Systemic peptide-mediated oligonucleotide therapy improves long-term survival in spinal muscular atrophy

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Abstract:

The development of antisense oligonucleotide therapy is an important advance in the identification of corrective therapy for neuromuscular diseases such as spinal muscular atrophy (SMA). However, current approaches utilizing invasive intrathecal single stranded oligonucleotide delivery fails to adequately treat affected peripheral tissues. Here we report an advanced peptide-oligonucleotide Pip6a-PMO that demonstrates potent systemic efficacy in severe SMA mice. Spinal muscular atrophy results from reduced levels of the ubiquitously-expressed survival motor neuron (SMN) protein due to loss-of-function mutations in the *SMN1* gene. Therapeutic splice-switching oligonucleotides (SSOs) modulate exon 7 splicing of the nearly identical *SMN2* gene to generate functional SMN protein. Pip6a-PMO yields SMN expression at high efficiency in peripheral and CNS tissues, resulting in profound phenotypic correction at doses an order of magnitude lower than required by standard naked SSOs. Survival is dramatically extended from 12 to > 550 days, with improvement in neuromuscular junction morphology, downregulation of transcripts related to programmed cell death in the spinal cord, and normalization of circulating insulin-like growth factor 1. The potent systemic efficacy of Pip6a-PMO, targeting essential peripheral as well as CNS tissues, demonstrates the high clinical potential of peptide-PMO therapy for SMA.

Significant Statement:

SSO treatment in SMA has quickly become a clinical reality, but without effective systemic delivery the results may never be strong enough to be considered a curative treatment. Our peptide-conjugated SSOs are being designed for clinical trials for the treatment of Duchenne muscular dystrophy. Here we report advanced Pip peptides that effectively deliver SSOs body-wide and at order of magnitude lower doses than required by naked SSOs in a mouse model of SMA. Furthermore, our peptide-SSO is able to deliver to the CNS of adult mice. This is thus the very first time that an oligonucleotide has shown activity in the CNS following a systemic route with peptide delivery.

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Introduction

Spinal muscular atrophy (SMA), a leading genetic cause of infant mortality primarily due to lower motor neuron degeneration and progressive muscle weakness, results from loss of the ubiquitous survival motor neuron 1 gene, *SMN1* (1, 2). Humans have a second nearly identical copy, *SMN2*, which differs from *SMN1* by a crucial nucleotide transition within exon 7 leading to the predominant generation of an alternative exon 7-excluded transcript and only marginally functional protein (3-7). *SMN2* therefore fails to compensate for loss of *SMN1* unless sufficient copies are present to generate functional levels of full length SMN protein (2).

A rational, gene therapy-based approach for SMA utilizes single-stranded antisense splice-switching oligonucleotides (SSOs) to enhance *SMN2* pre-mRNA exon 7 inclusion via steric block of splice regulatory pre-mRNA elements (8). Targeting the intron splice silencer N1 (ISS-N1) site within intron 7, by deletion or SSO-mediated splice-switching, improves exon 7 inclusion (9, 10). ISS-N1 targeted SSOs used to treat pre-symptomatic severely affected neonatal SMA mice, via systemic or intracerebroventricular administration, extend survival from 10 to >100 days (11, 12). While SSO targeting to the central nervous system (CNS) is essential, not all disease-relevant SMN roles are neuron-specific and there is mounting evidence that treating peripheral as well as CNS tissues will be essential for long term high efficacy of SSO therapy (13-24).

Although SSO therapy is currently at an advanced stage of development and one of the most promising approaches for SMA, a major challenge is efficient delivery. The current generation of SSOs do not cross the blood-brain barrier and must be administered by repeated intrathecal injection. This route of administration achieves high cerebrospinal fluid concentrations of SSOs yet provides no peripheral effect on SMN expression. Furthermore, while this mode of administration appears to be safe, nearly a third of treated patients experience the typical side effects associated with lumbar puncture, and patients who develop scoliosis, which frequently occurs in SMA, pose additional challenges associated with lumbar punctures (25). To dramatically improve delivery of neutrally charged SSOs, we have developed an advanced Pip peptide delivery technology. Pip peptides are covalently conjugated (26, 27) and capable of SSO delivery to a variety of adult tissues including liver, kidney, skeletal muscle, diaphragm, and heart (26, 28-30). Here we report that a highly active peptide, Pip6a, directly conjugated to a morpholino phosphorodiamidate oligomer (PMO), permits highly efficient systemic delivery, which enhances body-wide SMN expression, including in brain and spinal cord, and rescues the phenotype and dramatically prolongs lifespan of severe SMA mice. These data demonstrate powerful SMA disease modification by peptide-PMO therapy, a benefit that could be extended to many other neurodegenerative disorders (8, 31, 32).

Results

Systemic Pip6a-PMO treatment of severe SMA mice rescues the disease phenotype and enhances survival

Pip6a, our lead delivery peptide (28, 29), was directly conjugated to a 20-mer PMO sequence targeting the ISS-N1 element of *SMN2* intron 7 (12). We evaluated the efficacy of Pip6a-PMOs *in vitro* using the neuroblastoma cell line SH-SY5Y, which resulted in a significant, dose-

dependent increase of full-length *SMN2* (*FLSMN2)* expression (Fig. S1A). By contrast, PMO alone failed to increase *FLSMN2* expression even at the highest dose of 500 nM (Fig. S1B). Pip6a is thus able to improve cellular uptake of PMO by neurons.

For *in vivo* studies, we used the Taiwanese severe SMA model (33). Pups were treated at postnatal day 0 (PND0), with a single dose of 10 µg/g PMO or Pip6a-PMO via the facial vein. Survival was recorded till the humane endpoint was reached. Untreated and saline treated SMA mice survived a median of 12 days as is typical of this model (Table S1)(34). PMO-treated mice showed no change in survival, whereas Pip6a-PMO-treated mice survived to a median of 167 days (Fig. 1A and Table S1).

Pip6a-PMO-treated pups were significantly heavier than their untreated or PMO-treated littermates from day 7 (Fig. 1B). Movement and coordination were tested via the natural response to negative geotaxis. A greater percentage of Pip6a-PMO-treated pups were able to turn upright by day 8 compared with either untreated or PMO-treated pups (Fig. S2). Muscle strength was tested by the hind limb suspension 'tube test' scoring system (35). Pups treated with 10 µg/g PMO were not significantly different to untreated pups while Pip6a-PMO-treated pups were significantly stronger by day 12 (Fig. 1C).

While only Pip6a-PMO-treated pups showed an improved phenotype, both PMO- and Pip6a-PMO-treated pups demonstrated significantly increased *FLSMN* mRNA and SMN protein expression in all tissues tested (Fig. 1, D and E). Insulin-like growth factor 1 (IGF-1) is a neurotrophic factor reduced in severe SMA models (11, 36). Both Pip6a-PMO and PMO treatment rescued IGF-1 expression at PND7, Pip6a-PMO restoring IGF-1 to levels found in unaffected littermates (Fig. 1F). Impaired maturation of neuromuscular junctions (NMJs) is a hallmark of SMA, notable by small endplate area and complexity, denervation, poor terminal arborization and neurofilament accumulation at the pre-synaptic nerve terminal (37-41). We therefore studied the morphology of NMJs in the *transversus abdominis* (TVA), one of the most severely affected muscles, between untreated and treated SMA pups at PND12 (42). Here we observed the strongest difference between treatment groups. Pups treated with Pip6a-PMO had far fewer denervated or partially denervated NMJs, indicative of poor end-terminal arborization, in comparison to PMO-treated or untreated SMA pups (Fig. 1, G and H). While significantly greater than untreated SMA pups, both treatments resulted in similar restoration of post-synaptic motor endplates areas (Fig. 1I). In this side by side comparison using a low dose of compounds, Pip6a-PMO was clearly able to better correct the SMA phenotype over naked PMO-treated mice.

Pip6a-PMO treatment demonstrates dose-dependent phenotypic rescue and enhanced survival

As established above, pups treated with a single dose of 10 µg/g Pip6a-PMO survived a median of 167 days. We next evaluated lower doses of Pip6a-PMO demonstrating that survival of treated pups was dose-dependent. Untreated mice survived a median of 12 days, whereas a single dose of 5 µg/g Pip6a-PMO improved median survival to 55 days (Fig. 2A and Table S1). Furthermore, a single 2.5 µg/g dose of Pip6a-PMO-treated animals survived a median of 33 days while no increase in survival was recorded for 1 µg/g treated pups (Table S1). Reduced doses of 2.5 and 5 µg/g Pip6a-PMO still improved weight, muscle strength, movement and coordination over untreated SMA pups (Fig. S3). Delayed onset of peripheral ear and tail necrosis, a characteristic pathology in this model, was also found to be dose-dependent (Table S2).

Several seminal studies have reported that using two administrations of SSOs confers stronger phenotypic improvements than single doses (11, 43, 44). We therefore treated severe SMA pups at PND0 and PND2 with our most effective doses of Pip6a-PMO (5 and 10 µg/g) as well as 10 µg/g Pip6a-PMO scrambled and 10 µg/g naked PMO. Two doses of scrambled Pip6a-PMO showed no improvement on survival (Table S1). Median survival of pups treated with two doses of naked PMO was improved over single treatment (median survival of 54 and 11.5 days respectively) (Table S1). The second dose of naked PMO also moderately improved weight and motor function (Fig. S4). By contrast, all mice treated with two doses of 5 or 10 µg/g Pip6a-PMO survived at least 200 days, with median survival of 283 and 457 days respectively, the greatest lifespan extension published for any SSO-based therapy to date (Fig. 2B and Table S1). Importantly, doubling the doses did not generate a toxic response in these pups. Indeed, analysis of serum markers for liver and kidney toxicity revealed no differences between treated and untreated SMA pups 7 days post-administration (Fig. S5).

Early assessment of phenotypes after two administrations of 5 and 10 µg/g Pip6a-PMO in SMA pups showed an overall improvement over untreated pups (Fig. S6). Pre- and post-weaning weights for Pip6a-PMO treated animals were greater than untreated groups yet never reaching those of non-affected littermates (Fig. S6A and B). Response to negative geotaxis was similar between unaffected littermates and Pip6a-PMO treated pups, with the majority of treated pups completing the task by day 8. A much smaller percentage of untreated SMA pups were able to complete the task, even by day 12 (Fig. S6C). Hind limb strength of Pip6a-PMO treated pups was also similar to unaffected littermates (Fig. S6D).

To elucidate molecular efficacy, SMN mRNA and protein expression were determined in treated tissues (Fig. 2, C and D). Spinal cord, brain, lower limb skeletal muscle, heart, and liver were harvested at PND7 and *FLSMN2* transcripts analyzed by QPCR, normalized to total *SMN2* expression, while total SMN protein expression was determined by Western blot. A dose-dependent increase of *FLSMN2* mRNA as well as SMN protein was observed in nearly all tissues of mice treated with Pip6a-PMO. These results demonstrate that the efficacy of Pip6a-PMO is dose-dependent and improves with multiple administrations.

Pip6a-PMO improves neuromuscular junction structure and innervation

We previously described a rescue of NMJ pathology in pups treated with 10 µg/g Pip6a-PMO. To further understand the dose-dependent improvement on phenotype of Pip6a-PMO-treated animals, we analyzed the innervation and endplate area of NMJs from the TVA of pups treated with the reduced dose of 5 µg/g compared to 10 µg/g Pip6a-PMO (Fig. 3). Severe SMA mice have greater numbers of partially or fully denervated NMJs (45). A single dose of 10 µg/g rescued NMJs to normal levels of innervation and NMJs from pups treated while 5 µg/g Pip6a-PMO also showed an improvement over untreated pups (Fig. 3B). Similarly, post-synaptic endplate areas were significantly greater in both treatment groups compared with SMA affected mice (Fig. 3C). The extent of NMJ phenotypic correction therefore nicely correlates with lifespan extension and Pip6a-PMO concentration.

Loss of motor neurons in the spinal cord appears to be a late feature of SMA mouse models and there are no universally accepted methods for quantifying motor neuron loss, hindering

consistency across different studies. The health of neurons in the spinal cord was thus demonstrated by measuring two transcripts for apoptosis, *fas and Pmaip1*, which are dysregulated in the spinal cord of SMA mice (46). At PND7, spinal cords from unaffected littermates expressed significantly lower transcript levels of *fas* and *Pmaip1* than SMA affected pups (Fig. 3, D and E). Treatment with a single dose of 5 µg/g or 10 µg/g Pip6a-PMO reduced their transcript levels, indicating that early treatment protects against programmed cell death within the spinal cord.

Pip6a delivers SSO to the CNS in adult mice

While we have achieved systemic delivery in early postnatal pups, targeting the CNS of patients at all ages will be critical for a successful SMA therapy. To test this objective, we chose to use adult mice carrying the human *SMN2* transgene (*Smn1$^{tm1Hung/WT}$;SMN2$^{tg/tg}$*). These mice are indistinguishable from wildtype animals. At 7.5 weeks of age, mice were treated twice by tail vein administration two days apart with 18 µg/g Pip6a-PMO or saline. Seven days post-administration, tissues of the CNS and relevant peripheral tissues were harvested and SMN expression was assessed. Transcript levels of *FLSMN2* relative to total *SMN2* transcript levels were significantly increased in all parts of the brain and spinal cord as well as in skeletal muscle and liver (Fig. 4, A to C). SMN protein levels were augmented in skeletal muscles and liver only (Fig. 4D). Sub-optimal sensitivity in the protein detection method may have limited the observation of increased levels in CNS tissues, which are predicted to be lower than those observed in peripheral tissues. This is the first report of peptide delivery of antisense oligonucleotides causing changes in transcripts within the adult CNS.

Discussion

In developing an effective SMA therapy, it is important to consider the CNS and peripheral requirements for SMN restoration. Multiple studies in SMA mouse models have generated conflicting data regarding the importance of systemic versus CNS-only treatment (11, 12, 43, 44, 47, 48). The single most important pathology for SMA is the loss of lower motor neurons and denervation at the neuromuscular junction is the earliest pathological change in SMA mice (37-41). However, reduced SMN expression has also been observed to cause skeletal muscle (13, 14, 16) and vascular system (19, 20, 49) defects in SMA mice and SMN is involved in general cellular functions including snRNP biogenesis (13, 22-24) and glucose metabolism (17, 18, 21). Thus, while delivery to the CNS is primordial for SMA therapy, a combined targeting of both the CNS and the periphery will most likely be the most desired and optimal approach. We have shown that Pip6a-conjugated PMO heralds a potent therapeutic option combining the genetic precision of SSOs with the systemic delivery efficacy of a small molecule. Systemic Pip6a-PMO treatment demonstrates ultra-high potency with only a single 10 µg/g dose at PND0 yielding a median survival of 167 days in severe SMA pups, the longest-lived mouse surviving 428 days, far beyond the greatest extension recorded for naked PMO (12) (Fig. 2A and Table S1). This dramatic extension in survival is associated with increased SMN expression in brain, spinal cord and all examined peripheral tissues, rescued levels of circulating IGF-1, reduced NMJ denervation, and decreased expression of apoptotic markers in the spinal cord (Fig. 1, and 3). Further, we were able to enhance median survival to over 450 days with two 10 µg/g doses (Fig. 2B and Table S1). Significantly enhanced survival in severe SMA models has been published

using systemic administrations of SSOs targeting the ISS-N1 site. The best survival published to date includes Hua *et al*., (11) who reported a median survival of 248 days with MOE chemistry and Zhou *et al*., (44) who reported a median survival of 261 days with a 25 mer PMO. However, the former administered 40 mg/kg and the latter used two doses of 160 mg/kg. Pip6a delivery allows us to reduce the dose required for maximum survival by at least four fold, an important step for generating clinically relevant compounds.

Current clinical trials (Ionis Pharmaceuticals) use intrathecal delivery of a MOE SSO, primarily targeting SMN expression in the CNS. In an open label Phase 2 study, SMA infants were treated with SSO targeting ISS-N1 (Nusinerson, IONIS-SMN$_{RX}$) which modestly improved their median event-free age (age of permanent ventilation or death) (50). More encouragingly, treated infants showed improvement in muscle function and motor milestones. In a separate Phase 2 study in SMA children, the same SSO resulted in improved mobility (6 minute walk test) (51), upper limb mobility test (52) and Hammersmith functional motor scale-expanded function (53)) compared to pre-treatment functions. F. Hoffman-La Roche in Switzerland, PTC Therapeutics, and the SMA Foundation have partnered to develop a small molecule drug (RG7800) capable of positively influencing exon 7 splicing systemically. This trial, termed Moonfish, has been suspended because of safety concerns in treated animals. However, Roche has a new similarly acting drug (RG7916) for which they have initiated a phase 1 study to investigate safety, tolerability, pharmacokinetics and pharmacodynamics in healthy volunteers. A related trial with a Novartis-sponsored orally active small molecule that enhances *SMN2* splicing, elevates full-length SMN protein and extends survival in a severe SMA mouse model is also underway, sponsored by Novartis. The molecular mechanism of action of this drug is via stabilization of the transient double-strand RNA structure formed by the *SMN2* pre-mRNA and U1 small nuclear ribonucleic protein (snRNP) complex (54). It will be of great interest to compare results of the CNS restoration of SMN protein (intrathecal SSO) with the outcome of the combined peripheral and CNS restoration of SMN protein level, since these trials could provide the greatest indication in patients of the need of a systemic and/or CNS treatment.

While a previous study observed brain delivery of antisense oligonucleotides with tagged dyes (55), we are the first to report the *bona fide* activity of a CNS-peptide delivered SSO. We have demonstrated here the potent impact of peptide delivery on PMO SSO therapy. In addition to SMA, other diseases for antisense oligonucleotide therapies include, ALS (56, 57), Huntington's (58, 59) and Parkinson's disease (60, 61), which all ideally require both systemic and CNS SSO delivery. Future work will focus on extending further the clinical applications of Pip-PMOs.

Materials and Methods

Synthesis of Peptide-PMO Conjugates:

Pip6a Ac-(RXRRBRRXRYQFLIRXRBRXRB)-COOH, was synthesized and conjugated to PMO as described previously (28). The PMO sequence targeting ISS-N1 intron 7 (-10-27) (5'-ATTCACTTTCATAATGCTGG-3') and PMO scrambled (5'-ATTGTCTATCAAAATCCTGC-3') was purchased from Gene Tools LLC.

In vitro study

Human neuroblastoma SH-SY5Y cells were treated using 25, 50, 100, 250 and 500 nM oligonucleotides in 500 µl full serum DMEM and analyzed for *FLSMN2* mRNA expression 24 h later.

Animal models

Experiments were carried out in the Biomedical Sciences Unit, University of Oxford according to procedures authorized by the UK Home Office. SMA-like mouse strain FVB.Cg-Smn1tm1HungTg(SMN2)2Hung/J, was generated and maintained as previously described (33, 34). Intravenous administrations were performed by single (PND0) or double (PND0 and PND2) administrations via facial vein. Doses of 2.5 µg/g (0.25 nmol/g), 5.0 µg/g (0.5 nmol/g), and 10 µg/g (1 nmol/g) of Pip6a-PMO and 10 µg/g PMO (1.48 nmol/g) were diluted in 0.9% saline and given at a volume of 5 µl per gram body weight. Treated mice were given mash and jelly as food supplementation. Tail vein administration in adult mice were performed in unaffected mice (Smn1$^{tm1Hung/WT}$;SMN2$^{tg/tg}$) at 7.5 weeks of age. Two doses of 18 µg/g Pip6a-PMO or saline were given two days apart. Tissues were harvested seven days post-administration.

Measuring early disease phenotype

Weights were taken daily and overall health assessed. Following the humane endpoint, mice were euthanized according to the approved procedure of rising CO_2. Negative geotaxis was performed as previously described (35). Statistical significance was determined by a binomial test. The hind limb suspension 'tube test' was performed as described in procedures for pre-clinical studies by TREAT-NMD (35). For the purposes of reproducibility, factors such as leg pulls and time were left out of the analysis. Statistical significance was determined by Fisher's test with Bonferroni correction in SPSS.

NMJ immunohistochemistry

Animals treated with Pip6a-PMO were harvested 12 days post-administration (PND12). The TVA muscle was isolated and immunohistochemistry was performed on NMJs as previously described (42, 62). NMJs were imaged using an Olympus FV1000 confocal microscope. Images were taken at 40x objective as a z-stack. Only NMJs with fully observable post synapses were used for denervation counts. >300 NMJs were analyzed for full or partial denervation per group which was observed by the colocalization of presynaptic neuron with post-synaptic acetylcholine receptors. Area of post-synaptic motor endplates was measured using ImageJ as previously published (63).

QPCR

RNA extraction from harvested tissues was carried out using TRIzol® reagent (Invitrogen, Carlsbad, CA) and cDNA generated using ABI High Capacity cDNA Reverse Transcription Kits (Invitrogen, Carlsbad, CA) following manufacturer's instructions. QPCR reaction using Power SYBR® Green Master Mix (Life Technologies) was performed and analyzed on Applied Biosystems® StepOnePlusTM real-time PCR system (Life Technologies). *FLSMN2* and Total *SMN2* transcripts were amplified using gene-specific primers (Table S3). *Fas*, Pmaip1 and Gapdh was amplified using Integrated DNA Technologies derived probe and primer set (Table S3)

Protein extraction and western blot

Protein was harvested from approximately 300 mg of tissue homogenized into RIPA Buffer with complete mini proteinase inhibitors (Roche). 30-40 µg of protein was probed for human SMN protein using anti-SMN, clone SMN-KH monoclonal IgG1 (Millipore) and mouse β-tubulin using anti-β-actin monoclonal IgG2a (Sigma) and secondary antibody IRDye® 800CW goat anti-mouse IgG (LI-COR Biosciences, Lincoln, NE). Membranes were imaged on Li-Cor Odyssey® FC imager and analyzed with Image StudioTM software (LI-COR Biosciences, Lincoln, NE).

IGF-1 ELISA

Concentration of serum IGF-1 was determined using the Murine IGF-1 ELISA kit (900-K170; Peprotech) following manufacturer's instructions.

Clinical Biochemistry

Serum samples from mice administered with Pip6a-PMO or PMO at PND0 and PND0/2 were extracted from the jugular vein upon harvest at PND7 and analysis of toxicity biomarkers was performed by a clinical pathology laboratory, Mary Lyon Centre, MRC, Harwell, UK.

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Figure Legends

Fig. 1. Direct comparison of PMO versus Pip6a-PMO administration in severe SMA mice. (**A**) Survival curves for SMA untreated (n=9), 10 µg/g PMO (n=20) and 10 µg/g Pip6a-PMO (n=9) administered SMA pups. Pip6a-PMO treatment significantly enhanced survival (mean of 196.4 ± 114.9; median 167 days) ($p \leq 0.0001$ Log-rank Mante-Cox). (**B**) Weights of treated and untreated severe SMA pups plotted as mean ± SEM. Pip6a-PMO treated pups were significantly greater in weight from SMA untreated pups starting from PND7 (* $p \leq 0.005$, Student's *t*-test). (**C**) Hind limb suspension score plotted as mean ± SEM. (Fisher's tests with significance after Bonferroni correction *** $p = 3.55 \times 10^{-13}$). (**D**) Protein in tissues analyzed by western blots for human SMN and mouse β-tubulin. SMN expression of untreated SMA pups were normalized to one for comparative expression. (**E**) RNA Expression of *FLSMN* transcript analyzed by quantitative RT-PCR related to 'total *SMN'* transcripts, represented by exons 2a and 2b amplicon. Expression of *FLSMN* in each untreated SMA tissue was normalized to one for comparative expression. (**F**) Circulating serum levels of IGF-1 determined by murine IGF-1 ELISA plotted as mean ± SEM. Statistical significance determined by one-way ANOVA with multiple comparisons *$p \leq 0.01$(**G**) Representative confocal images of neuromuscular junctions (NMJs) at PND12. Several defects of the NMJs were observed in SMA untreated and PMO treated pups, including neurofilament accumulation (white arrow), denervated endplates (white arrow head) and reduced terminal arborization (open arrow head). Also observed in all treated and untreated pups, though not quantified, were multi-innervated endplates, indicative of normally developing NMJs (open arrow). Post-synaptic endplates were stained in red (alpha-bungarotoxin) and neurons were stained in green, (2H3) neurofilament and (SV2) synaptic vesicles. Scale bar, 10 µm. (**H**) Percentage of NMJs scored as partially or fully denervated. (**I**) Post-synaptic areas were quantified using ImageJ. (**D,E,H,I**) Data represented as mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Student's *t*-test in comparison to untreated SMA mice.

Fig. 2. Dose dependent Pip6a-PMO restoration of phenotype. (**A**) Survival curves for untreated severe SMA mice (n=9), pups treated with a single dose (PND0) of 2.5 µg/g Pip6a-PMO (n=11), 5 µg/g Pip6a-PMO (n=11), and 10 µg/g Pip6a-PMO (n=9). All administrations with Pip6a-PMO resulted in statistically significant improvement of survival over untreated SMA mice ($p \leq 0.0001$ Log-rank Mante-Cox). (**B**) Survival curves for untreated severe SMA mice (n=9) and two

doses (PND0/2) of 5 µg/g (n=5) or 10 µg/g Pip6a-PMO (n=5). Both administrations with Pip6a-PMO resulted in statistically significant improvement of survival over untreated SMA mice (p ≤ 0.0001 Log-rank Mante-Cox). (**C**) Protein expression of SMN in tissues following a single, PND0 (1x), or double, PND0 and 2 (2x) administration of Pip6a-PMO. SMN protein was analyzed with western blots for human SMN and mouse β-tubulin. SMN expression of untreated SMA pups were normalized to one for comparative expression. (**D**) *FLSMN2* transcript levels were measured by quantitative RT-PCR and. Expression of *FLSMN2* in each untreated SMA tissue was normalized to one for comparative expression. Samples are plotted as mean ± SEM and statistical significance compared to untreated SMA mice was determined by Student's *t*-test. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

Fig. 3. Rescue of SMA pathology within Pip6a-PMO administered SMA pups. (**A**) Representative confocal images of NMJs from untreated SMA, unaffected controls and PND0 administered 5 µg/g and 10 µg/g Pip6a-PMO pups. Post-synaptic endplates were stained in red (alpha-bungarotoxin) and neurons were stained in green, (2H3) neurofilament and (SV2) synaptic vesicles. Scale bar, 25µm. Reduced innervation (open white triangle) and accumulation of neurofilament (filled white triangle) was observed in SMA untreated pups. Partial innervation (open yellow triangle) and poor arborization (white arrow) was visible in Pip6a-PMO treated pups. The optimal complexity of terminal arborizations and fully innervated NMJs is observable in unaffected controls (yellow arrows). (**B**) NMJs were scored for denervation, either partial or full, and plotted as percentage of total NMJs analyzed. (**C**) Post-synaptic areas were quantified using ImageJ. Quantitative levels of markers for apoptosis; (**D**) Pmaip1 and (**E**) Fas relative transcripts in spinal cord of PND7 treated and untreated mice, normalized to *mGapdh* transcript levels. (**B,C,D,E**) Data represented as mean ± SEM. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005, Student's *t*-test in comparison to untreated SMA mice.

Fig. 4. Pip6a-PMO administration into unaffected adult mice harboring the human SMN2 allele (Smn1[tm1Hung/WT];SMN2[tg/tg]). (**A-C**) Quantitative RTPCR from tissues of 18 mg/kg administered via IV two days apart. Tissues from the (**A**) brain, (**B**) spinal cord, and (**C**) peripheral skeletal muscles and liver were harvested 7 days post administration. In all tissues, *FLSMN2* expression was significantly increased over saline treated mice. (**D**) Protein expression of SMN in tissues. Separate tissues spinal cord and skeletal muscles were combined. SMN protein was analyzed with western blots for human SMN and mouse β-tubulin. Data represented by mean ± SEM. * p ≤ 0.005, ** p ≤ 0.001, *** p ≤ 0.0001, student's *t*-test in comparison to saline treated mice.