Targeting muscle to treat Charcot-Marie-Tooth disease

David Villarroel-Campos, James N. Sleigh[*]

Charcot-Marie-Tooth disease (CMT) is a hereditary peripheral neuropathy causing muscle weakness/wasting and sensory dysfunction predominantly in limb extremities. CMT patients display gait abnormalities, foot deformities, loss of sensation and decreased/absent deep tendon reflexes, with motor symptoms usually being more prominent than sensory. Resulting from > 1500 different mutations across > 100 diverse genes, CMT affects 1 in ≈2500 people and is inherited in an autosomal recessive, autosomal dominant or X-linked fashion. Based on assessment of nerve conduction velocity, CMT is divided into Type 1/demyelinating CMT, in which perturbed Schwann cell homeostasis affects saltatory conduction and reduces nerve conduction velocity, and Type 2/axonal CMT, where motor and sensory axons are lost without affecting nerve conduction velocity. There are also intermediate forms of CMT that share features of demyelinating and axonal neuropathies, including intermediate nerve conduction velocity values.

CMT usually manifests during adolescence without curtailing lifespan, thus, the disease causes lifelong disability and a significant economic burden. It is therefore critical that the current lack of approved CMT treatments is rapidly improved upon. Fortunately, a plethora of promising therapeutics, including many gene therapies that address the genetic causes of CMT, have proven effective in preclinical CMT models (Bolino and D'Antonio, 2023). Given that Schwann cells and peripheral nerves are the primary sites of pathology in CMT1 and CMT2, respectively, it is unsurprising that many preclinical treatments are designed to selectively localize to or target disease pathways in these cell types. Recently, however, several encouraging preclinical approaches for CMT have been directed to the third major component of the neuromuscular system: muscle. Here, we briefly review these therapeutics (**Figure 1**), highlighting the value of using muscle as a source for systemic neurotrophic factors, touching upon non-cell autonomous pathomechanisms, and discussing the opportunity for combinatorial approaches to treat CMT.

Neurotrophins are a family of secreted neurotrophic factors that support neuronal development, differentiation, and survival by selectively interacting with different tropomyosin receptor kinase (Trk) receptors: nerve growth factor (NGF) signals through TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) via TrkB, and NT-3 through TrkC. Given its role in Schwann cell maintenance and peripheral nerve regeneration, NT-3 was identified by Zarife Sahenk and colleagues as a potential therapeutic protein for several different CMT subtypes, beginning with the most common form of genetic peripheral neuropathy, CMT1A. CMT1A is caused by duplications in the gene encoding the essential myelin protein, peripheral myelin protein 22 (*PMP22*). Initially, repeated subcutaneous injections (three times per week for eight weeks) of recombinant NT-3, but not BDNF, were shown to enhance nerve regeneration and myelination in immunodeficient mice hosting CMT1A patient sural nerve xenografts. A similar positive effect of NT-3 treatment was also shown post-nerve crush in Trembler[J] mice, which carry a point mutation in endogenous *Pmp22* (Sahenk et al., 2005). A double-blind, randomized, pilot clinical trial of subcutaneous NT-3 injections (three times per week for 24 weeks) was then performed in eight CMT1A patients (four receiving placebo and four receiving NT-3). Significant improvements in sural nerve regeneration, Neuropathy Impairment Score and sensory function were detected in patients treated with NT-3, but not placebo.

To circumvent issues of short serum half-life and the need for repeated injections, NT-3 cDNA was packaged into the muscle-tropic adeno-associated virus (AAV) serotype 1 under the control of either the constitutive CMV promoter (AAV1.CMV.NT3) or the muscle-specific tMCK promoter (AAV1.tMCK.NT3) (Sahenk et al., 2014). NT-3 is usually expressed by Schwann cells and acts in an autocrine fashion; however, targeting AAV to Schwann cells is difficult due to the lack of appropriate capsids, the requirement of crossing the blood-brain barrier and the need to transduce many myelinating cells. Consequently, muscle was chosen as the AAV target, as it can act as a secretory organ for systemic and long-lasting NT-3 distribution, which was confirmed in plasma for both AAV1.NT3 vectors. The gene therapies were then individually and unilaterally injected into the gastrocnemius or quadriceps muscles of Trembler[J] mice 3 weeks prior to sciatic nerve crush. NT-3 treatment improved myelinated nerve and Schwann cell density, neurofilament cytoskeleton integrity, neurophysiological parameters, and motor function (grip strength and rotarod), with greater effects observed upon extended treatment (40 > 20 weeks). In a separate study not involving nerve crush, both AAV1.CMV.NT3 and AAV1.tMCK. NT3 were also shown to restore muscle fiber cross-sectional area and fiber type proportions in gastrocnemius muscles of Trembler[J] mice 16 weeks post-treatment (Yalvac et al., 2018); this suggests that in addition to its systemic impact, NT-3 may also directly affect muscles. The CMV and tMCK promoters drive similar levels of transgene expression and equally affect nerve regeneration (Sahenk et al., 2014); thus, the use of tMCK is now being prioritized to reduce off-target NT-3 expression. Indeed, a Phase 1/2a clinical trial of multiple, single AAV1.tMCK.NT3 injections into lower leg muscles of CMT1A patients, with safety as the primary outcome, is planned; however, the trial is currently suspended due to vector production issues (clinical trial identifier: NCT03520751).

AAV1.tMCK.NT3 has also proven effective in mouse models of two additional CMT subtypes: CMTX1 and CMT2D. CMTX1 is an X-linked form of neuropathy, is the second most prevalent CMT and it is caused by deleterious mutations in the gene encoding Gap Junction Protein Beta-1/Connexin32 (*GJB1/Cx32*). GJB1 is a membrane-spanning protein that forms channels to enable ion and small molecule transfer between cells, including through the myelin sheath. CMT2D results from dominantly inherited mutations in *GARS1*, which encodes the housekeeping enzyme glycyl-tRNA synthetase (GlyRS) that charges the amino acid glycine to it cognate tRNA for protein synthesis. Following a similar paradigm of single, unilateral injections into the gastrocnemius at 3 months of age, AAV1.tMCK.NT3 was shown to preserve neurophysiology measurements, axon number and morphology, myelination, muscle fiber size and motor function of homozygous *GJB1* knockout mice (Ozes et al., 2022). In a slight variation in approach, $Gars^{P278KY/+}$ and $Gars^{\Delta ETAQ/+}$ mice modeling CMT2D received bilateral injections of AAV1.tMCK.NT3 at 4–6 and 8–10 weeks, respectively (Ozes et al., 2021). 12 weeks post-treatment, NT-3 improved neurophysiological parameters, neuromuscular junction innervation, myelin thickness, muscle fiber health and rotarod performance in $Gars^{P278KY/+}$ mice, while myelin thickness and myofibers were also improved in $Gars^{\Delta ETAQ/+}$ mice, albeit to a lesser extent. Together, studies from the Sahenk Laboratory indicate that muscle-specific NT-3 gene therapy can improve neuropathy across major CMT subtypes, and is thus a promising strategy for the treatment of CMT independently from the disease-causing genetic lesion.

A similar strategy of using muscle as an injection site for systemic therapy distribution has been employed for CMT2A, which is caused by dominantly inherited mutations in *mitofusin 2* (*MFN2*) and is the most common form of axonal CMT. Found in the outer mitochondrial membrane, MFN2 is crucial for the fusion of mitochondria and thus for their dynamics, distribution and
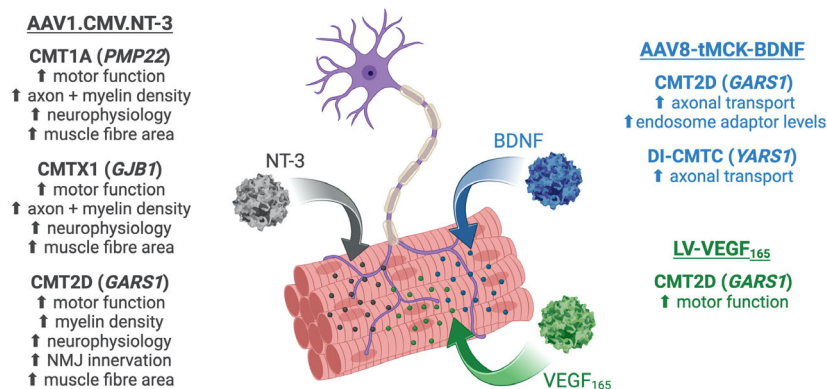

AAV1.CMV.NT-3

CMT1A (*PMP22*)
↑ motor function
↑ axon + myelin density
↑ neurophysiology
↑ muscle fibre area

CMTX1 (*GJB1*)
↑ motor function
↑ axon + myelin density
↑ neurophysiology
↑ muscle fibre area

CMT2D (*GARS1*)
↑ motor function
↑ myelin density
↑ neurophysiology
↑ NMJ innervation
↑ muscle fibre area

NT-3

BDNF

VEGF₁₆₅

AAV8-tMCK-BDNF

CMT2D (*GARS1*)
↑ axonal transport
↑ endosome adaptor levels

DI-CMTC (*YARS1*)
↑ axonal transport

LV-VEGF₁₆₅

CMT2D (*GARS1*)
↑ motor function

Figure 1 | Pre-clinical gene therapies targeting muscle in CMT.
AAV: Adeno-associated virus; BDNF: brain-derived neurotrophic factor; CMT: Charcot-Marie-Tooth disease; CMV: cytomegalovirus promoter; *GARS1*: glycyl-tRNA synthetase gene; *GJB1*: *gap junction protein beta-1*; LV: lentiviral vector; NMJ: neuromuscular junction; NT-3: neurotrophin-3; *PMP22*: *peripheral myelin protein 22*; tMCK: triple tandem muscle creatine kinase promoter; VEGF₁₆₅: vascular endothelial growth factor 165; *YARS1*: tyrosyl-tRNA synthetase gene. Created with BioRender.com.

function. MiM111 is a pharmacological activator of mitofusins capable of improving *in vitro* mitochondrial defects associated with MFN1 or MFN2 loss-of-function. MiM111 was injected into the biceps femoris muscle of CMT2A mice conditionally expressing human $MFN2^{T105M}$ in mouse neurons (Franco et al., 2020). Within 4 hours of treatment, MiM111 normalized mitochondrial axonal transport disruption in *ex vivo* sciatic nerves. Moreover, after 4 and 8 weeks of daily dosing (alternating between sides of the body), MiM111 improved neuronal physiology, motor function, neuromuscular junction morphology and axon health.

Returning to CMT2D, *GARS1* mutations cause the encoded mutant GlyRS to mis-interact with the extracellular domain of the transmembrane receptor neuropilin-1; this competitively interferes with vascular endothelial growth factor 165 ($VEGF_{165}$) binding and pro-survival signaling, contributing to neuropathy (He et al., 2015). GlyRS is secreted from several different cell types, including muscles, suggesting that antagonisation of the $VEGF_{165}$-neuropilin-1 interaction is non-cell autonomous in origin. To counteract this, $VEGF_{165}$ was overexpressed bilaterally in a variety of hindlimb muscles in 5-day-old $Gars^{P278KY/+}$ mice via injection of lentiviral vectors (LV). Treatment with LV-$VEGF_{165}$ resulted in improved neuromuscular function as shown by an inclined plane test at 4 weeks, as well as rotarod and gait analysis at 7 weeks. $Gars^{P278KY/+}$ mice were then subjected to unilateral LV-$VEGF_{165}$ injections versus LV-GFP control, followed by a leg extension test at 5 weeks. Muscle weakness was more prominent in the control-injected leg, suggesting that $VEGF_{165}$ functions locally in the injected muscles rather than having a systemic impact like NT-3.

We recently determined that a third neurotrophic factor, BDNF, is also a promising therapeutic for CMT2D mice when targeted to muscle (Sleigh et al., 2023). Mutant GlyRS aberrantly associates with the extracellular domains of the Trk receptors. By intravital imaging of intact sciatic nerves, we determined that dampened BDNF-TrkB signaling through a non-cell autonomous mechanism at the motor nerve terminal, is the cause of *in vivo* impairments in the axonal transport of neurotrophic signaling endosomes in CMT2D mice. We thus injected recombinant BDNF into CMT2D muscles and showed that this acutely rescued the endosome transport disruption. Perhaps surprisingly, NT-3 and $VEGF_{165}$ had no such effect, indicating that these neurotrophic factors act on different cellular processes to alleviate mutant GlyRS-associated pathology. Based on these discoveries, we created a gene therapy to selectively augment BDNF in muscle that combines the tMCK promoter with muscle-tropic AAV8. A month after intramuscular injections of AAV8-tMCK-BDNF, axonal transport was fully corrected in CMT2D mice and associated with increased availability of motor adaptor proteins critical to retrograde trafficking of endosomes.

We also recently confirmed the validity of boosting BDNF in muscle for a second CMT subtype. Dominant intermediate CMT subtype C (DI-CMTC) is caused by dominantly inherited mutations in tyrosyl-tRNA synthetase (TyrRS)-encoding *YARS1*. Like *GARS1*, *YARS1* also encodes an aminoacyl-tRNA synthetase, and is one of the seven genes of this family linked to CMT. Using the recently created $Yars^{E196K}$ mouse model for DI-CMTC, we determined that homozygous mutants display a muscle-selective and age-dependent decline in axonal transport of neurotrophic signaling endosomes, likely caused by the confirmed aberrant association of $TyrRS^{E196K}$ with the extracellular domain of TrkB (Rhymes et al., 2023). Given the conserved disease mechanism, we tested the effect of boosting BDNF in muscles of $Yars^{E196K/E196K}$ mice; we found that an acute treatment with recombinant BDNF or a prolonged exposure via AAV8-tMCK-BDNF was sufficient to fully rescue the signaling endosome trafficking disruption. However, similar to the local effect of $VEGF_{165}$, we found that BDNF only improves transport in motor axons innervating the injected muscle and not neurons in the contralateral sciatic nerve. It remains to be determined whether AAV-BDNF corrects additional features of CMT2D and DI-CMTC neuropathy and whether, like NT-3, it will be effective across CMT subtypes.

Conclusions: While most of the muscle-directed treatment strategies discussed above do not address the genetic cause of neuropathy, they can perhaps serve as complementary therapeutics that are delivered as part of a combinatorial approach; this has proven beneficial in preclinical models of other neuromuscular diseases and is currently being trialled in spinal muscular atrophy (e.g., NCT03921528). Moreover, mutation-independent therapies have the considerable benefit that they may treat several different CMT subtypes, as has already been shown for NT-3. It is therefore not surprising that similar growth factor-related strategies have been tested in CMT clinical trials. For instance, Engensis (VM202) is a hepatocyte growth factor-expressing DNA plasmid that was repeatedly injected into lower limb muscles of CMT1A patients in a Phase 1/2a trial (NCT05361031; outcome unreported), and CLZ-2002 is an allogenic mesenchymal stem-cell treatment in which Schwann cell-like cells capable of increasing neurotrophic factor availability are being injected into muscles of CMT1 patients in a Phase 1 trial (NCT05947578; results due in late 2024). Alternatively, it is possible to direct gene therapies to muscle for introducing viral vectors to select central and peripheral neurons and/or glia (Tosolini and Sleigh, 2020), as are muscle-protective strategies, such as inhibiting negative regulators of skeletal muscle, which has been shown to enhance total muscle volume when injected into the tibialis anterior of CMT1 and CMTX patients (Thomas et al., 2022). In summary, treating muscle in CMT appears to be a viable strategy to preserve neuromuscular function in several different subtypes of the disease – either by creating a secretory hub for systemic therapy delivery or by direct targeting for local benefits on the neuromuscular system.

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