expressing an empty vector control: empty(*RNAi*). Log-rank test. $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00019] FIGs. 3A-3C shows MEL-46(Gemin3) loss causes increased APT-4(AP2 α -adaptin) linear density. (FIG. 3A) *mel-46(tm1739)* animals had increased APT-4 (AP2 α -adaptin) linear density. Percent change from wild type control for APT-4 in the dorsal nerve cord of *mel-46(tm1739)* animals for ‘puncta analyzer’ parameters: puncta width (μm), intensity (AU), and linear density (number/ μm). Mann-Whitney *U*-test, two-tailed. (FIGs. 3B and 3C) Representative images of APT-4::GFP in the dorsal nerve cord of cholinergic DA MNs of wild type and *mel-46(tm1739)* animals. These images were taken as part of data collection. Scale bar, 5 μm . $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00020] FIGs. 4A-4J shows MEL-46 localization and levels are perturbed in *smn-1(lf)* animals. (FIG. 4A) Illustration: *mel-46* was tagged with GFP at the C-terminus and expression was driven by the cholinergic (ACh) *unc-17* promoter. Two lines were generated by UV integration. (FIG. 4B) *smn-1(ok355)* animals exhibited mislocalization and reduction of MEL-46::GFP in dorsal cord processes of cholinergic neurons. MEL-46::GFP localizes to granular punctate structures in dorsal cord processes. Percent change from *smn-1(+)* control for MEL-46::GFP in the dorsal cord of *smn-1(ok355)* animals for ImageJ parameters: puncta density (puncta/area), puncta intensity (AU), and puncta size (pixels/puncta). The ImageJ analysis was used instead of the ‘puncta analyzer’ program since MEL-46::GFP had a scattered non-linear pattern in *smn-1(ok355)* animals; a linear pattern is necessary for accurate ‘puncta analyzer’ analysis. Asterisks denote significance compared to wild type. Mann-Whitney *U*-test, two-tailed. (FIGs. 4C and 4D) Representative images of MEL-46::GFP in dorsal cord cholinergic DA MN processes for control *smn-1(+)* and *smn-1(ok355)* animals. These images were taken as part of data collection. Scale bar, 5 μm . (FIG. 4E) Increasing expression of *mel-46* rescued *smn-1(ok355)* aldicarb response defects. Time course for paralysis on 1mM aldicarb for *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);[mel-46(+)]#1*, and *smn-1(+);[mel-46(+)]#1* early larval stage L4 animals. *smn-1(+);[mel-46(+)]#1* animals were resistant to paralysis by aldicarb. Log-rank test. (FIG. 4F) Increasing *mel-46* rescued *smn-1(ok355)* RFP::SNB-1 synaptic localization defects. Percent change from *smn-1(+)* control for RFP::SNB-1 in the dorsal cord of *smn-1(ok355)*, *smn-1(ok355);[mel-46(+)]#1*, and *smn-1(+);[mel-46(+)]#1* animals for ‘puncta analyzer’ parameters: puncta width (μm), intensity (AU), and linear density (number/ μm). Asterisks denote significance compared to *smn-1(+)* control; shading indicates significant difference from *smn-1(ok355);[mel-46(+)]#1*. Mann-Whitney *U*-test, two-tailed. Expression of *mel-46* restored RFP::SNB-1 puncta width defects (*smn-1(+)* control versus *smn-1(ok355);[mel-46(+)]#1* $p = 0.05$; *smn-1(ok355)* versus *smn-1(ok355);[mel-46(+)]#1* $p = 0.001$), rescued SNB-1 puncta intensity defects (*smn-1(+)* control versus *smn-1(ok355);[mel-46(+)]#1* $p = 0.035$; *smn-1(ok355)* versus *smn-1(ok355);[mel-46(+)]#1* $p = 0.0004$), but did not rescue SNB-1 puncta linear density defects (*smn-1(+)*

control versus *smn-1(ok355);[mel-46(+)]#1* $p = 0.036$; *smn-1(ok355)* versus *smn-1(ok355);[mel-46(+)]#1* $p = 0.19$). (FIGs. 4G-4J) Representative images of cholinergic DA MN RFP::SNB-1 in the dorsal nerve cord of *smn-1(+)*, *smn-1(ok355)* and *smn-1(ok355);[mel-46(+)]#1*. These images were taken as part of data collection. Scale bar, 5 μ m. $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00021] FIGs. 5A-5E shows expressing *mel-46* restores neuronal defects in *smn-1(lf)* animals. (FIG. 5A) Decreased SMN-1 resulted in a 21% decrease in maximum MEL-46::GFP fluorescence in dorsal cord DA motor neuron processes. Histogram of maximum fluorescence (AU) for *smn-1(+)* and *smn-1(ok355)* animals. t -test. $p < 0.01$. (FIG. 5B) Increasing cholinergic expression using the *unc-17* (ACh) promoter of *mel-46* partially rescued *smn-1(ok355)* aldicarb response defects. Time course for paralysis on 1mM aldicarb for *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);[ACh::mel-46::GFP #1]*, and *smn-1(+);[ACh::mel-46::GFP #1]* early larval stage L4 animals. Log-rank test. (FIG. 5C) Increasing cholinergic expression of *mel-46* resulted in rescue of *smn-1(ok355)* aldicarb response defects. Time course for paralysis on 1mM aldicarb for *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);[ACh::mel-46::GFP #1]*, and *smn-1(+);[ACh::mel-46::GFP #1]* early larval stage L4 animals. *smn-1(+);[ACh::mel-46::GFP #1]* were hypersensitive to paralysis by aldicarb. Log-rank test. (FIGs. 5C and 5D) The same MEL-46::GFP integrated lines were used to evaluate levels and localization in FIGs. 2B-2D. Despite rescue *smn-1(ok355)* aldicarb resistance, these animals expressing cholinergic MEL-46::GFP still exhibit MEL-46 mislocalization likely leading to decreased function. This rescue may occur because enough MEL-46::GFP is expressed to overcome mislocalization-related functional deficits. (FIG. 5D) *smn-1(ok355)* animals had reduced pharyngeal pumping rates versus *smn-1(+)* control animals; defects were not rescued by increasing *mel-46* using the *[mel-46(+)]#1* array. *smn-1(+); [mel-46(+)]#1* animals had pharyngeal pumping rates indistinguishable from *smn-1(+)* controls. Mean $\pm$ SEM; Mann-Whitney U -test, two-tailed. (FIG. 5E) Broad expression of MEL-46 using the *mel-46* promoter ameliorated the APT-4 (AP2 α -adaptin) linear density defect in *smn-1(ok355)* animals. Percent change from *smn-1(+)* control for APT-4 in the dorsal cord of *smn-1(ok355)* and *smn-1(ok355);[mel-46(+)]#2* animals for 'puncta analyzer' parameters: puncta width (μ m), intensity (AU), and linear density (number/ μ m). Asterisks denote significance versus *smn-1(+)* control; shading indicates *smn-1(ok355)* is significantly different from *smn-1(ok355);[mel-46(+)]#2*. Mann-Whitney U -test, two-tailed. Expression of MEL-46 did not rescue APT-4 puncta width (*smn-1(+)* control versus *smn-1(ok355);[mel-46(+)]#2* $p = 0.006$; *smn-1(ok355)* versus *smn-1(ok355);[mel-46(+)]#2* $p = 0.48$), did not rescue APT-4 total puncta intensity (*smn-1(+)* control versus *smn-1(ok355);[mel-46(+)]#2* $p = 0.002$; *smn-1(ok355)* versus *smn-1(ok355);[mel-46(+)]#2* $p = 0.27$), but ameliorated APT-4 puncta linear density (*smn-1(+)* control versus *smn-1(ok355);[mel-46(+)]#2* $p = 0.93$; *smn-1(ok355)* versus *smn-1(ok355);[mel-46(+)]#2* $p = 0.05$). $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00022] FIGs. 6A-6C shows expressing *mel-46* does not increase SMN-1 levels. (FIG. 6A) Illustration: *smn-1(rt280)* was generated by CRISPR-mediated insertion of GFP upstream of *smn-1* exon 1. **(FIG. 6B)** Increasing MEL-46 expression using the [*mel-46(+)*#2] array led to decreased GFP::SMN-1 fluorescence. Quantification of mean *smn-1(rt280)* GFP fluorescence in wild type and [*mel-46(+)*#2] backgrounds. Mean $\pm$ SEM; Mann-Whitney *U*-test, two tailed. **(FIG. 6C)** Representative images of *smn-1(rt280)* GFP expression in wild type and [*mel-46(+)*#2] larval stage L4 animals. Scale bar, 50 μ m. **p* $\leq$ 0.05, ***p* < 0.01, ****p* < 0.001.

[00023] FIGs. 7A-7D shows miR-2 is required in cholinergic neurons for proper NMJ function. (FIG. 7A) *mir-2(gk259)* animals were resistant to paralysis by aldicarb. Expression of miR-2 behind the *unc-17* (ACh) cholinergic promoter partially restored *mir-2(gk259)* sensitivity to aldicarb compared to transgenesis controls expressing GFP behind the same promoter. Time course for paralysis on 1mM aldicarb for wild type (N2), *mir-2(gk259)*, *mir-2(gk259);[ACh::mir-2(+)]* and *mir-2(gk259);[ACh::GFP]* young adult animals. Log-rank test. **(FIG. 7B)** *mir-2(gk259)* animals had increased RFP::SNB-1 (synaptobrevin) linear density. Percent change from wild type (N2) control for RFP::SNB-1 in the dorsal nerve cord of *mir-2(gk259)* animals for ‘puncta analyzer’ parameters: puncta width (μ m), intensity (AU), and linear density (number/ μ m). *t*-test, two-tailed. **(FIGs. 7C and 7D)** Representative images of cholinergic DA MN RFP::SNB-1 in the dorsal cord of wild type and *mir-2(gk259)* animals. These images were taken as part of data collection. Scale bar, 5 μ m. **p* $\leq$ 0.05, ***p* < 0.01, ****p* < 0.001.

[00024] FIGs. 8A-8I shows miR-2 is required for NMJ function. (FIG. 8A) *mir-2(n4108)* animals were resistant to the acetylcholinesterase inhibitor, aldicarb. Time course for paralysis on 1mM aldicarb for wild type (N2) and *mir-2(n4108)* young adult animals. Log-rank test. **(FIG. 8B)** *mir-2(gk259)* animals had reduced pharyngeal pumping rates *versus* wild type control. Mean $\pm$ SEM; *t*-test, two-tailed. **(FIG. 8C)** *mir-2(gk259)* SYD-2 (α -liprin) levels were indistinguishable from wild type (N2). Percent change from wild type control for SYD-2 in the dorsal nerve cord of *mir-2(gk259)* animals for ‘puncta analyzer’ parameters: average puncta width (μ m), total intensity (AU), and linear density (number/ μ m). *t*-test, two-tailed. **(FIG. 8D)** *mir-2(lf)* had reduced expression of ITSN-1 (DAP160/Intersectin). Percent change from wild type (N2) control for ITSN-1 in the dorsal nerve cord of *mir-2(gk259)* and *mir-2(n4108)* animals as above. *t*-test, two-tailed. **(FIGs. 8E and 8F)** Representative images of ITSN-1::GFP expressed in the dorsal nerve cord of cholinergic DA motor neurons for wild type and *mir-2(n4108)* animals. These images were taken as part of data collection. Scale bar, 5 μ m. **(FIG. 8G)** *mir-2(gk259)* and *mir-2(n4108)* animals had reduced APT-4 (AP2 α -adaptin) levels. Percent change from wild type (N2) control for the APT-4 in *mir-2(gk259)* animals as above. *t*-test, two-tailed. **(FIGs. 8H-8I)** Representative images of cholinergic DA motor neuron APT-4::GFP in the dorsal cord of wild type (N2)

and *mir-2(gk259)* animals. These images were taken as part of data collection. $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00025] FIGs. 9A-9I shows miR-2 binds the gar-2 3'UTR and represses GAR-2 translation. (FIG. 9A) Loss of m2R ortholog, GAR-2, suppressed aldicarb response defects of animals lacking *mir-2(gk259)*. *gar-2(ok520)* animals were hypersensitive to paralysis by aldicarb. Time course for paralysis on 1mM aldicarb for wild type (N2), *mir-2(gk259)*, *gar-2(ok520)* and *mir-2(gk259);gar-2(ok520)* young adult animals. Log-rank test. (FIG. 9B) Schematic representation of changes made to the endogenous *gar-2* 3'UTR using CRISPR. For the wild type control (*gar-2* UTRwt^C), the miR-2 binding site remained intact, however, a C>T PAM site change was made. For the experimental condition (*gar-2* UTRscr^C), the miR-2 binding site was scrambled in addition to the C>T PAM site alteration. (FIG. 9C) *gar-2(rt318)*, referred to as *gar-2* UTRscr^C, animals were resistant to the acetylcholinesterase inhibitor, aldicarb, compared to *gar-2(rt317)*, referred to as *gar-2* UTRwt^C. Time course for paralysis on 1mM aldicarb for young adult animals. Log-rank test. (FIG. 9D) Scrambling the predicted endogenous *gar-2* 3'UTR miR-2 binding site increased *gar-2* messenger RNA levels. Quantification of *gar-2* mRNA levels in young adult *gar-2* UTRwt^C and *gar-2* UTRscr^C animals. *t*-test, two-tailed (n=4 for *gar-2* UTRwt^C and *gar-2* UTRscr^C). (FIG. 9E) Reporter constructs used to assess miR-2 regulation of *gar-2* 3'UTR in cholinergic neurons: *rtIs56* (*unc-17p*-ACh::GFP::*gar-2* 3'UTRwt) and *rtIs57* or *rtIs58* (*unc-17p*-ACh::GFP::*gar-2* 3'UTRscr). *unc-17p*-ACh::GFP::*gar-2* 3'UTRwt construct contains the *unc-17* promoter expressing NLS::GFP upstream of the *gar-2* 3'UTR, which has a predicted miR-2 binding site. Red text indicates intact seed region. *unc-17p*-ACh::GFP::*gar-2* 3'UTRscr is the same construct with the predicted miR-2 binding site scrambled identically to the sequence in *gar-2* UTRscr^C animals. (FIG. 9F) Representative images of *unc-17p*-ACh::GFP::*gar-2* 3'UTRwt expression in cholinergic neurons of wild type (N2) and *mir-2(gk259)* larval stage L4 animals. Scale bar, 50μm. (FIG. 9G) Representative images of *unc-17p*-ACh::GFP::*gar-2* 3'UTRscr (*rtIs57*) expression in cholinergic neurons of wild type (N2) and *mir-2(gk259)* larval stage L4 animals. (FIG. 9H) Ratio representation of mean GFP fluorescence for wild type and *mir-2(gk259)* animals. *t*-test, two-tailed. Ratio was calculated by dividing the mean GFP fluorescence of *unc-17p*-ACh::GFP::*gar-2* 3'UTRwt for each genotype by the corresponding mean GFP fluorescence of *unc-17p*-ACh::GFP::*gar-2* 3'UTRscr for that genotype. UTRwt represents mean fluorescence for each genotype expressing the *unc-17p*-ACh::GFP::*gar-2* 3'UTRwt reporter, while UTRscr represents mean fluorescence for each genotype expressing the *unc-17p*-ACh::GFP::*gar-2* 3'UTRscr control reporter. Error bars represent the cumulative SEM for each genotype across transgenes. (FIG. 9I) *gar-2* transcript levels did not increase in a *mir-2* loss of function background. Quantification of *gar-2* mRNA levels in young adult *mir-2(gk259)* animals compared to wild type (N2) controls. *t*-test, two-tailed (n=4 for *mir-2(gk259)* and N2). $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00026] FIGs. 10A-10C shows loss of predicted miR-2 mRNA targets suppresses *mir-2(lf)* aldicarb resistance. (FIG. 10A) Loss of DBL-1 suppressed *mir-2(gk259)* aldicarb resistance. Time course for paralysis on 1mM aldicarb for wild type (N2), *mir-2(n4108)*, *mir-2(n4108);dbl-1(nk3)*, and *dbl-1(nk3)* young adult animals. Log-rank test. **(FIG. 10B)** Loss of SEK-1 suppressed *mir-2(gk259)* aldicarb resistance. Time course for paralysis on 1mM aldicarb for wild type (N2), *mir-2(n4108)*, *mir-2(n4108);sek-1(km4)*, and *sek-1(km4)* young adult animals. Log-rank test. **(FIG. 10C)** Loss of VAB-2 suppressed *mir-2(gk259)* aldicarb resistance. Time course for paralysis on 1mM aldicarb for wild type (N2), *mir-2(n4108)*, *mir-2(n4108);vab-2(ju1)*, and *vab-2(ju1)* young adult animals. Log-rank test. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[00027] FIGs. 11A-11D shows miR-2 inhibits translation by binding the *gar-2* 3'UTR. (FIG. 11A) Loss of *miR-2* results in increased expression of *unc-17p-ACh::GFP::gar-2* 3'UTRwt. *unc-17p-ACh::GFP::gar-2* 3'UTRwt mean GFP fluorescence in wild type (N2) and *mir-2(gk259)* backgrounds. Mean $\pm$ SEM; *t*-test, two-tailed. **(FIGs. 11B and 11C)** Expression of *unc-17p-ACh::GFP::gar-2* 3'UTRscr was indistinguishable in *mir-2(gk259)* versus wild type (N2) animals for two independent integrated lines. *unc-17p-ACh::GFP::gar-2* 3'UTRscr mean GFP fluorescence in wild type (N2) and *mir-2(gk259)* backgrounds. Mean $\pm$ SEM; *t*-test, two-tailed. **(FIG. 11D)** Formula used to calculate the ratio and standard error of the mean (SEM) shown in FIG. 9. UTRwt represents mean fluorescence for each genotype expressing the *unc-17p-ACh::GFP::gar-2* 3'UTRwt reporter, while UTRscr represents mean fluorescence for each genotype expressing the *unc-17p-ACh::GFP::gar-2* 3'UTRscr control reporter. For SEM, *s* represents the standard deviation of the population and *n* represents the number of animals analyzed. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[00028] FIGs. 12A-12C shows *smn-1* loss of function abrogated miR-2 repression of GAR-2 expression. (FIG. 12A) Loss of *smn-1* caused a relative increase in *unc-17p-ACh::GFP::gar-2* 3'UTRwt expression. Expressing *mel-46* using the broadly expressed [*mel-46(+)*#2] array decreased relative *unc-17p-ACh::GFP::gar-2* 3'UTRwt expression in *smn-1(ok355)* animals. Ratio representation of mean GFP fluorescence for *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);[mel-46(+)]#2*, and *smn-1(+);[mel-46(+)]#2* animals. Mann-Whitney *U*-test, two-tailed. Ratio calculation was completed in the same manner as FIG. 9H and FIG. 11D **(FIG. 12B)** miR-2 levels were decreased in neurons when either SMN-1 or MEL-46 were decreased. Quantification of mature miR-2 for empty(*RNAi*), *smn-1(RNAi)*, and *mel-46(RNAi)* young adult animals relative to housekeeping miRNA miR-60. *t*-test, two-tailed (n=6 for each condition). **(FIG. 12C)** *gar-2* transcript levels did not change when SMN-1 or MEL-46 levels decreased in neurons. Quantification of *gar-2* mRNA for empty(*RNAi*), *smn-1(RNAi)*, and *mel-46(RNAi)* young adult animals. *t*-test, two-tailed (n=3 for each condition). **(FIGs. 12C and 12D)** Animals sensitive to RNAi in only neurons (TU3401) were fed bacteria expressing double-stranded RNA (dsRNA) against *mel-46* or *smn-1*.

Control animals were fed bacteria expressing an empty vector control: empty (*RNAi*). $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00029] FIGs. 13A-13D shows increasing MEL-46(Gemin3) ameliorates *smn-1(lf)* defective miR-2 activity. (FIG. 13A) Expression of mean *unc-17p-ACh::GFP::gar-2* 3'UTRscr (*rtIs57*) fluorescence was decreased in *smn-1(ok355)* compared to *smn-1(+)* control animals. Increasing MEL-46 levels using the [*mel-46(+)*#2] array did not alter expression *versus smn-1(+)* controls. Mean *unc-17p-ACh::GFP::gar-2* 3'UTRwt fluorescence in *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);[mel-46(+)]#2*, and *smn-1(+);[mel-46(+)]#2* animals. Mean $\pm$ SEM; Mann-Whitney *U*-test, two tailed. **(FIG. 13B)** Expression of mean *unc-17p-ACh::GFP::gar-2* 3'UTRwt fluorescence was indistinguishable between *smn-1(+)* and *smn-1(ok355)* animals; increasing MEL-46 using the [*mel-46(+)*#2] array reduced expression *versus* control. Mean *unc-17p-ACh::GFP::gar-2* 3'UTRwt fluorescence in *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);[mel-46(+)]#2*, and *smn-1(+);[mel-46(+)]#2* animals. Mean $\pm$ SEM; Mann-Whitney *U*-test, two tailed. **(FIG. 13C)** miR-2 levels were decreased in neurons when either SMN-1 or MEL-46 were decreased. Quantification of mature miR-2 for empty(*RNAi*), *smn-1(RNAi)*, and *mel-46(RNAi)* young adult animals relative to housekeeping gene *act-1*. *t*-test, two-tailed (n=6 for each condition). **(FIG. 13D)** miR-2 levels were decreased in neurons when either SMN-1 or MEL-46 were decreased. Quantification of mature miR-2 for empty(*RNAi*), *smn-1(RNAi)*, and *mel-46(RNAi)* young adult animals relative to housekeeping rRNA 18s. *t*-test, two-tailed (n=6 for each condition). $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00030] FIGs. 14A-14H shows decreasing GAR-2(m2R) levels rescues NMJ defects in *smn-1(lf)* and *mel-46(lf)* animals. (FIG. 14A) *smn-1(ok355)* animals had reduced pharyngeal pumping rates *versus smn-1(+)* control animals; defects were not rescued by loss of GAR-2. Mean $\pm$ SEM; Mann-Whitney *U*-test, two tailed. **(FIG. 14B)** Loss of GAR-2 ameliorated *smn-1(rt248)* aldicarb response. *smn-1(+);gar-2(ok520)* animals were hypersensitive to aldicarb. Time course for paralysis on 1mM aldicarb in *smn-1(+)*, *smn-1(rt248)*, *smn-1(rt248);gar-2(ok520)*, and *smn-1(+);gar-2(ok520)* early larval stage L4 animals. Log-rank test. **(FIG. 14C)** Loss of GAR-2 did not rescue *smn-1(ok355)* APT-4 (AP2 α -adaptin) defects. Loss of GAR-2 in the *smn-1(+)* background resulted in decreased APT-4 puncta width and intensity. Percent change from *smn-1(+)* control for APT-4 in the dorsal cord of *smn-1(ok355)*, *smn-1(ok355);gar-2(ok520)*, and *smn-1(+);gar-2(ok520)* animals for 'puncta analyzer' parameters: average puncta width (μ m), intensity (AU), and linear density (number/ μ m). Asterisks denote significance compared to *smn-1(+)* control. Mann-Whitney *U*-test, two-tailed. GAR-2 loss did not rescue APT-4 puncta width (*smn-1(+)* control *versus smn-1(ok355);gar-2(ok520)* $p = 0.12$; *smn-1(ok355)* *versus smn-1(ok355);gar-2(ok520)* $p = 0.48$), did not rescue APT-4 puncta intensity (*smn-1(+)* control *versus smn-1(ok355);gar-2(ok520)* $p = 0.08$; *smn-1(ok355)* *versus smn-1(ok355);gar-2(ok520)* $p = 0.54$) and did not

rescue APT-4 puncta linear density (*smn-1(+)* control versus *smn-1(ok355);gar-2(ok520)* $p = 0.66$; *smn-1(ok355)* versus *smn-1(ok355);gar-2(ok520)* $p = 0.07$). (**FIG. 14D**) Loss of GAR-2 ameliorated *smn-1(rt248)* SNB-1 (synaptobrevin) defects. Percent change from *smn-1(+)* control for SNB-1 in the dorsal nerve cord of *smn-1(rt248)*, and *smn-1(rt248);gar-2(ok520)* animals for all ‘puncta analyzer’ parameters: average puncta width (μm), total intensity (AU), and linear density (number/ μm). *smn-1(+);gar-2(ok520)* percent change was collected alongside this data and is shown in **FIG. 12**. Asterisks denote significance compared to *smn-1(+)* control; shading indicates significant difference from *smn-1(rt248);gar-2(ok520)*. Mann-Whitney *U*-test, two-tailed. Loss of GAR-2 rescued SNB-1 puncta width (*smn-1(+)* control versus *smn-1(rt248);gar-2(ok520)* $p = 0.83$; *smn-1(rt248)* versus *smn-1(rt248);gar-2(ok520)* $p = 0.004$), restored SNB-1 total puncta intensity (*smn-1(+)* control versus *smn-1(rt248);gar-2(ok520)* $p = 0.91$; *smn-1(rt248)* versus *smn-1(rt248);gar-2(ok520)* $p = 0.004$) and rescued SNB-1 puncta linear density (*smn-1(+)* control versus *smn-1(rt248);gar-2(ok520)* $p = 0.97$; *smn-1(rt248)* versus *smn-1(rt248);gar-2(ok520)* $p = 0.002$). (Martinez et al.) (**FIGs. 14E-14H**) Representative images of SNB-1::RFP expressed in the dorsal nerve cord of cholinergic DA motor neurons for *smn-1(+)*, *smn-1(rt248)*, *smn-1(rt248);gar-2(ok520)*, and *smn-1(+);gar-2(ok520)*. These images were taken as part of data collection. Scale bar, $5\mu\text{m}$. **FIG. 14E** and **14H** were taken from **FIG. 12** since this data was collected alongside both *ok355* and *rt248* SNB-1::RFP data. $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

[00031] FIGs. 15A-15G shows loss of *gar-2* ameliorated *smn-1(lf)* NMJ defects. (FIG. 15A) Loss of *gar-2* rescued *smn-1(ok355)* aldicarb response defect. Time course for paralysis on 1mM aldicarb for *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);gar-2(ok520)*, and *smn-1(+);gar-2(ok520)* early larval stage L4 animals. Log-rank test. (**FIG. 15B**) Loss of *gar-2* rescued *mel-46(tm1739)* aldicarb response defect. Time course for paralysis on 1mM aldicarb for *mel-46(+)*, *mel-46(tm1739)*, *mel-46(tm1739);gar-2(ok520)*, and *smn-1(+);gar-2(ok520)* early larval stage L4 animals. Log-rank test. For these experiments, *mel-46(tm1739)* was maintained over the *nT1* balancer and therefore, control *mel-46(+)* animals were obtained as ‘+/+’ animals from +/*nT1* heterozygous mothers. (**FIG. 15C**) Loss of *gar-2* rescued *smn-1(ok355)* RFP::SNB-1 synaptic localization defects. *gar-2* loss in the *smn-1(+)* background resulted in increased RFP::SNB-1 puncta width and intensity. Percent change from *smn-1(+)* control for RFP::SNB-1 in the dorsal nerve cord of *smn-1(ok355)*, *smn-1(ok355);gar-2(ok520)*, and *smn-1(+);gar-2(ok520)* animals for ‘puncta analyzer’ parameters: puncta width (μm), intensity (AU), and linear density (number/ μm). Asterisks denote significance compared to *smn-1(+)* control; shading indicates significant difference from *smn-1(ok355);gar-2(ok520)*. Mann-Whitney *U*-test, two-tailed. Loss of *gar-2* rescued RFP::SNB-1 puncta width defects (*smn-1(+)* control animals versus *smn-1(ok355);gar-2(ok520)* $p = 0.76$; *smn-1(ok355)* versus *smn-1(ok355);gar-2(ok520)* $p = 0.0001$), restored SNB-1 puncta intensity (*smn-1(+)* control animals versus *smn-1(ok355);gar-2(ok520)* $p = 1.00$; *smn-1(ok355)* versus *smn-*

l(ok355);gar-2(ok520) $p = 0.0001$) and rescued SNB-1 puncta linear density defects (*smn-1(+)* control animals versus *smn-1(ok355);gar-2(ok520)* $p = 0.08$; *smn-1(ok355)* versus *smn-1(ok355);gar-2(ok520)* $p = 0.02$). (FIGs. 15D-15G) Representative images of cholinergic DA MN RFP::SNB-1 in the dorsal nerve cord of *smn-1(+)*, *smn-1(ok355)*, *smn-1(ok355);gar-2(ok520)*, and *smn-1(+);gar-2(ok520)*. These images were taken as part of data collection. Scale bar, 5 μ m. FIGs. 15D and 15G are also shown in FIG. 13 since this control data was collected alongside both *ok355* and *rt248* RFP::SNB-1 data. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[00032] FIGs. 16A-16F shows increased m2R muscarinic receptor levels in SMA mouse model MNs contribute to axon outgrowth defects. (FIG. 16A) Alignment of predicted miR-2 or miR-128 binding sites for *C. elegans*, mouse and human *gar-2* or *CHRM2* 3'UTRs. *CHRM2* encodes the m2R muscarinic receptor (Paraskevopoulou et al., 2013; Reczko et al., 2012). Predicted nucleotide pairing shown by vertical lines. Red text indicates predicted miRNA seed region. A black line indicates potential seed region conservation. (FIG. 16B) Representative image for two E13.5 wild type and two *Smn*^{-/-}; *SMN2*^{tg0} DIV10 spinal MN immunoblots probed for m2R and control β -Actin. (FIG. 16C) Quantification of immunoblot, *t*-test, two-tailed, $p = 0.017$ (n=14 for WT and n=13 for SMA). (FIG. 16D) Quantification of miR-128 levels in DIV10 spinal MNs from E13.5 wild type and *Smn*^{-/-}; *SMN2*^{tg0} animals. *t*-test, two-tailed (n=24 from 12 mice for WT; n=20 from 10 mice for SMA). (FIG. 16E) Longest axon length for E13.5 wild type and *Smn*^{-/-}; *SMN2*^{tg0} DIV5 spinal MNs treated with 0nm, 50nm, and 500nm methoctramine. *t*-test, two-tailed. (n=103 for WT 0 nM, n=131 for WT 50 nM, n=98 for WT 500 nM, n=102 for SMA 0 nM, n=67 for SMA 50 nM and n=53 for SMA 500 nM) (FIG. 16F) Proposed model: SMN acts via MEL-46 to influence microRNAs that play important roles in NMJ function and MN survival. SMN loss affects other pathways as well. *C. elegans* genes shown on left side; human genes on right side. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[00033] FIG. 17 shows m2R inhibition by methoctramine increases axon length in SMA mouse model MNs. Total axon length for E13.5 wild type and *Smn*^{-/-}; *SMN2*^{tg0} DIV5 spinal MNs treated with 0nm, 50nm, and 500nm methoctramine. 'Total axon length' is a measurement of all axon branches. *t*-test, two-tailed. n=103 for WT 0 nM, n=131 for WT 50 nM, n=98 for WT 500 nM, n=102 for SMA 0 nM, n=67 for SMA 50 nM and n=53 for SMA 500 nM. Neurons are from at least three biological samples. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DETAILED DESCRIPTION

[00034] As described herein, the inventors have discovered defects in neuromuscular and/or synaptic defects associated with the disease Spinal Muscular Atrophy (SMA) is/are ameliorated following the administration of an agent that inhibits muscarinic acetylcholine receptor M2. Without wishing to be

bound by a theory, the inhibition of muscarinic acetylcholine receptor M2 results in an increase in miRNA activity in the cells, specifically miR-128. The inventors have discovered that targeting muscarinic acetylcholine receptor M2 with a M2 antagonist effectively rescues defects associated with aberrant muscarinic acetylcholine receptor M2 activity. Administering an M2 antagonist reversed defects associated with SMA in motor neuron cells derived from an SMA mouse model. Thus, targeting muscarinic acetylcholine receptor M2 is an effective method for treating and/or preventing SMA. Accordingly, provided herein are methods for treating SMA.

[00035] The spinal muscular atrophies (SMAs) comprise a group of autosomal-recessive disorders characterized by progressive weakness of the lower motor neurons. SMA is described as a disorder of progressive muscular weakness beginning in infancy that resulted in early death, though the age of death was variable. In pathologic terms, the disease was characterized by loss of anterior horn cells. The central role of lower motor neuron degeneration was confirmed in subsequent pathologic studies demonstrating a loss of anterior horn cells in the spinal cord and cranial nerve nuclei. Several types of spinal muscular atrophies have been described based on age when accompanying clinical features appear. The most common types are acute infantile (SMA1, or Werdnig-Hoffman disease), chronic infantile (SMA2), chronic juvenile (SMA3 or Kugelberg-Welander disease), and adult onset (SMA4) forms. The genetic defects associated with SMA1, SMA2 and SMA3 are localized on chromosome 5q11.2-13.3.

[00036] Muscarinic acetylcholine receptors belong to a class of metabotropic receptors that use G proteins as their signaling mechanism. In such receptors, the signaling molecule (the ligand) binds to a receptor that has seven transmembrane regions; in this case, the ligand is ACh. This receptor is bound to intracellular proteins, known as G proteins, which begin the information cascade within the cell.

[00037] In some embodiments herein, the muscarinic acetylcholine receptor is muscarinic acetylcholine receptor M2, also known as the cholinergic receptor, muscarinic 2. In one embodiment, the muscarinic acetylcholine receptor is encoded by the human *CHRM2* gene. Multiple alternatively spliced transcript variants have been described for this gene. M2 muscarinic receptors act via a G_i type receptor, which causes a decrease in cAMP in the cell, generally leading to inhibitory-type effects. They appear to serve as autoreceptors. In addition, they modulate muscarinic potassium channels. In the heart, this contributes to a decreased heart rate. They do so by the G beta gamma subunit of the G protein coupled to M2. This part of the G protein can open K^+ channels in the parasympathetic nodes in the heart, which causes an outward current of potassium, which slows down the heart rate.

[00038] Spinal muscular atrophy is linked to a genetic mutation in the *SMN1* gene. Human chromosome 5 contains two nearly identical genes at location 5q13: a telomeric copy *SMN1* and a centromeric copy *SMN2*. In healthy individuals, the *SMN1* gene codes the survival of motor neuron

protein (SMN) which, as its name says, plays a crucial role in survival of motor neurons. The *SMN2* gene, on the other hand - due to a variation in a single nucleotide (840.C→T) - undergoes alternative splicing at the junction of intron 6 to exon 8, with only 10-20% of *SMN2* transcripts coding a fully functional survival of motor neuron protein (SMN-fl) and 80-90% of transcripts resulting in a truncated protein compound (SMNΔ7) which is rapidly degraded in the cell.

[00039] In individuals affected by SMA, the *SMN1* gene is mutated in such a way that it is unable to correctly code the SMN protein - due to either a deletion occurring at exon 7 or to other point mutations (frequently resulting in the functional conversion of the *SMN1* sequence into *SMN2*). Almost all people, however, have at least one functional copy of the *SMN2* gene (with most having 2-4 of them) which still codes small amounts of SMN protein - around 10-20% of the normal level - allowing some neurons to survive. In the long run, however, reduced availability of the SMN protein results in gradual death of motor neuron cells in the anterior horn of spinal cord and the brain. Muscles that depend on these motor neurons for neural input now have decreased innervation (also called denervation), and therefore have decreased input from the central nervous system (CNS). Decreased impulse transmission through the motor neurons leads to decreased contractile activity of the denervated muscle. Consequently, denervated muscles undergo progressive atrophy.

[00040] In one embodiment, SMA is caused by a reduction of the SMN protein. In another embodiment, SMA is caused by a mutation in the *SMN1* gene.

[00041] In one embodiment, the type of SMA can be SMA1, SMA2, SMA3, SMA4, SMARD, SBMA, or DSMA.

[00042] SMA1 (also known as Werdnig-Hoffmann disease) is believed to be the most common form. It causes severe muscle weakness, which can result in problems moving, eating, breathing and swallowing. These symptoms are usually apparent at birth or during the first few months of life. The muscles of babies with SMA1 are thin and weak, which makes their limbs limp and floppy. They're usually unable to raise their head or sit without support. Breathing problems can be caused by weakness in the baby's chest muscles, and difficulty swallowing can be made worse by weakness of the muscles in the tongue and throat. Because of the high risk of serious respiratory problems, most children with SMA1 die in the first few years of life.

[00043] Symptoms of SMA2 usually appear when an infant is 7-18 months old. The symptoms are less severe than SMA1, but become more noticeable in older children. Infants with SMA2 are usually able to sit, but cannot stand or walk unaided. They may also have the following symptoms: breathing problems, weakness in their arms and, particularly, their legs, swallowing or feeding problems, and/or a slight tremor (shaking) of their fingers. In some cases, deformities of the hands, feet, chest and joints develop as the muscles shrink. As they grow, many children with SMA2 develop scoliosis. This is an

abnormal curvature of the spine caused by the muscles supporting the bones of the spine becoming weaker. A child with SMA2 has weak respiratory muscles, which can make it difficult for them to cough effectively. This can make them more vulnerable to respiratory infections. Although SMA2 may shorten life expectancy, improvements in care standards mean most people can live long, fulfilling and productive lives. The majority of children with SMA2 are now expected to survive into adulthood.

[00044] SMA3 (also known as Kugelberg-Welander disease) is the mildest form of childhood SMA. Symptoms of muscle weakness usually appear after 18 months of age, but this is very variable and sometimes the symptoms may not appear until late childhood or early adulthood. Most children with SMA3 are able to stand unaided and walk, although many find walking or getting up from a sitting position difficult. They may also have: balance problems, difficulty walking, difficulty running or climbing steps, and /or a slight tremor (shaking) of their fingers. Over time, the muscles of children with SMA3 become weaker, resulting in some children losing the ability to walk when they get older. Breathing and swallowing difficulties are very rare and the condition doesn't usually affect life expectancy.

[00045] SMA4 is a less common form that begins in adulthood. The symptoms are usually mild to moderate, and may include: muscle weakness in the hands and feet, difficulty walking, and/or muscle tremor (shaking) and twitching. SMA4 doesn't affect life expectancy.

[00046] Spinal muscular atrophy with respiratory distress (SMARD) is a very rare form of SMA that severely affects the muscles used in breathing. It's usually diagnosed within the first year of life.

[00047] Kennedy's syndrome, or spinobulbar muscular atrophy (SBMA), is a rare type of adult SMA. SBMA only affects men. It usually develops very gradually between the ages of 20 and 40. Rarely, it can affect teenage boys or sometimes only become obvious after 40. The initial symptoms of Kennedy's syndrome may include tremor (shaking) of the hands, muscle cramps on exertion, and/or muscle twitches and weakness of the limb muscles. As the condition progresses, it may cause other symptoms, including: weakness of the facial and tongue muscles, which may cause difficulty swallowing (dysphagia) and slurred speech, and/or recurring pneumonia (infection of lung tissue). Some people with Kennedy's syndrome also develop enlarged male breasts (gynaecomastia), diabetes, and a low sperm count or infertility. Kennedy's syndrome doesn't usually affect life expectancy.

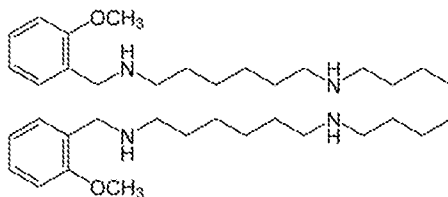
[00048] Distal spinal muscular atrophy (DSMA) is a rare form of SMA that affects the distal muscles, such as the hands, feet, lower arms and lower legs. This leads to reduced mobility and range of movement. Some types of DSMA can affect the muscles used for speaking or swallowing.

[00049] Generally, the method comprises administering an agent that inhibits muscarinic acetylcholine receptor M2. As used herein, the term "inhibiting" with respect to targeting of a muscarinic acetylcholine receptor M2 refers to attenuating an activity of said muscarinic acetylcholine receptor M2.

The agent can be an antagonist of said muscarinic acetylcholine receptor M2. In one embodiment, the agent can be antibody that binds to said muscarinic acetylcholine receptor M2 and inhibits its activity. In another embodiment, the agent can be a small molecule that targets said muscarinic acetylcholine receptor M2 and inhibits its activity. In yet another embodiment, the agent can be a polypeptide that targets said muscarinic acetylcholine receptor M2 and inhibits its activity. In yet another embodiment, the agent can be an oligonucleotide or analog thereof, that targets said muscarinic acetylcholine receptor M2 and inhibits its activity. In one embodiment, the agent can be an inhibitory nucleic acid molecule that targets said muscarinic acetylcholine receptor M2 and inhibits its activity.

[00050] As used herein, the term “inhibitor” refers to an agent which can decrease the expression and/or activity of the targeted expression product, e.g. by at least 10% or more, e.g. by 10% or more, 50% or more, 70% or more, 80% or more, 90% or more, 95% or more, or 98 % or more. The efficacy of an inhibitor of a particular target e.g. its ability to decrease the level and/or activity of the target can be determined, e.g. by measuring the level of an expression product and/or the activity of the target. Methods for measuring the level of a given mRNA and/or polypeptide are known to one of skill in the art, e.g. RT-PCR with primers can be used to determine the level of RNA and Western blotting with an antibody (e.g. an anti- muscarinic acetylcholine receptor M2 antibody, e.g. Cat No. ab2805; Abcam; Cambridge, MA) can be used to determine the level of a polypeptide. The activity of a target can be determined using methods known in the art, e.g. measuring the expression level of a genes regulated by muscarinic acetylcholine receptor M2 as described herein.

[00051] In some embodiments the M2 antagonist is a small molecule that selectively inhibits muscarinic acetylcholine receptor M2. Non-limiting examples of small molecule inhibitors of muscarinic acetylcholine receptor M2 include atropine, hyoscyamine, dimethindene, otenzepad, AQRA-741, AFDX-384, dicycloverine, thorazine, diphenhydramine, dimenhydrinate, tolterodine, oxybutynin, ipratropium, methoctramine, tripitramine, gallamine, and chlorpromazine. In some embodiments of all aspects described herein, the muscarinic acetylcholine receptor M2 antagonist is the small molecule methoctramine.



Methoctramine, Structure 1

[00052] N,N'-bis[6-[(2-methoxyphenyl)methylamino]hexyl]octane-1,8-diamine (methoctramine) is a polymethylene tetraamine that acts as a highly selcective muscarinic antagonist. It preferently binds to

the pre-synaptic receptor M2, a muscarinic acetylcholine ganglionic protein complex present basically in heart cells. In normal conditions -absence of methoctramine-, the activation of M2 receptors diminishes the speed of conduction of the sinoatrial and atrioventricular nodes thus reducing the heart rate. Thanks to its apparently high cardioselectivity, it has been studied as a potential parasympatholytic drug, particularly against bradycardia.

[00053] In some embodiments of all aspects described herein, the method for SMA comprises administering the agent that inhibits muscarinic acetylcholine receptor M2 to a subject in need thereof. In one embodiment, the subject whom the agent will be administered to will have been diagnosed as having a neuromuscular and/or synapse defect. A skilled clinician can diagnose a subject as being in need of treatment and/or as having neuromuscular and/or synapse defects using standard SMA diagnostic tests. Common methods for diagnosing neuromuscular or synaptic defects include, but are not limited to, the identification of hypotonia associated with absent reflexes, the use of electromyogram to identify fibrillation and muscle denervation, assessing serum from a patient for an increase in creatine kinase. In addition, genetic testing can be done to identify bi-allelic deletion of exon 7 of the *SMN1* gene and to establish the number of *SMN2* gene copies. In one embodiment, the method of administering the agent that inhibits muscarinic acetylcholine receptor M2 ameliorates symptoms and/or defects resulting from SMA. Specifically, neuromuscular defects and synaptic defects. Non-limiting examples of neuromuscular defects resulting from SMA include loss of motor neurons and muscle wasting. Synaptic defects in SMA models and/or patients are characterized by one skilled in the art as a synapse differing in morphology and/or function from a wild-type or control synapse. A non-limiting example of a synaptic defect would be a synapse that grows longer and/or more disorganized from a wild-type or control synapse. The efficacy of the agent in ameliorating neuromuscular and synaptic defects can be determined by one skilled in the art.

[00054] As used herein, “ameliorates symptoms and/or defects” is improving any defect or symptom associated with SMA. As compared with an equivalent untreated control, such reduction is by at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, 99% or more as measured by any standard technique.

[00055] In another embodiment, the method described herein further comprises administering to a subject in need thereof additional anti-SMA therapeutics and/or symptom management therapy.

[00056] Nusinersen (Spinraza) is currently the only drug approved to treat children (including newborns) and adults with SMA. Nusinersen is an 2'-O-methoxyethyl modified antisense oligonucleotide (ASO) designed to treat SMA caused by mutations in chromosome 5q that lead to SMN protein deficiency. Using *in vitro* assays and studies in transgenic animal models of SMA, nusinersen was shown to increase exon 7 inclusion in SMN2 messenger ribonucleic acid (mRNA) transcripts and production of

full-length SMN protein. This therapeutic is administered to a subject directly into the spinal canal via intrathecal administration.

[00057] Non-limiting examples of SMA symptom management include (1) Orthopaedic treatment. Weak spine muscles may lead to development of kyphosis, scoliosis and other orthopaedic problems. Spine fusion is sometimes performed in people with SMA1 and SMA2 once they reach the age of 8-10 to relieve the pressure of a deformed spine on the lungs. People with SMA might also benefit greatly from various forms of physiotherapy and occupational therapy. (2) Mobility support. Orthotic devices can be used to support the body and to aid walking. For example, orthotics such as AFO's (ankle foot orthosis) are used to stabilize the foot and to aid gait, TLSO's (thoracic lumbar sacral orthosis) are used to stabilize the torso. Assistive technologies may help in managing movement and daily activity, and greatly increase the quality of life. (3) Respiratory care and treatment. Respiratory system requires utmost attention in SMA as once weakened it never fully recovers. Weakened pulmonary muscles in people with SMA1 and SMA2 can make breathing more difficult and pose a risk of hypoxiation, especially in sleep when muscles are more relaxed. Impaired cough reflex poses a constant risk of respiratory infection and pneumonia. Non-invasive ventilation (BiPAP) is frequently used and tracheostomy may be sometimes performed in more severe cases; both methods of ventilation prolong survival in a comparable degree, although tracheostomy prevents speech development. (4) Nutritional therapy. Difficulties in jaw opening, chewing and swallowing food might put people with SMA at risk of malnutrition. A feeding tube or gastrostomy can be necessary in SMA1 and people with more SMA2. Additionally, metabolic abnormalities resulting from SMA impair β -oxidation of fatty acids in muscles and can lead to organic acidemia and consequent muscle damage, especially when fasting. It is suggested that people with SMA, especially those with more severe forms of the disease, reduce intake of fat and avoid prolonged fasting (i.e., eat more frequently than healthy people). (5) Cardiology treatment. Although the heart is not a matter of routine concern, a link between SMA and certain heart conditions has been suggested. (6) Mental health treatment. SMA children do not differ from the general population in their behaviour; their cognitive development can be slightly faster, and certain aspects of their intelligence are above the average.

Dosage forms and administration

[00058] The dosages to be administered can be determined by one of ordinary skill in the art depending on the clinical severity of the disease, the age and weight of the patient, the exposure of the patient to conditions that may precipitate outbreaks of psoriasis, and other pharmacokinetic factors generally understood in the art, such as liver and kidney metabolism. The interrelationship of dosages for animals of various sizes and species and humans based on mg/m^3 of surface area is described by E. J.

Freireich et al., "Quantitative Comparison of Toxicity of Anticancer Agents in Mouse, Rat, Hamster, Dog, Monkey and Man," *Cancer Chemother. Rep.* 50: 219-244 (1966). Adjustments in the dosage regimen can be made to optimize the therapeutic response. Doses can be divided and administered on a daily basis or the dose can be reduced proportionally depending on the therapeutic situation.

[00059] Typically, these drugs will be administered orally, and they can be administered in conventional pill or liquid form. If administered in pill form, they can be administered in conventional formulations with excipients, fillers, preservatives, and other typical ingredients used in pharmaceutical formations in pill form. Typically, the drugs are administered in a conventional pharmaceutically acceptable formulation, typically including a carrier. Conventional pharmaceutically acceptable carriers known in the art can include alcohols, e.g., ethyl alcohol, serum proteins, human serum albumin, liposomes, buffers such as phosphates, water, sterile saline or other salts, electrolytes, glycerol, hydroxymethylcellulose, propylene glycol, polyethylene glycol, polyoxyethylenesorbitan, other surface active agents, vegetable oils, and conventional anti-bacterial or anti-fungal agents, such as parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. A pharmaceutically-acceptable carrier within the scope of the present invention meets industry standards for sterility, isotonicity, stability, and non-pyrogenicity.

[00060] The pharmaceutically acceptable formulation can also be in pill, tablet, or lozenge form as is known in the art, and can include excipients or other ingredients for greater stability or acceptability. For the tablets, the excipients can be inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc, along with the substance for muscarinic acetylcholine receptor inhibition and other ingredients.

[00061] The drugs can also be administered in liquid form in conventional formulations that can include preservatives, stabilizers, coloring, flavoring, and other generally accepted pharmaceutical ingredients. Typically, when the drugs are administered in liquid form, they will be in aqueous solution. The aqueous solution can contain buffers, and can contain alcohols such as ethyl alcohol or other pharmaceutically tolerated compounds.

[00062] A variety of means for administering the compounds and compositions described herein to subjects are known to those of skill in the art. Such methods can include, but are not limited to oral, parenteral, intravenous, intramuscular, subcutaneous, transdermal, airway (aerosol), pulmonary, cutaneous, topical, or injection. Administration can be local or systemic.

[00063] In some embodiments, the inhibitory agent is administered intrathecally. The blood brain barrier is a highly selective semipermeable membrane barrier that separates the circulating blood from the brain extracellular fluid in the central nervous system (CNS). For therapeutics needed to be delivered to

the CNS, a skilled clinician can directly deliver a therapeutic to the spinal canal. For direct administration into the spinal canal, the compounds and compositions described herein will be administered via intrathecal administration by a skilled clinician. Intrathecal administration is a route of drug administration in which the drug is directly injected in the spinal canal or in the subarachnoid space, allowing it to directly reach the cerebrospinal fluid (CSF). Non-limiting examples of other drugs that are administered via intrathecal administration are spinal anesthesia, chemotherapeutics, pain management drugs, and therapeutics that cannot pass the blood brain barrier.

[00064] In one aspect described herein, a subject is administered a composition comprising an agent that inhibits muscarinic acetylcholine receptors (e.g., methoctramine) and a second agent that facilitates passage through the blood brain barrier. In one embodiment, the second agent is a pharmaceutically acceptable carrier that has the capacity to pass through the blood brain barrier.

[00065] In another embodiment, the second agent is a pharmaceutically acceptable nanoparticle that can pass through the blood brain barrier. An example of a nanoparticle that can pass through the blood brain barrier is radiolabeled polyethylene glycol coated hexadecylcyanoacrylate nanospheres.

[00066] In another embodiment, the second agent is a pharmaceutically acceptable compound that can permeabilize the blood brain barrier. Non-limiting examples of pharmaceutically acceptable compounds that permeabilize the blood brain barrier are RMP-7, histamine, leukotrienes, and 5-hydroxytryptamine.

[00067] In yet another embodiment, the second agent is a pharmaceutically acceptable peptide nucleic acid molecule. Peptide nucleic acid molecules promote influx of therapeutics, passing the therapeutic through the blood brain barrier. Peptide nucleic acid molecules are designed to mimic proteins known to pass through the blood brain barrier, e.g. casomorphin. A “peptide nucleic acid molecule” artificially synthesized polymer similar to DNA or RNA, ~20-25 base pairs long. Peptide nucleic acid molecules bind complementary DNA with high specificity.

[00068] The drugs can be administered from once per day to up to at least five times per day, depending on the severity of the disease, the total dosage to be administered, and the judgment of the treating physician. In the case of intrathecal administration, the drug can be administered once per treatment. In some cases, the drugs need not be administered on a daily basis, but can be administered every other day, every third day, or on other such schedules. However, it is generally preferred to administer the drugs daily.

[00069] Agents useful in the methods and compositions described herein can be administered topically, intravenously (by bolus or continuous infusion), orally, by inhalation, intraperitoneally, intramuscularly, subcutaneously, intracavity, and can be delivered by peristaltic means, if desired, or by other means known by those skilled in the art.

[00070] Therapeutic compositions containing the compound inhibiting muscarinic acetylcholine receptors can be conventionally administered in a unit dose. The term “unit dose” when used in reference to a therapeutic composition refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required physiologically acceptable diluent, *i.e.*, carrier, or vehicle.

[00071] A therapeutically effective amount is an amount of an agent that is sufficient to produce a statistically significant, measurable change in *e.g.*, muscle growth, etc. (see “Efficacy Measurement” below). Such effective amounts can be gauged in clinical trials as well as animal studies for a given inhibitory agent.

Efficacy measurement

[00072] The efficacy of a given treatment for SMA can be determined by the skilled clinician. However, a treatment is considered “effective treatment,” as the term is used herein, if any one or all of the signs or symptoms of, as but one example, muscle wasting are altered in a beneficial manner, other clinically accepted symptoms or markers of disease are improved or ameliorated, *e.g.*, by at least 10% following treatment with an inhibitor. Efficacy can also be measured by failure of an individual to worsen as assessed by hospitalization or need for medical interventions (*e.g.*, progression of the disease is halted or at least slowed). Methods of measuring these indicators are known to those of skill in the art and/or described herein. Treatment includes any treatment of a disease in an individual or an animal (some non-limiting examples include a human, or a mammal) and includes: (1) inhibiting the disease, *e.g.*, arresting, or slowing muscle atrophy; or (2) relieving the disease, *e.g.*, causing regression of symptoms, reducing the muscle waste; and (3) preventing or reducing the likelihood of the further muscle atrophy.

[00073] An effective amount for the treatment of SMA means that amount which, when administered to a mammal in need thereof, is sufficient to result in effective treatment as that term is defined herein, for that disease. Efficacy of an agent can be determined by assessing physical indicators of SMA, such as *e.g.*, synaptic function, muscle function, muscle size, etc.

[00074] The term “effective amount” as used herein refers to the amount of a compound or composition (*e.g.* methoctramine) described herein needed to alleviate at least one or more symptom of the disease or disorder, and relates to a sufficient amount of pharmacological composition to provide the desired effect. The term “therapeutically effective amount” therefore refers to an amount of a composition that is sufficient to provide a particular anti-SMA effect when administered to a typical subject. An effective amount as used herein, in various contexts, would also include an amount sufficient to delay the development of a symptom of the disease, alter the course of a symptom disease (for example but not

limited to, slowing the progression of a symptom of the disease), or reverse a symptom of the disease. Thus, it is not generally practicable to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation.

[00075] Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the active ingredient, which achieves a half-maximal inhibition of symptoms) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay, *e.g.*, synaptic function. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

Definitions

[00076] The term "gene" used herein can be a genomic gene comprising transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (*e.g.*, introns, 5'- and 3'- untranslated sequences and regulatory sequences). The coding region of a gene can be a nucleotide sequence coding for an amino acid sequence or a functional RNA, such as tRNA, rRNA, catalytic RNA, siRNA, miRNA and antisense RNA. A gene can also be an mRNA or cDNA corresponding to the coding regions (*e.g.* exons and miRNA) optionally comprising 5'- or 3' untranslated sequences linked thereto. A gene can also be an amplified nucleic acid molecule produced *in vitro* comprising all or a part of the coding region and/or 5'- or 3'- untranslated sequences linked thereto. The term "gene product(s)" as used herein refers to include RNA transcribed from a gene, or a polypeptide encoded by a gene or translated from RNA.

[00077] The terms "lower", "reduced", "reduction" or "decrease", "down-regulate" or "inhibit" are all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, "lower", "reduced", "reduction" or "decrease" or "inhibit" means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least

about 80%, or at least about 90% or up to and including a 100% decrease (i.e. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level. When “decrease” or “inhibition” is used in the context of the level of expression or activity of a gene or a protein, it refers to a reduction in protein or nucleic acid level or activity in a cell, a cell extract, or a cell supernatant. For example, such a decrease may be due to reduced RNA stability, transcription, or translation, increased protein degradation, or RNA interference.

[00078] The terms “up-regulate”, “increase” or “activate” are all used herein to generally mean an increase by a statically significant amount; for the avoidance of any doubt, the terms “up-regulate”, “increase” or “higher” means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or a 100% increase or more, or any increase between 10-100% as compared to a reference level, or an increase greater than 100%, for example, an increase at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. When “increase” is used in the context of the expression or activity of a gene or protein, it refers to a positive change in protein or nucleic acid level or activity in a cell, a cell extract, or a cell supernatant. For example, such an increase may be due to increased RNA stability, transcription, or translation, or decreased protein degradation. Preferably, this increase is at least 5%, at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 80%, at least about 100%, at least about 200%, or even about 500% or more over the level of expression or activity under control conditions. In some embodiments, an agent for inhibition of muscarinic acetylcholine receptor is a small-molecule as disclosed herein can inhibit muscarinic acetylcholine receptor. Preferably, this inhibition is at least about 5%, at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 80%, or even at least about 90% of the level of expression and/or activity under control conditions.

[00079] The terms “significantly different than”, “statistically significant,” and similar phrases refer to comparisons between data or other measurements, wherein the differences between two compared individuals or groups are evidently or reasonably different to the trained observer, or statistically significant (if the phrase includes the term “statistically” or if there is some indication of statistical test, such as a p-value, or if the data, when analyzed, produce a statistical difference by standard statistical tests known in the art).

[00080] The term “effective amount” is used interchangeably with the term “therapeutically effective amount” and refers to the amount of at least one agent, e.g., muscarinic acetylcholine receptor inhibitor of a pharmaceutical composition, at dosages and for periods of time necessary to achieve the desired

therapeutic result, for example, to reduce or stop at least one symptom of SMA, for example a symptom of decreased muscle mass, known as muscle wasting, in the subject. For example, an effective amount using the methods as disclosed herein would be considered as the amount sufficient to reduce a symptom of SMA by at least 10%. An effective amount as used herein would also include an amount sufficient to prevent or delay the development of a symptom of the disease, alter the course of a symptom disease (for example but not limited to, slow the progression of a symptom of the disease), or reverse a symptom of the disease. Accordingly, the term "effective amount" or "therapeutically effective amount" as used herein refers to the amount of therapeutic agent (e.g. at least one muscarinic acetylcholine receptor inhibitor as disclosed herein) of pharmaceutical composition to alleviate at least one symptom of SMA. Stated another way, "therapeutically effective amount" of a muscarinic acetylcholine receptor inhibitor as disclosed herein is the amount of a muscarinic acetylcholine receptor inhibitor which exerts a beneficial effect on, for example, the symptoms of SMA. The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factors, including pharmacokinetic properties of the muscarinic acetylcholine receptor inhibitor, the route of administration, conditions and characteristics (sex, age, body weight, health, size) of subjects, extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. A therapeutically effective amount is also one in which any toxic or detrimental effects of the therapeutic agent are outweighed by the therapeutically beneficial effects. The effective amount in each individual case can be determined empirically by a skilled artisan according to established methods in the art and without undue experimentation. In general, the phrases "therapeutically-effective" and "effective for the treatment, prevention, or inhibition", are intended to qualify the muscarinic acetylcholine receptor inhibitor as disclosed herein which will achieve the goal of reduction in the severity of at least one symptom of SMA.

[00081] As used herein, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with, a disease or disorder. The term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with SMA. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but can also include a cessation or at least slowing of progress or worsening of symptoms that would be expected in absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s) of a malignant disease, diminishment of extent of a malignant disease, stabilized (i.e., not worsening) state of a malignant disease, delay or slowing of progression of a malignant disease, amelioration or palliation of the malignant disease state, and remission (whether partial or total), whether detectable or

undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[00082] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00083] The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation, for example the carrier does not decrease the impact of the agent on the treatment. In other words, a carrier is pharmaceutically inert. The terms "physiologically tolerable carriers" and "biocompatible delivery vehicles" are used interchangeably.

[00084] The terms "administered" and "subjected" are used interchangeably in the context of treatment of a disease or disorder. Both terms refer to a subject being treated with an effective dose of pharmaceutical composition comprising muscarinic acetylcholine receptor inhibitor of the invention by methods of administration such as parenteral or systemic administration.

[00085] The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection, infusion and other injection or infusion techniques, without limitation. The phrases "systemic administration," "administered systemically", "peripheral administration" and "administered peripherally" as used herein mean the administration of a pharmaceutical composition comprising at least an muscarinic acetylcholine receptor inhibitor as disclosed herein such that it enters the animal's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

[00086] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) below normal, or lower, concentration of the marker. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value.

[00087] The term “optional” or “optionally” means that the subsequent described event, circumstance or substituent may or may not occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

[00088] As used herein, the term “comprising” means that other elements can also be present in addition to the defined elements presented. The use of “comprising” indicates inclusion rather than limitation. The term “consisting of” refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment. As used herein the term “consisting essentially of” refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00089] Agents inhibiting muscarinic acetylcholine receptors can also include nucleic acids. For example, nucleic acids that can bind with and reduce or inhibit expression of a nucleic acid encoding the receptor. Without wishing to be bound by a theory, reduction or inhibition of the expression can inhibit activity muscarinic acetylcholine receptor, resulting in an increase in miR-128. Exemplary nucleic acid for reducing or inhibiting expression of a muscarinic acetylcholine receptor include, but are not limited to, small interfering RNAs (siRNAs) and antisense oligonucleotides.

[00090] The term “siRNA” refers to any non-endogenous and synthetic RNA duplex designed to specifically target a particular mRNA for degradation. Accordingly, “siRNA” refers to an RNA capable of down-regulating its target expression level via activation of the DICER complex. The term “mRNA” refers to a nucleic acid transcribed from a gene from which a polypeptide is translated, and can include non-translated regions such as a 5'UTR and/or a 3'UTR. An siRNA can include a 21 base-pair nucleotide sequence that is completely complementary to any sequence of an mRNA molecule, including translated regions, the 5'UTR, the 3'UTR, and sequences that include both a translated region and a portion of either 5'UTR or 3'UTR

[00091] The term “oligonucleotide” as used herein refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof, as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. An oligonucleotide preferably includes two or more nucleomonomers covalently coupled to each other by linkages (*e.g.*, phosphodiesters) or substitute linkages. The term “antisense oligonucleotides” refers a 15-20 base-pair polymer comprising chemically-modified deoxynucleotides. Its sequence in antisense (3'-5') such that it is complementary to its target mRNA. Accordingly, “antisense

oligonucleotides” refers to a polymer that, upon mRNA binding prevents synthesis of the target and promotes degradation of the target.

[00092] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00093] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[00094] All patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

EXAMPLES

[00095] Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease and the leading genetic cause of infant death in the US (Cusin et al., 2003; Pearn, 1978). SMA is caused by homozygous deletion or mutation of the *SMN1* (*Survival Motor Neuron 1*) gene, resulting in reduced Survival of Motor Neuron (SMN) protein levels (Lefebvre et al., 1995). SMN expression is ubiquitous, but particularly essential for motor neuron survival (Lefebvre et al., 1997). Disease severity, as well as spinal cord α -MN dysfunction and degeneration, correlates with the extent of SMN loss (Lefebvre et al., 1997). Understanding why SMN loss impairs function should offer insight into SMA and may reveal therapeutic targets. SMN is conserved across species (Miguel-Aliaga et al., 1999). Studies of various SMA models suggest a role for SMN in several cellular processes including snRNP assembly (Golembe et al., 2005; Yong et al., 2002), messenger RNA (mRNA) transport (Fallini et al., 2011), and local translation (Dimitriadi et al., 2010; Kye et al., 2014). SMN function, however, has not been linked definitively to MN degeneration or synaptic transmission defects caused by SMN loss.

[00096] microRNAs (miRNAs) are non-coding RNAs that often repress protein translation, by a mechanism that requires miRNA binding to the 3'UTR of mRNA targets. Disruption of the miRNA

pathway in spinal MNs leads to severe degeneration (Haramati et al., 2010). SMN loss alters levels and/or activity of specific miRNAs (Haramati et al., 2010; Kye et al., 2014; Valsecchi et al., 2015; Wang et al., 2014), but the cellular mechanisms leading to altered miRNA expression and/or function are unknown. The RNA helicase Gemin3 associates with both SMN and RNA-induced silencing complex components (Charroux et al., 1999; Hock et al., 2007; Hutvagner and Zamore, 2002; Meister et al., 2005; Mourelatos et al., 2002; Murashov et al., 2007). Gemin3 and SMN levels decrease concomitantly, indicative of a functional link (Feng et al., 2005; Helmken et al., 2003).

[00097] *C. elegans* SMA model was used to examine the connection between SMN, Gemin3, and miRNA function. *SMN1*, *Gemin3*, and multiple miRNA pathway components are conserved in *C. elegans* (Grishok et al., 2001; Miguel-Aliaga et al., 1999; Minasaki et al., 2009). Loss-of-function (*lf*) mutations in *smn-1*, the *C. elegans* ortholog of *SMN1*, cause behavioral and morphological abnormalities, premature death, and sterility (Briese et al., 2009; Sleight et al., 2011). *smn-1(lf)* animals also have neuromuscular junction (NMJ) defects, indicating a functional role for SMN-1 in MNs (Briese et al., 2009). MNs in *smn-1(lf)* animals do not die, likely because of their short lifespan. However, *smn-1(lf)* neuromuscular defects may correspond to the early stages of SMA pathogenesis, characterized by NMJ dysfunction prior to MN degeneration (Miguel-Aliaga et al., 1999; Yoshida et al., 2015). The *C. elegans* Gemin3 ortholog, MEL-46, is perturbed by SMN-1 loss, impacting miR-2 suppression of the M2 muscarinic receptor ortholog, GAR-2 (Lee et al., 2000). Across species in SMA mouse model MNs, levels of miR-128 decreased, a potential miR-2 ortholog, and increased expression of the GAR-2 ortholog, m2R. Notably, m2R inhibition ameliorates axon outgrowth defects in mouse SMA MNs, consistent with findings in *C. elegans*.

[00098] *MEL-46 (Gemin3) is required for NMJ function.*

[00099] The *C. elegans* Gemin3 ortholog is MEL-46. Homozygous loss of *smn-1* or *mel-46* results in lethality (Briese et al., 2009; Miguel-Aliaga et al., 1999), but maternal loading of *smn-1* or *mel-46* mRNA and protein allows many homozygous, loss of function animals to survive into the last larval stage, called L4 (Miguel-Aliaga et al., 1999; Minasaki et al., 2009). Loss of *smn-1* results in neuromuscular defects including decreased pharyngeal pumping rates, followed by overtly altered locomotion and subsequent death (Briese et al., 2009). Like *smn-1* loss in L4 stage animals, *mel-46(tm1739)* homozygous loss of function animals had severely decreased pharyngeal pumping rates. Pharyngeal pumping was restored to normal rates in *mel-46(tm1739)* animals using a previously described, broadly expressed *mel-46* rescue array (also referred to as [*mel-46(+)*#1]), which utilizes the *mel-46* promoter (FIG. 1A) (Minasaki et al., 2009). *mel-46* partial loss of function alleles, *yt5* and *ok3760*, also caused pumping defects as did global *mel-46* RNA inhibition (*RNAi*) or cholinergic neuron-specific *mel-46(RNAi)* (FIG. 2A-2E). Thus, MEL-46 is necessary for normal neuromuscular function.

[000100] SMN-1 is required for normal NMJ function in *C. elegans* cholinergic MNs (Dimitriadi et al., 2016). Aldicarb is an acetylcholinesterase inhibitor that leads to acetylcholine accumulation in the NMJ and consequently, paralysis (Mahoney et al., 2006). The time course of aldicarb-induced paralysis was slowed by decreased SMN-1 activity (Dimitriadi et al., 2016). It was determined if a decrease in MEL46 function causes similar resistance to aldicarb and found that *mel-46* loss of function resulted in aldicarb resistance across multiple alleles (FIG. 1B; FIGs. 2F and 2G), reminiscent of *smn-1* loss. Reintroduction of *mel-46* using the [*mel-46(+)*#1] rescue array restored aldicarb sensitivity in *mel-46(tm1739)* animals. Tissue-specific knock-down of *mel-46* in cholinergic neurons resulted in aldicarb resistance, thus confirming that MEL-46 function is required in cholinergic neurons, as is SMN-1 (FIG. 1C) (Dimitriadi et al., 2016). In addition, knock-down of *mel-46* or *smn-1* in inhibitory GABAergic neurons resulted in aldicarb hypersensitivity (FIG. 2H). Taken together with previous work, this shows that MEL-46 and SMN-1 are required in both cholinergic and GABAergic neurons for normal NMJ function.

[000101] *smn-1* loss causes changes in presynaptic protein localization (Dimitriadi et al., 2016). Do similar changes occur in *mel-46(tm1739)* animals? The localization of presynaptic proteins SNB-1 (synaptobrevin) and APT-4 (AP2 α -adaptin) were examined in cholinergic dorsal A-type (DA) MNs of *mel-46(tm1739)* animals (Ch'ng et al., 2008; Sieburth et al., 2005). SNB-1 is a v-SNARE protein required for SV exocytosis, while APT-4 associates with clathrin-coated endocytic vesicles (Kamikura and Cooper, 2006; Nonet et al., 1998). In the dorsal cord, cholinergic DA MNs do not have presynaptic inputs; they form *en passant* presynaptic connections in a punctate pattern (Ch'ng et al., 2008; White et al., 1976). Three parameters were measured to evaluate fluorescently labeled SNB-1 and APT-4 localization to presumptive synapses: puncta width (μ m), intensity (AU), and linear density (puncta/ μ m), as previously described (Kim et al., 2008). Loss of *smn-1* or *mel-46* resulted in similar SNB-1 synaptic localization defects: decreased SNB-1 puncta width, intensity and linear density (FIGs. 1D-1G) (Dimitriadi et al., 2016). Loss of *smn-1* leads to decreased APT-4 puncta width and intensity, but increased linear density (Dimitriadi et al., 2016). *mel-46(tm1739)* animals also had increased APT-4 linear density, but no changes in puncta width or intensity compared to controls (FIGs. 3A-3C). Therefore, decreased *mel-46* causes synaptic protein defects that overlap partially with defects observed when SMN-1 levels decrease. Given the similarities between SMN-1 and MEL-46 loss in aldicarb resistance, decreased pharyngeal pumping rates, and defective synaptic protein localization, whether SMN-1 and MEL-46 act in common pathways required for NMJ function was explored.

[000102] *Perturbed MEL-46 (Gemin3) function likely contributes to synaptic defects in smn-1(lf) animals.*

[000103] MEL-46 might act together with or downstream of SMN-1 in pathways necessary for NMJ function. To test these and other possibilities, integrated multicopy transgenic lines expressing GFP-tagged MEL-46 expressed under control of the *unc-17* cholinergic-specific promoter were generated (FIG. 4A). MEL-46::GFP was found in both the cell bodies and processes of neurons. No obvious changes were seen in cytoplasmic MEL-46::GFP, leading to evaluation of the MEL-46::GFP localization in MN dorsal cord processes in *smn-1(ok355)* animals. Because *ok355* deletion in *smn-1* leads to a complete loss of function, *smn-1(ok355)* animals were maintained over an *hT2* balancer and sterile *smn-1(ok355)* homozygous progeny carry some maternally-loaded SMN-1 protein (Briese et al., 2009). It was found that MEL-46::GFP localizes to small granular structures in dorsal cord processes in control (*smn-1(+)*) and *smn-1(ok355)* animals. This finding is consistent with previous work showing that Gemin3 localizes to granular structures in mammalian neurites; Gemin3 co-localizes with SMN in 50-60% of these granules, along with multiple mRNAs (Todd et al., 2010a; Todd et al., 2010b; Zhang et al., 2006). In *smn-1(ok355)* animals, it was identified that the density of MEL-46::GFP-positive granular structures was doubled compared to *smn-1(+)* controls (FIGs. 4B-4D). Furthermore, the mean intensity of MEL-46::GFP fluorescence and the maximum fluorescence for each sample were decreased in *smn-1(ok355)* animals (FIGs. 4B-4D; FIG. 5A). These results indicate that decreased SMN-1 leads to MEL-46 mislocalization in cholinergic MN processes and diminished MEL-46 levels in granules. These findings indicate that SMN-1 impairs MEL-46 function, which could contribute to *smn-1(ok355)* synaptic defects (Dimitriadi et al., 2016). To test this hypothesis, *mel-46* gene dosage in *smn-1(ok355)* animals was increased using the [*mel-46(+)*]*#1*] rescue array and showed that this ameliorated *smn-1(ok355)* aldicarb resistance defects (FIG. 4E). Increasing *mel-46* specifically in cholinergic neurons, using the cholinergic-specific *unc-17* (ACh) promoter in an integrated array, referred to as [ACh::*mel-46*::GFP], rescued *smn-1(ok355)* aldicarb resistance (FIGs. 5B and 5C). The aldicarb resistance observed with broad expression of *mel-46* in control animals (FIG. 4E) was not observed when *mel-46* was overexpressed in cholinergic neurons only. Under this condition mild hypersensitivity was observed in one integrated line (referred to as [ACh::*mel-46*::GFP*#1*]) (FIG. 5C) and no difference from control animals in a second line (referred to as [ACh::*mel-46*::GFP*#2*]) (FIG. 5B). High levels of MEL-46 in cholinergic neurons may cause aldicarb hypersensitivity, whereas broad overexpression of MEL-46 may impact NMJ function independent of cholinergic neurons. Taken together, these results indicate that loss of SMN-1 negatively impacts MEL-46 function, resulting in perturbed NMJ signaling. These findings are consistent with observations in humans that reduced human SMN levels result in Gemin3 downregulation (Feng et al., 2005; Helmken et al., 2003),

[000104] The interdependency reported herein, between SMN-1 and MEL-46 levels, can indicate specificity to specific tissues and/or neural circuits. For example, pharyngeal pumping rate defects were

not ameliorated in *smn-1(ok355)* animals by increased *mel-46* levels ([*mel-46(+)*#1] rescue array, (FIG. 5D), indicating a privileged relationship between SMN-1 and MEL-46 in cholinergic NMJ signaling. Increasing *mel-46* did rescue *smn-1(ok355)* synaptic protein localization defects. Using the [*mel-46(+)*#1] rescue array, *smn-1(ok355)* defective SNB-1 puncta width and intensity were rescued to normal levels, but did not ameliorate linear density defects (FIGs. 4F-4J). Notably, increased *mel-46* in an *smn-1(+)* control background did not increase SNB-1 levels, indicating that *mel-46*-induced up-regulation of SNB-1 is specifically beneficial in a *smn-1(ok355)* background. Increasing *mel-46* with a second broadly-expressed *mel-46* rescue array line, [*mel-46(+)*#2], also restored APT-4 puncta linear density to normal levels (FIG. 5E), without rescuing other metrics. These results are consistent with the aldicarb/NMJ functional rescue studies presented here and indicate increasing *mel-46* improves neuromuscular signaling in *smn-1(ok355)* animals by partially restoring levels and localization of synaptic proteins. Furthermore, these results determine that *mel-46* may act with or downstream of *smn-1* in a pathway essential for NMJ function.

[000105] Alternatively, the possibility that increasing *mel-46* stabilizes maternally-loaded SMN-1 protein and mRNA was considered, a scenario which can also explain *mel-46* rescue of *smn-1(ok355)* NMJ function. Since mammalian Gemin3 directly binds SMN (Charroux et al., 1999), increasing MEL-46 (Gemin3) can decrease the rate of SMN-1 loss. To test this possibility, CRISPR/Cas9-targeted genome editing was used to insert GFP coding sequences at the N-terminus of *smn-1* on chromosome I, resulting in fluorescent SMN-1 protein (FIG. 6A) (Dickinson et al., 2015). The GFP-tagged protein was functional; animals were viable and fertile. It was found that increasing *mel-46* using the [*mel-46(+)*#2] rescue array did not increase GFP::SMN-1 levels, but unexpectedly caused a modest overall decrease (FIGs. 6B and 6C). It therefore seems unlikely that *smn-1(ok355)* rescue by increased *mel-46* is due to stabilization of maternally-loaded SMN-1.

[000106] *C. elegans* miR-2 is required for NMJ function

[000107] The pathways in MNs downstream of SMN and Gemin3 that are linked to SMA are unknown. Herein, a role for these two proteins in miRNA regulation was considered. As mammalian Gemin3 co-localizes and co-purifies with RISC pathway components (Hock et al., 2007; Hutvagner and Zamore, 2002; Meister et al., 2005; Mourelatos et al., 2002; Murashov et al., 2007), a role for miRNA regulation in NMJ function was considered. miRNA miR-2 is enriched in neurons, expressed at all developmental stages, and predicted to regulate expression of many proteins involved in neuronal development and function (Marco et al., 2012; Martinez et al., 2008). Without wishing to be bound by theory, it is hypothesized that miR-2 is necessary for proper NMJ function and that it might be perturbed by loss of either SMN-1 or MEL-46. To test this possibility, the aldicarb response of *mir-2(lf)* animals was examined. Two different deletion alleles, *gk259* and *n4108*, caused resistance to aldicarb paralysis

compared to wild type animals (FIG. 7A; FIG. 8A). This defect was partially rescued by expressing miR-2 under the control of a the *unc-17* (ACh) cholinergic neuron-specific promoter (referred to as ([ACh::miR-2(+)])) (FIG. 7A). Loss of miR-2 also resulted in a mild pharyngeal pumping defect (FIG. 8B). Taken together, these findings conclude that miR-2 is required in cholinergic neurons for proper NMJ signaling.

[000108] As a first step towards evaluating how miR-2 loss impacts cholinergic MN presynaptic function, the effects of miR-2 loss on localization of presynaptic proteins in DA MNs was examined. Four fluorescently-tagged presynaptic proteins were examined: SNB-1 (synaptobrevin), SYD-2 (α -liprin), ITSN-1 (DAP160/Intersectin), and APT-4 (AP2 α -adaptin). Analysis of tagged SNB-1 in *mir-2(gk259)* animals revealed increased SNB-1 puncta linear density, but no change in puncta width or intensity compared to wild type animals (FIGs. 7B-7D). Additionally, *mir-2(gk259)* animals were indistinguishable from wild type control animals with respect to SYD-2 synaptic localization for all metrics analyzed (FIG. 8C), indicating that pre-synaptic active zones are unchanged in number and size; thus, synaptic changes are likely not the result of altered active zone number or size (Zhen and Jin, 1999). ITSN-1 puncta width and intensity, but not linear density, were decreased in *mir-2(gk259)* and *mir-2(n4108)*, animals compared to wild type controls (FIGs. 8D-8F). Finally, both *mir-2(gk259)* and *mir-2(n4108)* had decreased APT-4 puncta width, intensity, and linear density (FIGs. 8G-8I). ITSN-1, similar to APT-4, is involved in vesicle recycling at the NMJ (Wang et al., 2008). Together with results from aldicarb resistance studies, these results determine that loss of miR-2 results in synaptic dysfunction at the NMJ, consistent with decreased cholinergic synaptic release (Ch'ng et al., 2008; Sieburth et al., 2005). Additionally, a considerable overlap between synaptic protein localization defects resulting from miR-2 loss with those of *smn-1(ok355)* animals (Dimitriadi et al., 2016) was observed, a finding consistent with miR-2 and SMN-1 acting in partially redundant pathways at the NMJ.

[000109] *C. elegans* miR-2 targets *gar-2* mRNA in cholinergic neurons.

[000110] To address mechanistically how miR-2 loss impacts NMJ function, mRNA targets of miR-2 were searched. Canonically, miRNA loss results in overexpression of direct mRNA targets (Elbashir et al., 2001). Since miR-2 loss leads to aldicarb resistance, loss of the target(s) is expected to cause hypersensitivity to aldicarb. Following a literature search for genes whose loss of function results in hypersensitivity, the following genes with putative miR-2 3'UTR binding sites for study were identified: *gar-2*, *dbl-1*, *sek-1*, and *vab-2* (Jan et al., 2011; Lewis et al., 2005; Paraskevopoulou et al., 2013; Reczko et al., 2012; Vashlishan et al., 2008). Loss of a *bona fide* target gene is predicted to suppress aldicarb resistance caused by miR-2 loss. Therefore, a deletion allele for each gene was crossed into the *mir-2(gk259)* background. Loss of any of these four genes suppressed *mir-2(gk259)* to some extent, but *gar-2(ok520)*, which contains a large deletion removing *gar-2* exons 6 and 7, resulted in the most complete suppression, thus indicating that GAR-2 acts downstream of miR-2 (FIG. 9A; FIGs. 10A-10C). GAR-2 is

a G protein-coupled acetylcholine receptor orthologous to the mammalian M2 muscarinic receptor (m2R) (Lee et al., 2000).

[000111] To determine if miR-2 regulates GAR-2 expression directly, the consequences of perturbing the putative miR-2 binding site in the *gar-2* 3'UTR was examined. Using CRISPR/Cas9-targeted genome editing, the 18 base pair *gar-2* 3'UTR region corresponding to the endogenous miR-2 binding site was scrambled (FIG. 9B). Compared to control animals (*gar-2* UTRwt^C) carrying the disrupted PAM site mutation (C>T), it was found that animals with the scrambled miR-2 binding site (*gar-2* UTRscr^C) were resistant to aldicarb, similar to miR-2 loss (FIG. 9C). To evaluate whether disruption of the miR-2 binding site influenced *gar-2* transcript levels, *gar-2* mRNA levels in *gar-2* UTRscr^C animals and *gar-2* UTRwt^C controls were compared. A 40% increase in *gar-2* transcript in *gar-2* UTRscr^C was found (FIG. 9D). Without wishing to be bound by theory, disruption of the 3'UTR site likely inhibits binding of other miR-2 family members, possibly contributing to the effect observed (Ibanez-Ventoso et al., 2008).

[000112] An *in vivo* GFP reporter analysis of GAR-2 expression determined effects of miR-2 loss on GAR-2 function in cholinergic neurons. A construct encoding GFP with a *gar-2* 3'UTR, whose expression is driven under the control of a cholinergic-specific promoter (referred to as *unc-17p*-ACh::GFP::*gar-2* 3'UTRwt), was generated. A second control version of the construct contained the same scrambled UTR sequence as used in *gar-2* UTRscr^C animals (referred to as *unc-17p*-ACh::GFP::*gar-2* 3'UTRscr) (FIG. 9E). Transgenic lines were created by multicopy insertion for each construct. Increased GFP levels were observed in *mir-2(gk259)* animals expressing the intact 3'UTR construct as compared to control animals (FIG. 9F; FIG. 11A). Whereas, loss of the *mir-2* gene did not affect GFP levels in animals expressing the scrambled 3'UTR construct compared to control (FIG. 9G; FIGs. 11B and 11C). To quantify impact of miR-2 on expression of these GFP reporters in various genetic backgrounds, relative changes in GFP levels of transgenic lines were compared using a ratiometric strategy; GFP expression for lines carrying *unc-17p*-ACh::GFP::*gar-2* 3'UTRwt were determined and compared these values to lines carrying *unc-17p*-ACh::GFP::*gar-2* 3'UTRscr in the same genetic background (FIG. 11D). By this method, a ~13% increase in relative GFP expression associated with loss of miR-2 (FIG. 9H) was observed, indicating that miR-2 directly suppresses translation of *gar-2* mRNA by binding the *gar-2* 3'UTR at this 3'UTR site. The effect of miR-2 loss on *gar-2* transcript levels was assessed and no significant difference in *gar-2* mRNA levels between wild type animals and *mir-2(gk259)* loss of function animals was found (FIG. 9I). These results, combined with the reporter data, indicate that miR-2 binds and inhibits *gar-2* mRNA translation, but does not reduce transcript levels. Previous studies have reported that miRNAs can influence protein synthesis of targets without destabilizing mRNA levels (Cloonan, 2015; Selbach et al., 2008).

[000113] *miR-2 suppression of gar-2 is disrupted by smn-1 loss*

[000114] Next, it was determined if *smn-1* loss altered miR-2 regulation of the *gar-2* 3'UTR. Both GFP reporter transgenes were crossed into the *smn-1(ok355)* background. Using the same ratiometric strategy from FIG. 9H, a small increase in relative GFP reporter expression (~5%) in *smn-1(ok355)* animals compared to *smn-1(+)* controls was observed (FIG. 12A; FIGs. 13A and 13B). This finding indicated that *smn-1(ok355)* animals may have decreased miR-2 function and, as a consequence, an increase in GAR-2 translation. Increased *mel-46* levels ameliorates *smn-1(ok355)* NMJ defects (FIG. 4; FIG. 5), assessed the impact of increased *mel-46* on miR-2 activity using the [*mel-46(+)*#2] rescue array was assessed. In control *smn-1(+)* animals, increasing *mel-46* did not alter relative expression of the miR-2 GFP reporter. However, in *smn-1(ok355)* animals, increasing *mel-46* caused decreased relative reporter expression by ~15%, compared to *smn-1(ok355)* animals lacking the *mel-46* rescue array (FIG. 12A; FIGs. 13A and 13B). These data indicate that MEL-46 overexpression decreases GAR-2 levels in *smn-1(ok355)* by increasing miR-2 activity.

[000115] Increased GAR-2 translation in animals lacking SMN-1 might be due to decreased mature miR-2 levels. To test this possibility, quantitative RT-PCR studies were used. After neuron-specific RNAi knock-down of either SMN-1 or MEL-46, decreases in mature miR-2 levels were found (FIG. 12B; FIGs. 13C and 13D), but no change in *gar-2* transcript levels (FIG. 12C). This result is consistent with the finding that *gar-2* transcript levels were unchanged in miR-2 complete loss conditions (FIG. 9I), despite alterations in GFP reporter expression (FIG. 9H). These results indicate neuronal miR-2 levels are decreased when MEL-46 or SMN-1 levels decrease.

[000116] Collectively, the data above are consistent with a model where diminished SMN-1 leads to decreased miR-2 levels and activity, resulting in increased GAR-2 expression. Since M2 receptors inhibit synaptic release at cholinergic NMJs across species (Dittman and Kaplan, 2008; Parnas et al., 2005; Slutsky et al., 2003), overexpression of these receptors in *smn-1(ok355)* MNs can contribute to the NMJ defects previously observed in these animals (Dimitriadi et al., 2016; Sleight et al., 2011). Furthermore, without wishing to be bound by a particular theory, increased MEL-46/Gemin3 might have an ameliorative effect in animals lacking SMN-1 by stimulating miR-2 activity, thus decreasing GAR-2 levels and disinhibiting cholinergic release.

[000117] *gar-2 loss ameliorates smn-1(lf) neuromuscular defects*

[000118] If increased GAR-2 levels exacerbate NMJ dysfunction in animals lacking SMN-1, then decreasing GAR-2 function should ameliorate the NMJ defects caused by *smn-1* loss of function. Loss of GAR-2 did not improve pharyngeal pumping rates in *smn-1(ok355)* animals (FIG. 14A), similar to the results in animals with increased *mel-46* levels (FIG. 5D). However, similar to increasing *mel-46*, *gar-2(ok520)* restored normal response to aldicarb in both *smn-1(ok355)* and *smn-1(rt248)* animals (FIG.

15A; FIG. 14B), consistent with improved NMJ function. The *rt248 smn-1* allele causes a frameshift and loss of SMN-1 function similar to *ok355* (Dimitriadi et al., 2016). Additionally, *gar-2(ok520)* restored normal response to aldicarb in *mel-46(tm1739)* animals (FIG. 15B). The results indicate that decreasing GAR-2 likely improves presynaptic function in animals with decreased SMN-1 or MEL-46. Consistent with this conclusion, *gar-2(ok520)* also rescued numerous presynaptic protein localization defects caused by SMN-1 loss; SNB-1 puncta width, intensity and linear density defects were rescued in both *smn-1(ok355)* and *smn-1(rt248)* backgrounds (FIGs. 15C-15G; FIG. 14H). GAR-2 loss in *smn-1(+)* control animals resulted in increased SNB-1 puncta width and intensity, but did not alter SNB-1 puncta linear density.

[000119] Research in vertebrates has demonstrated m2R internalization normally occurs in response to chronic m2R stimulation, either by pharmacological agonist application or acetylcholinesterase inhibition (Clancy et al., 2007). Since endocytosis is defective in animals lacking SMN-1, perturbed endocytosis, in combination with miRNA misregulation, could contribute to GAR-2 accumulation at the membrane leading to decreased SNB-1 in *smn-1(ok355)* motor neurons (Dimitriadi et al., 2016). Loss of GAR-2 did not rescue *smn-1(ok355)* APT-4 puncta defects (FIG. 14C), but decreased APT-4 puncta width and intensity in *smn-1(+)* control animals. As loss of GAR-2 did not restore APT-4 synaptic defects, it was concluded that there are additional pathways affected by *smn-1* loss, beyond GAR-2 misregulation. Nevertheless, these results indicate that *C. elegans* GAR-2 levels are increased by *smn-1* loss, which might contribute to NMJ defects in *smn-1(lf)* animals.

[000120] The GAR-2 mammalian ortholog, m2R, is increased in SMA mouse model motor neurons.

[000121] The closest human ortholog of GAR-2 is the M2 muscarinic receptor (m2R), encoded by the *CHRM2* gene. GAR-2 and m2R are functionally conserved, as activation of these presynaptic receptors by acetylcholine in different species results in hyperpolarization and decreased NMJ acetylcholine release across species (Dittman and Kaplan, 2008; Dudel, 2007; Parnas et al., 2005; Slutsky et al., 2003). Previous research suggests decreased SMN function across species might impact miRNA activity across species, which could increase m2R levels consistent with work reported herein in *C. elegans*. The *CHRM2* mRNA is a predicted target of miR-128 in mice and humans (FIG. 16A) (Jan et al., 2011; Lewis et al., 2005; Paraskevopoulou et al., 2013). Without wishing to be bound by a particular theory, based on results from *C. elegans* it was predicted that vertebrate SMN loss might disrupt miR-128 activity, also leading to increased m2R. m2R protein levels were examined in MNs isolated from E13.5 SMA mice (*Smn*^{-/-}; *SMN2*^{tg0}). A ~50% increase in m2R levels was observed, compared to wild type control MNs (FIGs. 16B and 16C). miR-128 levels in SMA mouse MNs were decreased compared to wild type (FIG. 16D). Combined, these results indicate that diminished SMN protein causes decreased levels of mature miR-128, thus disinhibiting m2R expression in MNs across species.

[000122] *Inhibition of m2R by methoctramine rescued axon outgrowth defects in SMA mouse model MNs*

[000123] Decreased SMN levels results in axon outgrowth defects in MNs derived from SMA model mice (Rossoll et al., 2003) and increased m2R can contribute to this functional defect. To test this, the impact of m2R pharmacological inhibition on axon length for DIV5 MNs from E13.5 wild type (FVB) and SMA mice (*Smn*^{-/-}; *SMN2*^{tg/0}) were examined (FIG. 16E). Wild type and SMA MNs were cultured in the presence of 50nm or 500nm methoctramine, an m2R antagonist. In wild type MNs, methoctramine decreased mean longest axon length. Conversely, methoctramine treatment in SMA MNs increased both mean longest axon length and total axon length (FIG. 16E; FIG. 17A). This concludes that m2R inhibition rescues MN axon outgrowth defects in a SMA mouse model, consistent with a deleterious impact of increased m2R activity in SMA model MNs.

[000124] *Materials and Methods*

[000125] *C. elegans strains, constructs and transgenes.* Strains listed in Supplementary File 2a were maintained under standard conditions at 25°C (Brenner, 1974); complete genotypes with unique strain identifiers were provided, consistent with the rigorous standards of the *C. elegans* community. Abbreviated names are sometimes used for arrays, integrated lines or alleles in Figures 1-5; additional information about abbreviations can be found in Supplementary File 2c. For experiments with *smn-1(ok355)* and *smn-1(rt248)*, animals assayed were first generation progeny of hermaphrodites heterozygous for the *hT2* balancer. To maintain a common genetic background, control *smn-1(+)* animals were also derived from *+/hT2* parents. Similarly, for APT-4::GFP synaptic localization (Figure 2 – figure supplement 1E) and aldicarb response studies (Figure 6B), *mel-46(tm1739)* animals were first generation progeny of parents heterozygous for the *nT1* balancer. Control *mel-46(+)* animals were derived from *+/nT1* animals. For all other assays involving *mel-46(tm1739)*, animals were first generation progeny of parents carrying the *ytEx211[mel-46(+)]* rescue array; animals tested did not carry the array unless specified. For these experiments, N2 animals served as wild type controls.

[000126] The pHA#756 (*unc-17p::mir-2::unc-54 3'UTR*) plasmid was generated by excising a 867 bp fragment from pHA#755 (*aex-3p::mir-2::unc-54 3'UTR*) using *NheI* and *SpeI*. This fragment, containing the genomic *mir-2* pre-miRNA sequence along with *unc-54 3'UTR* sequence, was subcloned into pPD95.77 (pPD95.77 was a gift from Andrew Fire; Addgene plasmid 1495) between *NheI* and *SpeI* sites, resulting in removal of the GFP sequence. Additionally, a 4466 bp fragment corresponding to the *unc-17* promoter was inserted between pPD95.77 *SphI* and *AscI* sites. Information for all amplification primers can be found in Supplementary File 2b. pHA#757 (*unc-17p::GFP::unc-54 3'UTR*) was generated by inserting the *unc-17* promoter fragment between pPD95.77 *SphI* and *AscI* sites, without altering the GFP sequence. Plasmid pHA#758 (NLS::GFP::*gar-2 3'UTR*wt) contains a 269 bp fragment

corresponding to the *gar-2* 3'UTR that was subcloned into pPD95.67 (pPD95.67 was a gift from Andrew Fire; Addgene plasmid 1490) as a EcoRI and SpeI product. pHA#759 (*unc-17p::NLS::GFP::gar-2* 3'UTRwt) was generated by excising a 1286 bp fragment containing the NLS::GFP sequence and *gar-2* 3'UTR from pHA#758 using MscI and SpeI and ligating this fragment into pHA#756, thus removing the genomic *mir-2* pre-miRNA and *unc-54* 3'UTR sequences. pHA#760 was generated by ligating the *gar-2* 3'UTR fragment into pBluescript KS+ (Stratagene) using EcoRI and SpeI. To construct pHA#761, the last 85 bp of the *gar-2* 3'UTR were removed from pHA#760 using NcoI and SpeI. This fragment was replaced with an identical 85bp sequence, but with 19 bp scrambled at the predicted miR-2 binding site sequence (*gar-2* 3'UTRscr). Primers were annealed to produce this 85 bp sequence (Supplementary File 2c). Plasmid pHA#762 (NLS::GFP::*gar-2* 3'UTRscr) was generated by subcloning the 269 bp *gar-2* 3'UTRscr fragment from pHA#761 into pPD95.67 with EcoRI and SpeI. pHA#763 (*unc-17p::NLS::GFP::gar-2* 3'UTRscr) was produced by subcloning the 1286 bp fragment containing NLS::GFP and *gar-2* 3'UTRscr sequences from pHA#762 into pHA#756 using MscI and SpeI, while removing the genomic *mir-2* pre-miRNA and *unc-54* 3'UTR sequences. pHA#790 (*unc-122p::mel-46::unc-54* 3'UTR) was created by amplifying the MEL-46 coding region from the pRM8 plasmid (Minasaki et al., 2009) and inserting this fragment into the pHA#729 EcoRI site (Dimitriadis et al., 2016). Using SphI and MscI restriction enzymes, the *unc-122* promoter was then excised and replaced with the 4466 bp *unc-17* promoter fragment excised from pHA#763 with the same enzymes, thus generating pHA#791 (*unc-17::mel-46::unc-54* 3'UTR). To create pHA#792 (*unc-17p::mel-46::GFP::unc-54* 3'UTR), a 906 bp GFP sequence was amplified from pHA#763 and subcloned by Gibson assembly into pHA#791 just before the MEL-46 stop codon (TGA). The small guide RNA (sgRNA) plasmids targeting the *smn-1* gene (pHA#764 and pHA#765) and the sgRNA plasmid targeting the *gar-2* 3'UTR (pHA#793) for CRISPR/Cas9-mediated genome editing were produced by amplification of *PU6::klp-12* (Friedland et al., 2013). Plasmid pHA#766 contains a GFP insertion template and self-excising cassette flanked by *smn-1* arms of homology that were subcloned by Gibson assembly into pDD282 following a protocol from Dickinson et al. 2015 (Dickinson et al., 2015).

[000127] Integrated arrays *rtIs64* and *rtIs65* [*unc-17p::mel-46::GFP::unc-54* 3'UTR] were created by UV irradiation of *rtEx871*, which were generated by standard injection of pHA#792 at 50ng/μl, alongside 5 ng/μl *myo-3p::mCherry* (pCFJ104 - *myo-3p::mCherry::unc-54utr* was a gift from Erik Jorgensen (Frokjaer-Jensen et al., 2008), and 75 ng/μl pBluescript KS+. To generate *rtEx855*[pRM8(*mel-46*(+); *myo-2p::RFP*], wild type animals were injected with 133ng/μl PRM8 plasmid (Minasaki et al., 2009), 5 ng/μl *myo-3p::mCherry*; Addgene plasmid 19328) and 75 ng/μl pBluescript KS+. Animals injected with *rtEx855*[pRM8(*mel-46*(+); *myo-3p*-RFP)] were crossed into a *mel-46(tm1739)* background to assure rescue of viability before further experiments were undertaken. Notably, expression of either

rtEx855 or *ytEx211* in *smn-1(lf)* animals did not rescue lethality or adult survival, further emphasizing a privileged relationship between SMN-1 and MEL-46 in cholinergic NMJ signaling. Lines for *rtEx853[unc-17p::mir-2; myo-2p::mCherry]* and *rtEx854[unc-17p::GFP; myo-2p::mCherry]* were produced by injecting *mir-2(gk259)* animals with *pHA#756* or *pHA#757*, respectively, at 40 ng/μl alongside 2.5 ng/μl *myo-2p::mCherry* (pCFJ90 - *myo-2p::mCherry::unc-54utr* was a gift from Erik Jorgensen (Frokjaer-Jensen et al., 2008); Addgene plasmid 19327) and 77.5 ng/μl pBluescript KS+. *rtIs56[unc-17p::GFP::gar-2 3'UTRwt; myo-2p::mCherry]* was integrated by UV irradiation into the genome and is derived from extrachromosomal array *rtEx856*, containing *pHA#759*, which was injected into wild type animals at 20 ng/μl with 2.5 ng/μl *myo-2p::mCherry* and 77.5 ng/μl pBluescript KS+. Integrated arrays *rtIs57* and *rtIs58 [unc-17p::GFP::gar-2 3'UTRscr; myo-2p::mCherry]* are two separate lines generated by UV irradiation of extrachromosomal array *rtEx857*, containing *pHA#763*, which was injected into wild type animals at 20 ng/μl with 2.5 ng/μl *myo-2p::mCherry* and 77.5 ng/μl pBluescript KS+. *gar-2(rt317)* and *gar-2(rt318)* alleles were generated by injecting *pha-1(e2123)* animals with the *pHA#793* sgRNA plasmid targeting the *gar-2 3'UTR* at 25 ng/μl with either 50 ng/μl of a mutant single-strand oligo DNA (ssODN) repair template (*rt318*) or a control ssODN repair template (*rt317*), alongside the injection cocktail as described in Ward *et al.* 2015. Progeny from this injection were screened as described (Ward, 2015). Information on ssODN template sequences can be found in Supplementary File 2c. To generate *smn-1(rt280)*, which contains a GFP N-terminal insertion, wild type animals were injected with both *pHA#764* and *pHA#765* sgRNA plasmids targeting *smn-1* at 50 ng/μl alongside 20 ng/μl of the GFP template plasmid *pHA#766* and the standard injection cocktail described in Dickinson et al. 2015. Progeny from this injection were screened as described (Dickinson et al., 2015). Consistent with Miguel-Aliaga et al., tagged-SMN protein was expressed in all blastomeres throughout embryonic development with redistribution from the nucleus to the cytoplasm during mitotic stages. The presence of GFP::SMN during such early stages indicates that GFP::SMN is maternally transmitted during germline development (Miguel-Aliaga et al., 1999).

[000128] RNAi studies: RNAi studies involved animals from an RNAi-enhanced background (KP3948) (Kennedy et al., 2004), neuron-specific RNAi-sensitized background (TU3401) (Calixto et al., 2010), cholinergic neuron-specific RNAi-sensitized background (XE1581), or GABA neuron-specific RNAi-sensitized background (XE1375) (Firnhaber and Hammarlund, 2013). Aldicarb response, pumping rates, and RNA quantification were evaluated in animals that had been reared for at least two generations on HT115 bacteria containing control vector L4440, C41G7.1/*smn-1(RNAi)*, C26C6.2/*goa-1(RNAi)*, T06A10.1/*mel-46(RNAi)*, or Y37D8A.23/*unc-25(RNAi)* (Kamath and Ahringer, 2003). Primer sequences used to generate the PCR products specific to each gene of interest can be found on the Kim Lab Stanford University website (<http://cmgm.stanford.edu/~kimlab/primers.12-22-99.html>).

[000129] *C. elegans* behavioral assays. *Aldicarb resistance assay:* 1mM aldicarb assays were completed in at least three independent trials blinded to genotype ($n \geq 30$ animals/genotype) as described in previous work (Mahoney et al., 2006; Sato et al., 2009). Paralysis induced by aldicarb was scored as inability to move or pump in response to prodding with a platinum wire. Experiments involving *smn-1(ok355)*, *smn-1(rt248)* or *mel-46(tm1739)* animals were completed at the early L4 stage. All other aldicarb experiments were done with young adult animals. *Pharyngeal pumping:* Assays were performed blinded to genotype as previously described (Dimitriadi et al., 2010). Pumping events were scored as grinder movement in any axis. Average pumping rates ($\pm$ Standard Error of the Mean (SEM)) were pooled from at least two independent trials ($n > 20$ animals/genotype). Experiments involving *smn-1(ok355)*, *smn-1(rt248)* or *mel-46(tm1739)* animals were completed at day 3 post-hatching (animals were kept at 25°C for 2 days and then 20°C for 1 day). Pumping experiments involving all other genotypes were done with young adult animals.

[000130] *C. elegans* light level microscopy. Animals were mounted on 2% agar pads and immobilized using 30 mg/mL BDM (Sigma) in M9 buffer. *Dorsal cord protein localization:* Images were obtained as Z-stacks of the dorsal cord above the posterior gonad reflex (100x objective, Zeiss AxioImager ApoTome and Axiovision software v4.8). For MEL-46::GFP analysis, a set area was defined for each image along the dorsal cord (25 μ m x 5 μ m). Using ImageJ (RRID:SCR_003070), a uniform threshold was used to eliminate background. The number (density), mean fluorescence (intensity) and area (size) for MEL-46::GFP granular structures were calculated using the ImageJ ‘particle analyzer’ program. For synaptic protein localization, mean puncta width (meanfixedwidth), intensity (meanfixedvolume) and linear density (fixedwidthlineardensity) were quantified with an in-house developed program called ‘Punctaanalyser’ using MatLab software (v6.5; Mathworks, Inc., Natick, MA, USA; RRID:SCR_001622) (Kim et al., 2008). At least three independent trials ($n > 17$ animals/genotype) were performed. For data sets involving *smn-1(ok355)*, *smn-1(rt248)*, or *mel-46(tm1739)* animals, all genotypes were examined at the early L4 stage, while other data sets were collected with young adult stage animals. *GFP Fluorescence Quantification:* GFP images of L4 animals were acquired (10x objective, Zeiss V20 stereoscope and Axiovision software v4.8). Mean GFP fluorescence was quantified using ImageJ (RRID:SCR_003070). A threshold was set to eliminate background fluorescence. For each data set, thresholds were kept constant. Average fluorescence values ($\pm$ SEM) were combined from at least three independent trials for $n > 25$ animals/genotype; however certain backgrounds containing *rtEx855[mel-46(+)]* had a lower n (reported in legends) as these animals went sterile and/or did not throw many progeny carrying the *mel-46* array. Ratios in Figure 4H and Figure 5A were calculated as average mean fluorescence for each genotype in the *rtIs56* background and divided by their respective average mean fluorescence in the control *rtIs57* background. Ratio SEM was calculated by summing the SEM for

each population (see Figure 4 – figure supplement 2D). All representative images shown were analyzed as part of data collection.

[000131] *C. elegans* total RNA isolation, cDNA synthesis and qPCR. For each RNA sample, animals were synchronized by collecting eggs for 6 hours from gravid adults on large seeded NGM plates. After two days at 25°C, young adult progeny were washed off, rinsed and flash frozen. Total RNA was extracted after a 15 minutes Trizol (Thermo Fisher) incubation. 1 ng total RNA was used for reverse transcription with either the miScript II RT kit (Qiagen #218160) for miRNA or the SuperScript® III First-Strand Synthesis Supermix kit (Invitrogen #11752050) for mRNA. Methodology followed manufacturer's instructions. miRNA levels were determined in a 10 µl reaction using miScript SYBR Green PCR kit (#218073, Qiagen) and 300 nM of mature miR-2 primer/probe. miR-60 was used to normalize miR-2 expression as it is not expressed in the nervous system where SMN-1 or MEL-46 were knocked-down. Forward primer sequences for miR-2 and miR-60 were, respectively: 5'-TATCACAGCCAGCTTTGATGTGC-3' (SEQ ID NO. 12) and 5'-TATTTATGCACATTTTCTAGTTCA-3' (SEQ ID NO. 13). A universal reverse probe was provided by Qiagen. Primer sequences for *act-1*: 5'-acgccaaacactgttcttcc-3' and 5'-gatgatcttgatcttcattggtga-3' (Ly et al., 2015). Primer sequences for 18S rRNA: 5'-TTGCTGCGGTTAAAAAGCTC-3' (SEQ ID NO. 14) and 5'-CCAACCTCAAACCAGCAAAT-3' (SEQ ID NO. 15) (Essers et al., 2015). The stability of miR-60, 18S rRNA, and *act-1* housekeeping RNAs were evaluated using the 'model-based approach to estimation of expression variation' (Andersen et al., 2004). mRNA levels were determined in a 10 µl reaction using Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific # 4368706), and 300 nM of each primer. PGK-1 was used to normalize *gar-2* expression, as the mammalian orthologue has been used previously as a housekeeping gene for experiments involving SMN (Abera et al., 2016; Simard et al., 2007). Primer sequences for *gar-2*: 5'-CCTGAAGTCTCATTGCCCTTTATTGATGC-3' (SEQ ID NO. 16) and 5'-CTAGCAGTCCCTTGCAATTGAAAC-3' (SEQ ID NO. 17). Primer sequences for *pgk-1*: 5'-GGCCCTCGACAACCCAGCTCGTC-3' (SEQ ID NO. 18) and 5'-CGGCGGAGGAATGGCCTATACC-3 (SEQ ID NO. 19). All reactions were performed in triplicate. Melting curve analysis and electrophoresis in agarose gel of every PCR product was conducted after each qRT-PCR to control amplification specificity. Gene expression level was calculated as the fold change of relative DNA amount of a target gene in a target sample and a reference sample normalized to a reference gene using the comparative $\Delta\Delta CT$ method as previously described (Kurrasch et al., 2004).

[000132] Embryonic spinal MN culture, miR-128a quantification and Western blot. E13.5 mouse MNs were isolated from WT (FVB/NJ; RRID:IMSR_JAX:001800) and SMA mice (FVB, *Smn*^{-/-}; *SMN2*^{tg0}; generated by crossing lines RRID:IMSR_JAX:005058 and RRID:IMSR_JAX:005024) (Riessland et al., 2010) as described (Wiese et al., 2001). Isolated mouse MNs were differentiated 10 days

in NB/B27 media supplemented with growth factors to promote survival; brain derived neurotrophic factor (BDNF) 10ng/ml, ciliary neurotrophic factor (CNTF) 10 ng/ml and glial-derived neurotrophic factor (GDNF) 50 ng/ml. Fifty percent of medium was replaced every 3 days. To reduce the amount of glia and fibroblasts in culture, 1μM cytosine arabinoside (AraC) was added at day 3.

[000133] After 10 days *in vitro* culture, total RNA was extracted from E13.5 MNs using the mirVana total RNA isolation kit (Thermo Scientific). Nanodrop was used to measure RNA amount. Using 100 ng of total RNA, miR-128 expression levels were determined by real time PCR with mature miR-128a primer/probe (TaqMan MicroRNA Assays, #4427975, Thermo Scientific). Actin-beta was used to normalize miR-128a expression. Primer sequences for actin-beta: 5'-agccatgtacgtagccatcc-3' (**SEQ ID NO. 20**) and reverse 5'-ctctcagctgtggtggtgaa-3' (**SEQ ID NO. 21**). Methodology followed manufacturer's instruction (Kye et al., 2014).

[000134] Proteins were extracted from motor neurons, after 10 days *in vitro* culture, using RIPA buffer and protease inhibitor cocktail (Smith et al., 2014). Expression of m2R and β-actin was measured using Western blot. Antibodies against m2R (ABCAM, ab109226; RRID:AB_10858602; 1:1000) and β-actin (Santa Cruz, sc-47778; RRID:AB_626632; 1:1000) were used to detect proteins. Methocramine (Sigma, M105) was treated 48 hours from DIV 3 to DIV5 in various concentrations. After 5 days of *in vitro* culture, neuronal morphology was visualized with Tau (Santa Cruz, A-10) staining. Axon length was analyzed with ImageJ (RRID:SCR_003070).

[000135] **Statistical Analysis.** Log-rank test, two-tailed Mann-Whitney *U*-test, or *t*-test were used for *C. elegans* statistical analysis. The Mann Whitney *U*-test was chosen over *t*-test for experiments where homogeneity could not be assured (i.e. RNAi; extrachromosomal arrays; or potential maternal loading from a heterozygous parent). *t*-test was used to determine significance for spinal motor neuron Western blot quantification and qPCR quantification.

[000136] All publications cited herein expressly incorporated herein by reference in their entireties.

[000137] *References for Example*

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SEQUENCE LISTING

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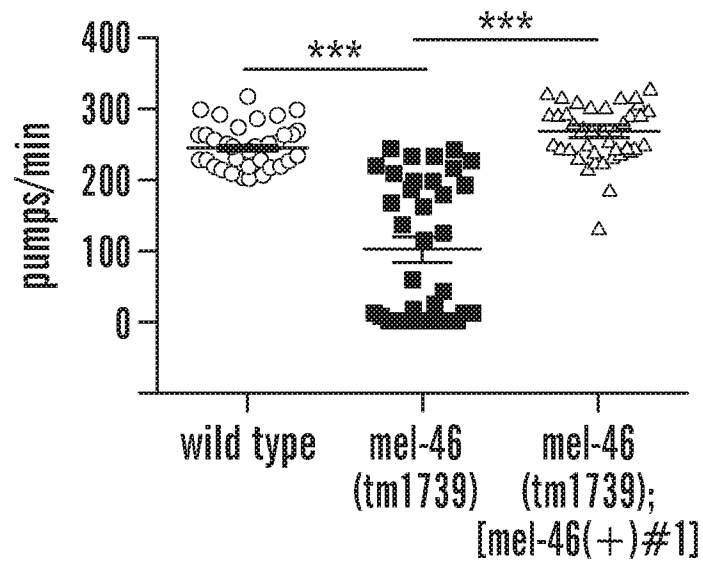
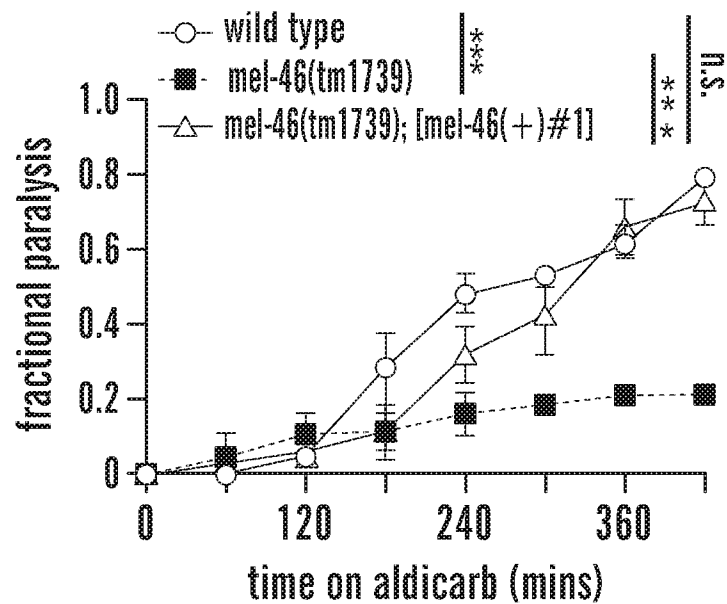
CLAIMS

What is claimed is:

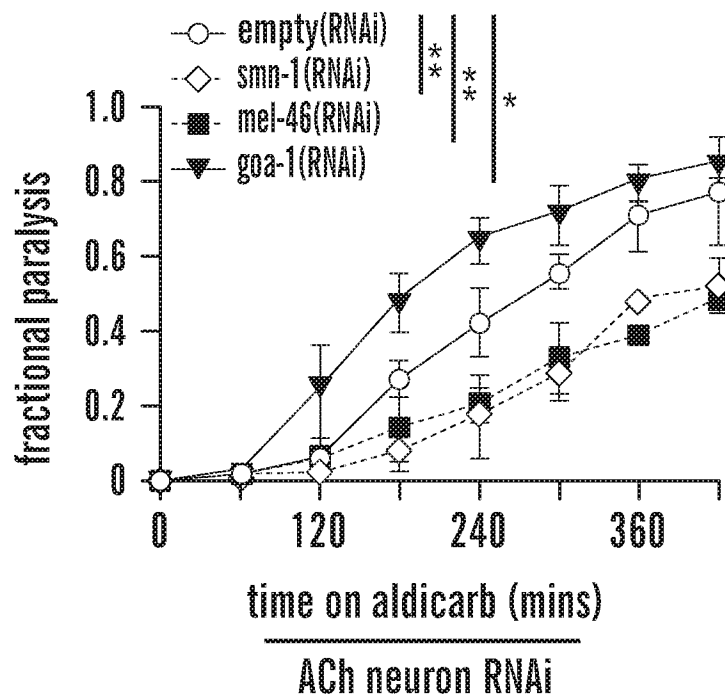
1. A method for treating Spinal Muscular Atrophy (SMA), the method comprising: administering to a subject in need thereof an agent that inhibits a muscarinic acetylcholine receptor.
2. The method of claim 1, wherein the subject has SMA1, SMA2, SMA3, SMA4, Spinal muscular atrophy with respiratory distress (SMARD), Kennedy's syndrome (SBMA), or distal SMA (DSMA).
3. The method of claim 1, wherein SMA is caused by a mutation in the *SMN1* gene.
4. The method of claim 1, wherein SMA is caused by a reduction of SMN protein.
5. The method of claim 1, wherein the muscarinic acetylcholine receptor is muscarinic acetylcholine receptor M2.
6. The method of claim 1, wherein the muscarinic acetylcholine receptor M2 is the human M2 gene, *CHRM2*.
7. The method of claim 1, wherein the agent is a muscarinic acetylcholine receptor M2 antagonist.
8. The method of claim 1, wherein the agent inhibits the function of the muscarinic acetylcholine receptor M2.
9. The method of claim 1, wherein the agent prevents muscarinic acetylcholine receptor M2 from binding neurotransmitter acetylcholine.
10. The method of claim 7, wherein the agent comprises an antibody, a small molecule, a polypeptide, an oligonucleotide or an analog thereof, or an inhibitory nucleic acid molecule.
11. The method of claim 7, wherein the muscarinic acetylcholine receptor M2 antagonist is selected from the group consisting of: atropine, hyoscyamine, dimethindene, otenzepad, AQRA-741, AFDX-384, dicycloverine, thiorazine, diphenhydramine, dimenhydrinate, tolterodine, oxybutynin, ipratropium, methoctramine, tripitramine, gallamine, and chlorpromazine.
12. The method of claim 7, wherein the muscarinic acetylcholine receptor M2 antagonist is methoctramine.
13. The method of any one of claims 1 to 12, wherein the subject is a mammal.
14. The method of any one of claims 1 to 12, wherein the subject is human.
15. The method of claims 1 to 14, further comprising administering an additional anti-SMA therapeutic and/or therapy to said subject.
16. The method of any one of the claims 1 to 15, wherein the subject has been diagnosed as having neuromuscular defects and/or synaptic defects.
17. The method of any one of claims 1 to 16, wherein administration of the agent ameliorates neuromuscular defects and/or synaptic defects.
18. The method of any one of claims 1 to 17, wherein the agent is administered intrathecally.

19. A method for treating Spinal Muscular Atrophy (SMA), the method comprising: administering to a subject in need thereof a therapeutically effective amount of methoctramine.
20. The method of claims 19, further comprising administering an additional anti-SMA therapeutic and/or therapy to said subject.
21. The method of any one of the claims 19 and 20, wherein the subject has been diagnosed as having neuromuscular defects and/or synaptic defects.
22. The method of any one of claims 19 and 20, wherein administration of methoctramine ameliorates neuromuscular defects and/or synaptic defects.
23. The method of any one of claims 19 to 22, wherein methoctramine is administered intrathecally.
24. A composition comprising: methoctramine and an agent that facilitates the passage through a blood brain barrier.
25. The composition of claim 24, wherein the agent is a pharmaceutically acceptable carrier that passes through the blood brain barrier.
26. The composition of claim 24, wherein the agent is a pharmaceutically acceptable nanoparticle that passes through a blood brain barrier.
27. The composition of claim 24, wherein the agent is a pharmaceutically acceptable compound that permeabilizes a blood brain barrier.
28. The composition of claim 24, wherein the agent is a pharmaceutically acceptable peptide nucleic acid molecule that passes through a blood brain barrier.

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**FIG. 1A****FIG. 1B**

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**FIG. 1C**

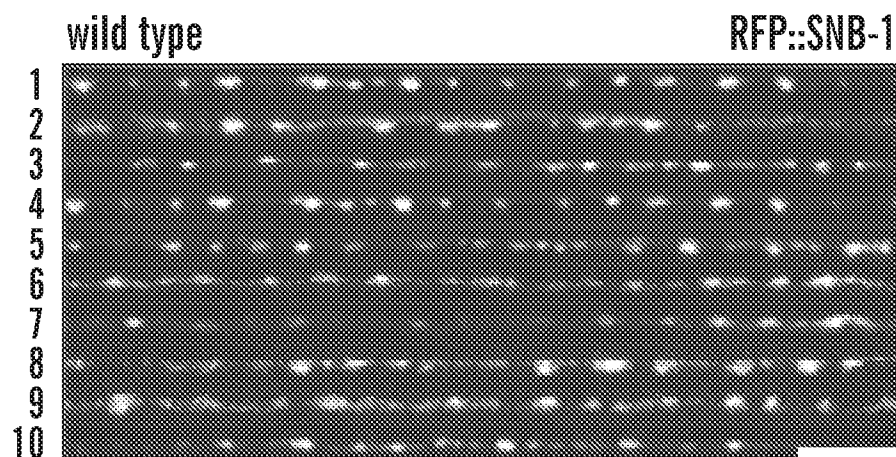
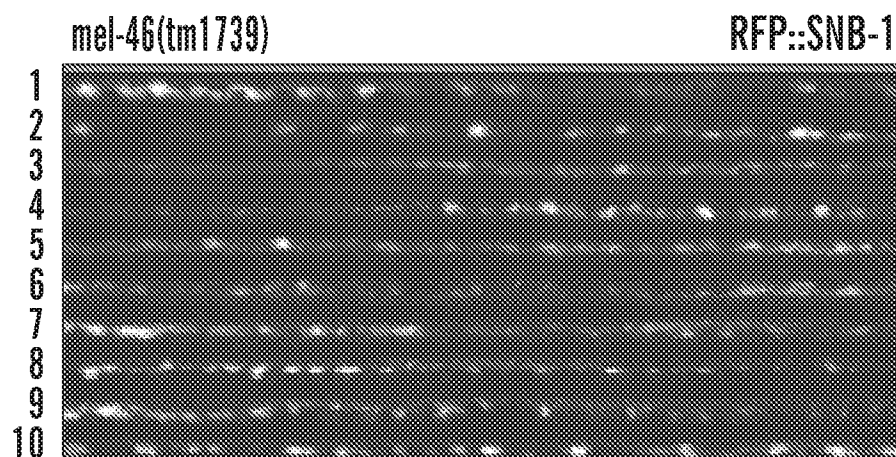
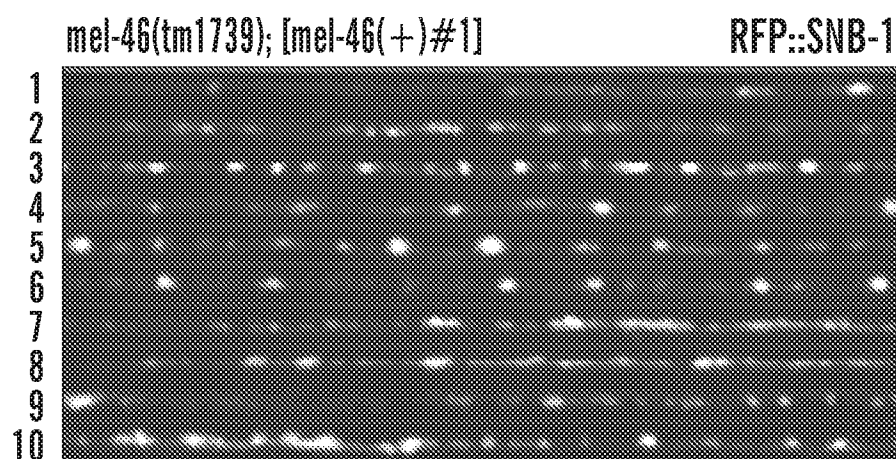
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mel-46(tm1739)	-29%**	-45%***	-81%***
mel-46(tm1739); [mel-46(+)]#1	10%	19%	-36%***

Asterisks: significance vs. control

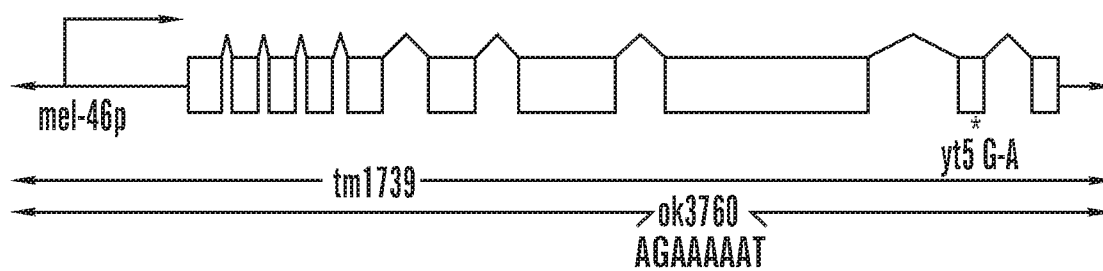
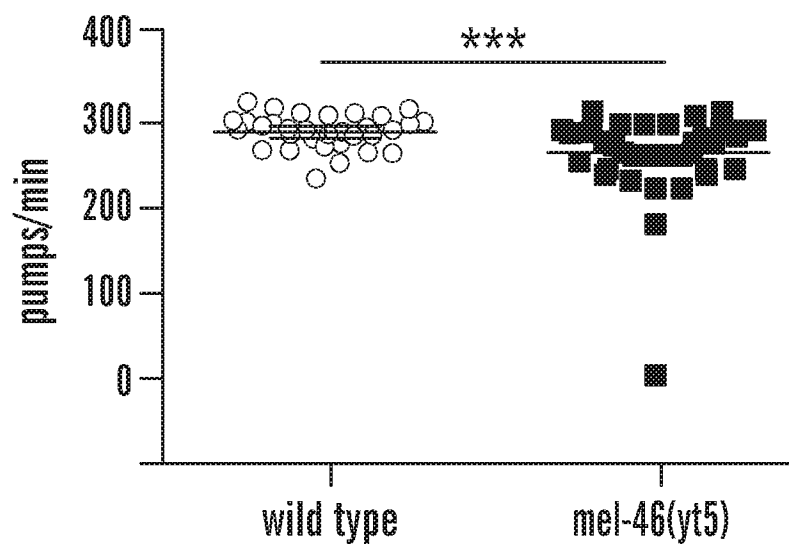
Shading: significance vs. shaded genotype

FIG. 1D

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**FIG. 1E****FIG. 1F****FIG. 1G**

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**FIG. 2A****FIG. 2B**

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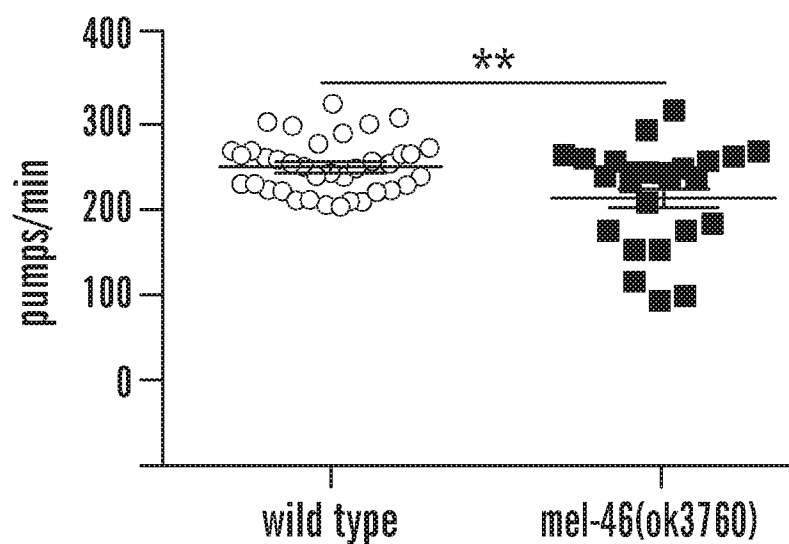


FIG. 2C

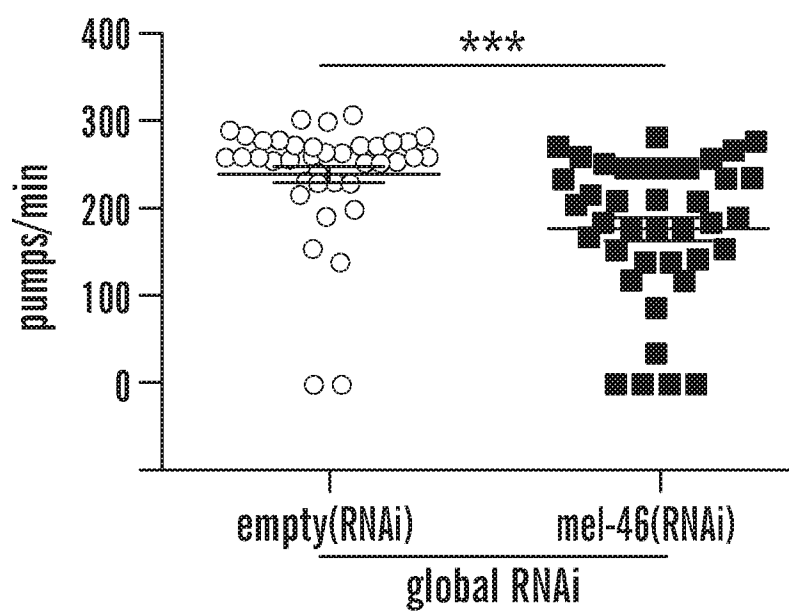
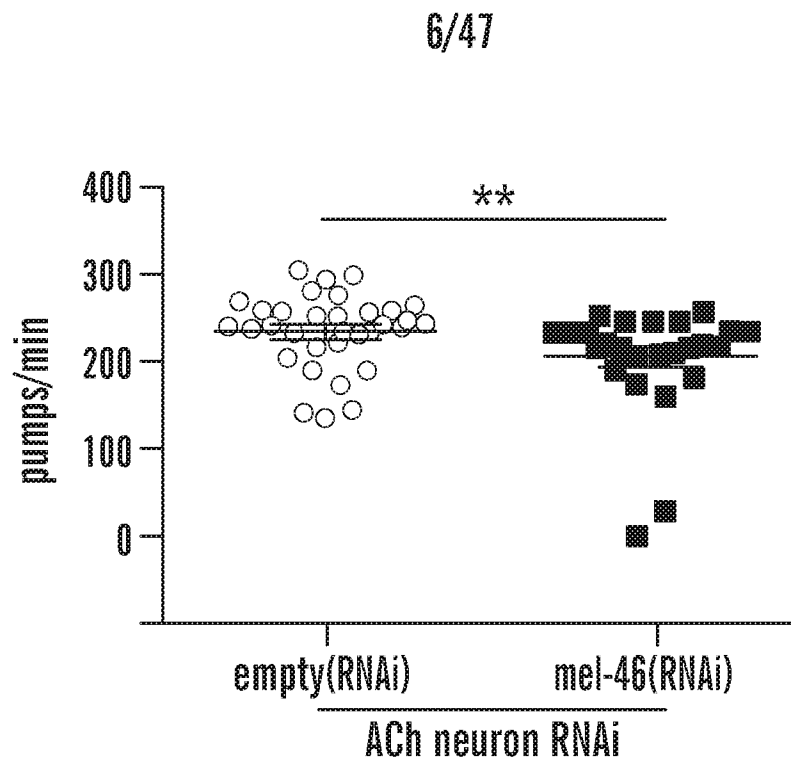
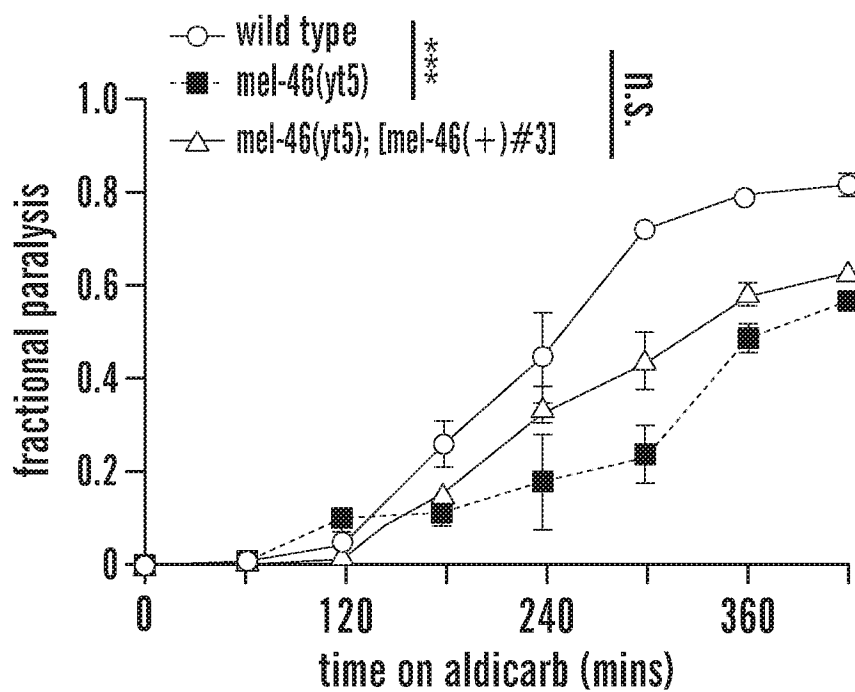
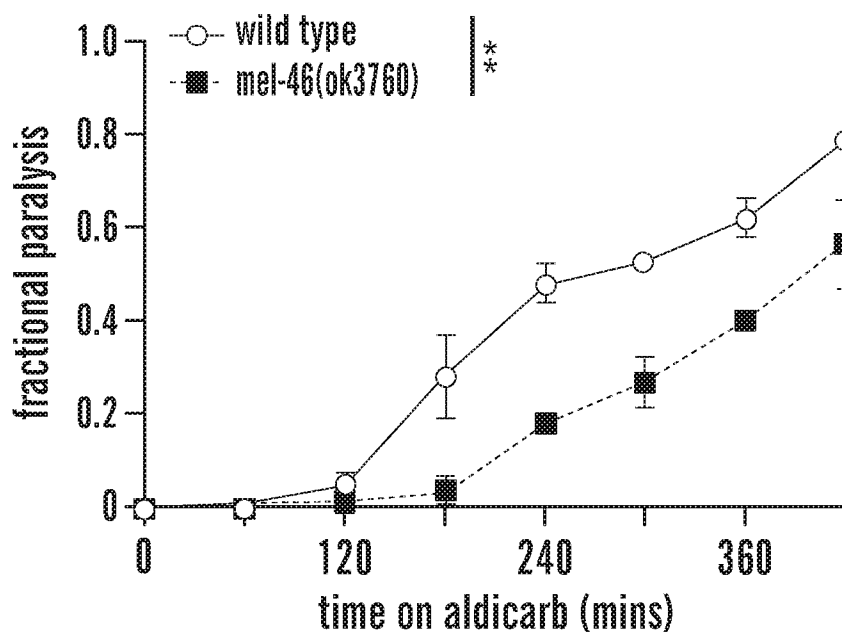
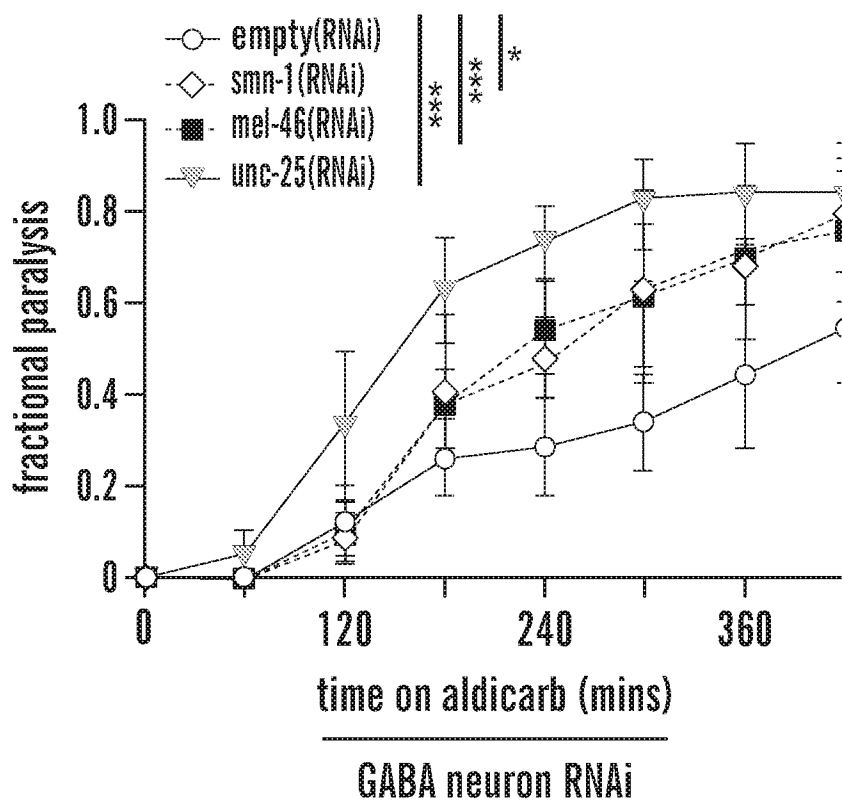


FIG. 2D

**FIG. 2E****FIG. 2F**

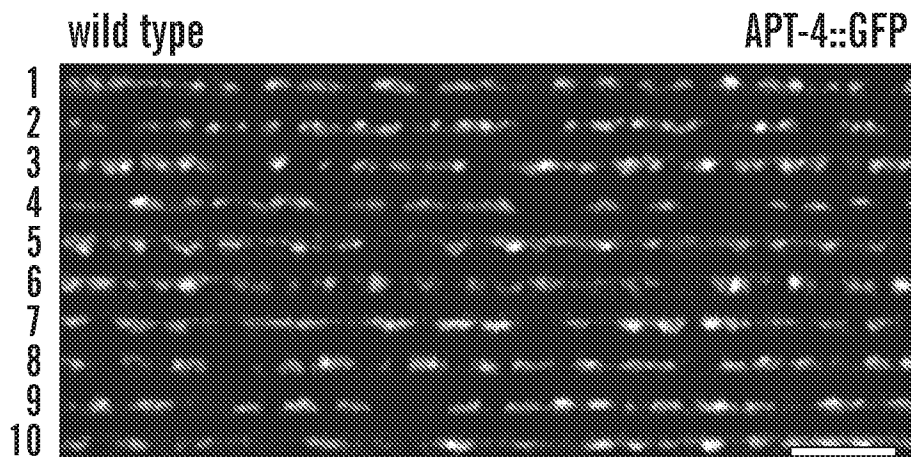
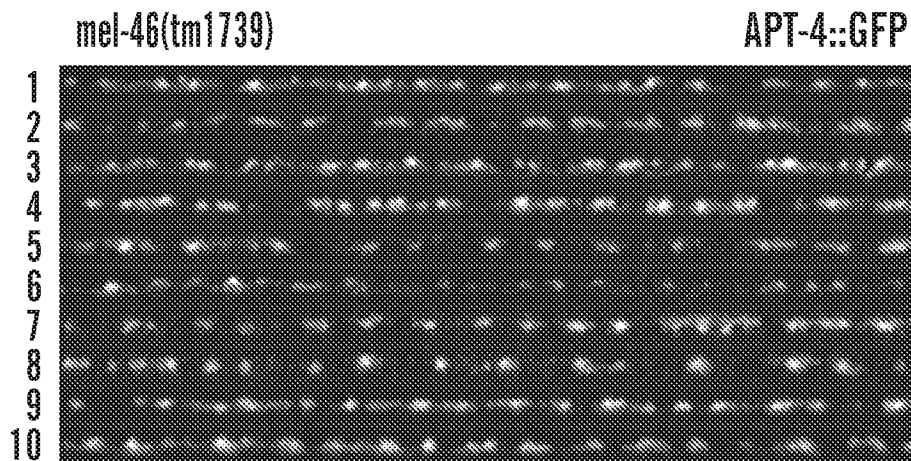
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**FIG. 2G****FIG. 2H**

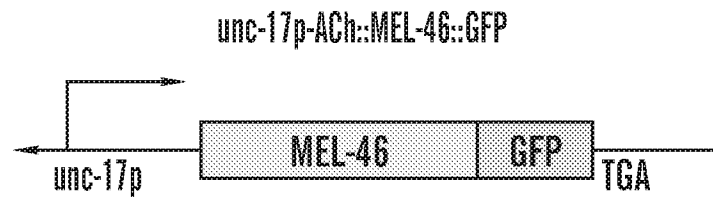
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	APT-4::GFP		
	Width	Intensity	Linear Density
mel-46(tm1739)	-2%	2%	23%*

Asterisks: significance vs. control

FIG. 3A**FIG. 3B****FIG. 3C**

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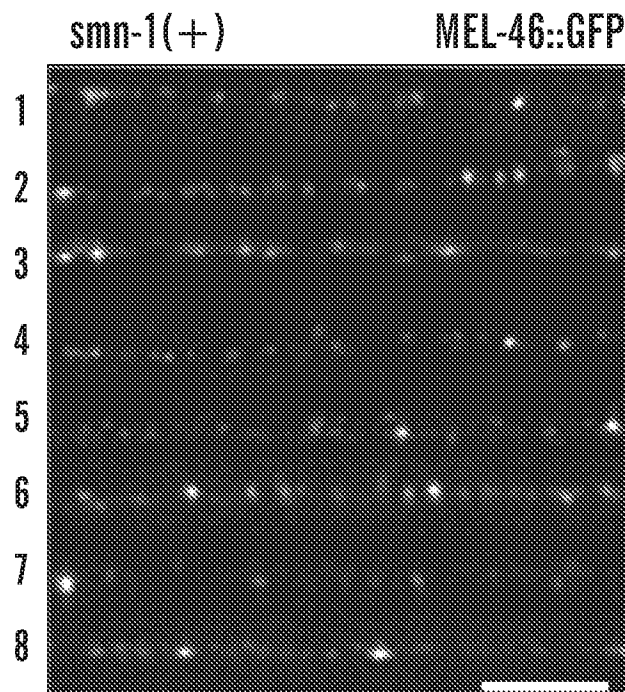
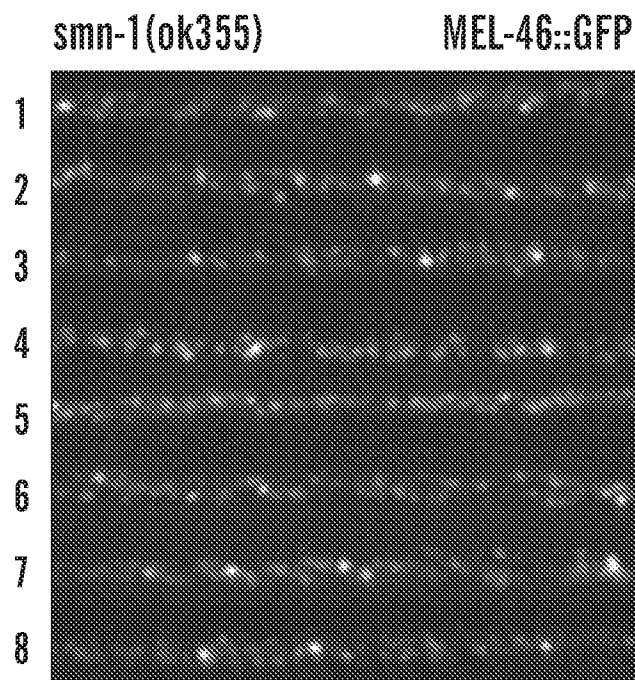
**FIG. 4A**

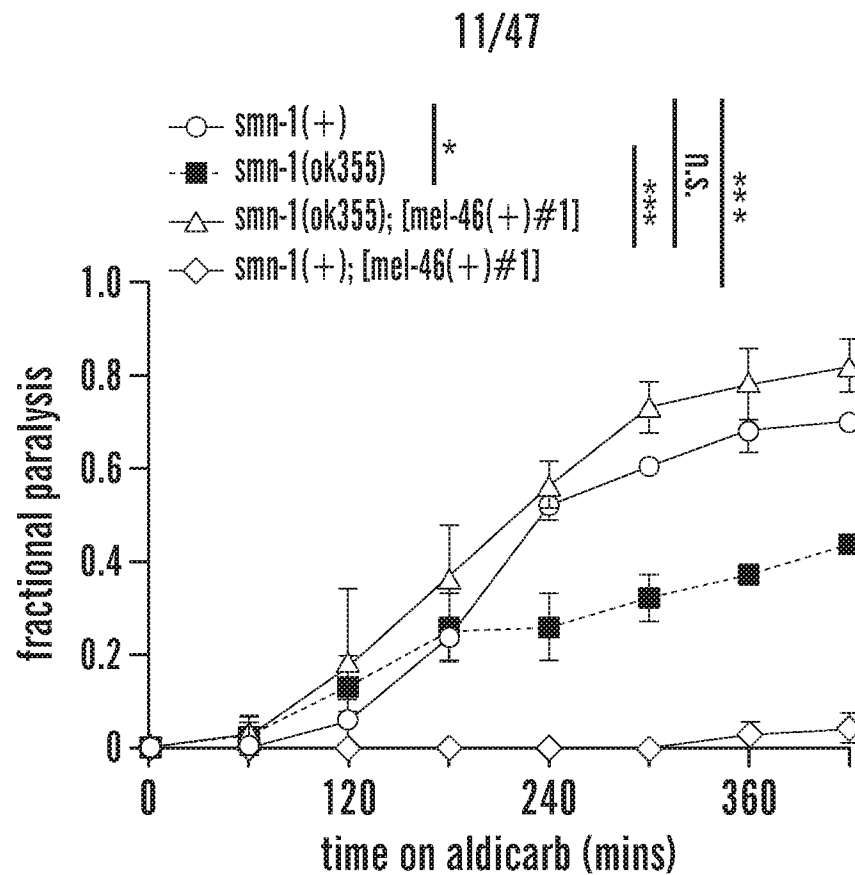
ImageJ	MEL-46::GFP		
	Density	Intensity	Size
smn-1(ok355)	116%***	-12%**	-10%

Asterisks: significance vs. control

FIG. 4B

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***FIG. 4C******FIG. 4D***

**FIG. 4E**

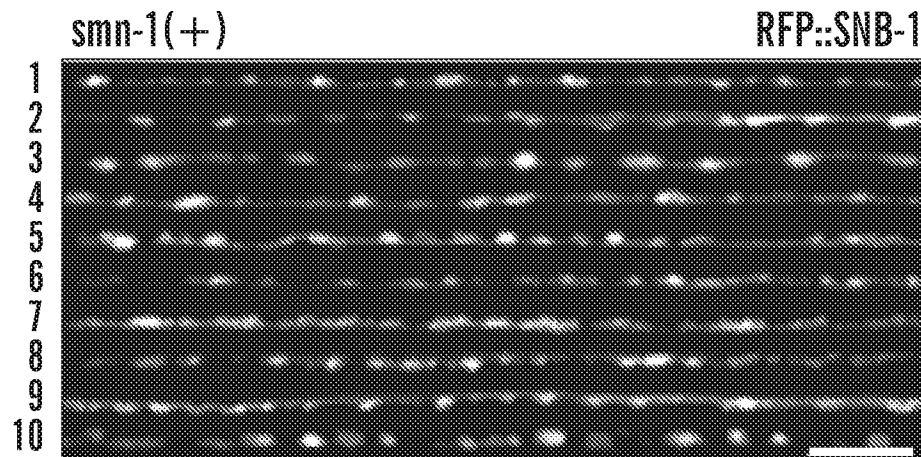
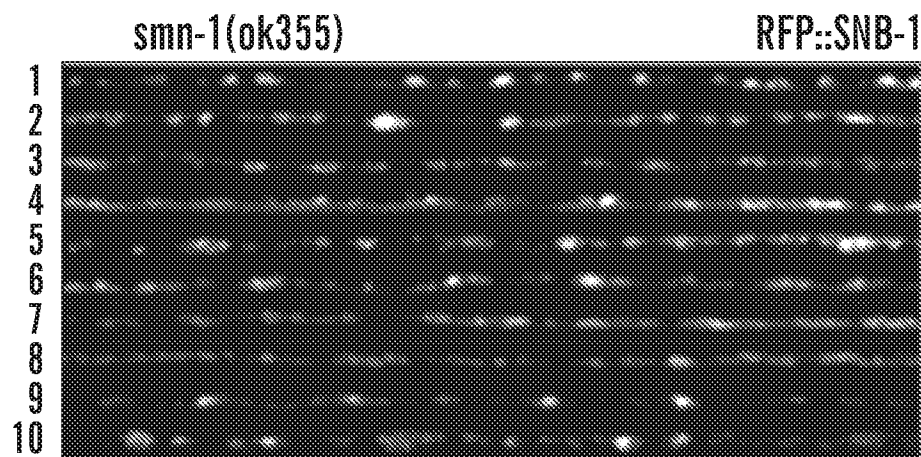
Puncta analyzer	RFP::SNB-1		
	Width	Intensity	Linear Density
smn-1(ok355)	-26%*	-35%**	-31%**
smn-1(ok355); [mel-46(+)]#1	59%*	76%*	-17%*
smn-1(+); [mel-46(+)]#1	13%	16%	-17%

Asterisks: significance vs. control

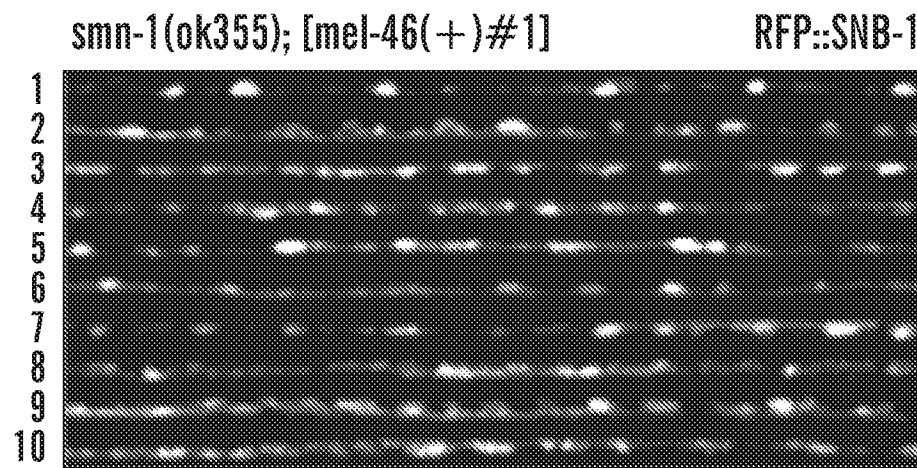
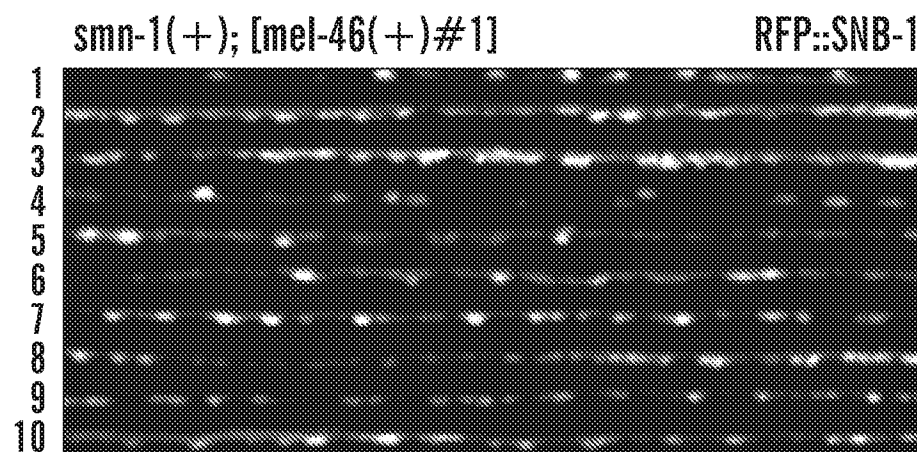
Shading: significance vs. shaded genotype

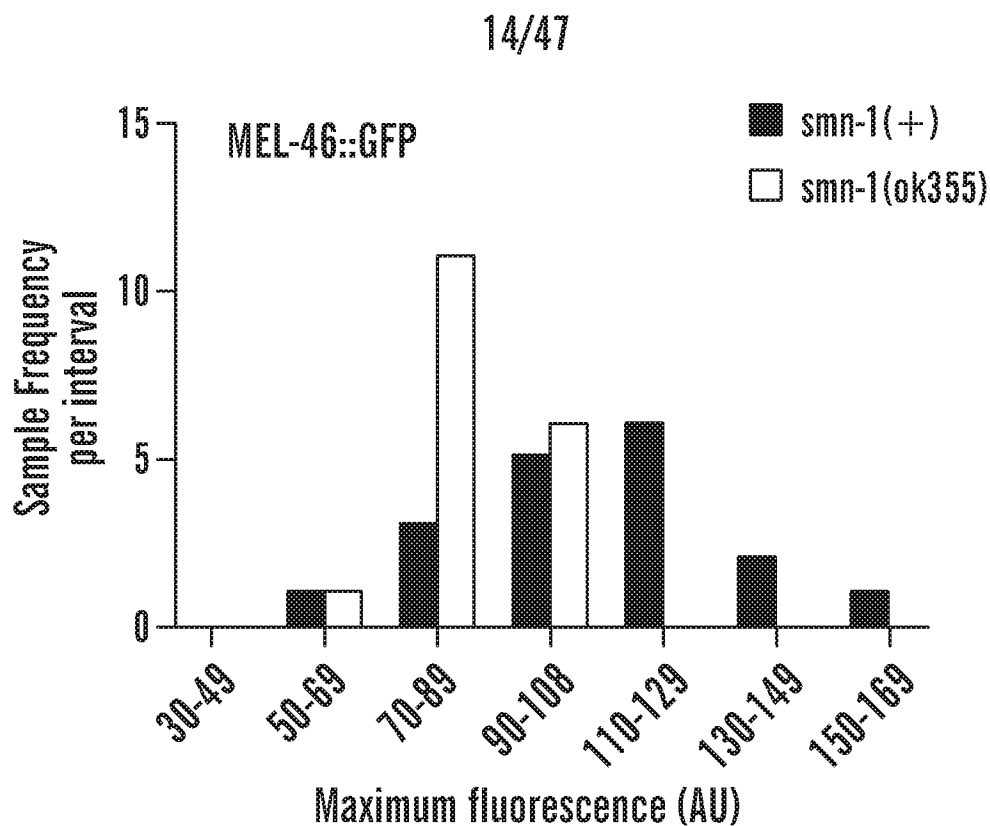
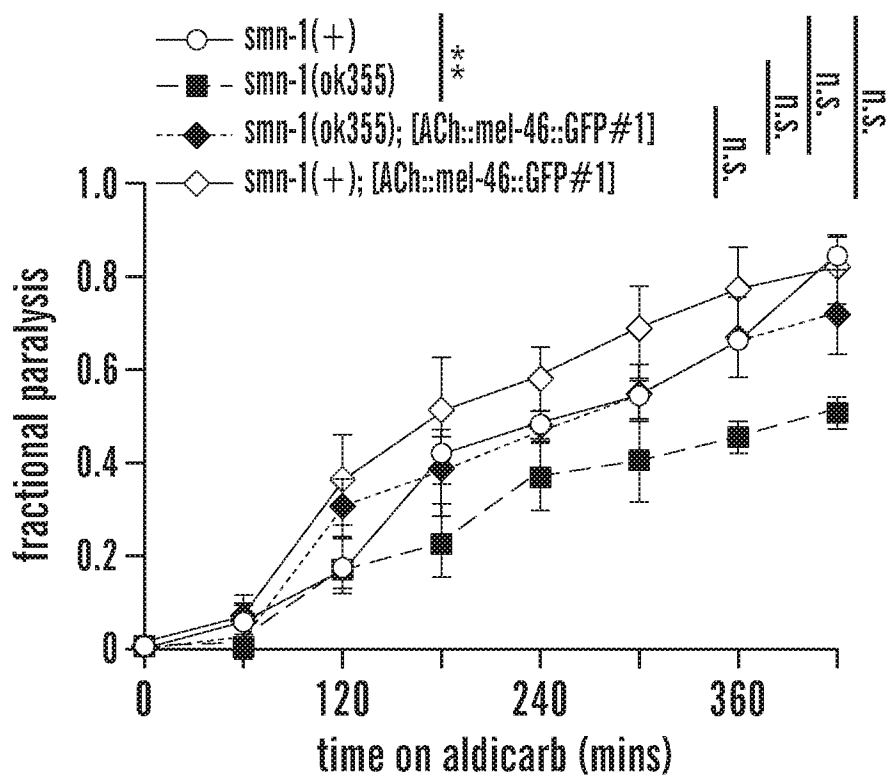
FIG. 4F

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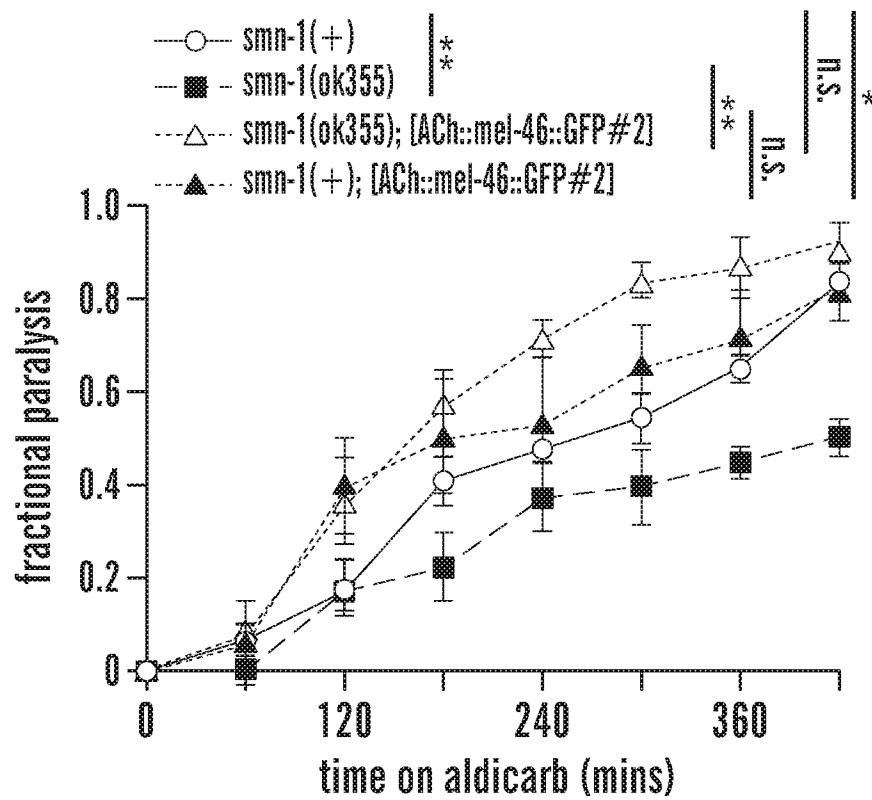
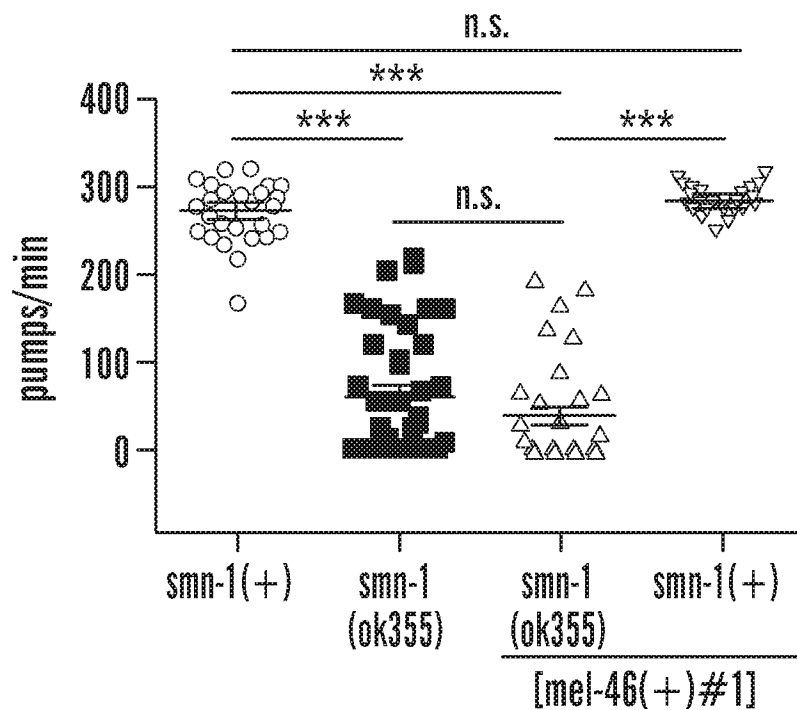
*FIG. 4G**FIG. 4H*

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***FIG. 4I******FIG. 4J***

**FIG. 5A****FIG. 5B**

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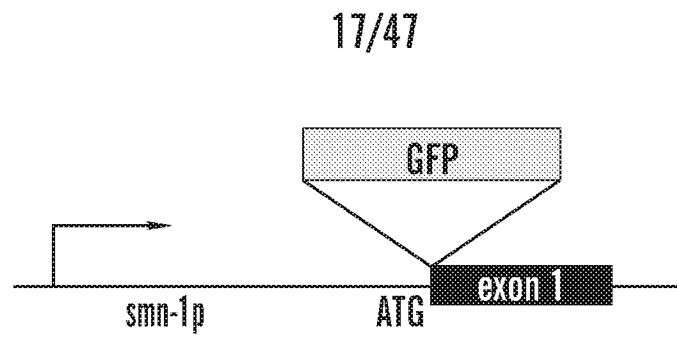
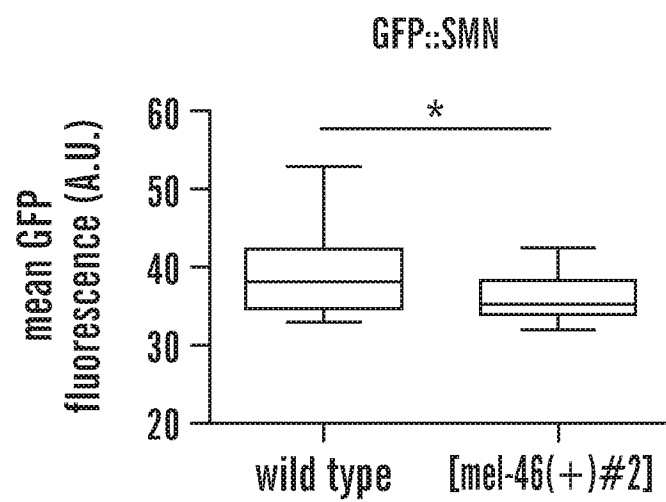
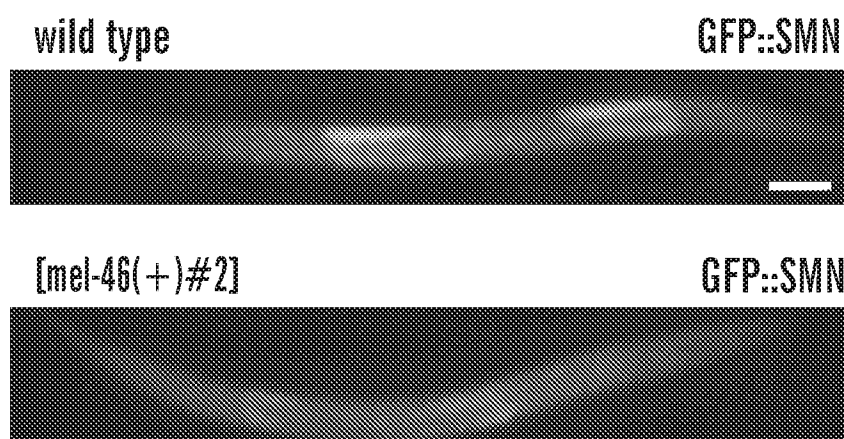
**FIG. 5C****FIG. 5D**

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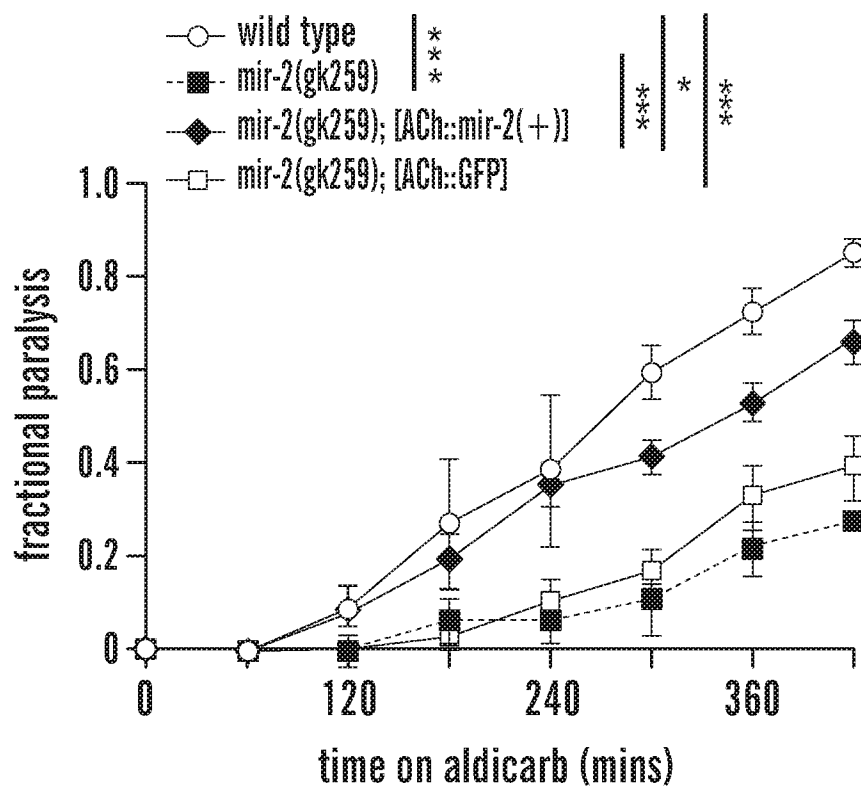
	APT-4::GFP		
	Width	Intensity	Linear Density
smn-1(ok355)	-16%*	-20%*	41%*
smn-1(ok355); [mel-46(+)]#2]	-18%**	-25%**	1%

Shading: significance vs. shaded genotype

FIG. 5E

**FIG. 6A****FIG. 6B****FIG. 6C**

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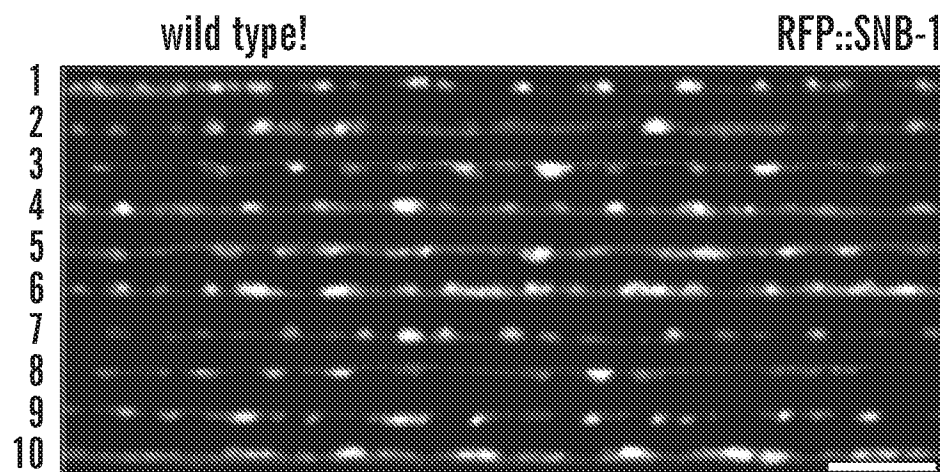
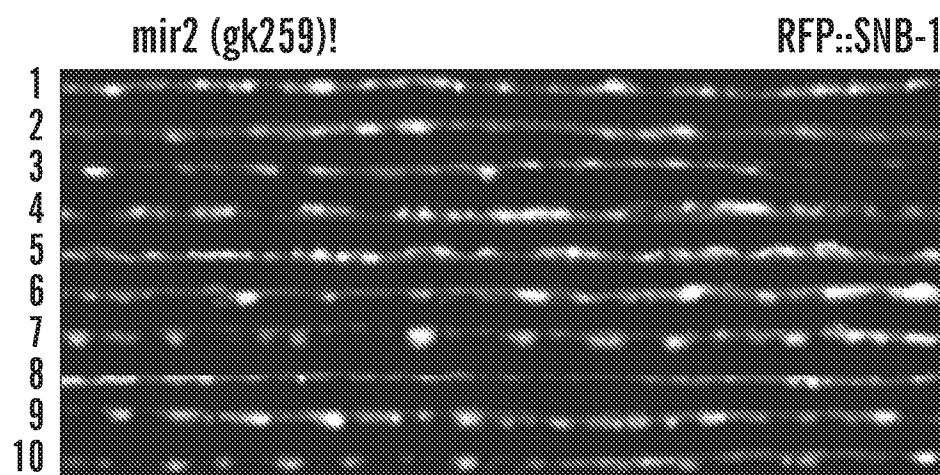
**FIG. 7A**

	RFP::SNB-1		
	Width	Intensity	Linear Density
mir-2(gk259)	-7%	-5%	70%*

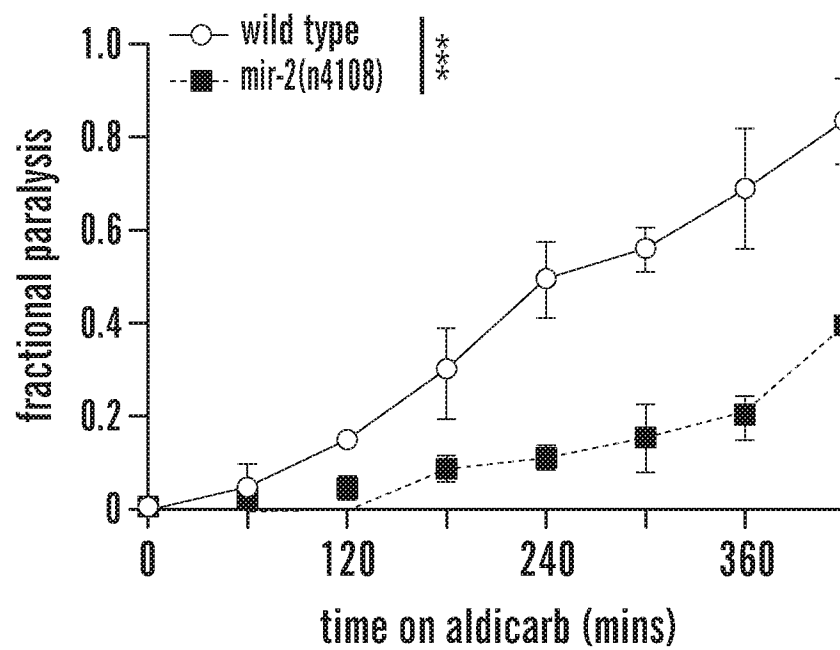
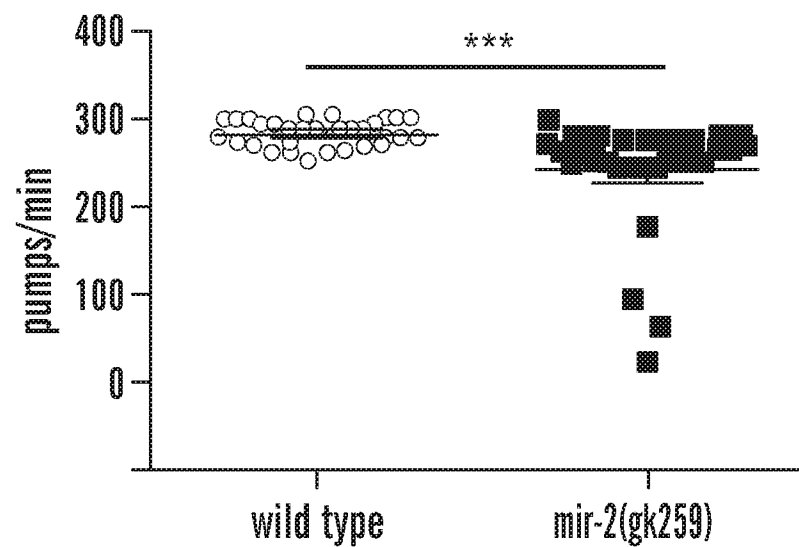
Asterisks: significance vs. control

FIG. 7B

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**FIG. 7C****FIG. 7D**

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**FIG. 8A****FIG. 8B**

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	SYD-2::GFP		
	Width	Intensity	Linear Density
mir-2 (gk259)	7%	5%	8%

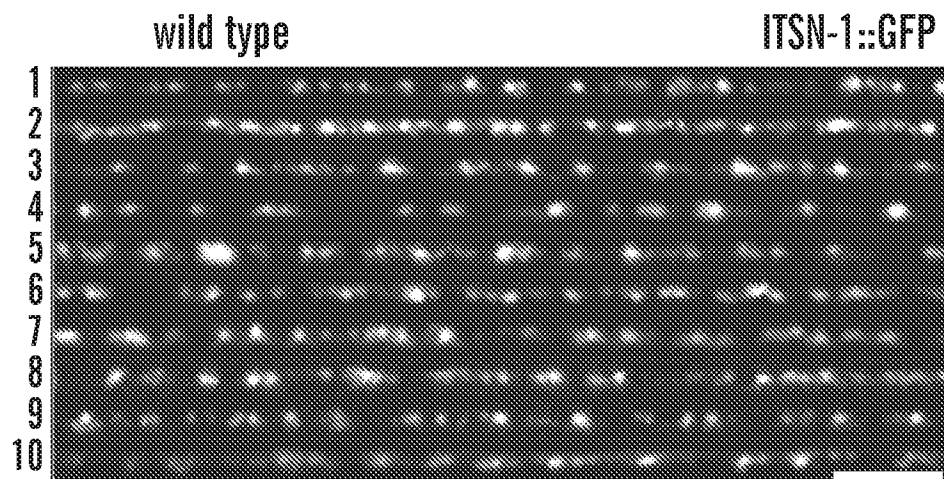
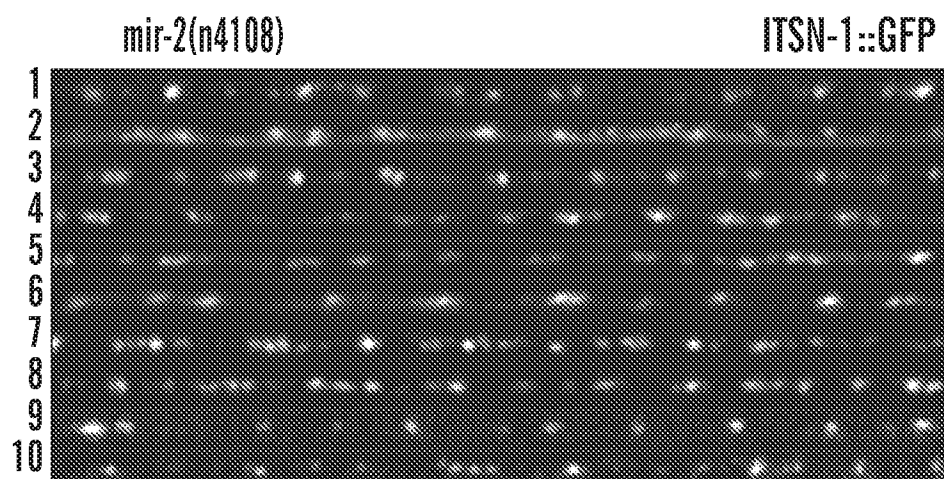
Asterisks: significance vs. control

FIG. 8C

	ITSN-1::GFP		
	Width	Intensity	Linear Density
mir-2 (gk259)	-20%**	-20%*	1%
mir-2 (n4108)	-22%**	-25%**	-9%

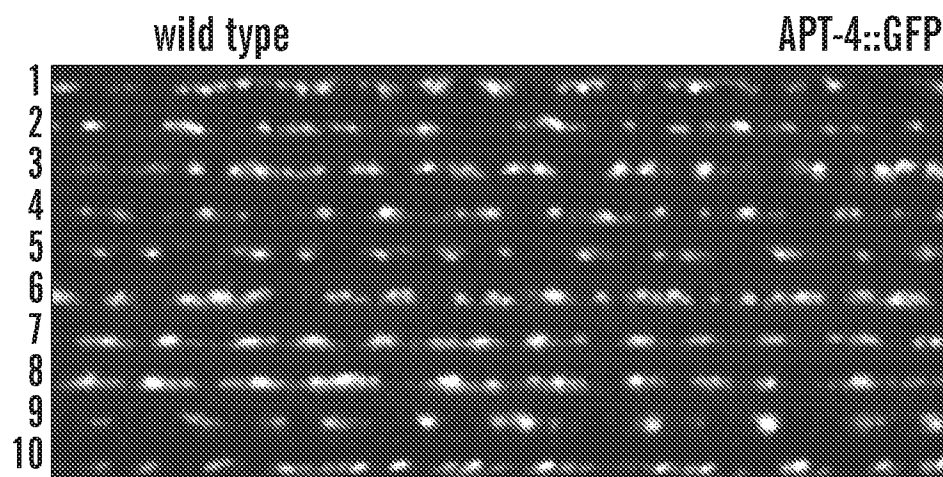
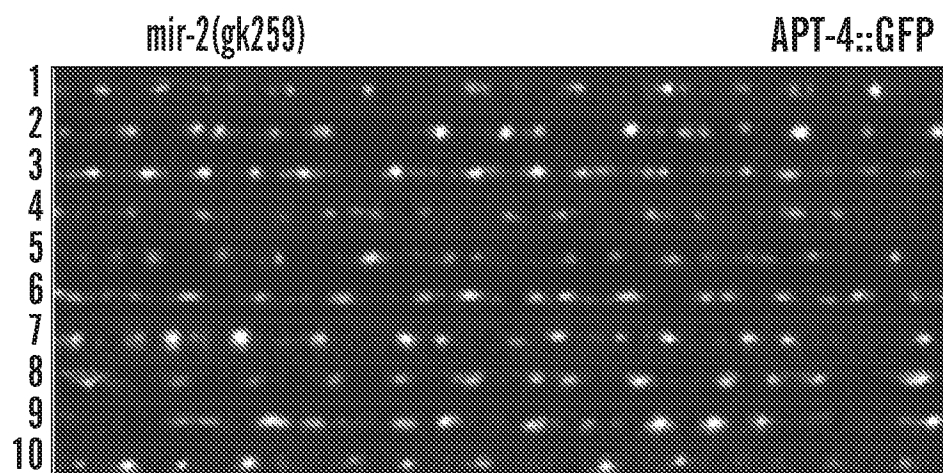
FIG. 8D

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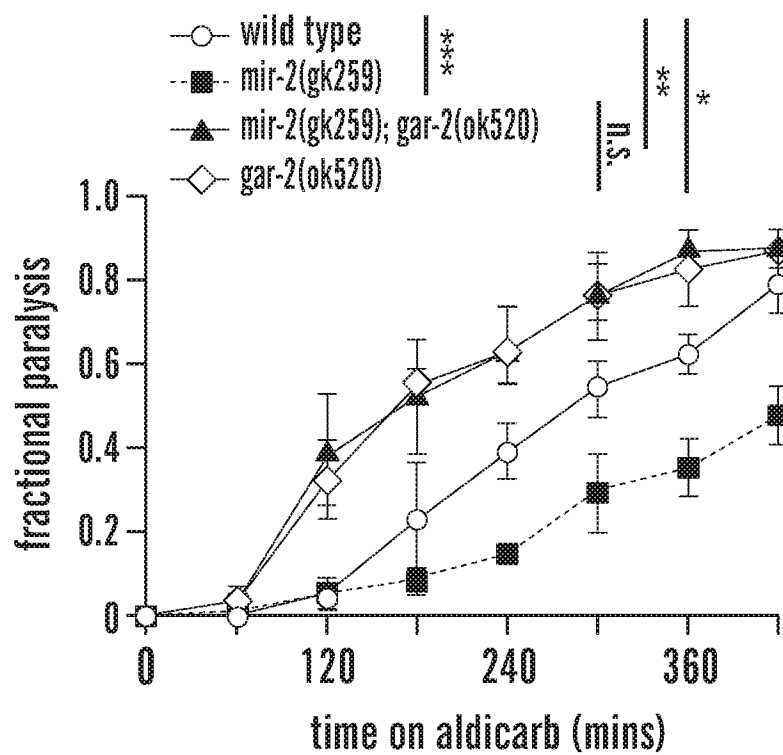
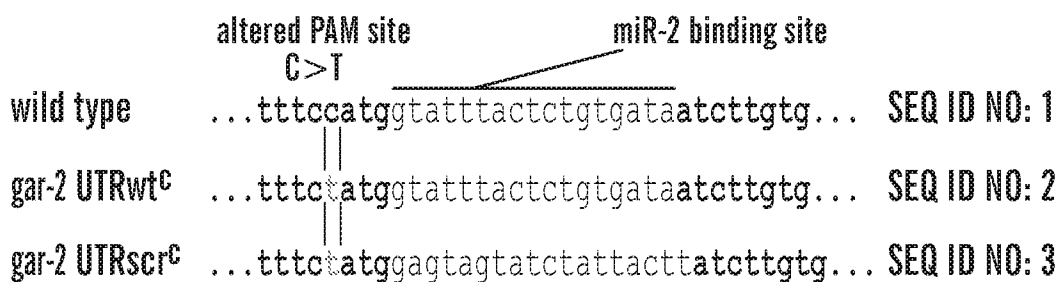
**FIG. 8E****FIG. 8F**

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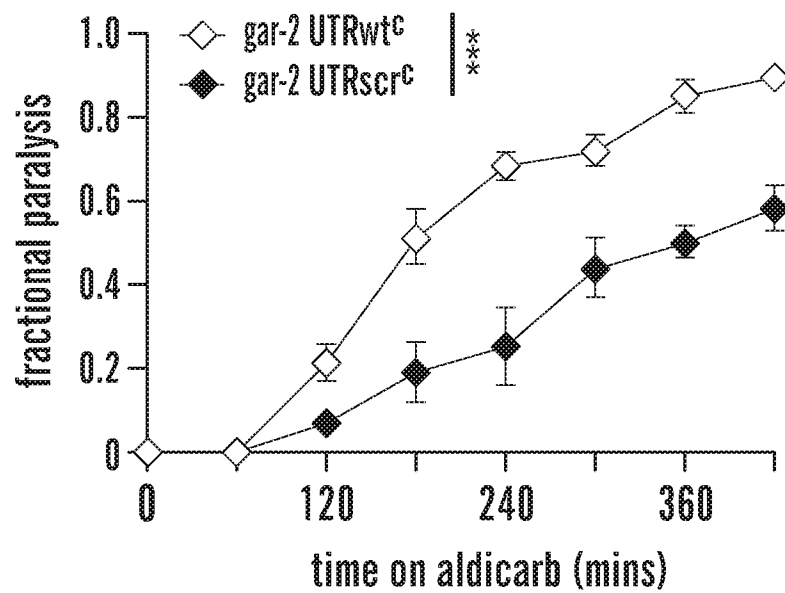
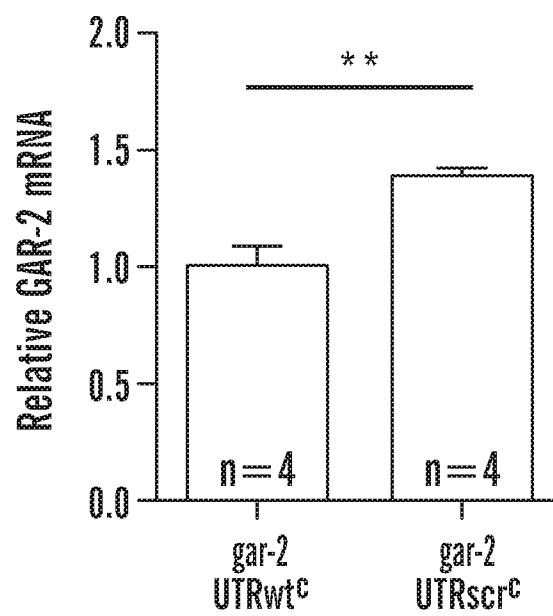
	APT-4::GFP		
	Width	Intensity	Linear Density
mir-2(gk259)	-22%**	-25%*	-32%**
mir-2 (n4108)	-20%**	-20%*	-37%***

FIG. 8G**FIG. 8H****FIG. 8I**

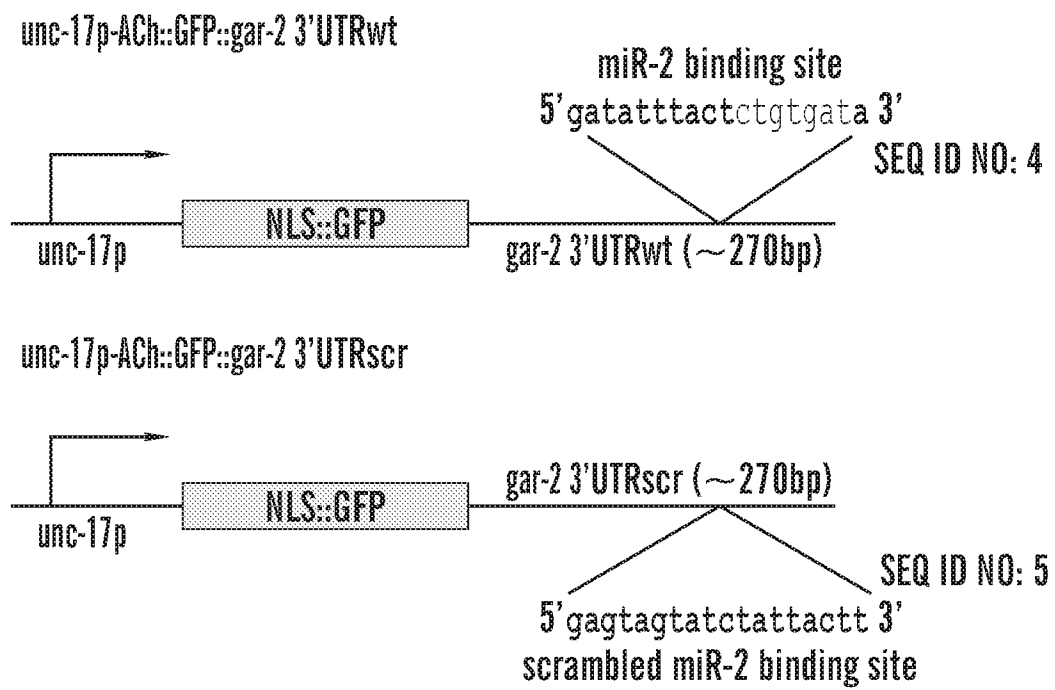
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**FIG. 9A****FIG. 9B**

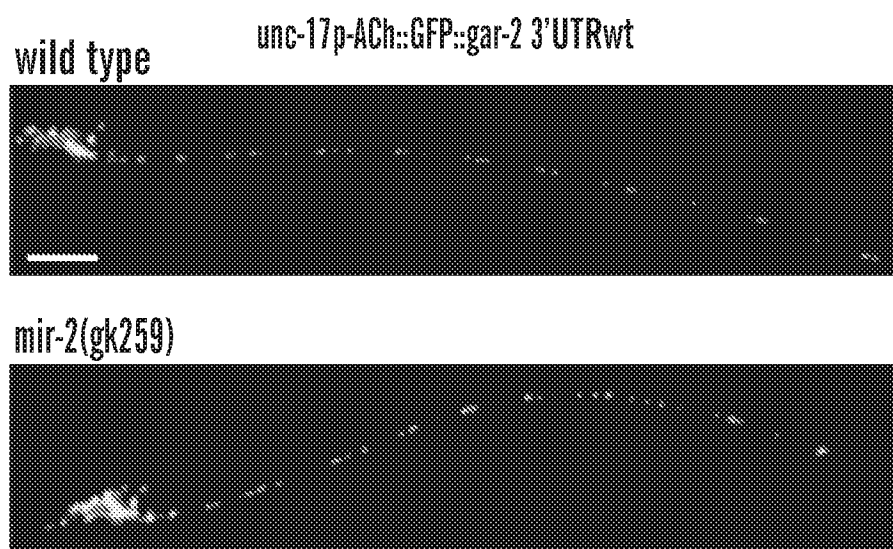
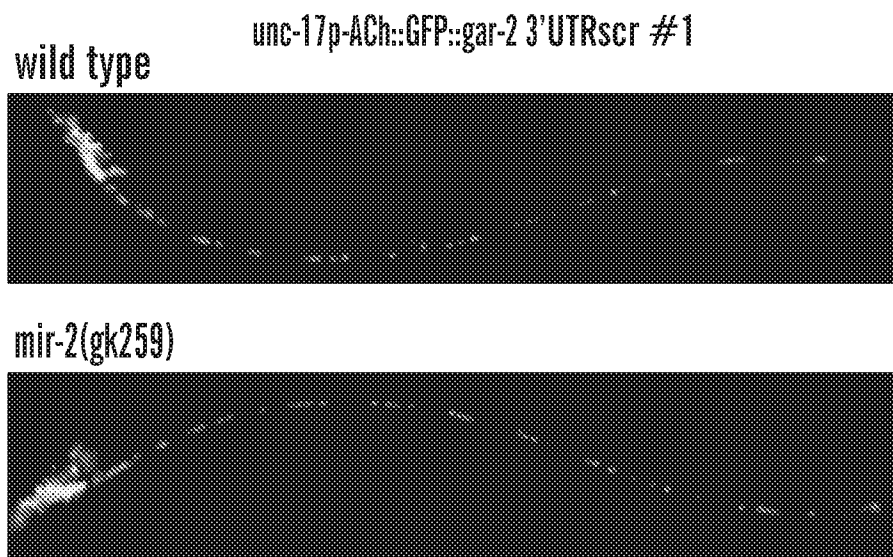
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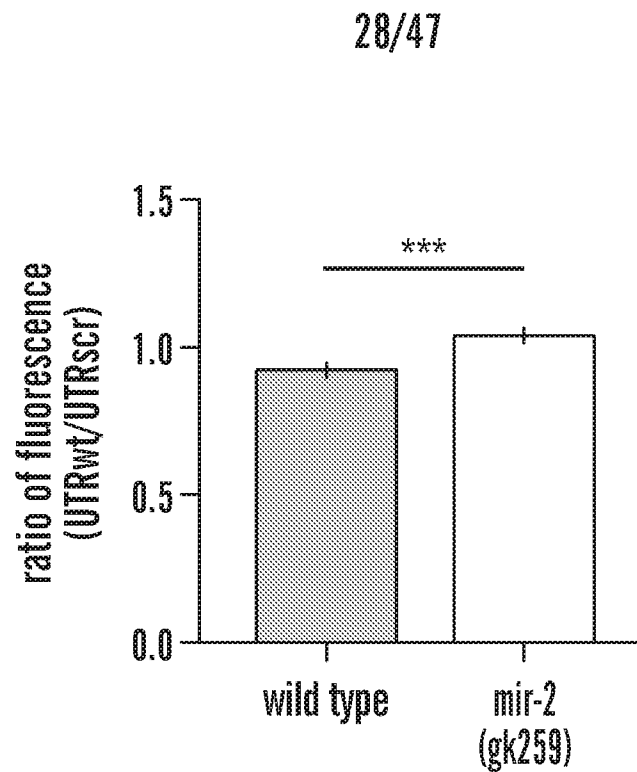
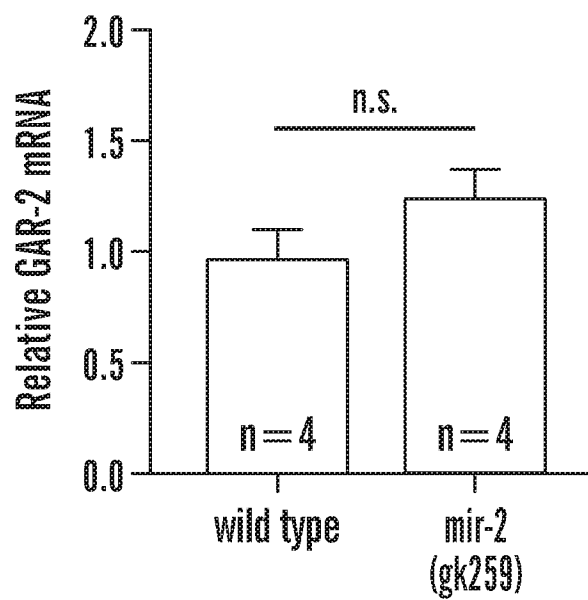
**FIG. 9C****FIG. 9D**

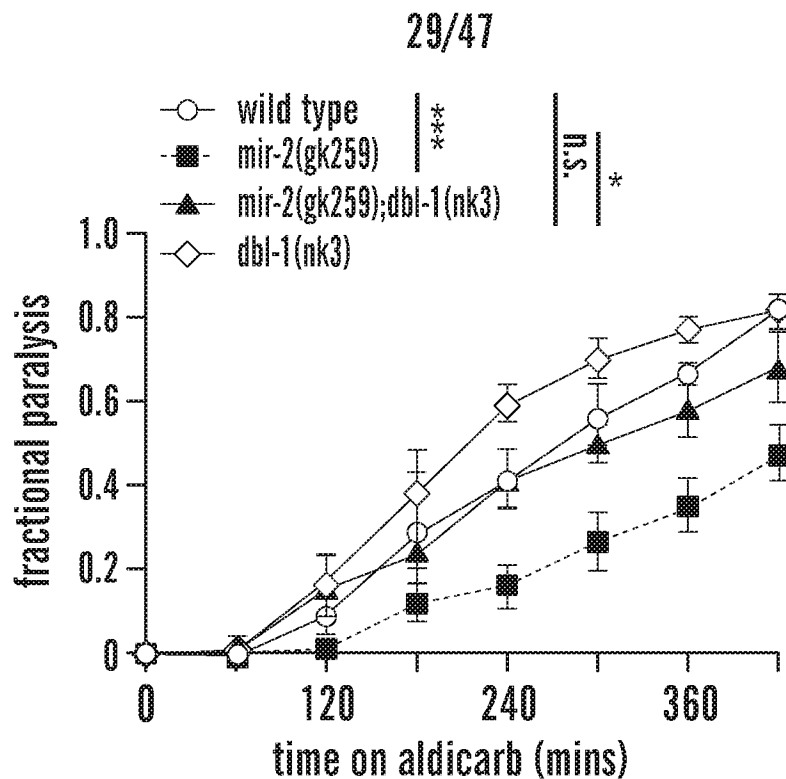
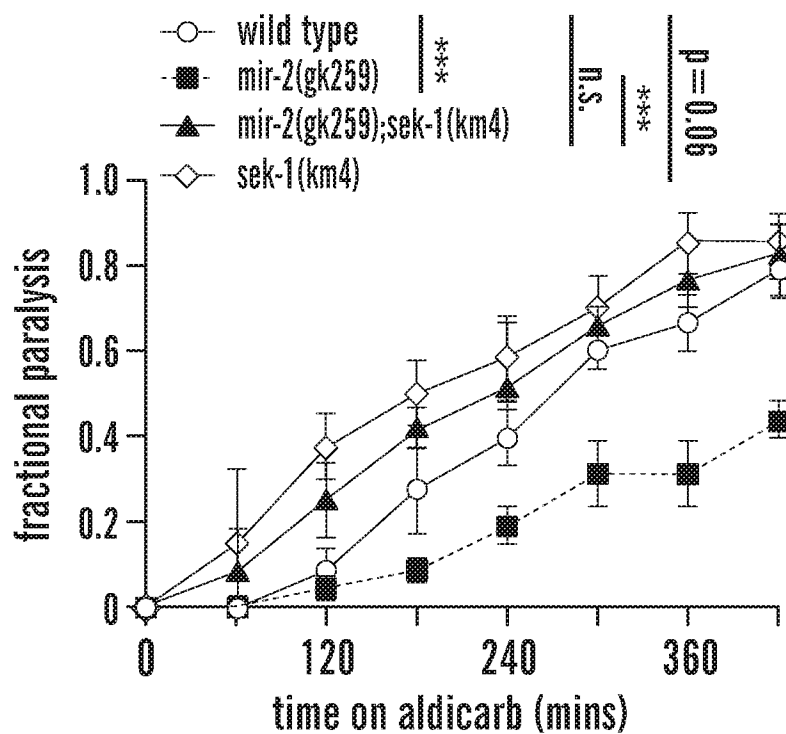
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**FIG. 9E**

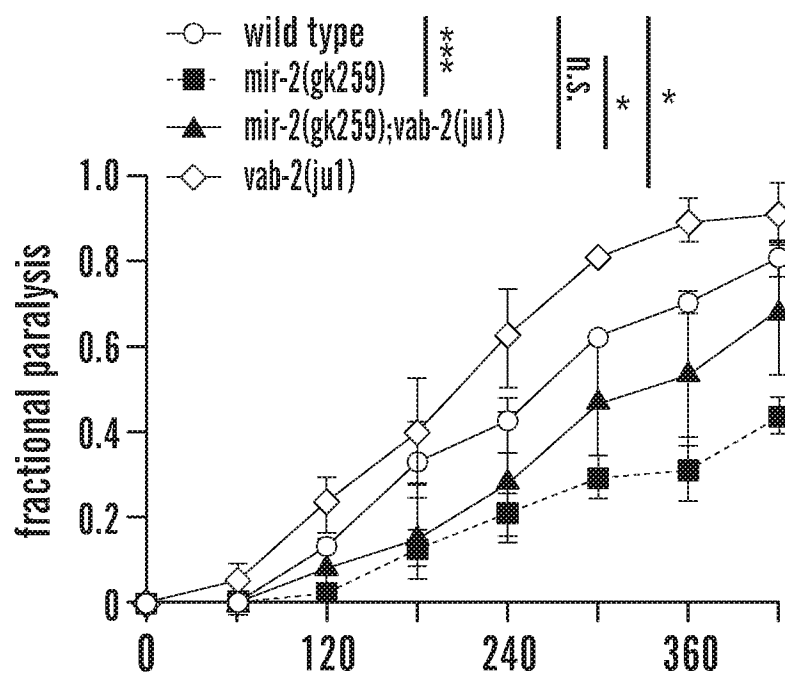
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**FIG. 9F****FIG. 9G**

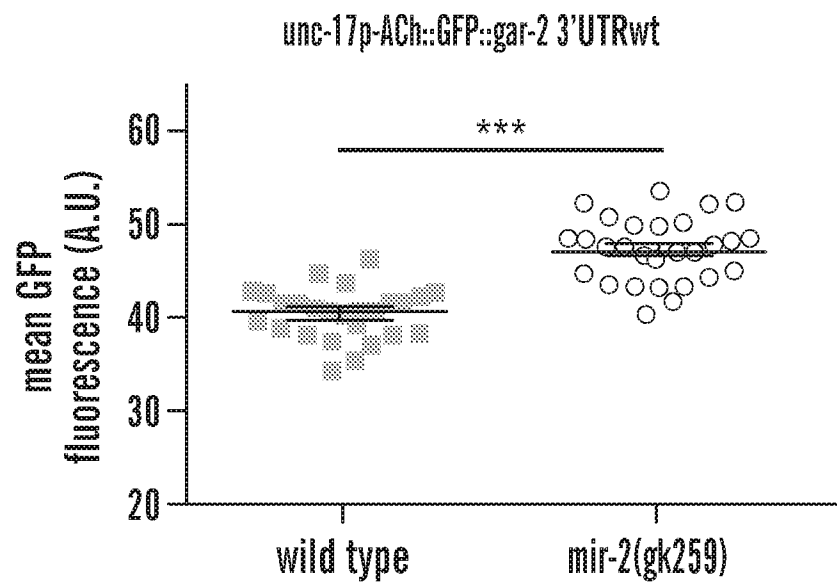
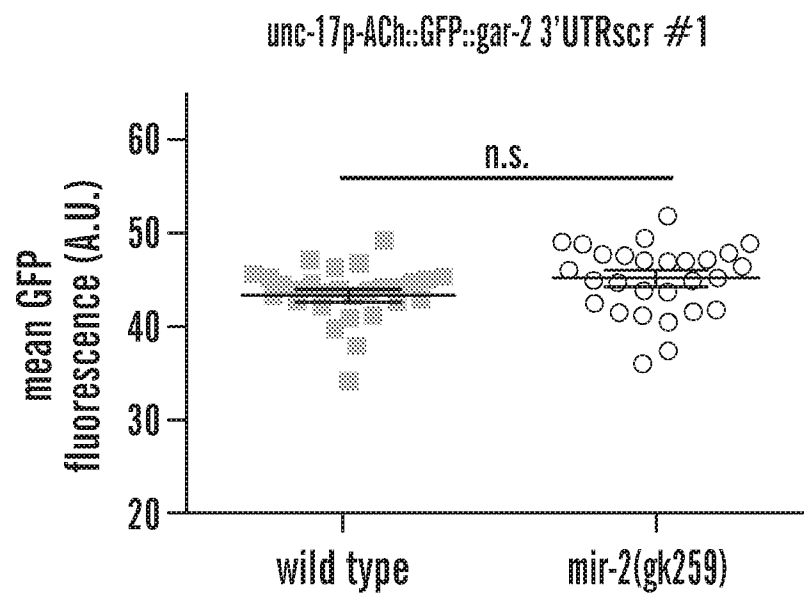
**FIG. 9H****FIG. 9I**

**FIG. 10A****FIG. 10B**

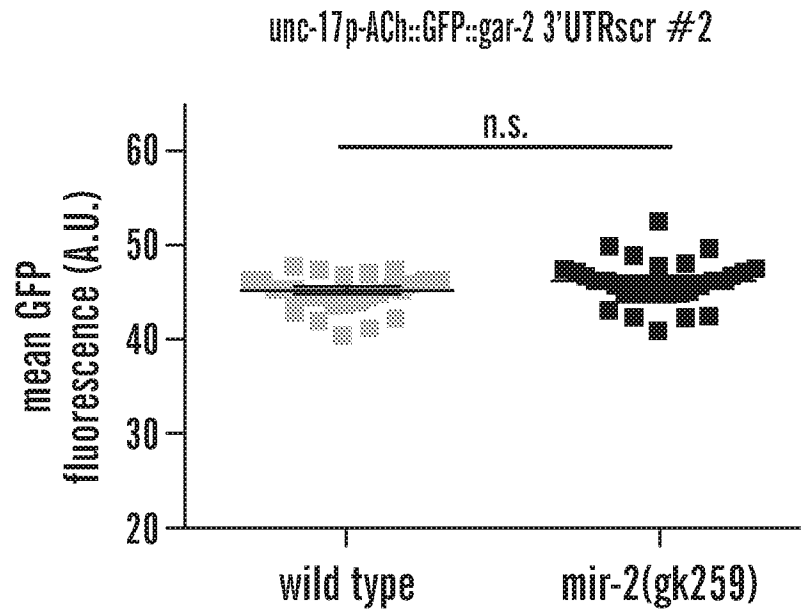
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**FIG. 10C**

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**FIG. 11A****FIG. 11B**

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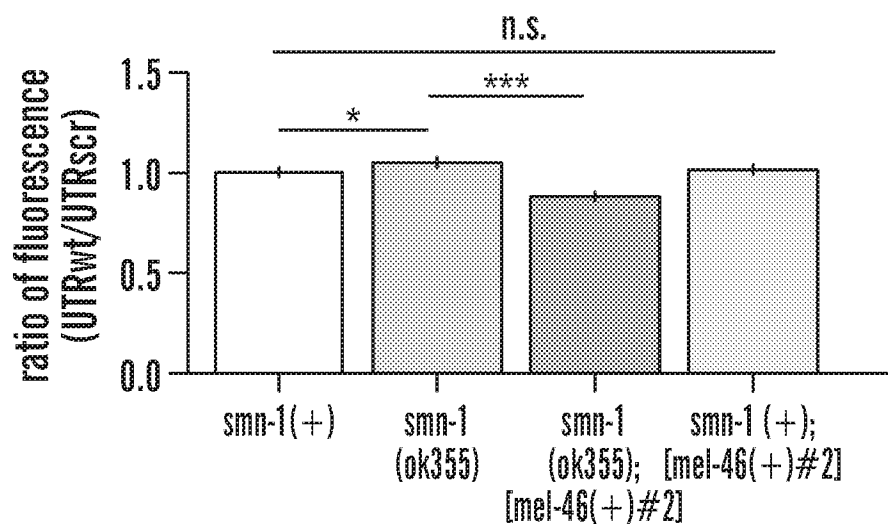
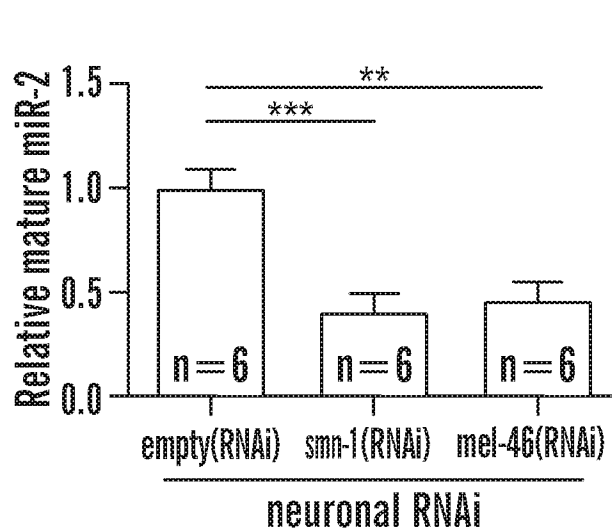
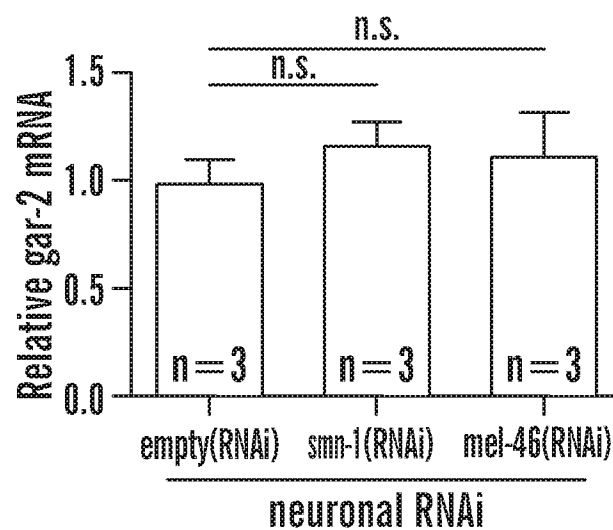
**FIG. 11C**

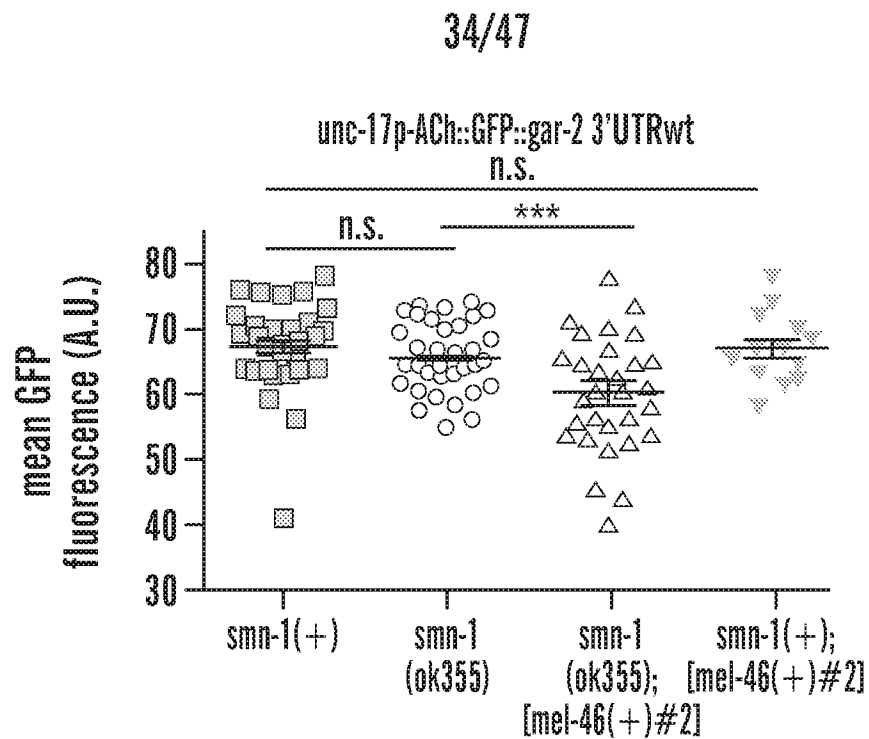
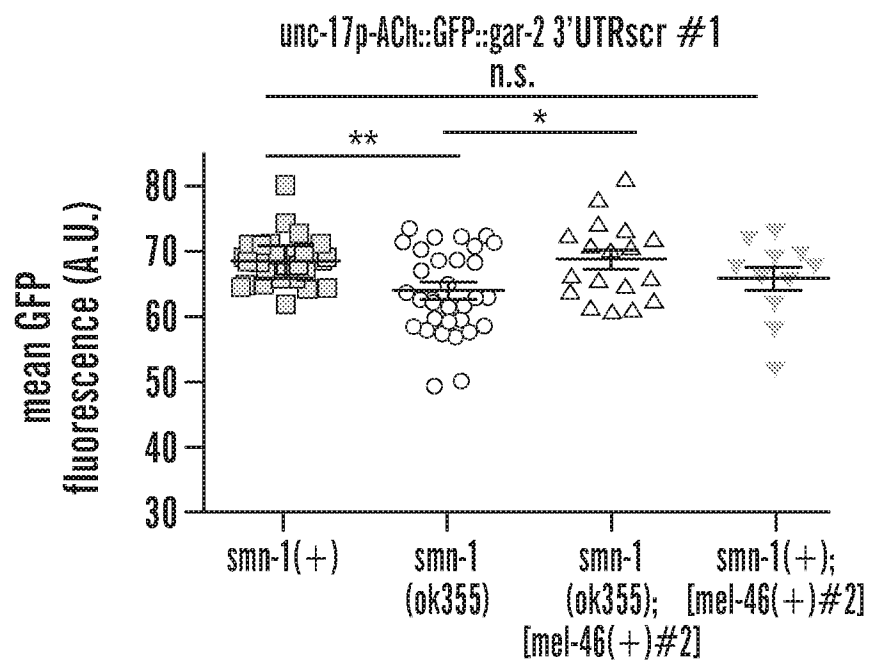
$$\text{Ratio} = \frac{\overline{\text{UTRwt}}}{\overline{\text{UTRscr}}}$$

$$\text{SEM} = (s/\sqrt{n})_{\text{UTRwt}} + (s/\sqrt{n})_{\text{UTRscr}}$$

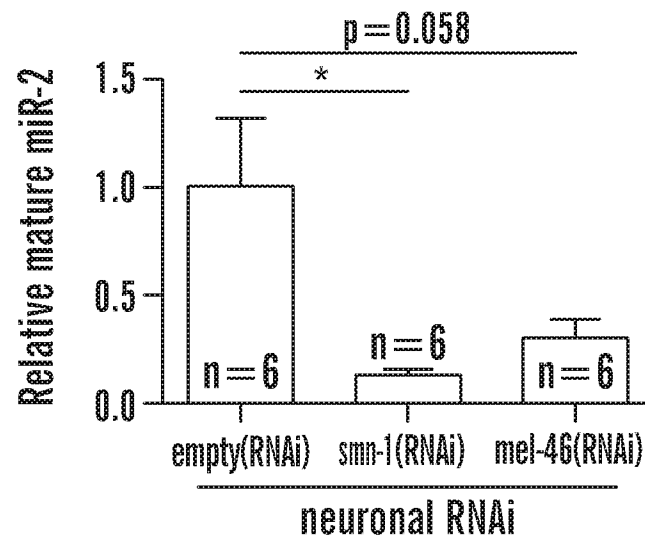
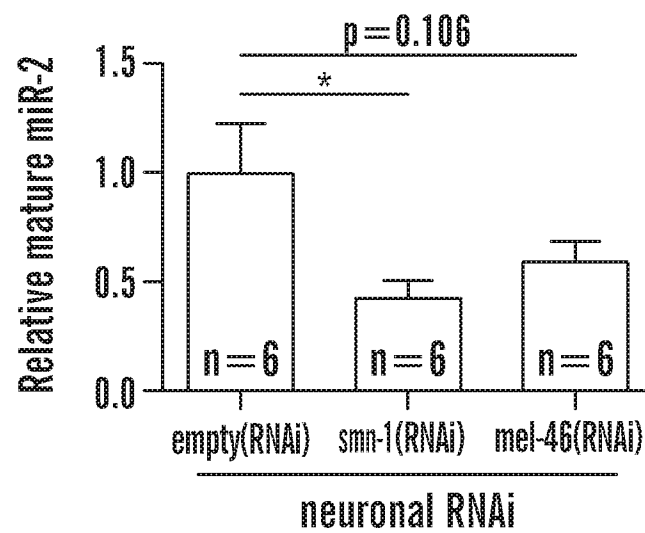
FIG. 11D

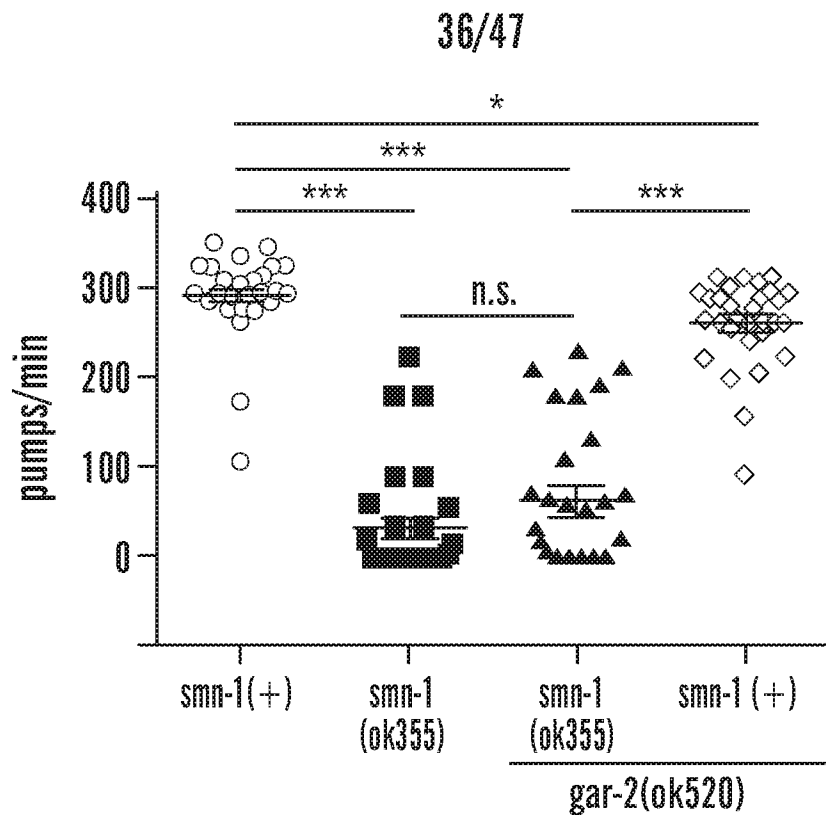
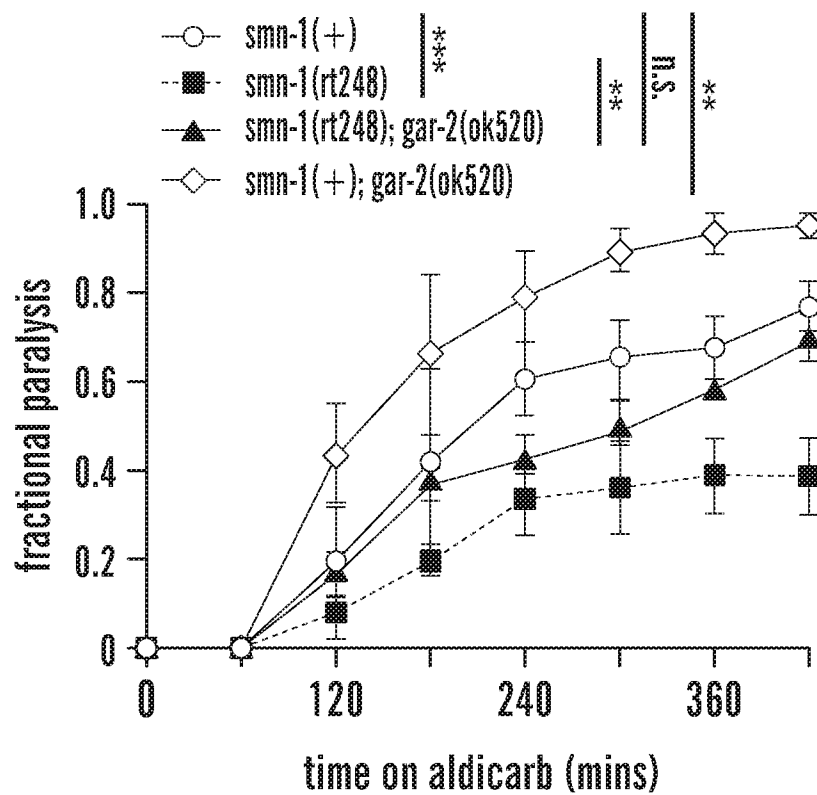
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**FIG. 12A****FIG. 12B****FIG. 12C**

**FIG. 13A****FIG. 13B**

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**FIG. 13C****FIG. 13D**

**FIG. 14A****FIG. 14B**

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	APT-4::GFP		
	Width	Intensity	Linear Density
smn-1(ok355)	-16%*	-20%*	42%*
smn-1(ok355); gar-2(ok520)	-13%	-16%	7%
smn-1(+); gar-2(ok520)	-19%**	-23%**	-17%

Asterisks: significance vs. control

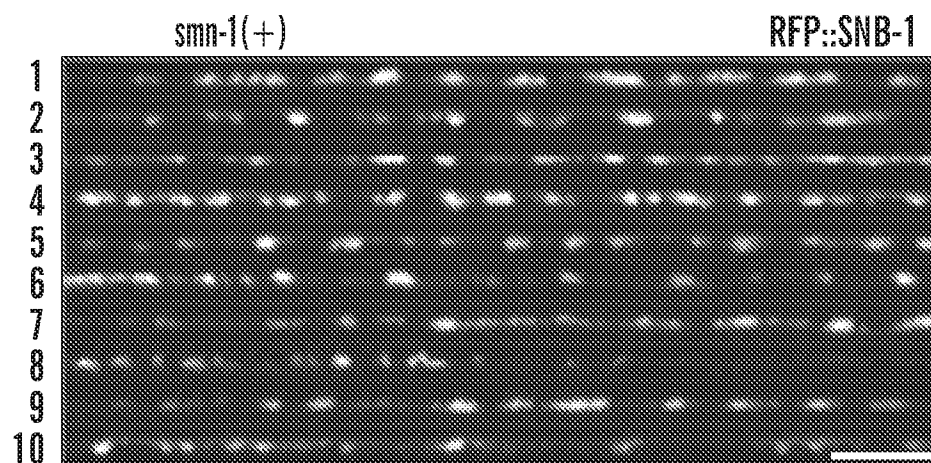
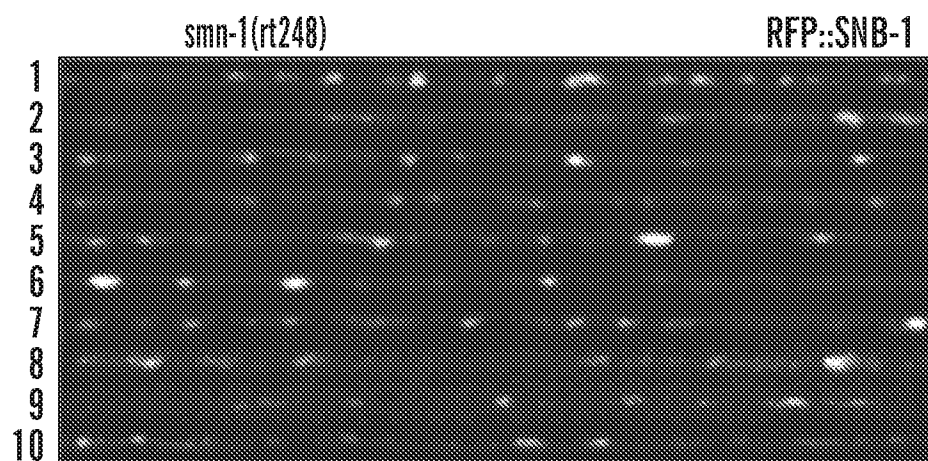
Shading: significance vs. shaded genotype

FIG. 14C

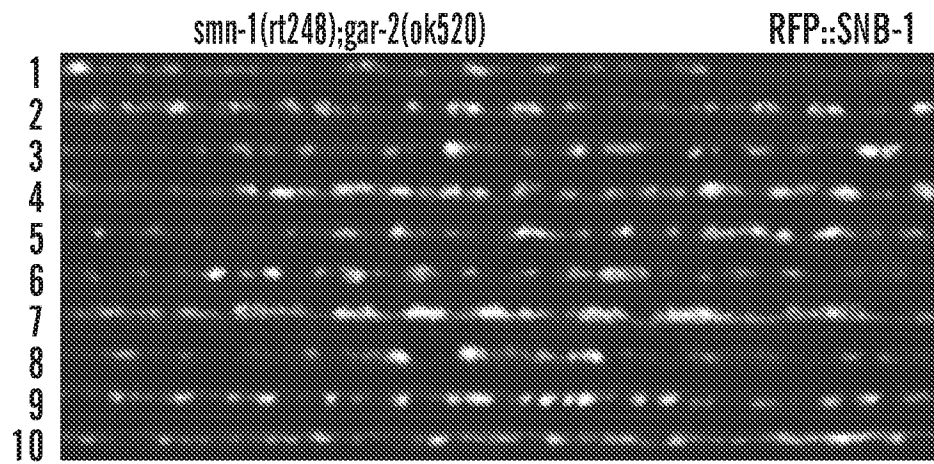
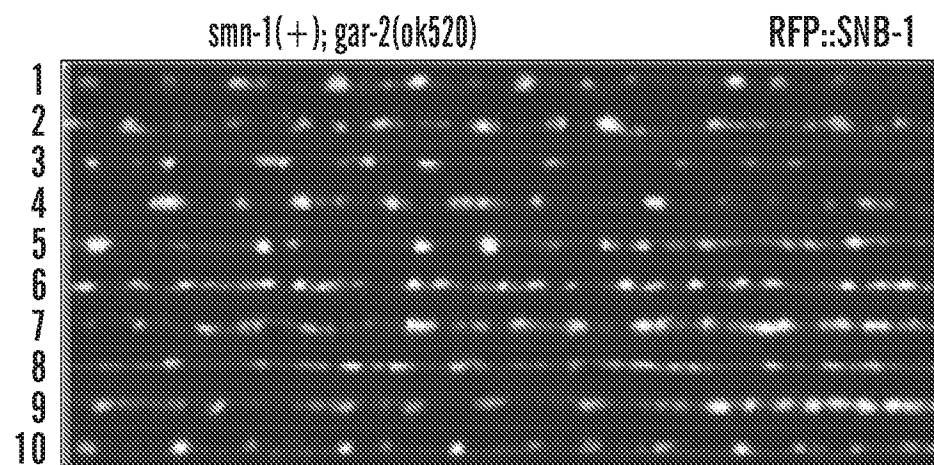
	RFP::SNB-1		
	Width	Intensity	Linear Density
smn-1(rt248)	-24%***	-33%**	-50%***
smn-1(rt248); gar-2(ok520)	1%	0%	-6%

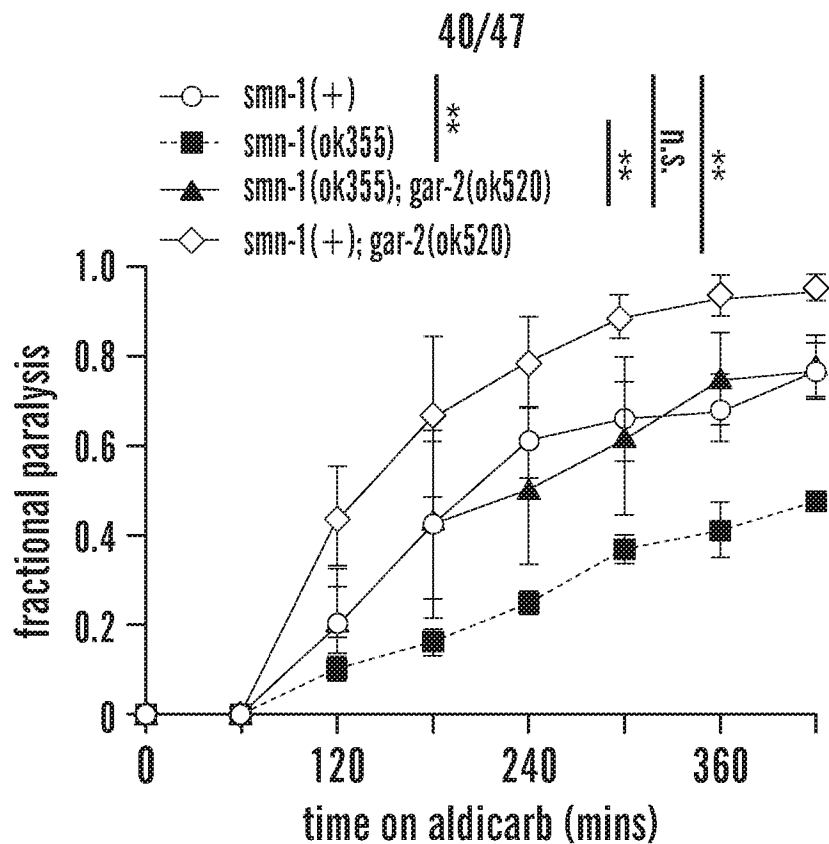
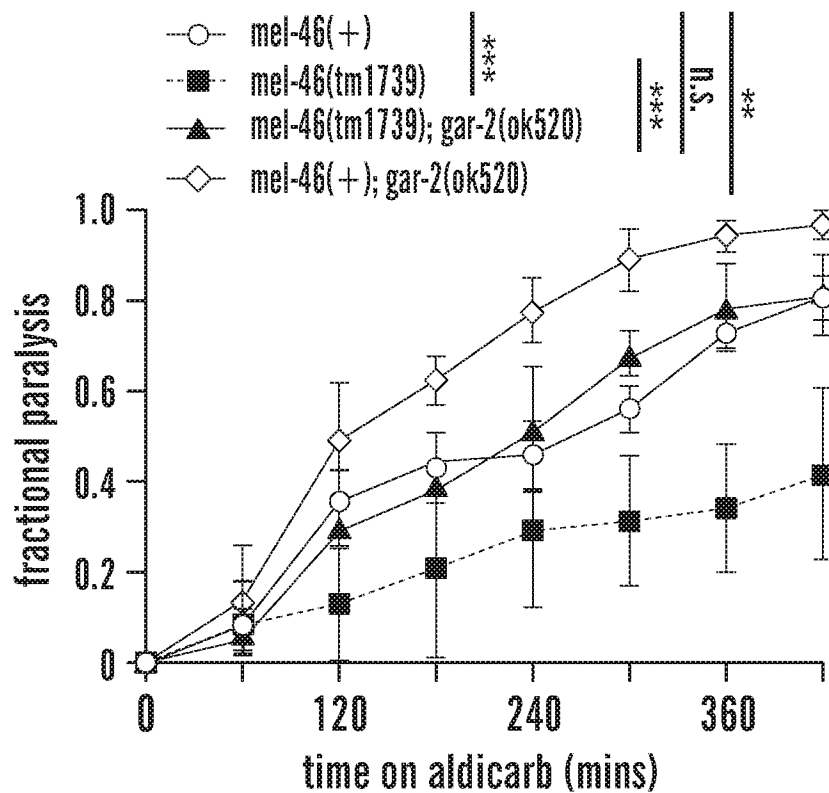
FIG. 14D

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***FIG. 14E******FIG. 14F***

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**FIG. 14G****FIG. 14H**

**FIG. 15A****FIG. 15B**

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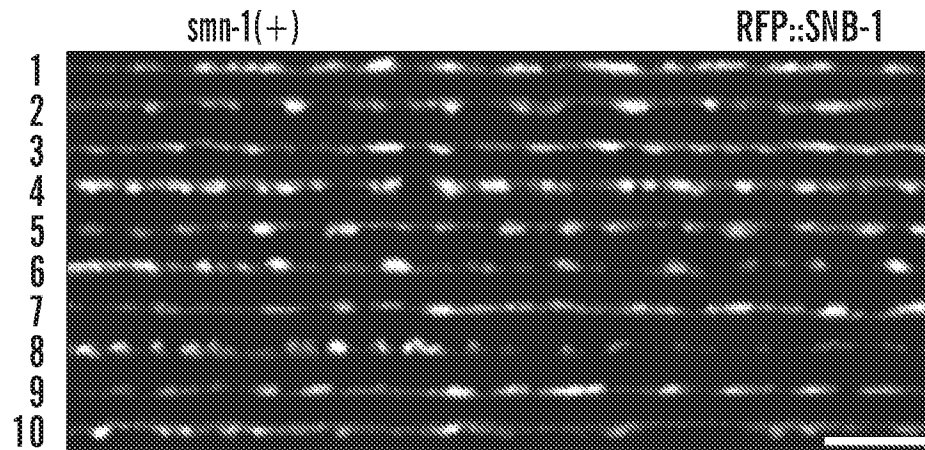
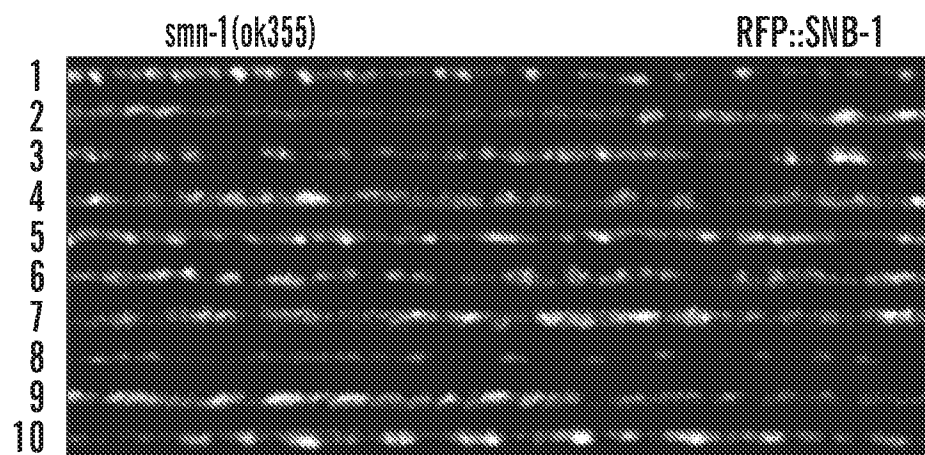
	RFP::SNB-1		
	Width	Intensity	Linear Density
smn-1(ok355)	-43%***	-53%***	-52%***
smn-1(ok355); gar-2(ok520)	6%	4%	20%
smn-1(+); gar-2(ok520)	25%*	31%*	-13%

Asterisks: significance vs. control

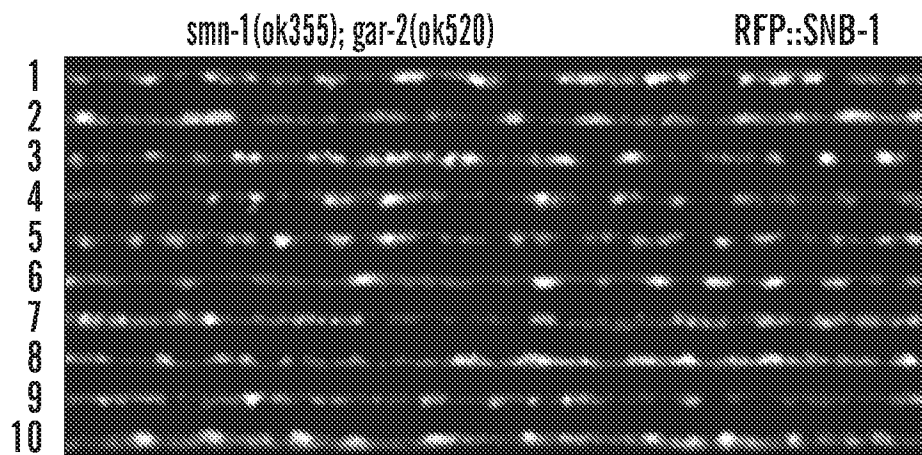
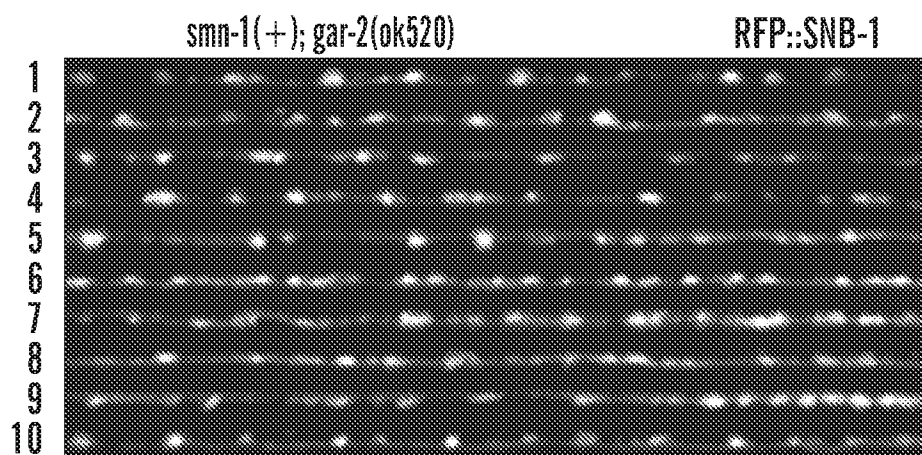
Shading: significance vs. shaded genotype

FIG. 15C

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**FIG. 15D****FIG. 15E**

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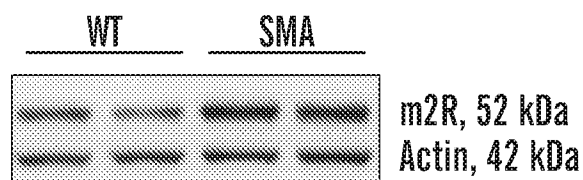
***FIG. 15F******FIG. 15G***

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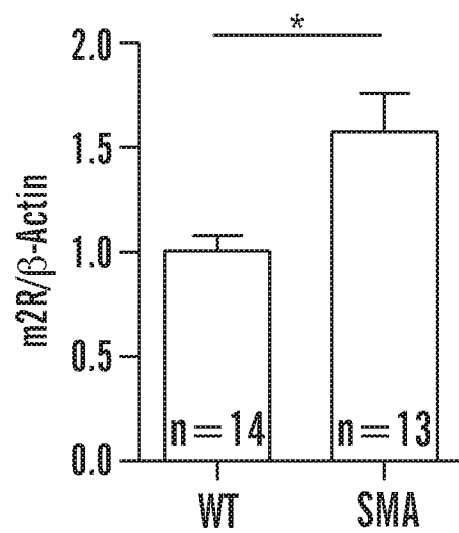
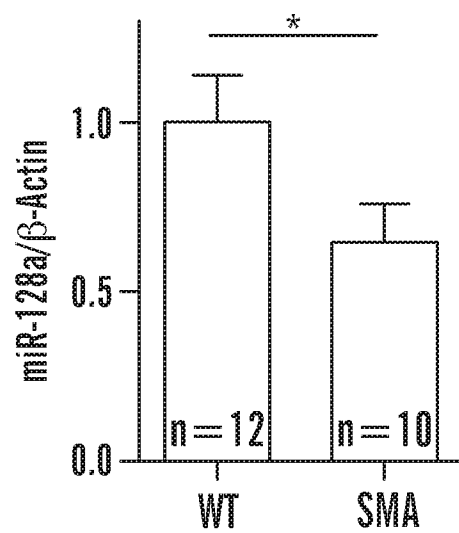
Cel-miR-2	3' cguguaguuuucgaccgacacua	5'	SEQ ID NO: 6
Cel-gar-2 3'UTR	5' ggauauuuu--cu-- <u>cugugau</u>	3'	SEQ ID NO: 7
Mmu-miR-128	3' uuucucuggccaagugacacu	5'	SEQ ID NO: 8
Mmu-Chrm2 3'UTR	5' cacucugccauccc <u>acugugu</u>	3'	SEQ ID NO: 9
Hsa-miR-128	3' uuucucuggccaa--gugacacu	5'	SEQ ID NO: 10
Hsa-CHRM2 3'UTR	5' cguuuc <u>acccccuccuacugugu</u>	3'	SEQ ID NO: 11

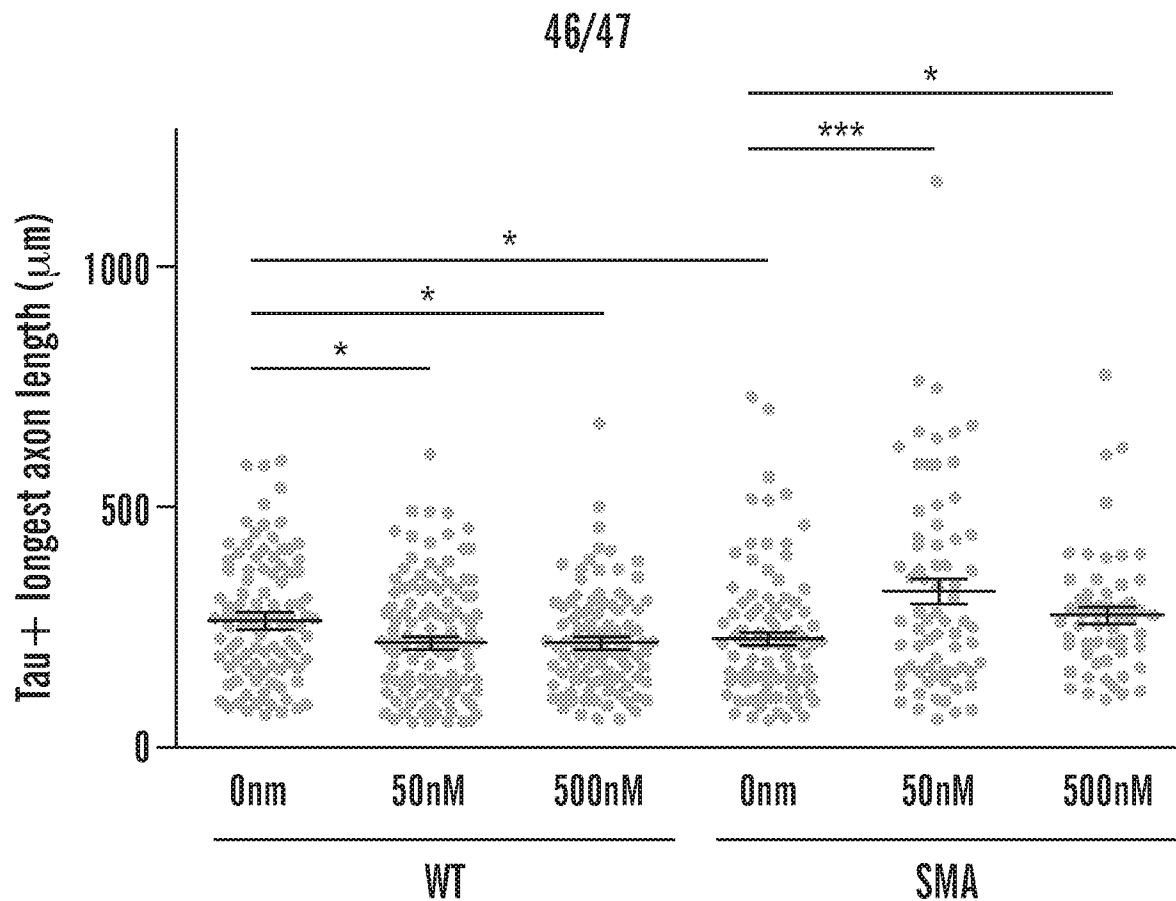
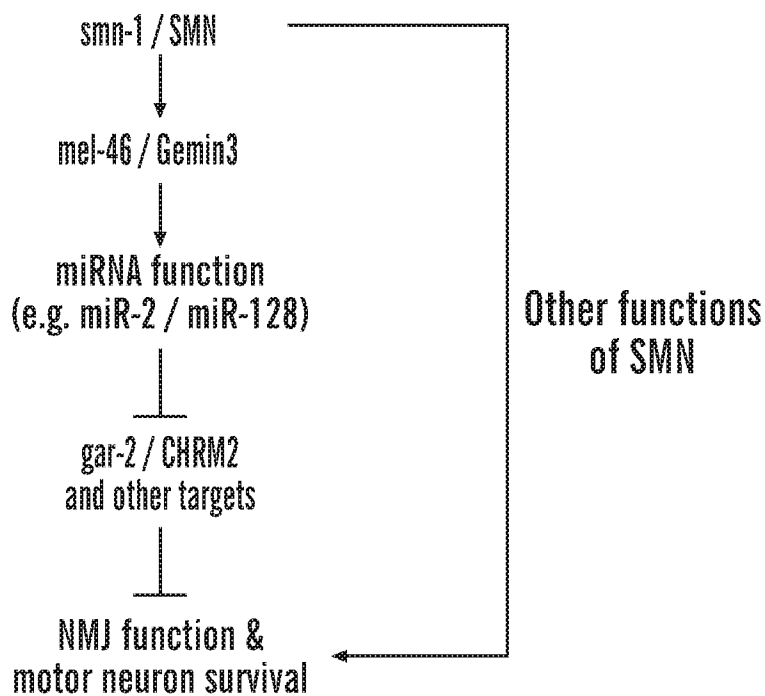
FIG. 16A

Spinal motor neurons, DIV10

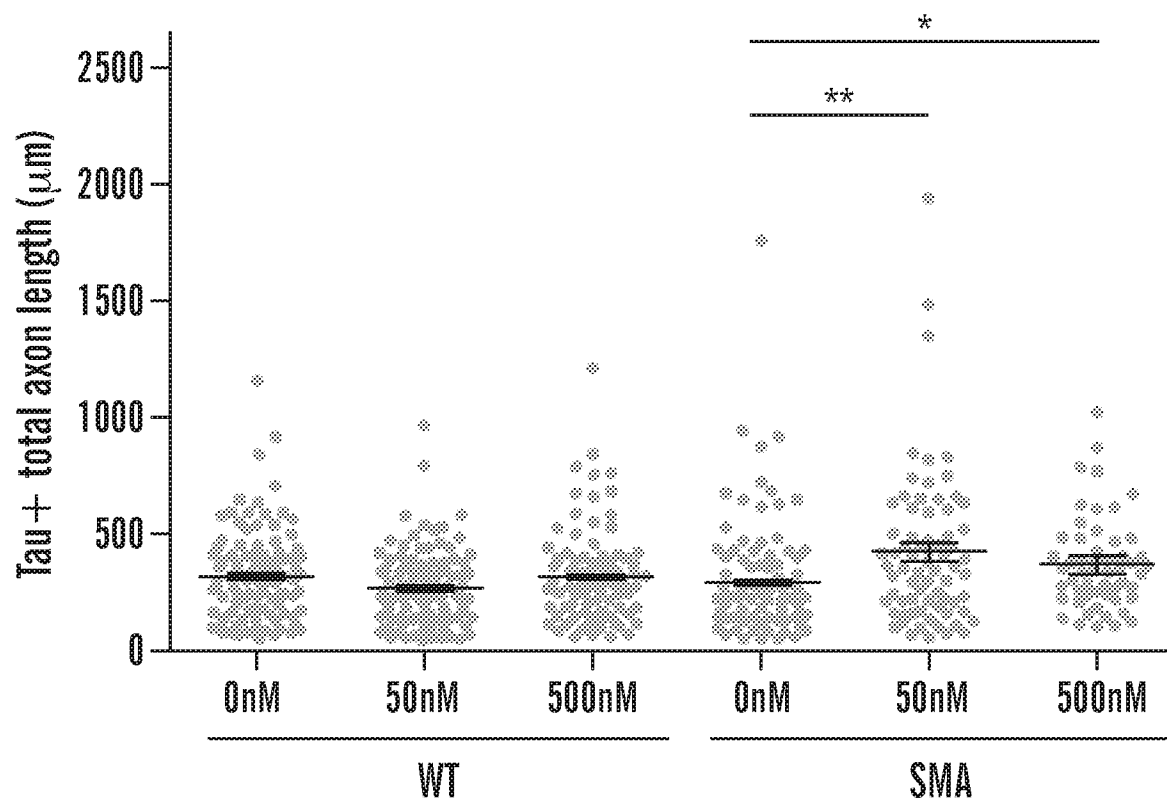
**FIG. 16B**

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**FIG. 16C****FIG. 16D**

**FIG. 16E****FIG. 16F**

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**FIG. 17**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/37894

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/5513, A61K 31/14, A61K 31/455, A61K 31/46, A61K 31/4995, A61P 25/22 (2017.01)
CPC - A61K 31/5513, A61K 31/46, A61K 31/455, A61K 31/14, A61K 31/4995, A61K 31/439, A61K 45/06, A61K 31/5386, A61K 2300/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Saito, et al. A Study of valproic acid for patients with spinal muscular atrophy. Neurology and Clinical Neuroscience 2015, 3(2):49-57; Abstract; pg 50, col 1 and Table 1,	1-10, (13,14)/(1-10)
A	Gibbons, et al. Changes in Muscarinic M2 Receptor Levels in the Cortex of Subjects with Bipolar Disorder and Major Depressive Disorder and in Rats after Treatment with Mood Stabilisers and Antidepressants. Int J Neuropsychopharmacol. April 2016, 19(4):pyv118; Abstract, pg 5, col 2; pg 6, Figure 3, and its legend	1-10, (13,14)/(1-10)
X	Gregoretti, et al. Survival of Patients With Spinal Muscular Atrophy Type 1. PEDIATRICS 2013, 131(5): e1509; pg 1 [according to the posted document]; pg 2, col 3	1, 7, 10, 11, (13,14)/(1,7,10,11)

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 August 2017

Date of mailing of the international search report

28 NOV 2017

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Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/37894

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item I.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

- a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
 - ☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/37894

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 15-18, 23
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+: claims 1-14, 19-22, directed to a method for treating Spinal Muscular Atrophy (SMA) by administering to a subject an agent that inhibits a muscarinic acetylcholine receptor. The method will be searched to the extent that the inhibitor of a muscarinic acetylcholine receptor encompasses atropine. It is believed that claims 1-11, (13,14)/(1-11) encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass a method for treating SMA by administering atropine to a subject in need thereof. Additional inhibitor(s) of a muscarinic acetylcholine receptor will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected inhibitor(s) of a muscarinic acetylcholine receptor. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a method for treating SMA by administering methoctramine, i.e., claims 1-14, 19-22. ***** See Supplemental Sheet to continue

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11, (13,14)/(1-11), restricted to atropine

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/37894

In Continuation of Box III. Observations where unity of invention is lacking:

Group II: claims 24-28, directed to a composition comprising: methoctramine and an agent that facilitates the passage through a blood brain barrier.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

The special technical feature of each invention of Group I+ is a specific muscarinic acetylcholine receptor inhibitor recited therein.

The inventions of Group I+ do not include the shared or common technical feature of a composition comprising: methoctramine and an agent that facilitates the passage through a blood brain barrier, as required by Group II.

The inventions of Group II do not include the shared or common technical feature of a method for treating SMA by administering to a subject an agent that inhibits a muscarinic acetylcholine receptor, as required by Group I+.

Common Technical Features

Some inventions of Groups I+ and II share the technical feature of methoctramine. However, this shared technical feature does not represent a contribution over prior art as being anticipated by a paper titled "Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses" by Wright, et al. (J Neurosci. 2009, 29(47):14942-55) (hereinafter "Wright").

Wright discloses methoctramine, an M2/4-preferring muscarinic acetylcholine receptor antagonist, as well as a role of muscarinic acetylcholine receptors in the functioning of neuromuscular junctions (NMJs) (Abstract, "Muscarinic acetylcholine receptors (mAChRs) modulate synaptic function... At neuromuscular junctions (NMJs), mAChRs have been implicated in compensatory sprouting of axon terminals in paralyzed or denervated muscles. Here we used pharmacological and genetic inhibition and localization studies of mAChR subtypes at mouse NMJs to demonstrate their roles in synaptic stability and growth but not in compensatory sprouting. M2 mAChRs were present solely in motor neurons, whereas M1, M3, and M5 mAChRs were associated with Schwann cells and/or muscle fibers... methoctramine, an M2/4-preferring antagonist, induced terminal sprouting and terminal withdrawal, but no muscle fiber atrophy. Consistent with this observation, M2-/- but no other mAChR mutant mice exhibited spontaneous sprouting..."; pg 14943, col 1, "Administration of mAChR antagonists. Local in vivo blockade of one or more mAChR subtypes was achieved... antagonists, dissolved in 50 ml of sterile physiological saline, were administered by subcutaneous injection over the right levator auris longus (LAL) muscle. Sources and doses of the antagonists were as follows: atropine (Sigma-Aldrich)..., 0.2-20 mg/kg; methoctramine (Sigma-Aldrich)..."). As said technical feature was known in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the groups.

The inventions of Group I+ share the technical feature of a method for treating Spinal Muscular Atrophy (SMA) by administering to a subject an agent that inhibits a muscarinic acetylcholine receptor. However, this shared technical feature does not represent a contribution over prior art as being anticipated by a paper titled "A Study of valproic acid for patients with spinal muscular atrophy" by Saito, et al. (Neurology and Clinical Neuroscience 2015, 3(2):49-57) (hereinafter "Saito").

Saito discloses a method for treating SMA, the method comprising: administering valproic acid to a subject in need thereof (Abstract, "Valproic acid (VPA) is expected to become an effective therapeutic agent for spinal muscular atrophy (SMA)... Methods: Seven consecutive Japanese SMA patients (three males, four females) were recruited. Of those, six were type 2 (cases A-E, G) and one was type 3 (case F). One female patient (case E) was aged 2 years and 10 months, whereas the others ranged in age from 15 to 42 years. VPA was administered for 6 months with L-carnitine. We ... evaluated using the Modified Hammersmith Functional Motor Scale for SMA (MHFMS), vital capacity (VC), maximum insufflation capacity (MIC), and cough peak flow (CPF) before and at 1, 3 and 6 months after starting treatment. Results: Cases B-E and G completed the study. The final VPA dosage in cases B-D and G was 400 mg/day, whereas that in case E was 100 mg/day... Case E showed a remarkable improvement in MHFMS, and gained motor function to turn from side to side during the study period. Although no significant changes were observed in MHFMS in the older cases, VC, MIC and CPF were improved in those. Conclusion: Our findings suggest that VPA treatment is effective for improving MHFMS [Modified Hammersmith Functional Motor Scale] and respiratory function in some SMA patients").

Saito does not specifically disclose that valproic acid inhibits a muscarinic acetylcholine receptor. However, said limitation is met by/inherently present in Saito for the following reasons:

a paper titled "M-current preservation contributes to anticonvulsant effects of valproic acid" by Kay, et al. (J Clin Invest. 2015, 125(10): 3904-3914) discloses that "... effects of nonacute VPA treatment involve preservation of the M-current, a low-threshold noninactivating potassium current, during seizures. In a wide variety of neurons, activation of Gq-coupled receptors, such as the m1 muscarinic acetylcholine receptor, suppresses the M-current and induces hyperexcitability. We demonstrated that VPA treatment disrupts muscarinic suppression of the M-current and prevents resultant agonist-induced neuronal hyperexcitability..." (Abstract, see also pg 3904, col 2 to pg 3905, col 1, "We demonstrate that VPA treatment disrupted muscarinic acetylcholine receptor-induced M-current suppression, preventing receptor-induced hyperexcitability in neurons. Consistently, pharmacological blockade of M-current diminished the anticonvulsant effect of VPA but not that of diazepam"), thereby disclosing that VPA is an inhibitor of the activity of a muscarinic acetylcholine receptor.

In addition or in the alternative, a paper titled "Changes in Muscarinic M2 Receptor Levels in the Cortex of Subjects with Bipolar Disorder and Major Depressive Disorder and in Rats after Treatment with Mood Stabilisers and Antidepressants" by Gibbons, et al. (Int J Neuropsychopharmacol. April 2016, 19(4):pyv118) discloses that VPA potentiates binding of AF-DX 384, the M2 muscarinic receptor antagonist (Abstract, instant application, claim 11), to the M2 receptor (pg 5, col 2, "Mood Stabilisers... Compared to vehicle, there were higher levels of [3H]AF-DX 384 binding in inner layer of the cingulate cortex, the outer and middle layer of the parietal cortex and the striatum from rats treated with valproate"; pg 6, Figure 3, and its legend, "(B) The specific binding ... of [3H]AF-DX 384 to rat CNS following 28 days treatment with vehicle... or valproate (12 g sodium valproate/day)", thereby disclosing that valproate is an allosteric inhibitor of Muscarinic M2 Receptor. As said technical feature was known in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the inventions.

The inventions of Groups I+ and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.