Viral vector-mediated overexpression of the repulsive axon guidance cue semaphorin 3A in mouse skeletal muscle does not induce changes in motor function or alter the integrity of the neuromuscular junction
Chapter 4.

Abstract

Amyotrophic Lateral Sclerosis (ALS) is characterized by a progressive degeneration of motor neurons leading to loss of muscle function. The early molecular events are continuing to be deciphered, but much evidence now points to ALS as a distal axonopathy, whereby neuromuscular junctions (NMJs) show pathological changes prior to motor neuron degeneration and onset of clinical symptoms. The chemorepulsive guidance cue semaphorin3A (SEMA3A) is upregulated in the terminal Schwann cells (TSCs) at the NMJs of presymptomatic ALS mice where it may be acting to potentiate the dying-back phenotype. In the current study we used adeno-associated viral (AAV) vectors to overexpress SEMA3A in non-pathological skeletal muscle and evaluated motor function and morphological changes at the NMJ. We observed expression of SEMA3A in targeted muscles, but the levels were insufficient to induce a detectable change in motor behavior or alter NMJ integrity. One interpretation of our data is that muscle-mediated overexpression of SEMA3A alone is unable to cause a progressive pathological-like state at the NMJ. SEMA3A may thus function as a component of a larger network of molecular changes occurring during ALS disease progression, which are not modelled in our intact muscle paradigm. Alternatively the lack of a phenotype may indicate that SEMA3A is not involved in NMJ degeneration. Although technically challenging, perhaps a more appropriate way to study the role of SEMA3A at the neuromuscular synapse would be to directly target the overexpression to the TSCs themselves in order to more closely mimic the molecular situation necessary for NMJ degeneration.
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Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease in which motor neurons selectively degenerate leading to loss of muscle function. The incidence is high (1 in 100,000) and life expectancy after diagnosis is approximately 5 years, with the majority of patients dying of respiratory failure. Since 1997, Riluzole has been the only available treatment for ALS, but shows a very limited beneficial effect in the majority of patients (Miller et al., 2012). Many of the genetic causes for familial ALS (fALS) have now been identified: mutations in the SOD1, TDP-43, FUS and C9ORF72 genes account for approximately 70% of fALS cases (reviewed in Robberecht and Philips, 2013). However, the majority of ALS cases are sporadic (sALS) in nature, with a variety of environmental and (unknown) genetic factors thought to influence the pathophysiology. Interestingly, although the initial triggers may be different, the progression of sALS and fALS show a remarkable similarity (reviewed in Robberecht and Philips, 2013). The “dying-back” hypothesis has gained much strength over the last decade and it may provide the link between sALS and fALS pathophysiology (Fischer et al., 2004; Frey et al., 2000; Pun et al., 2006): independent of the molecular trigger, motor neurons and nerve terminals show pathological changes prior to motor neuron degeneration and onset of clinical symptoms. As a result, efforts are being made to decipher the (early) molecular mechanisms leading to ALS, with many modifier genes being involved in common pathways such as inflammation and cytoskeletal activities (reviewed in Riboldi et al., 2011).

Semaphorin 3A (SEMA3A) is a well-described chemorepulsive guidance molecule mediating its effects on growth cone collapse by modulating pathways involved in cytoskeletal function (Gu and Ihara, 2000; Hung et al., 2010; Kruger et al., 2005; Schmidt and Strittmatter, 2007). Previous evidence has suggested a role for SEMA3A in the presymptomatic stages of ALS. First, SEMA3A expression in Terminal Schwann cells (TSCs) is present at the neuromuscular junction (NMJ) of G93A-hSOD1 ALS mice as early as 5 weeks post-birth, but is not observed in wild-type littermates (De Winter et al., 2006). Second, ALS mice with a conditional neuron-specific knockout of neuropilin-1 (NRP1, a neuronal membrane-bound SEMA3A receptor) show improved motor function (Moloney et al., 2012)Third, administration of a humanized monoclonal antibody that specifically blocks Sema3A binding to NRP1 improved lifespan and motor function in hSOD1 ALS mice (submitted Venkova et al., 2014). Fourth, interference of SEMA3A-NRP1 signaling with soluble NRP1 receptors also improve or worsen motor function dependent on the type of receptor body applied (this thesis, Chapter 3). Additional evidence for a role of SEMA3A signaling in ALS pathology comes from Pettmann and colleagues who showed that overexpression of a variant of Collapse Response Mediator Protein 4 (CRMP4) in motor neurons triggered axon degeneration, while
knock-down of CRMP4 in motor neurons of hSOD1 ALS mice had a protective effect (Duplan et al., 2010). Collectively these data point to SEMA3A having a negative role in ALS pathophysiology: by creating a repulsive environment at the NMJ SEMA3A may induce motor neurons to retract from their muscle target, potentiating the dying-back phenotype. Furthermore, the idea of SEMA3A having a role in ALS is supported by the recent discovery of anti-sema3A antibodies in the serum/CSF of ALS patients which may indicate an immune mechanism triggered by the aberrant re-expression of the SEMA3A protein during the disease process (Hernández et al., 2010).

Evidence also exists to support a role for other repulsive guidance molecules in the pathophysiology of ALS. Nogo-A is a well-known repulsive molecule and increased expression of Nogo-A has been described in the skeletal muscle of ALS patients (Jokic et al., 2005). Jokic and colleagues subsequently showed that by overexpressing Nogo-A in non-pathological muscle (using an expression plasmid and electroporation), Nogo-A was able to induce NMJ destabilization and motor neuron destruction in the soleus muscle of wild-type mice (Jokic et al., 2006). With this approach in mind, and to increase our knowledge about the role of SEMA3A at the NMJ in ALS, we investigated the effects of viral vector-mediated overexpression of SEMA3A in non-pathological skeletal muscle and evaluated motor function and morphological changes at the NMJ.

Materials and methods

Expression constructs

Expression plasmids were created using the pTR-CMV-GFP-WPRE (pTR-CGW) backbone. The pTR-CGW plasmid contains an expression cassette flanked by two inverted terminal repeats of AAV2; the GFP gene, followed by a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is under the control of the cytomegalovirus (CMV) promoter (Ruitenberg et al., 2002). The GFP-SEMA3A fragment from pAG-GFP-SEMA3A expression plasmid (De Wit et al., 2005) was excised and inserted into the pTR-CGW backbone taking the place of the pre-existing GFP fragment, creating the pTR-CMV-GFP-SEMA3A-WPRE expression construct.

Virus production and quantification

The pTR transfer plasmids containing transgenes GFP or GFP-Sema3A were used to produce adeno-associated viruses (AAVs). The packaging plasmid containing cap and rep genes for serotype 6 (pDP6) was kindly provided by Jeurgen Kleinschmidt (Grimm et al., 2003). A batch of AAV6 was made using the following protocol; 6 15-cm petri dishes containing 1.25x10^7 HEK 293T cells in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS; all GIBCO-Invitrogen Corp., New York, NY, USA) were
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Prepared one day before transfection. The medium was refreshed 1 hour prior to transfection to Iscoves modified Eagle medium (IMEM) containing 10% FCS, 1% PS and 1% Glutamine. Transfer plasmids were co-transfected using polyethylenimine (PEI, MV25000; Polysciences Inc., Warrington, PA, USA) in a ratio of 1:3 with pDP6 plasmid resulting in a total amount of 50ug of DNA per plate. The day after transfection, medium was replaced with fresh IMEM containing 10% FCS, 1% PS and 1% Glutamine. Two days later (3 days post-transfection), cells were harvested in D-phosphate buffered saline (D-PBS, GIBCO) and lysed with 3 freeze-thaw cycles. Genomic DNA was digested by adding 10ug/ml DNAsel (Roche Diagnostics GmbH, Mannheim, Germany) into the lysate and incubating for 1 hour at 37°C. The crude lysate was cleared by centrifugation at 4000rpm for 30 minutes. Virus was purified from the crude lysate using the iodixanol gradient method (Hermens et al., 1999; Zolotukhin et al., 1999), diluted in D-PBS/5% sucrose and concentrated using an Amicon 100kDa MWCO Ultra-15 device (Millipore). Concentrated AAV6 vectors were stored at -80°C until use. Titers (genomic copies/ml) were determined by quantitative PCR on viral DNA using primers directed against enhancer portion of the CMV promoter (Forward: CCCACTTGGCAGTACATCAA; Reverse: GGAAAGTCCCATAAGGTCATGT).

**Experimental animals**

Male C57BL/6 mice (Harlan, The Netherlands) were housed in littermate groups under a 12 h light/dark cycle with ad libitum access to food and water. Starting at 4 weeks of age, animals were weighed weekly to monitor weight loss for humane endpoint euthanasia (20% loss of maximum weight). All interventions and behavioral tests were approved and carried out in compliance with the Institutional Animal Care and Use Committee of the Royal Netherlands Academy of Sciences. Euthanasia was performed by intra-peritoneal administration of Nembutal (sodium pentobarbital, Sanofi Sante) or CO2-inhalation followed by decapitation.

**Experimental procedure**

The experimental setup fell into two categories: unilateral or bilateral intramuscular injections of the gastrocnemius muscles. Animals were placed under isoflurane anesthesia prior to injection (5% induction, 3% maintenance) and allowed to recover in a heated (37°C) cage before being returned to their home cage.

Animals were unilaterally injected (into right gastrocnemius muscle) with AAV6-GFP (n=6) or AAV6-GFP-SEMA3A (1.0E+11 viral particles/animal diluted in D-PBS/5% sucrose, two injection sites [medial and lateral sides of muscle], 50ul per injection site) at 5 weeks of age, and euthanized with Nembutal 3 weeks after injection. Animals were perfused intracardially with ice-cold 0.9% saline followed
by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH7.4). Left and right gastrocnemic muscles were harvested and placed in 250mM EDTA in 0.1M PB overnight. The following day, muscles were transferred to 25% sucrose in 0.1M PB to prepare for cryopreservation. Once samples had equilibrated in the sucrose solution, muscles were snap-frozen in dry-ice cooled isopentane and stored at -80oC until required for \textit{in situ} hybridization or immunohistochemistry.

For the bilateral set-up, isoflurane-anaesthetized mice were injected with AAV6-GFP (n=15) or AAV6-GFP-SEMA3A (n=15) in both gastrocnemic muscles at 5 weeks of age. Virus samples were diluted in D-PBS/5% sucrose to injected a total volume of 200ul per animal (two injection sites per muscle [medial and lateral sides of muscle], 50ul per injection site), with a final dose of approximately $3.0\times10^{11}$ viral particles/animal. For the following 15 weeks, animals were tested weekly with the Rotarod and PaGE behavioral paradigms (see below for details). At 20 weeks of age, animals were euthanized in a CO2-chamber, and the left and right gastrocnemic muscles were harvested and immediately frozen in dry-ice cooled isopentane. Tissue samples were kept at -80oC until required for acetylcholinesterase /silverstaining or Western blotting.

\textbf{Immunohistochemistry}

Immunohistochemistry was performed on longitudinal cryosections (40um thick; collected in a droplet of 250mM EDTA in 0.1M PB) of the left and right gastrocnemic muscles as follows. Sections were defrosted for 30 minutes at room temperature (RT) and post-fixed. Sections were rinsed in 0.1M Tris-buffered saline pH 7.4 (TBS) and blocked for 1 hour in 5% Fetal Calf Serum in TBS with 0.2% Triton-X (block-mix). Goat-anti SEMA3A (Santa Cruz C17, 1:200) was diluted in block mix and added to the sections for an overnight incubation at 4oC. Biotinylation of the primary antibody was performed by incubation of the sections in biotinylated anti-goat antibodies. The following day sections were rinsed with TBS and incubated with the appropriate streptavidin conjugated and fluorescently-labelled secondary antibody (Alexa Fluor 488-labelled, Molecular Probes, 1:1400) in combination with fluorescently-tagged bungarotoxin (Alexa Fluor 594-labelled, Molecular Probes, 1:1000) for 2 hours at RT. Sections were rinsed with TBS again prior to mounting in Mowiol for imaging (Zeiss Axioplan).

\textbf{\textit{In situ} hybridization}

\textit{In situ} hybridization (ISH) was performed on longitudinal cryosections (40µm thick) of the left and right gastrocnemic muscles as follows. Sections were defrosted for 30 minutes at RT and subsequently fixed for 5 minutes in 4% PFA in Phosphate Buffered Saline (PBS, pH7.4). Following a 10 minute incubation in 10µg/ml proteinase K, sections were fixed for an additional 15 minutes in 4% PFA. Sections were rinsed thoroughly with three 5 minute washes in PBS, then Milli-Q
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...water, before acetylation in 0.25% acetic anhydride in 1% triethanolamine (10 minutes at RT).Slides were rinsed in PBS, then in 2x Salt Sodium-Citrate buffer (SSC, pH 7) in preparation for hybridization of the RNA probe.A hybridization mix was prepared by diluting the RNA probe in hybridization solution (5x Denhardt's, 250µg/ml tRNA, 5x SSC, 50% formamide), denatured for 5 minutes at 85 oC, cooled for 5 minutes on ice, and subsequently applied to the slides for an overnight incubation at 60 oC. After a series of stringency washes at 60 oC (5x SSC, 2x SSC), sections were incubated for 30 minutes in 0.2% SSC in 50% formamide, followed by a wash in 0.2x SSC at RT.

The RNA probe was prepared for detection by treating the sections in 1% blocking reagent (Roche) in 100mM Tris/150mM NaCl (Buffer 1, pH 7.5) for 1 hour at RT. Subsequently, sections were incubated with anti-DIG-AP Fab (1:3000, Roche) in Buffer 1 for 3 hours. After several washes in Buffer 1, sections were treated with 100mM Tris/100mM NaCl/5mM MgCl2 (Buffer 2, pH 9.5) in preparation for development. A color development solution comprised of 335ug/ml nitro-blue-tetrazolium and 175ug/ml BCIP-Phosphate in Buffer 2 was applied to the sections for up to 20 hours. The reaction was halted by the addition of Tris-EDTA solution. The sections were then dehydrated with a series of ethanol, cleared with xylene and mounted with Entellan (Millipore) for imaging.

**Acetylcholinesterase and silverstaining**

Longitudinal cryosections of fresh frozen gastrocnemius muscles (40um thick, collected in a droplet of 250mM EDTA/0.1M PB) were air dried at RT. Slides were subjected to acetylcholine-esterase staining (to visualize the neuromuscular synaptic cleft) and silver staining (to visualize presynaptic terminals and motor fibers) as outlined previously (Pestronk and Drachman, 1978), without treating the sections with 0.5% celloidin. Following dehydration of the sections, slides were cleared with xylene and mounted using Entellan (Millipore). Neuromuscular junction (NMJ) morphology was scored as innervated (full overlap of blue and brown stain), intermediate (some overlap, but not complete) or denervated (no overlap of blue and brown staining). One section per animal was quantified and data

**Western blotting**

Gastrocnemius muscles were dissected from CO2-euthanized mice and immediately frozen in dry-ice cooled isopentane. When required for western blotting, several serial sections (40um; approximately 3 sections per muscle) obtained on the cryostat were homogenized in 100ul of 1x SDS loading buffer (containing 10% SDS and 10% b-mercaptoethanol). Samples were boiled for 5 minutes, then loaded (10ul) alongside the Protein-Precision All Blue Standard (BioRad) onto 8% SDS-PAGE gels and electrophoresed using the Mini-PROTEAN®
Tetra-Cell (BioRad). After wet-transferring to nitrocellulose membranes, the membranes were blotted with rabbit-anti-Sema3A (1:1000; Santa Cruz C-17) and anti-beta-actin (A5316, Sigma) followed by anti-rabbit-HRP or anti-mouse-HRP (1:1000, Dako). Blots were developed with an enhanced chemiluminescence reaction and exposed to Lumi-light films (Roche Diagnostics, The Netherlands).

Behavioral testing

Rotarod

To assess overall motor coordination, an accelerating Rotarod paradigm was used (model 47600, Ugo Basile Biological Research Apparatus, Italy). Animals were placed on a rotating beam (3 cm diameter) and the latency to fall (in seconds) was measured. An arbitrary cut-off time of 180 s was chosen (Miana-Mena et al., 2005; Weydt et al., 2003; Zhou et al., 2007) during which the rotation of the beam increased from 5 to 15 rpm in the first 60 seconds and was then held constant until the end of the trial. Each animal was given three attempts and the longest period achieved prior to falling was recorded. At 4 weeks of age, the animals were allowed to familiarize with the Rotarod for three 180 s trials with the rod rotating at a constant speed of 5 rpm. Starting at 5 weeks of age, the animals were tested weekly for 15 weeks.

Paw grip endurance

This test measures muscular strength of the limbs (Crawley, 1999). Each mouse was placed on the wire-lid of a conventional housing case. The lid was gently shaken to prompt the mouse to hold onto the grid before the lid was swiftly turned upside down, approximately 50 cm above the surface of soft bedding material to avoid injuries. The latency until the mouse falls of the grid was timed, with an arbitrary maximum of 90s (Weydt et al., 2003). Each mouse was given three attempts and the longest latency to fall was recorded. Starting at 5 weeks of age, the animals were tested weekly for 15 weeks.

Results

SEMA3A overexpression in skeletal muscle

Male C57/Bl6 mice were injected into the right gastrocnemius muscle at 5 weeks of age with AAV6-GFP-SEMA3A (1.00E+11 viral particles). Tissue was harvested at either 3 weeks post-injection, for in situ hybridization (ISH) or immunohistochemistry (IHC) or at 15 weeks post-injection for Western blot analysis to characterize the expression patterns and levels of SEMA3A respectively.

After ISH for SEMA3A there is a clear presence of SEMA3A mRNA in the injected muscle (Figure 1; A1) compared to the non-injected muscle (Figure 1; A2). The in situ signal is localized around nuclei as expected for an mRNA signal (Figure 1; A1; arrow) specifically as a punctate stain surrounding the nucleus (Figure 1; A1; inset,
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Figure 1: AAV6-mediated SEMA3A expression in skeletal muscle of C57BL/6 mice. (A) The right gastrocnemius muscle was injected with 1.00E+11 viral particles, and was processed 3 weeks later for ISH or IHC. Positive signal for SEMA3A RNA was found throughout the injected muscle (panel A1) compared to the non-injected muscle (panel A2). SEMA3A protein was detected in injected muscle (panel A3) but not in non-injected muscle (panel A4) as hotspots of positive signal (arrows). These hotspots were generally aligned along the muscle fiber direction (panel A3, inset, arrowhead) and did not co-localize with neuromuscular junctions (data not shown). Faint SEMA3A signal was detected throughout the muscle fibers of injected muscle illustrated by the greenness of panel A3 compared to panel A4 (both photos taken with 500ms exposure time for the 470BP filter).

(B) Fresh tissue was harvested from mice injected with AAV6-GFP or AAV6-GFP-SEMA3A at 15 weeks post-injection. Western blot analysis revealed SEMA3A positive bands in the AAV6-GFP-SEMA3A group (n=3 animals) compared to the AAV6-GFP group (n=3 animals). Scale bars = 50µm.

C57/Bl6 male mice were injected with AAV6-GFP (n=15) or AAV6-GFP-SEMA3A (n=15) into both gastrocnemius muscles at 5 weeks of age, and tested weekly for 15 weeks. Both Rotarod (A) and Paw Grip Endurance (PaGE, B) testing showed that there were no behavioral differences between the groups. Treatment groups also showed no differences in weight, as both groups progressively gained weight in a similar fashion over the course of the experiment (C).

Figure 2: Motor function in wild-type mice is not altered by skeletal muscle administration of AAV6-GFP-SEMA3A.

arrowhead). IHC on adjacent sections using a SEMA3A antibody (C17, Santa Cruz) resulted in a sparse pattern of protein hot spots along some but not all muscle fibers (Figure 1; A3; arrows). These hotspots did not co-localize with neuromuscular junctions (data not shown), and were generally orientated longitudinally along the muscle fiber (Figure 1; A3; inset, arrowhead). Injected muscle generally had a
greener hue (Figure 1; A3) than non-injected muscle (Figure 1; A4) indicative of a muscle-wide diffuse localization of the secreted protein, making that IHC signal consistent with the ISH results.

Western blot analysis revealed SEMA3A-positive bands in AAV6-GFP-SEMA3A injected muscle of the expected molecular weight of approximately 125kDa, with an absence of such bands in AAV6-GFP injected muscle (Figure 1; B).

Motor function is not compromised after AAV6-GFP-SEMA3A administration

In order to characterize the functional effects of overexpressing SEMA3A in skeletal muscle, we performed a behavioral study whereby male C57/Bl6 mice were injected into both gastrocnemius muscles at 5 weeks of age with either AAV6-GFP (n=15) or AAV6-GFP-SEMA3A (n=15), with a total dose of 3.00E+11 viral particles per animal. Motor function was measured weekly using the Rotarod and Paw Grip Endurance (PaGE) tests until the animals were 20 weeks old. Rotarod (Figure 2; A) and PaGE (Figure 2; B) tests revealed no behavioral differences between the GFP or SEMA3A treated groups. Animals were weighed several times weekly, and both groups gained weight in a similar fashion over time (Figure 2; C), indicating that the presence of SEMA3A in the injected muscle did not have an effect on overall muscle loss.

**Figure 3:** Neuromuscular integrity in wild-type mice is not altered by skeletal muscle overexpression of AAV6-GFP-SEMA3A.

(A) Representative images of innervated, intermediate and denervated neuromuscular junctions after acetylcholinesterase (AChE; blue stain) and silverstaining (brown stain) on gastrocnemius muscles of AAV6-injected C57/Bl6 mice, 15 weeks post-injection.

(B) Quantification of NMJ integrity in AAV6-GFP (n=6) versus AAV6-GFP-SEMA3A (n=5) injected animals revealed no differences between groups. Scale bar = 25µm.
Neuromuscular junction integrity is not altered after intramuscular AAV6-GFP-SEMA3A administration

Given the lack of behavioral differences with AAV6-GFP-SEMA3A we sought to determine if minor changes in neuromuscular junction integrity could be identified as a result of the presence of SEMA3A in the injected muscle. Gastrocnemius muscle sections were subjected to acetylcholine-esterase (AChE)- and silver-staining to label the post-synaptic and pre-synaptic regions of the neuromuscular junction respectively. NMJs were scored as innervated, intermediate or denervated depending on the amount of overlap between the blue AChE- and brown silver-staining (Figure 3; A). Quantification revealed that there was no difference in NMJ integrity between the GFP and SEMA3A treated groups (Figure 3; B), indicating that SEMA3A has no effect on nerve-muscle innervations or that the levels of SEMA3A obtained by overexpression were not sufficient to alter the synapse.

Discussion

Increasing evidence suggests that motor neuron death in amyotrophic lateral sclerosis (ALS) is preceded by pathological changes in motor axons and their nerve terminals (Fischer et al., 2004; Frey et al., 2000; Pun et al., 2006). Previous studies have identified an increase in SEMA3A expression in Terminal Schwann cells (TSCs) in presymptomatic ALS mice (De Winter et al., 2006) which may contribute to NMJ destabilization, and could constitute a trigger for the progressive dying-back nature of motor neuron degeneration in ALS. In addition, various manipulations of the SEMA3A signaling pathway have resulted in improvement of motor function in ALS mice (Duplan et al., 2010; Venkova et al., 2014), illustrating a possible link between SEMA3A and the disease process. Recently, anti-SEMA3A antibodies were discovered in the CSF and serum of ALS patients (Hernández et al., 2010), which indicates a possible immune mechanism to overcome the effect of aberrant expression of the protein. In order to strengthen the link between SEMA3A and neuromuscular dysfunction, the current study sought to identify the effects of muscle-mediated (over)expression of SEMA3A on NMJ integrity and motor behavior to further illuminate the role of this protein in motor neuron degeneration.

Intramuscular injection of an adeno-associated viral (AAV) vector harboring a transgene of interest is an ideal approach to investigate the effect of the transgene product on the NMJ. The muscle itself has a large capacity for protein synthesis and secretion, and with the ability of AAV vectors to maintain stable gene expression over time without associated toxicity, long term and safe expression of a (therapeutic) protein can be achieved in muscles of healthy mice or supplied over the course of a disease such as ALS to study changes in motor neuron function and/or muscle tissue survival.

Previous studies have shown that muscle-mediated (over)expression of
certain genes can induce motor neuron degeneration and an ALS-like phenotype in otherwise healthy mice. Wong and colleagues generated transgenic mice in which mutant hSOD1 was selectively expressed in skeletal muscle, and showed that this was sufficient to induce various ALS-like pathological phenotypes, including motor neuron dysfunction and neuromuscular junction abnormalities (Wong and Martin, 2010). Using an alternative approach, Jokic and colleagues delivered Nogo-A expression plasmids via electroporation to the skeletal muscle of wild-type mice and showed that overexpression of Nogo-A resulted in destabilization of the NMJ and progressive axonal loss similar to that observed in ALS mice (Jokic et al., 2006). Both these studies provided insight into the role of skeletal muscle in the pathophysiology of the disease, and illustrated the use of muscle-mediated expression to deliver proteins which can influence NMJ integrity and motor neuron health.

Conversely, muscle-mediated protein expression has been exploited to deliver therapeutic proteins aimed at preserving the NMJ in ALS. Various neurotrophic factors have been assessed for their ability to preserve NMJ integrity and improve motor neuron survival and function in ALS mouse models (reviewed in Gould and Oppenheim, 2011; Henriques et al., 2010). Skeletal muscle-mediated overexpression of glial cell line-derived neurotrophic factor (GDNF) using AAV vectors prevented motor neuron degeneration and delayed the progression of motor dysfunction (Wang et al., 2002). Similarly, insulin-like growth factor-1 (IGF-1) has been shown to protect motor neuron terminals after viral vector-mediated overexpression in muscles (Dobrowolny et al., 2005; Kaspar et al., 2003). Vascular endothelial growth factor (VEGF) has also been shown to promote motor neuron survival and enhance motor performance, whether it is administered via intramuscular viral injections (Azzouz et al., 2004; Zheng et al., 2004) or as a recombinant protein (Storkebaum et al., 2005; Zheng et al., 2007). These studies illustrate the potential of skeletal muscle as the vehicle by which proteins can be successfully delivered to the NMJ in order to preserve its function and improve motor neuron survival in the context of ALS.

Here we used this approach to study the effects of the expression of SEMA3A on the neuromuscular junction in wild-type mice. AAV6-GFP-SEMA3A was administered to the gastrocnemius muscles of wild-type mice. Consistent with previous findings (this thesis, Chapter 3) direct intramuscular injection resulted in muscle-wide expression of the transgene. Most, if not all, nuclei were surrounded by a punctate SEMA3A RNA signal. However, there were difficulties in detecting the anticipated protein levels. Two distinct patterns of protein staining were observed after immunohistochemistry; most predominant was the diffuse stain, while hotspots were also detected scattered throughout the muscle. Muscle cells are large and AAV-mediated expression of SEMA3A may have resulted in the
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Diffuse localization of the protein due to the physical size of the cell. In addition, SEMA3A is a secreted protein which may have precluded the visualization of the protein in the transduced cells since it may not build-up to high enough levels within the cell for accurate detection. The faint, diffuse staining obtained for SEMA3A through immunohistochemistry reflects these possible scenarios. Further analysis is required to identify the subcellular location of the hotspots of SEMA3A immunostaining obtained as they do not co-localize with NMJs, and appear to be randomly spread throughout the muscle fiber. Western blot analysis performed on muscle samples confirms the presence and overexpression of SEMA3A protein in SEMA3A-treated animals, but not in GFP-treated animals, for at least 15 weeks after injection.

Our behavioral data indicate that the current levels of AAV-mediated SEMA3A overexpression were unable to induce a detectable change in behavior. This is probably not due to motor neuron insensitivity to SEMA3A, as it has been shown that adult motor neurons continue to express the necessary receptor components for SEMA3A signaling throughout adulthood (De Winter et al., 2006). It may be difficult to translate a local effect of SEMA3A into a functional outcome that resembles muscle weakness as observed in ALS given that only the gastrocnemius muscles were targeted, and not the entire skeletal musculature. In our study, one could assume that there were local effects in terms of NMJ integrity in the transduced muscle which failed to manifest into a functional defect; however, upon quantification, there was no difference in NMJ integrity between GFP and SEMA3A treated muscle at 15 weeks post-injection. However, a recent study by Rocha and colleagues showed that in presymptomatic ALS mice neuromuscular transmission was enhanced prior to signs of denervation (Rocha et al., 2013). Perhaps electrophysiological recordings of AAV6-GFP-SEMA3A injected muscle would indicate initial changes in neuromuscular transmission due to the presence of SEMA3A, which fail to cause anatomical or behavioral changes. This could be due to the fact that other mechanisms involved in NMJ denervation and ALS pathology are not activated in our experimental approach (Ferraiuolo et al., 2011; Van Hoecke et al., 2012; Jokic et al., 2006; Moloney et al., 2014). Thus, our data indicate that muscle-mediated overexpression of SEMA3A alone is unable to cause a progressive pathological-like state at the NMJ.

At this point it is important to mention that SEMA3A re-expression at the muscle only occurs after injury or within the context of a disease (Sato et al., 2013; De Winter et al., 2006), while under normal circumstances, SEMA3A is not present. Moreover, in the context of ALS, SEMA3A is specifically induced in terminal Schwann cells (TSCs) that are intimately associated with the motor axon terminal at the NMJ and not in the muscle cells per se (De Winter et al., 2006). It is possible that without a previous insult to the muscle or motor neuron, the amount of SEMA3A present...
at the NMJ after AAV administration does not reach the levels usually found at the NMJ after injury or in disease situations. In addition it is likely that healthy muscle is very efficient in removing unwanted proteins from its environment, resulting in rapid removal and/or degradation of the SEMA3A protein to maintain normal homeostasis of the muscle. It is also plausible that SEMA3A expression in an intact muscle does not produce changes in NMJ integrity as anticipated because other molecular features which are usually activated in injury or disease contexts are not present, thus limiting the potential effects of SEMA3A alone. This may mean that SEMA3A has a function in NMJ denervation, but only as a component of a larger network of molecular changes occurring during ALS disease progression. In line with our hypothesis, it would be interesting to study the effects of AAV-mediated overexpression of SEMA3A in the skeletal muscle of ALS mice. In this scenario, it is conceivable that the ALS phenotype would be worsened by the combined presence of SEMA3A from TSCs (intrinsic source) and from muscle cells (“extrinsic”, AAV source).

An alternative approach we considered for studying the role of SEMA3A at the NMJ was to specifically target the overexpression to the TSCs themselves as this would lead to much higher levels of SEMA3A locally at the neuromuscular synapse and provide a more effective trigger for axonal dye back than muscle-wide SEMA3A expression. This approach would therefore mimic ALS pathophysiology more closely, allowing us to gain a deeper insight into the role of SEMA3A at the NMJ. However, it is very difficult to achieve TSC-specific gene targeting with viral vectors or in transgenic mice. Thompson and colleagues generated transgenic mice whereby GFP or YFP, under the control of the S100-B promoter, is selectively expressed in glia including the TSCs in the muscle (Zuo, 2004). Using these transgenic mice they were able to image the post-natal development of NMJs over time. These mice may be useful for our study in terms of targeting expression to the TSCs, but because our approach includes behavioral analysis, care must be taken in drawing conclusions based on the effects seen due to the potential off-target expression of the transgene in other glial cells such as macrophages, astrocytes or Bergmann glia which could affect the behavioral phenotype. Following this rationale, viral vector mediated TSC targeted gene-insertion or deletion also raises some technical issues; there is no promoter that selectively works in TSCs alone (Zuo, 2004) and targeting TSCs with viral vectors requires intramuscular administration as one cannot inject directly into the TSC itself given the anatomy of the NMJ. Nonetheless, it may be interesting to investigate S100-B mediated SEMA3A-expression using muscle-mediated viral vector administration, but as cell-specific promoters are usually less efficient than the generic CMV promoter (which we used in our study), perhaps the levels of SEMA3A will not surpass those obtained using the CMV promoter. However, S100-B mediated SEMA3A expression
would be localized at the NMJ itself, and may locally reach higher levels there than what we observed using the CMV-promoter. In conclusion, studying the role of a protein in TSCs at the NMJ is (technically) challenging in the context of SEMA3A, and requires careful interpretation if specific targeting to the TSCs cannot be achieved.

Ultimately, the current approach we took, i.e. muscle-mediated (over) expression of SEMA3A using AAVs, did not provide additional evidence for a role for SEMA3A in NMJ degeneration and may illustrate the possibility that SEMA3A is in fact not involved in NMJ degeneration, or may be due to technical obstacles in mimicking the molecular situation necessary for NMJ degeneration.