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Gene therapy to promote regeneration in Charcot-Marie-Tooth disease

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1. Introduction

Peripheral nerve disorders are relatively common conditions with increasing prevalence up to 8% of population with advancing age (Hughes, 2002). Those with underlying genetic causes include Charcot-Marie-Tooth (CMT) disease, one of the most common inherited conditions occurring in populations worldwide with a prevalence of about 1 in 3,300 individuals (ghr.nlm.nih.gov). During the last several decades, significant advances have been made in understanding the pathobiology of peripheral nerve disorders. The major challenge the investigators and clinicians have been facing is to develop effective treatment strategies to improve the function, or at least halt the progression of the disease process.

The classical approach to peripheral neuropathies has been to subdivide them into four groups according to the primary anatomical site of involvement: 1) cell body disease (neuronopathy), 2) myelin disease (myelinopathy), 3) distal non-terminal axonopathy which typically affects the distal parts of the longest axons (length-dependent distal axonal disease, dying back neuropathy or distal axonal neuropathy) and 4) distal terminal axonopathy starting with nerve terminal degeneration (Schaumburg and Spencer, 1979; Schroder, 2006; Stojkovic, 2006; Thomas et al., 1984; Watanabe et al., 2013). Although this is a prologue to understanding the neuropathic process, it is important not to overlook the final common path leading to Wallerian-like degeneration and axonal loss for the vast majority of neuropathies, regardless of the primary anatomical site of involvement (Glass, 2004). A striking example

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for this is the primary Schwann cell (SC) genetic defects resulting in secondary axonal pathology presenting with a length-dependent axonal loss, which directly correlates with the clinical severity (Dyck et al., 1974; Krajewski et al., 2000; Sahenk and Chen, 1998; Sahenk, 1999).

Wallerian-like degeneration resulting from axonal pathology should be viewed as final common path, directly linked to the maintenance of bioenergetics homeostasis, essential for cell survival. This is particularly important for structural and functional integrity of distal axons. The unique morphology of axons, especially those with the longest extensions from the cell body are destined to require extraordinary demand for ATP to support energy consuming activities, including axoplasmic transport and generation of ion gradients. Therefore, the currently known numerous CMT subtypes and the continuously increasing numbers of newly discovered disease-causing genes need to be evaluated from this perspective: “**All paths lead to the center of things**”. This approach is important for understanding how a specific gene defect fits into the final common pathway. The critical point of commonality alters one or more cellular processes that compromises axonal structural integrity. In addition to determining the molecular consequences, the morphological correlates of such defects are important to understanding the entire disease process which offers clues for how to bypass a defect that can prevent or slowdown axonal loss.

There is no doubt that technology today permits us to design state-of-the art molecular approaches to manipulate or theoretically correct individual gene defects in these disorders. However, the formidable challenge now is the clinical translation for the most common CMT subtypes, so called “demyelinating CMT” or CMT1, caused by primary SC genetic defects. This task is made difficult by the paucity of vectors endowed with the ability to transduce myelinating SCs through the blood-nerve barrier. This task is further challenged given that SCs sequentially line the axon, extending the length of the nerve from axon hillock to the terminal bulb. Alternatively, a surrogate gene therapy approach, not directed to correct a specific gene mutation but to improve cellular processes compromised by this defect may offer therapeutic potential. One such approach that we have used in our laboratory is to improve regeneration capacity of distal axons, common to all longstanding neuropathic conditions including CMT disease. The focus in this review will be on the preclinical studies showing the potential therapeutic use of neurotrophin 3 (NT-3) to improve nerve regeneration and associated myelination in Trembler^J (Tr^J) mouse model for CMT1. In this context, SC genetic defects and secondary axonal pathology, impaired regeneration and the essential role of SCs in nerve regeneration process will also be emphasized.

2. Role of Schwann cells in axonal regeneration

Injury to axon, either intrinsic-resulted from slowly developed local pathology or induced by an external physical factor, gives way to a regeneration process that involves complex interactions between axon, SCs, and the extracellular matrix. Axonal pathology may occur as membranous organelles and mitochondria increase, usually most conspicuous on the proximal and distal paranodal areas when bidirectional transport is compromised. Defects affecting cytoskeletal components may present with abnormalities such as organizational

disarray, increased neurofilament (NF) content with axonal enlargement, axonal atrophy with increased NF density, or decreased NF content if their synthesis is impaired. The term “Wallerian-like” is used for axonal breakdown that follows a slowly evolving intrinsic axonal pathology in contrast to the classical definition that includes changes in distal stump following an acute injury, such as transection or compression (Glass, 2004). Regardless of the nature of injury identical changes take place distal to the injury site. Myelin degradation products boosted by secretory products from infiltrating macrophages -stimulate the first wave of SC proliferation within their basal lamina tubes, forming the bands of Bungner (Salzer and Bunge, 1980). SCs are essential for the process of axonal regeneration (Hall, 1986; Hall, 1989). In addition to clearing the myelin debris, SCs are the source of neurotrophic factors that diffuse from the distal stump across the injury area and to stimulate the elongation of regenerating axons (Boyd and Gordon, 2003; Fu and Gordon, 1997; Reynolds and Woolf, 1993; Terenghi, 1999). In the absence of axons, either in the developing nervous system or during regeneration in adult nerve, SC-derived autocrine survival factors are essential for their survival, proliferation and differentiation. (Mirsky et al., 2002) Maturing SC progressively switches its trophic dependence to a set of autocrine-derived factors consisting of NT-3, insulin-like growth factor-1 (IGF1) and platelet derived growth factor-BB (PDGF-BB) which are secreted by the SC, and for which it has high-affinity receptors (Jessen and Mirsky, 1999; Lobsiger et al., 2000; Meier et al., 1999). The contact with regenerating axons induces a second phase of SC proliferation, which is mediated by neuron-derived glia-specific neurotrophic factors. Delay in axonal contact leads to a progressive decrease in number of SCs and a loss of responsiveness to regeneration (Li et al., 1998; Terenghi et al., 1998).

3. Schwann cell genetic defects, axonal pathology and impaired regeneration

CMT1A, the most common form of hereditary motor sensory neuropathy, results from 1.4-Mb tandem DNA duplications on the short arm of the chromosome 17 encompassing peripheral myelin protein 22 (PMP22) gene (Lupski et al., 1991; Raeymaekers et al., 1991). This SC-specific mutation represents the “classical CMT clinical phenotype” with slowly progressive weakness and atrophy of distal limb muscles, more pronounced in lower extremities with frequent foot deformity and distal sensory loss and no or few sensory symptoms. Therefore, the evolution of the disease process is typical for length-dependent distal axonal loss, which was first illustrated more than 40 years ago in “dominantly inherited hypertrophic neuropathy” (diagnostic term prior to identification of gene defect in CMT1A) by using quantitative analysis of saphenous nerves which showed markedly severe myelinated fiber loss at the ankle level compared to more proximal sites (Dyck et al., 1974). Although at the time, axonal loss was best explained with a “neuronal disease/atrophy” as the underlying disease mechanism, this observation remained significant, paving the way to understanding the pathobiology of axonal loss in CMT neuropathies with primary SC genetic defects.

The classical xenograph paradigm has been instrumental in determining the pathogenesis of the disease state: a defective intrinsic axonal function, or a loss of function or a toxic gain-

of-function in the ensheathing SCs, causing loss of a critical interaction between these neuronal components.(Aguayo et al., 1977; de Waegh and Brady, 1991; Sahenk, 1999). This model enabled us to transfer mutant SCs to the nude mouse to create an *in vivo* regeneration-associated remyelination system. (Sahenk and Chen, 1998; Sahenk et al., 1998; Sahenk et al., 1999). With this approach, not only can the local influence of mutant SCs derived from patients on axonal properties be studied, but also the entire regeneration-associated myelination process starting from promyelinating SC can be examined in a sequential manner. Sural nerve segments from individuals with PMP22 duplications or deletions and point mutations, as well as point mutations in the gap junction B1 (*GJB1*, encoding connexin 32) were grafted into cut ends of the sciatic nerve of nude mice and studied at different time intervals after grafting. *GJB1* mutations in SCs are responsible for the second most common CMT, an X-linked dominant disorder.(Saporta and Shy, 2013) Female carriers have milder phenotype compared to affected males. Males usually present on the first two decades of life with a classic length-dependent neuropathy phenotype.(Hahn et al., 1999) Nude mice axons engulfed by mutant SCs showed profound alterations in the axonal cytoskeleton and an increase in membranous organelle/mitochondria density. A preferential distal axonal loss associated with a perpetual axonal atrophy, degeneration, and axonal sprouting was observed over time with increasing frequency at later time points. A distal summation of the pathology, as evidenced by a greater amount of fiber loss in the distal graft segments, was present similar to that observed in patients. These alterations were seen to a lesser extent in PMP22 deletion or point mutation xenografts and were not observed in controls. Collectively, these studies are the first to show that SCs bearing the PMP22 or Cx32 genetic defects cause major perturbations in SC-axon interactions emphasizing the role of axonal component in the pathobiology of hereditary neuropathies (Sahenk, 1999).

Subsequent studies using detailed quantification and temporal analysis of the growth and myelination dynamics of the axons through PMP22 duplication xenografts revealed a delay at the onset of myelination and an impairment of axon tip elongation, indicating that the PMP22 duplication in SCs have profound effects upon the regeneration process. This defect was most pronounced for large diameter axons, which is likely a contributing factor to distally prominent myelinated fiber loss(Sahenk et al., 2003). In addition, we showed that the peripheral nerve regeneration is severely impaired in a naturally occurring animal model harboring a primary SC genetic defect, the Tr^J mouse, which we used extensively in our subsequent studies to assess the efficacy of NT-3 (Sahenk et al., 2005; Sahenk et al., 2014).

Moreover, similar to Tr^J model, previously unpublished studies from this laboratory have shown that regeneration in connexin 32 null (Cx32 KO) mice is also severely impaired. In the nerve segments distal to the crush site, the number of myelinated fibers in the regenerating Cx32 KO nerves was significantly lower than the wild type, therefore justifying the use of this model to establish the efficacy of NT-3 gene therapy. Cx32 KO mouse with targeted ablation of *GJB1* in SCs is a model for X-linked CMT (Vavlitou et al., 2010). Similar to Tr^J model, there is secondary axonal pathology including decreased axonal diameters of large myelinated fibers and decreased phosphorylation of NFs which precedes demyelination.(Vavlitou et al., 2010)

4. NT-3 improves Impaired regeneration in CMT mouse models

Prolonged denervation with accompanying SC atrophy, a model that simulates a chronic distal axonal neuropathic process in humans has a series of consequences that include decreased regeneration capacity related to loss of receptors and reduced expression of growth factors, a gradual decline in SC number with inability to maintain bands of Bungner (Li et al., 1997; Sulaiman and Gordon, 2000). From a translational viewpoint, these observations emphasize the importance of targeting denervated SCs in order to maintain a state of readiness for regeneration for chronic neuropathic conditions, especially for disorders with primary SC genetic defects.

In theory, an improvement in a length-dependent distal axonal disease can be accomplished if the rate of regeneration from the nascent axon tips exceeds the rate of “dying back” degeneration within the distal nerve segments (Sahenk, 2006). The premise for our studies is that in long standing neuropathic conditions, the prolonged denervation status of SCs could lead to decreased regeneration capacity; therefore, for efficient regeneration, SCs distal to the axonal injury site must remain in a growth-supporting mode. This can potentially be achieved by transforming chronically denervated SCs to a competent pre-myelinating state, enabling complete myelination. We hypothesized that during the initial phase of myelination, at a time of high metabolic demand (for myelin protein synthesis), primary SC genetic defects interfere with SC differentiation into a competent state capable of inducing axonal growth and myelination. Given that NT-3 is an important component of the SC autocrine loop, exogenous administration of this neurotrophic factor could overcome this failure (Sahenk et al., 2005). Endogenous NT-3 is expressed by SCs and takes part in autocrine loop that allows SCs to mature and survive without the axon and stimulates neurite outgrowth and myelination. (Jessen and Mirsky, 1999; McTigue et al., 1998; Meier et al., 1999; Sterne et al., 1997)

Another confirmatory study from this laboratory has shown that NT-3 heterozygous knockout mice (NT3^{+/-}) displayed retardation of the myelination process, and this defect was found to be associated with decreased SC survival and increased NF packing density of regenerating axons. Allograft paradigms indicated that the reduced NT-3 status of the SCs, but not of the axons, is responsible for impaired nerve regeneration and that NT-3 is essential for SC cell survival during early stages of regeneration-associated myelination in the adult peripheral nerve. Moreover, as shown in the NT-3 homozygous knockout mice (NT3^{-/-}) postnatal absence of NT-3 resulted in a rapidly evolving motor neuropathy with degeneration of intramuscular nerves and loss of nerve terminals emphasizing its protective effect as a vital survival factor on the periterminal SC, “the last player in the neuromuscular system”. (Woolley et al., 2005) These studies provided insights in understanding the impaired regeneration in CMT disorders and laid the groundwork for the development of rational translational approach to promote nerve regeneration (Sahenk et al., 2008). Figure 1 schematically illustrates the biological effects of NT-3 upon impaired regeneration in long-standing neuropathic conditions.

5. From NT-3 peptide to path forward to NT-3 gene therapy in CMT1A

Our initial proof of principle data came from two sets of studies using NT-3 in peptide form via subcutaneous injections: 1) NT-3 pharmacological treatment of nude mice harboring CMT1A xenografts improved axonal growth and the associated-myelination process within the grafts; 2) in Tr^J mice improved SC survival and differentiation resulting in increases in the available SC pool and the number of myelinated fibers within the regenerating and the uncrushed-intact nerves. These studies were crucial for future clinical attempts to repair the diseased axons resulting from ensheathment by faulty SCs (Sahenk et al., 2005). Based on these encouraging results, we conducted a double blind, placebo-controlled, randomized pilot clinical trial. In a small cohort of CMT1A patients, we demonstrated that exogenously delivered NT-3 by a subcutaneous route promotes nerve regeneration in sural nerves and improves the Mayo Clinic Neuropathy Impairment Score (NIS) and reduces the clinical sensory deficit after 24 weeks of treatment (Sahenk et al., 2005). However, further clinical trials of subcutaneously injected NT-3 are thwarted by limitations that include: a short serum half-life, high production costs, need for repeated dosing, and discontinuation of the product. Our attention then was directed toward delivery of NT-3 by adeno-associated virus serotype 1 (AAV1); in the Tr^J mouse, this approach proved successful. We chose AAV1 due to its well-recognized muscle tropism, determined by its capsid protein VP1 amino acids 213 to 423 (Hauck and Xiao, 2003).

Several observations support the rationale for using Tr^J mouse as model for CMT1A. Tr^J mice carry a naturally occurring point mutation (L16P) in PMP22 gene which is the gene duplicated in CMT1A patients. This mouse model recapitulates the axonal pathology of CMT1A, but causes more severe hypomyelination and nerve conduction slowing than CMT1A (Meekins et al., 2004). In both conditions, impaired SC-axon interaction results in the secondary axonal pathology leading to a distally prominent, length-dependent axonal loss (de Waegh and Brady, 1991; de Waegh et al., 1992; Dyck et al., 1974; Meekins et al., 2007; Sahenk, 1999; Sahenk et al., 1999). The fiber loss is most predominant for the large myelinated fiber population in both Tr^J and CMT1A nerves (Dyck et al., 1974; Sahenk et al., 2003; Sahenk et al., 2005). The axonal pathology is highlighted with abnormalities in axonal cytoskeleton associated with hypophosphorylated NFs with increased NF packing density (de Waegh and Brady, 1991; de Waegh et al., 1992; Sahenk et al., 2014). Both Tr^J mutation and the PMP22 duplication (overexpression) are thought to cause similar cellular dysfunctions which are likely to interfere with SCs ability to support regeneration (Clayton and Popko, 2016; Fortun et al., 2006; Liu et al., 2004). In both conditions there is SC loss by apoptosis, which occurs throughout the life of Tr^J mice while it is more prominent in the older age group as we showed in the CMT1A patient-biopsies (Erdem et al., 1998; Sancho et al., 2001). Most importantly, these studies in the Tr^J mice and the xenografts from patients with CMT1A as well as in a pilot study examining sural nerve biopsies from CMT1A patients have shown that NT-3 has the same biological effects both in Tr^J and CMT1A nerves (Sahenk et al., 2005). NT-3 in both conditions improved mutant SC survival and differentiation resulted in increases in the available SC pool, which in turn increased the number of myelinated fibers. In addition, exogenous NT-3 normalized axonal NF cytoskeleton which we believe contributes to improving axonal NF transport as well as

regeneration. These features provided strong support for the Tr^J mice model to be used in our preclinical studies testing the efficacy of AAV1.NT-3 gene therapy. It should be emphasized that Tr^J nerves have more severe electrophysiological and histopathological findings than CMT1A and in spite of the severe pathology, our preclinical studies using AAV1.NT-3 gene therapy have established efficacy in every parameter measured: histopathological, functional and electrophysiological. A summary of efficacy of NT-3 in trembler nerves follows (Sahenk et al., 2005).

5.1 Preclinical functional and histopathological studies in regenerating and intact nerves following intramuscular injection of single strand (ss)AAV1.CMV.NTF3

The premise for these studies is that intramuscular injection of AAV1 vector carrying human *NTF3* cDNA under the control of CMV promoter will transduce muscle to produce NT-3 peptide, which is then secreted into the circulation and induces systemic effects (*NTF3*, neurotrophic factor 3, the official designation of gene encoding NT-3, encodes the pre-pro-NT-3 peptide).

This initial set of studies revealed several findings critical for the success of NT-3 gene therapy program. First of all, intramuscular injection of AAV1.CMV.NTF3 in Tr^J mice resulted in persistent NT-3 expression as evidenced by serum levels easily detectable by ELISA three weeks after gene transfer. The levels remained elevated for up to 10 months, the last time point that was tested. Additionally, circulating NT-3 Tr^J in mice at 20 weeks produced histopathological and functional improvements in trembler nerves. The nerve segments distal to the crush site of the sciatic nerves displayed significantly greater number of myelinated fibers indicative of increased nerve regeneration. In the contralateral intact sciatic nerves, the number of myelinated fibers as well as myelin thickness was increased. Similar to previous observations that NT-3 in peptide form improved SC proliferation and survival in Tr^J nerves as well as in sural nerves from patients with CMT1A, the AAV1.NT-3 gene therapy at 20 weeks post injection resulted in a more robust SC density increase effect in crushed or intact Tr^J nerves (Sahenk et al., 2014). Moreover, axonal pathology (increased NF packing density associated with hypophosphorylation state of NFs) in trembler nerves is improved towards normalization. The improvements in Tr^J pathology in the other extremity which did not get intramuscular gene transfer provides evidence supporting a systemic biological effect of NT-3. This is based on our previous quantitative studies comparing retrograde labeling of spinal cord neurons in the ipsilateral and contralateral side following the delivery of plasmid vectors containing the Lac Z gene under the control of both the Rous sarcoma virus (RSV) and Simian virus (SV)40 promoters (Sahenk et al., 1993). B-galactosidase activity was observed in alpha and gamma motor neurons only on the ipsilateral side; the internuncial small neurons of the ipsilateral and contralateral side were labeled; however, the numbers were negligible. Therefore, retrograde transport at the site of gene transfer affecting motor neurons on the contralateral extremity via transsynaptic transport is highly unlikely.

Functionally, hindlimb grip strength on the side of nerve crush correlated with improved morphological findings. Studies in a parallel group without nerve crush showed that ssAAV1.NTF3 gene transfer could maintain hindlimb grip strength while a control group

treated with empty capsids declined over 20 weeks. Because the sciatic nerve crush injury paradigm generates nascent axon tips, we predict that uptake of NT-3 from the circulation at the crush site and its retrograde transport into the spinal cord motor neurons should occur effectively in the ipsilateral limb. One could also assume similar event could take place in patient nerves; individual growing nascent axon tips should be able to uptake NT-3 from its microenvironment in the nerve.

5.1.1 Relevance of crush paradigm to CMT1A—It can be argued that an acute crush paradigm applied to entire sciatic nerve has no direct anatomical/pathological relevance to CMT1A patients. One can also take into consideration that the pathology of each individual axon at one point along the length results in Wallerian-like degeneration distal to the site of injury. This is an acute axonal event, and the behavior of SCs distal to that point will be similar to crush model where the nerve is surgically severed to generate a concentrated population of nascent axon tips. What is different in patients is obviously the slow frequency of these individual acute events, as supported by the rare occurrence of Wallerian degeneration in human biopsy specimens and that the summation of the individual nerve fiber degeneration gives rise to a slowly progressive clinical course (Benedetti et al., 2010; Hanemann et al., 2001; Schroder, 2006).

The crush paradigm is a versatile model that generates a large population of regenerating axons tips, locally concentrated at the crush site that enables study of the proximodistal progression of regeneration-associated myelination through mutant or wild type SC tubes in a temporal manner (Sahenk et al., 2008; Sahenk et al., 2010). In our hands, the crush paradigm has been very useful to assess the efficacy of NT-3 gene therapy in T^J mouse (Sahenk et al., 2014). Using the same paradigm we previously showed that regeneration was impaired in this model and that subcutaneous injection of NT-3 in peptide form improved regeneration (Sahenk et al., 2005). Using xenografts we also showed that regeneration of nude mice axons through grafted-CMT1A nerve segments where myelination is provided by the mutant SCs from patients was abnormal (Sahenk et al., 2003). In addition, it needs to be emphasized that the paradigm best suited to uncover impaired regeneration in a transgenic model with no obvious pathology is a forced regeneration model, as we showed in NT3 heterozygous ($NT3^{+/-}$) mice with qualitative and quantitative assessments of post-crush myelinated fiber counts and SC survival distal to the crush site (Sahenk et al., 2008). Basic findings from these and others indicate that acute crush experiments in animal models could have relevance to ongoing individual nerve fiber regeneration attempts in all chronic neuropathic conditions (Li et al., 1997; Sulaiman and Gordon, 2000; Sunderland, 1952).

5.1.2 Efficacy of ssAAV1.NTF3 gene transfer in Tr^J mice peripheral nerves assessed by electrophysiology and correlative functional studies—In a parallel group, these studies were conducted to examine the efficacy of NT-3 gene therapy by assessing alterations in the sciatic nerve conduction parameters and correlative ipsilateral and bilateral hindlimb grip strength. For these experiments, Tr^J mice received AAV1.CMV.NTF3 (1×10^{11} vg) I.M. injection of the vector or PBS into the right quadriceps muscle and sciatic nerve conduction studies were carried out on the opposite extremity at baseline, 20 and 40 weeks. Results showed significantly greater compound muscle action

potential (CMAP) amplitude in the NT-3-treated Tr^J corresponding to a 37% difference compared to the PBS control group at 20 weeks. This CMAP amplitude increase correlated with hindlimb grip strength corresponding to a 39.7% improvement (10.53 g (grip force) difference; $P = 0.0001$) in simultaneous bilateral grip strength and a 29% improvement (6.4 g difference; $P = 0.0009$) when tested on only the ipsilateral side (Sahenk et al., 2014). Endpoint electrophysiological studies at 40 weeks post treatment revealed a mean CMAP increase of 84% greater amplitude in the NT-3 group compared to the PBS. At this time point, there was a small but statistically significant increase in the sciatic nerve conduction velocity. Moreover, compared with baseline at 20 weeks post injection demonstrated 28% increase of CMAP amplitude, which increased to 52% at 40 weeks ($P < 0.05$) while in the PBS-Tr^J controls, over the same period, there was a decline in the CMAP amplitude corresponding to a 27% reduction without reaching statistical significance suggesting a correlation with the natural progression of the neuropathic process in this model. The results of these studies validate that the CMAP parameter can be used as a reliable exploratory outcome measure as we proposed for the clinical trial. In addition, we collected additional functional data from these mice and recorded their rotarod performance weekly between 20 to 40 weeks post injection and found a continuous improvement in the rotarod performance of the NT-3 treated group compared to the PBS controls (Sahenk et al., 2014). This data clearly showed that we have a long lasting functional improvement with NT-3 in this model (last data collection is at 10 months post gene transfer).

5.2 Studies with self-complementary (sc) AAV1 and the use of a muscle specific truncated creatine kinase (tMCK) promoter

scAAV permits lower dosing that adds up to enhanced safety and dosing levels that will meet production standards. The use of tMCK promoter is a valued objective again offering greater safety by avoiding off target effects, which has already been used in a clinical trial (Mendell et al., 2019). A tMCK vector was also tested previously in non-human primates in safety studies by the same group. In the next set of experiments, we compared the efficacy of scAAV1.NTF3 under control of the CMV promoter versus the muscle specific tMCK promoter both given at three doses, within a half-log range (3×10^9 vg, 1×10^{10} vg and 3×10^{10} vg) (Sahenk et al., 2014). The efficacy the AAV1.NTF3 variants in Tr^J mice peripheral nerves was assessed by electrophysiological and morphological studies 24 weeks post gene transfer. The evidence of transgene expression was shown by measuring serum NT-3 levels using ELISA (Sahenk et al., 2014). At high dose (3×10^{10} vg), both CMV and tMCK vectors produced statistically highly significant improvements in CMAP amplitudes and myelinated fiber densities compared to PBS-control group. There was significant difference in serum NT-3 levels for highest and intermediate doses of vectors for both promoters and control and a strong Spearman correlation between MF densities and NT-3 levels for all samples. Neither CMAP amplitudes nor NT-3 levels are statistically different for these two promoters at high dose when they were compared with each other; therefore, we decided to use the tMCK promoter in the clinical trial. Upon completion of the pre-clinical studies we held a pre-IND (Investigational New Drug) meeting with FDA and mapped out a course to obtain an IND. This included a toxicology-biodistribution studies in C57BL/6 wild type and the Tr^J mice using scAAV1.tMCK.NTF3 delivered by intramuscular injection. No toxicity was encountered and the FDA awarded the IND (#16815) for the

clinical trial. By all indications, including safety and efficacy in pre-clinical studies, a favorable path forward to enroll patients in a clinical trial has been established. Since the high dose of 3×10^{10} vg (1.5×10^{12} vg/kg for 20 g animal) was required for significant improvement in CMAP and myelinated fiber densities, 1.5×10^{12} vg/kg is our minimally efficacious dose which guided the designation of 2×10^{12} vg/kg as the low dose in the proposed clinical trial, which will be distributed between both legs, anterior and posterior compartment muscles.

It also needs to be emphasized here that the long term toxicology studies conducted in the animal model, the Tr^J mouse provided additional supportive data showing a continuous efficacy with time from one time intramuscular delivery of the vector. Figure 2. illustrates notable improvements in histopathology of the sciatic nerve samples from Tr^J mouse received scAAV1.tMCK.NTF3 at 2×10^{11} vg at 48 weeks post injection compared to earlier time points published previously. In addition, the rotarod data collected from these cohorts provided additional supportive functional data showing a continuous efficacy of NT-3 in the Tr^J mice (Figure 3). Moreover, as shown before, NT-3 levels did not show a significant decline over time when the mean values at 48 weeks were compared to levels obtained at 24 weeks and gender had no effect on the circulating NT-3 levels.

6. The promise of NT-3 gene therapy for patients with CMT1A

The primary outcome of the “Phase I/IIa Trial Evaluating scAAV1.tMCK.NTF3 for Treatment of Charcot-Marie-Tooth Neuropathy Type 1A (CMT1A)” is safety and the expectation at minimum is to halt the decline in abilities by directly targeting regeneration capacity of damaged axons. This will be done by creating a continuous and low level NT-3 delivery system via intramuscular AAV1.NT-3 gene injection. The muscle will be transduced to produce and release NT-3 into circulation, and via this systemic approach, NT-3 will exert its known biological effects to: 1) improve regeneration capacity of nascent axon tips by targeting denervated mutant SCs to increase competent SC pool, ready to engulf and myelinate growing axons. NT-3 will accomplish this by improving mutant-SC survival and differentiation, and via Akt/mTOR activation increasing protein synthesis crucial for efficient myelination; 2) improve SC support to axons and prevent axonopathy by normalizing NF phosphorylation and cytoskeletal organization. Improving regeneration along with normalizing NF cytoskeleton could make a significant difference in the proposed clinical trial. This approach is crucial for halting progression of CMT1A because the disability is not due to abnormal myelin but directly correlating with the extent of axonal loss. In this respect, it is different from previous clinical trials attempting to lower PMP22 expression within the mutant SCs (Ekins et al., 2015), but focusing directly on nerve regeneration. In addition, intramuscular injection of the vector ensuring NT-3 expression under tMCK promoter provides a long term continuous reservoir for NT-3 source with the addition of local axonal sprouting within the anterior and posterior leg muscle groups. This regional effect along with direct NT-3 effect improving muscle protein synthesis should contribute to our goal, halting the decline in functional abilities which will be measured using CMT Pediatric Scale (CMTPedS).

In the proposed open-label clinical trial, we will deliver the scAAV1.tMCK.NTF3 to the medial and lateral heads of the gastrocnemius and tibialis anterior muscles in both legs. Due to the length-dependent nature of axonal degeneration in CMT1A these two muscles of the anterior and posterior leg compartments would be appropriate for NT-3 to exert its direct regional effects on the muscle including increased axonal sprouting and reinnervation. Our pre-clinical studies showed significant increase of muscle fiber diameter both in gastrocnemius and tibialis anterior muscles from Tr^J mice at 40 weeks post AAV1.NT3 gene therapy (Sahenk et al., 2014). Moreover, additional *in vivo* and *in vitro* studies in our laboratory have shown that NT-3 stimulates Akt/mTOR pathway in myotubes through Trk-C receptors indicating its direct effect in fiber diameter increase in muscles of Tr^J mice (Yalvac et al., 2018). Similar studies (unpublished) in our laboratory have also shown that NT-3 stimulates Akt/mTOR pathway in SCs cells giving rise to improved myelination.

This will be a novel treatment approach to CMT1A, which is not a conventional manipulation of the causative gene but targeting the consequence, axonal damage common to all length dependent neuropathies with direct implications for other CMT subtypes. As noted above, vector delivery via injection into the medial and lateral heads of gastroc and tibialis anterior muscles is a unique approach, taking advantage of the biological effects of this versatile molecule (systemic and local) in the appropriate sites to induce the most meaningful functional changes.

Future studies may focus on enhancing nerve regeneration by improving bioenergetics through combinatorial approaches to increase readily available ATP pool or bypass a defect to alleviate cellular stress in both SC and axon to create an additive or potentiating effect on regeneration process. In this respect, it needs to be noted that a simple pyruvate supplementation improved regeneration and myelination of trembler nerves and that there is a potentiating effect of the combinatorial therapy when exogenous pyruvate is combined with NT-3 gene therapy simultaneously (Sahenk et al., 2018). For the same reasons, future studies could explore possibilities of combining SC survival strategies with manipulation of intrinsic axonal factors that will boost nicotinamide adenine dinucleotide (NAD⁺) levels for an additive or potentiating treatment effect (Llobet Rosell and Neukomm, 2019; Meyer zu Horste et al., 2011)

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Abbreviations

SC	Schwann cell
NT-3	neurotrophin 3

Tr^J	trembler-J
NF	neurofilament

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HIGHLIGHTS

- AAV1 with NT-3 transgene shown to be efficient for gene therapy of a CMT mouse model
- Preventing or slowing axonal loss might be possible by enhancement of regeneration
- Therapeutic potential of NT-3 to improve nerve regeneration in CMT subtypes

Figure 1.

Schematic representation of nerve regeneration. Cross sectional schemes represent the NF cytoskeleton. A and B represents regeneration following Wallerian-degeneration in normal conditions. SC proliferation leads to formation of bands of Bungner that directs growing axon to muscle (A). Myelination of the axon and muscle reinnervation are established (B). Note that regeneration associated myelination results in shorter internodes and reduced axon size. Normal NF cytoskeleton is represented in the cross section of axon. (C) Impaired regeneration in longstanding neuropathic conditions including primary SC genetic defects. Atrophied SCs that are also reduced in number fail to form bands of Bungner. Loss of axon elongation further triggers the chronic state of distal axonal neuropathy and increased NF density in the axon. D and E depicts the biological effects of NT-3 on regeneration. NT-3 improves the nerve regeneration by supporting SC survival, proliferation and differentiation, enhancing axonal growth and regeneration associated myelination (D). NT-3 also improved NF phosphorylation state and normalized the NF cytoskeletal organization (E). Independent of its effect on nerve regeneration, NT-3 has direct effect on muscle protein synthesis resulting in increases in muscle fiber size.

Figure 2.

Representative cross sections from plastic embedded left sciatic nerves from PBS- or scAAV1.tMCK.NTF3-injected C57BL6 wild type (A and B) and Tr^J mice (C and D) at 48 weeks post treatment are shown. Age-related changes such as myelin corrugation/infoldings and outfoldings suggesting axonal atrophy (arrows) are seen in the nerves from saline (A) and scAAV1.tMCK.NTF3- injected C57BL6 wild type mice (B). In the saline-injected Tr^J nerves (C) there is a notable dropout in the myelinated fibers and numerous hypomyelinated or nude axons (arrows) while scAAV1.tMCK.NTF3- injected Tr^J nerves (D) show a visible increase in the small myelinated fibers, myelin thickness and a decrease in the nude axons (arrow). Scale bar = 10 μ m.

Figure 3.

Rotarod test. Both PBS and scAAV1.tMCK.NTF3 vector injected mice were tested for motor functions by performing rotarod test. In C57Bl/6 mice, there was no significant difference between PBS and NT-3 groups (A) while in Tr^J mice (B), the NT-3 group showed significant improvement in rotarod performance compared to the PBS group starting at 16 weeks post-treatment which was continuous (data collection was terminated at 38 weeks post-injection). Error bars represent SEM. Statistical significance: *P<0.05; Multiple t test was performed, n=10–23 per group.