Review

CNTF, a pleiotropic cytokine: emphasis on its myotrophic role

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Abstract

Ciliary neurotrophic factor (CNTF) is a cytokine whose neurotrophic and differentiating effects over cells in the central nervous system (CNS) have been clearly demonstrated. This article summarizes the general characteristics of CNTF, its receptor and the signaling pathway that it activates and focuses on its effects over skeletal muscle, one of its major target tissues outside the central nervous system. The evidence for the existence of other molecules that signal through the same complex as CNTF is also reviewed.

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As it would be very difficult to cover the whole literature about ciliary neurotrophic factor (CNTF), a member of the interleukin-6 (IL-6) family, in this review, we will summarize some general aspects, and will refer to some recently identified new members of the group. We will also refer to its effects on skeletal muscle, a topic that has not been reviewed; its clinical use and its role as a modulator of body weight. Excellent reviews about the neurotrophic role of CNTF are available [29,34,46,101].

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1. CNTF, general aspects

CNTF is a member of a group of signaling molecules, the cytokines, that act as chemical communicators between cells by binding to a complex of proteins in the target tissues. This complex is formed by a specific alpha-receptor subunit and one or more beta subunits that are coupled to signal transduction pathways whose activation affect survival, proliferation, differentiation, activation or cell death in different cell types that include neurons and glia. When referring to neurons, factors that affect neuronal survival are considered as trophic while molecules that stimulate neurite growth, control expression of neurotransmitters and affect regeneration are considered as differentiating factors. Signaling mediated by cytokines is a complex process: a single cytokine can elicit different responses in different tissues, a property known as pleiotropy and also, different cytokines can trigger similar responses in a given tissue, a property described as redundancy. Redundancy is explained in part because beta subunits are shared between several cytokines. Important cellular functions are backed up in such a way that a given cellular response can be achieved by several different cytokines; this fact highlights the importance of cytokine-mediated signaling. Few individual cytokines are absolutely essential for life because one cytokine can compensate for the loss of another [26].

An example of synergic interactions between CNTF and leukemia inhibitory factor (LIF) over the trophic support of motoneurons has been studied by Sendtner et al. They found that knockout mice for either LIF or CNTF, sharing the same genetic background, showed no abnormalities of motor neuron functions. Nevertheless, significant functional motor deficits were found in the double knockouts (CNTF\(^{-/-}\), LIF\(^{-/-}\)) [97].

CNTF was originally described as a factor that supported in vitro survival of parasympathetic neurons from the chick ciliary ganglia [1] and it was later shown that it also has trophic and differentiating effects on different types of peripheral and central neurons, glia and cells outside the nervous system. CNTF is also an endogenous pyrogen, induces acute-phase protein expression in hepatocytes and has cachectic or anorectic effects (reviewed in Refs. [26,101]).

CNTF is included in the structurally related family of the interleukin-6 cytokines together with leukemia inhibitory factor (LIF), interleukins 6 and 11 (IL-6, IL-11), oncostatin M (OSM), cardiotrophin 1 (CT-1) [26] and the recently identified new members: cardiotrophin-like cytokine (CLC; also referred to as novel neurotrophin-1 (NNT-1) or B-cell stimulating factor 3 (SF-3)) [94,99] and neuropoietin (NP) [24].

CNTF was initially purified from rabbit sciatic nerve [60] and the rat [103] and human forms were soon cloned [64]. It is a 200 amino-acid peptide of around 23 kD with no glycosylation sites and only one cysteine residue in the whole sequence. The gene for CNTF is localized to chromosome 11q12 in humans. There is an 86% sequence identity between the rat and human proteins and it has no signal peptide sequence. The lack of a secretory signal and the cytosolic localization of the protein are considered as evidences that CNTF is not a secreted cytokine [86,96]. Nevertheless, there are reports of detectable circulating levels of CNTF in apparently healthy individuals [88,116] and also in patients with septic shock [38], systemic lupus erythematosus, [87] rheumatoid arthritis [88], renal failure and malaria [115], multiple myeloma [116] and ALS [45]. The range of concentrations reported for humans, assuming the extracellular water is 40% of total body weight [93] goes from around 6 pg/ml to 1 ng/ml [38,115]. This leaves open the question of what are the conditions that trigger CNTF release and how this comes about. One possibility is that CNTF would be released by a mechanism like the one proposed for interleukin-1\(\beta\) that involves regulated exocytosis of endocytic vesicles [8,91] or via a nonclassical pathway as described for chick CNTF [85].

After cloning of cytokine receptors, several patterns of primary sequence homology among them became evident and these patterns have been used to classify the cytokines. In this context, the terms superfamilly refers to proteins with sequence homology of 50% or less and family to the proteins with higher homologies [21]. CNTF belongs to the largest group called cytokine receptor superfamilly type I or hematopoietin superfamilly [26]. The extracellular region of this superfamilly contains combinations of cytokine domains, fibronectin III and in some cases also immunoglobulin (Ig) domains. The cytokine domain is a segment of \(~200\) amino acids where it is possible to define two subdomains of \(~100\) amino acids each. The N-terminal subdomain has four positionally conserved cysteines and the C-terminal subdomain has a Trp–Ser–X–Trp–Ser motif. All these cytokine receptors have a single transmembrane domain composed by 22–28 amino acids and an intracellular signaling domain, except for CNTF receptor (CNTFR\(\alpha\)). This receptor is anchored to the membrane by a glycosyl–phosphatidylinositol (GPI) linkage and lacks the intracellular signaling region. Due to its GPI linkage, it can be cleaved from the membrane generating a soluble and functional form of the receptor (sCNTFR\(\alpha\)) [19].

The receptor for CNTF is composed of an \(~70\) kD protein, the CNTFR\(\alpha\) subunit, and two “beta” components, gp130 and LIFR, also named gp190 [26]. In the absence of CNTF, these three proteins are not associated in the cell surface. The first step in signaling is the binding of CNTF to CNTFR\(\alpha\) that triggers the association of gp130 and finally the recruiting of LIFR. Heterodimerization of the beta components initiates signaling by activation of the cytoplasmic JAK/TYK tyrosine kinases. These kinases are constitutively associated with the cytoplasmic domains of gp130 and LIFR; they phosphorylate each other as well as
the beta components, generating a docking site for the transcription factor STAT3 (signal transduction activator of transcription). STAT3 is also phosphorylated by JAK kinases, and in this condition, it forms a dimer that translocates to the nucleus where it activates transcription of target genes [20,49,102,105]. The activation of this signaling pathway is negatively regulated by protein tyrosine phosphatases, like SHP-2, and members of the suppressor of cytokine signaling (SOCS) family of proteins [14,54,57,104]. Depending on the cell type, the regulation of the JAK-STAT-SOC signaling pathways can be quite complex [54]. It is important to note that all members of this family share the use of gp130 as a transducing subunit and most of them also use LIFR.

Recently, an atypical signaling mode of CNTF was revealed by the work of Schuster et al. [93]. They showed that human CNTF, at difference from rat CNTF, can bind and signal through the receptor for interleukin-6 (IL-6R) in its soluble or membrane bound forms. This means that the number of potential target cells for CNTF is much wider than initially estimated and it provides a frame to reevaluate the side effects that have been reported after CNTF’s use in clinical trials. This observation may explain, for instance, the acute-phase response triggered by CNTF in liver cells [13] or the protection of striatal neurons [56] neither of which express the CNTFRα.

The crystal structure of CNTF shows four helices, named A to D, that are arranged in a left-handed antiparallel manner with two long loops connecting segments AB and CD and one short loop connecting segment BC. There are three different binding sites located in the surface of the molecule for binding CNTFRα, gp130 and LIFR. Sequence alignment of CNTF and CLC shows 23–27% identity among them but importantly, the residues that have been identified as implicated in binding to CNTFRα are well conserved, an indication of a possible common mechanism of binding to their specific receptor subunit [16,66,78]. Primary sequence alignment between most members of the IL-6 family shows low levels of homology, but the “four-helix bundle structure” is shared among them. Moreover, the alpha- and beta-receptor recognition sites are organized as modules that can be experimentally interchanged generating chimeric cytokines [16,52]. Other molecules sharing the general four-helix bundle structure are granulocyte colony-stimulating factor (G-CSF), erythropoietin, interleukin 12 (IL-12), growth hormone (GH), prolactin and leptin. Understanding the interactions between CNTF, CNTFRα and beta members of the receptor complex is important in order to introduce changes in the CNTF sequence that could optimize its therapeutical use [82]. A recent in vitro study that examined the association between the different components of the complex, considering wild type and mutated sequences, favors the view of an hexameric asymmetric complex with two CNTF molecules, two CNTFRα, one LIFR and one gp130 molecule [61].

2. New heteromeric ligands for the CNTF receptor complex

Despite the clear role of exogenous CNTF as an ontogenic rescue factor or as a protective factor after axotomy on embryonic and adult motor neurons (reviewed in Ref. [101]), it is not clear if it has a role during normal development. On the one hand, at the same developmental stage where the expression levels of CNTFRα are easily detectable by in situ hybridization (rat embryonic day 11, E-11), CNTF expression levels are barely detectable [47]. On the other hand, knockout mice for CNTF are viable and only show some motor problems and muscle weakness in later adulthood [65]. Moreover, a genetic study in the Japanese population showed that ~2.5% of the individuals are homozygous for a null mutation A/A in the CNTF gene and they do not express the cytokine. Nevertheless, this study did not reveal any association of the heterozygous (G/A) or homozygous (A/A) mutated genotype to neurologic abnormalities [106]. Although individuals lacking CNTF are viable, mice lacking the CNTFRα die during the first 24 h after birth, are unable to suckle and have severe losses in the number of motor neurons in the brainstem and spinal cord motor nuclei. Besides, the cross-sectional area of the surviving spinal cord motor neurons is also decreased [22]. All these data indicate that CNTF is not essential during development but acts later in life and that there must be one or more ligands that binds CNTFRα whose role are critical early in embryonic life [22,24]. The first step towards the description of other ligands for CNTFRα came after the identification and cloning of the human and murine orphan cytokine-like factor receptor (CLF-1) by two independent groups [4,27]. This is a secreted soluble protein that was identified by expressed sequence tags using amino-acid sequences from conserved regions of the cytokine receptor family type I. The amino-acid identity between the human and murine proteins is 96% and they both have the four conserved cysteines and the Trp–Ser–X–Trp–Ser motif typical of the type I cytokine receptor family [27].

Because the knockout mice for CLF-1 or CNTFRα had a similar phenotype, i.e., they died within the first 24 h due to a suckling defect, it was hypothesized by Elson et al. [28] that CLF could participate in the formation of a second ligand for CNTFRα and therefore looked for proteins that would interact with CLF. One of the proteins tested for interactions with CLF-1 was the newly recognized member of the IL-6 family, CLC. This cytokine has a putative signal sequence but when its DNA was transfected into COS cells, CLC was synthesized but not released to the culture media. Nevertheless, when CLC and CLF-1 were cotransfected, a stable heterocomplex of these two molecules could be detected in the cell media, an indication that CLC secretion is controlled by CLF-1. To test which cells would be sensitive to this complex, they used a cell line that can be made responsive to cytokines of the IL-6 family by transfection with the appropriate receptors. They found that
the CLF/CLC complex induces proliferation only on cells that express the complete CNTF receptor complex, i.e., CNTFRα, gp130 and LIFR. In addition, the activation of the signaling pathway, i.e., tyrosine phosphorylation of gp130, LIFR and STAT3 was tested. Phosphorylation of these proteins was only detected in cell lines that express the complete CNTF receptor complex. This work shows that the interaction of the nonsecreted CLC cytokine with the soluble receptor CLF-1 leads to the secretion of an heterodimer that binds to CNTFRα and triggers the same signaling pathway as CNTF does. These ideas were further reinforced in the work of Lelièvre et al. [59] that characterized the signaling pathways triggered by CLC/CLF in human cell lines of neural origin. They showed that neither LIFR nor gp130 could bind directly CLC/CLF that was only bound by CNTFRα. However, they do contribute to CNTF binding apparently by increasing the whole complex affinity for the cytokine. The exposure of neuroblastoma cells to CLC/CLF triggered activation of the kinases JAK1, JAK2 and to a lesser extent of TYK2 and the downstream activation of STAT3 and STAT1. A major difference between CLC/CLF and CNTF is that the heterodimer has an absolute requirement for the membrane bound form of CNTFRα, whereas CNTF can signal through both the soluble and the membrane bound forms [19]. In addition, CLC/CLF is secreted and CNTF stays mainly in the cytoplasm until cell damage occurs. An interesting fact is that STAT3 is essential for development of mouse embryos [108]. Because CLC and CLF are apparently expressed early during embryonic life, it is likely that the complex CLC/CLF (or CNTF-2) is one of the developmentally important ligands for CNTFRα [59]. Accordingly, in an analysis of spinal cord and brainstem neurons, Forger et al. found region specific decreases in motoneurons in clf\(^{-/-}\) knockouts newborn mice. The mRNA for clc and clf was detected in normal mice on day E16.5 in skeletal muscle, indicating that the complex CLC/CLF most probably behaves as a target-derived factor for motoneuron survival that signals through the CNTF receptor [32].

Recently, Derouet et al. identified in the mouse genome neuropoietin (NP) a new member of the family. The identity of NP to CNTF is 16% and 11–27% with the other members of the family. They used a computational screening that considered structural similarities among the IL-6 family members. NP signaling was studied in the murine cell line Ba/F3 transfected with different combinations of the receptors for this family; proliferation occurred only on cells that expressed the complete functional receptor for CNTF (i.e., gp130, LIFR and CNTFRα). Orthologs of this new cytokine were identified in rat, chimpanzee and human genome. In humans, a deletion in one of the putative exons of the gene results in loss of the reading frame and, consistently with this, no transcripts for NP were detected. The mRNA for mouse NP is apparently expressed in the central nervous system (CNS) and some peripheral tissues only during the embryonic period with a time course that closely matches the expression of mRNA for CNTFRα [24]. During development, signaling through the CNTF complex is important, but the signaling molecule in this period does not seem to be CNTF but the newly described members of the family.

3. CNTF’s effects on skeletal muscle: in vivo studies

3.1. Protective effects of CNTF upon denervation induced changes?

The first observation that CNTF could have a myotrophic role was that of Helgren et al. [42], and this report led other researchers to investigate the effects of this cytokine on muscle fibers. They reasoned that if skeletal muscle expresses CNTFRα and the receptor expression level increased after muscle denervation, it was possible that CNTF would behave as a nerve-derived myotrophic factor after its release upon nerve injury. To assess whether this was the case, they performed a unilateral 5-mm resection of the sciatic nerve to a group of rats that afterwards received daily subcutaneous injections of CNTF (0.1–3 mg/kg). It is important to mention that later studies have established that doses higher than 0.3 mg/kg can induce cachexia in mouse and rats [43,63,72,73]. The contralateral leg was sham operated and used as control. After 4, 7, 14 or 42 days, the animals were sacrificed and wet weight of the soleus, a slow muscle, was determined. They also measured the soleus cross-sectional area after 4, 7 or 14 days of CNTF treatment and the contractile parameters after 14 days. They expressed their results as the ratio of the values obtained for the denervated over the innervated (control) muscle and found that CNTF increased the ratio as compared to rats that did not receive the cytokine for up to 14 days of treatment. Therefore, they concluded that CNTF partially protected the soleus from the denervation-induced changes in all the parameters evaluated. The protective effect on muscle weight was not seen after 42 days of exposure to the cytokine.

Using similar doses and experimental design, but quantifying the effects of CNTF mainly over the gastrocnemius weight, a fast muscle, Martin et al. [63] found that the increased ratio in muscle weight in CNTF-treated animals was not due to CNTF-induced sparing of muscle mass in denervated muscles but to a muscle-wasting effect of CNTF on the control-innervated muscles. They did not measure fiber area or twitch parameters. As a first approximation, therefore, we could interpret the discrepancy about CNTF’s effect on muscle weight as reflecting possible differential effects of this cytokine over different muscle fiber types or to a misinterpretation of the experimental results. Pointing to differential effects of CNTF not only over different muscle fiber types but also over their respective motoneurons is the work of Mousavi et al. [72]. They performed a unilateral sciatic nerve
anatomy to newborns (P5) and measured the extent of protection offered by the injection of CNTF, combined or not with neurotrophins 3 and 4 (NT-3, NT-4) on muscle mass, muscle fiber type, average fiber cross-section and tetanic tension. In addition, the number of motor units innervating the soleus and EDL in the control group and the neurotrophin-exposed animals was determined. Axotomy caused loss of muscle fibers in both muscles and a selective loss of motoneurons innervating the EDL, despite the fact that soleus motoneuron numbers were not altered. Tested after 3 months, neither CNTF alone nor the NT by themselves reduced the extent of loss of muscle mass after denervation relative to that occurring in the unoperated contralateral muscles. However, CNTF combined with either NT prevented the loss of mass for the EDL and soleus, and enhanced the survival of muscle fibers, but only in EDL and not in soleus. CNTF combined with the NTs partially prevented the loss of EDL motoneurons. The normal distribution of fiber types in both muscles was altered after the combined treatment; that is, specific subpopulations of developing muscle fibers were affected by these drugs. From this work, it is clear that the protective effect on muscle of CNTF combined with the NT is mediated in part by the motoneurons. Therefore, another point to consider besides the possible differential effects on different muscle fiber types is that in some experimental arrangements, there is also a nerve-mediated effect over the whole system: motoneuron, including the Schwann cell and muscle. For experiments with unilateral denervation, the effects of CNTF on denervated muscle are direct, but the effects on the contralateral control muscle could also be mediated by the motoneuron. It should also be considered that the motoneuron–muscle cell interaction changes along aging ([23]; see discussion in Ref. [73]).

The CNTF dose and application method are comparable in the works mentioned above. Helgren et al. [42] and Martin et al. [63] used human recombinant CNTF (hrCNTF) and Mousavi et al. [72] used a modified version of CNTF (axokine-1; see discussion in Ref. [93]).

Because most of the experiments of Helgren et al. and Martin et al. used 1 mg CNTF/kg, some muscle-wasting effect should have been present in both sets of data.

In addition, effects over the innervated soleus with respect to twitch kinetics went unnoticed by Helgren’s group. We used a low dose of CNTF (0.5 μg/ml, rrCNTF released systemically at 0.5 μl/h by an osmotic pump) that did not affect the weight of either innervated nor denervated fast or slow rat leg muscles: tibialis anterior, EDL, gastrocnemius or soleus, but that affected the muscle twitch kinetics [83]. Even with the dose we used, the slowing of the contraction time triggered by CNTF on the innervated soleus was evident (see Fig. 6c in Ref. [42] and Fig. 5 in Ref. [83]). Expressed as a ratio, both sets of data are identical. Therefore, the use of ratios to express results should be avoided, because ratios (treated/control) do not allow disclosing whether the treatment affects the measured parameter from the innervated (control), the denervated or both preparations.

3.2. CNTF and skeletal muscles fiber area

Guillet et al. [39] found that the average cross-sectional area of innervated soleus muscle fibers of aged rats treated with rCNTF was significantly higher than for aged matched saline-treated control muscles. The cytokine was delivered locally with an osmotic pump at a rate of 16 μg/kg/h. With a systemic, but otherwise identical delivery system, we applied a much lower dose of CNTF (1.3 ng/kg/h) to 40-day-old rats. We also observed an increase in soleus fibers area for both innervated and denervated muscles, but only in some animals. In contrast, in the same animals, CNTF did not affect the fast EDL muscle fiber area for either innervated nor denervated muscles (Vergara and Ramirez, unpublished data). These results reinforce the idea that CNTF effects can be different on different types of muscles.

In addition, Mitsumoto et al. [70] described the effects of CNTF alone or combined with BDNF on wobbler mice. These animals, which show a progressive and fast decrease in muscle fiber area as they age, are a model for human motoneuron diseases. They found that CNTF or CNTF+BDNF protected the biceps muscles from the progressive decrease in fiber area. They attributed most of the protection to a neurotrophic effect of the cytokine on the spinal motoneurons, although they did not discard a direct action on the muscles.

3.3. CNTF effect on muscle proteins

The effect of CNTF on muscle proteins that are regulated by both nerve activity and neurotrophic molecular factors has also been studied. The rationale for these studies was based on the report of CNTF’s trophic effect on muscle [42] that hinted that muscle enzymes or synaptic proteins that depend, at least in part, on neural factors, could be affected by CNTF. The effect of CNTF on the activity and the accumulation site of SDH, an enzyme related to muscle energetic metabolism [67], and the expression level of acetylcholinesterase (AChE) and the acetylcholine receptor, that are synaptic proteins [15], was studied. It was found that, in soleus rat muscles, CNTF prevents the denervation-associated decrease of SDH activity within the myofibrillar compartment but not at the endplate. In contrast, the cytokine further reduced the mRNA and enzyme level for AChE in denervated muscles and did not affect the transcription level of the ε subunit of the acetylcholine receptor either in innervated or in denervated soleus muscles [15]. Therefore, as the authors claimed, CNTF does indeed affect several but not all muscle proteins in the soleus, and in different ways. It is interesting to note that these authors [15,67] applied high doses (0.3 mg/kg) of both human and rat recombinant CNTF to treat rats, and reported that the effects were similar. This point is
interesting, because it has been recently described that rat CNTF differs from human CNTF in its receptor specificity [93]. Rat CNTF is unable to interact with human interleukin-6 receptor (IL-6R) but at high concentration can directly induce a signaling heterodimer of gp130 and human LIFR, in the absence of the specific CNTFRα. Human recombinant CNTF, in contrast, can use both the membrane bound and the soluble form of IL-6 receptor, besides its cognate CNTFRα, but cannot induce a heterodimer of human gp130 and LIFR. It remains to be established if there are also differences in the rrCNTF and rhCNTF interaction with different rat receptors.

3.4. Nerve-mediated effects of CNTF on muscle

The levator ani muscle (LA) in rats is a sexually dimorphic muscle that attaches to the base of the penis in adult male rodents but is vestigial in adult females. The motor neurons that innervate this muscle are located in the spinal nucleus of the bulbocavernousus (SNB). This sex difference is dependent on androgens and it appears perinatally. If female rats are treated with testosterone or CNTF early after birth, the muscle volume is equivalent to that in males. Forger et al. [31] reported that exogenous rrCNTF maintains the SNB neurons and their target muscles when given to female rats from embryonic day 22. They propose that CNTF acts directly on the motoneurons (that express CNTFRα) and indirectly on the muscles. In addition, Peroulakis and Forger [80] evaluated the number of muscle fibers and total LA muscle area after daily injections of rrCNTF (in the vicinity of the LA muscle) to newborn female rats. They found that total muscle area was more than twice in CNTF-treated animals than in control (vehicle injected) rats, despite no difference was found in the mean cross-sectional area of individual fibers. The difference in total area was due to an increase in the number of fibers per muscle. An increase in the number of fibers could result from a decreased degeneration of muscle fibers or from increased myogenesis mediated by CNTF. To our knowledge, there is no evidence that CNTF induces replication of muscle fibers; thus, probably, this is an indirect effect mediated by the preservation of the corresponding motor neurons. In this connection, Varela et al. [114] found that hrCNTF also provoked an increase in the number of surviving motor neurons in the SNB in female rats; nevertheless, the effects observed with hrCNTF in this article are less intense than when rrCNTF was used [80].

A possible role for CNTF in the signaling between the motor neuron, the Schwann cell and the muscle fiber at the time of synapse formation comes from the work of Barlett et al. [12] in rapsyn-deficient mice. Rapsyn is a protein that participates in the clustering of AchR and the rapsyn null mice shows an increased axonal branching at the diaphragm’s neuromuscular junction. Apparently this leads to an increased access to trophic factors because the number of motoneurons in the brachial lateral columns is higher than in wild type mice (at embryonic day 18). RT-PCR from samples of diaphragm (embryonic days 16–18) showed a decrease of 50% in the mRNA for CNTF in the rapsyn-deficient mice as compared to wild types; nevertheless, the mRNA levels for CNTFRα did not differ. On the other hand, the JAK2 kinase protein levels were not altered but the negative regulator of CNTF signaling, SOC3 was also decreased in rapsyn-deficient mice. The decreased levels of SOC3 could maintain active for a longer time the CNTF signaling pathway. This in turn could explain the increase in nerve muscle branching and survival of motoneurons. The mRNA for other trophic factors (NGF, BDNF, NT3, TGF-β2 and NT4 and their receptors (trkA, trkB, trkC and p75) did not differ between wild type and the rapsyn null mice [12].

In case of partial denervation, some muscle fibers become devoid of neural input. The neighboring motoneurons grow new processes that can reinnervate some of the denervated fibers, a process known as sprouting. This also occurs after paralysis caused by blockade of the neuromuscular junction by toxins, and until now, the cellular and molecular events controlling this response are not completely understood [29]. The original description for a role of exogenously added CNTF in inducing sprouting in adult animals [40] was confirmed by Siegel et al. [100] for endogenous CNTF. They quantified the sprouting response to partial denervation or to the injection of botulinum toxin in the gastrocnemius of normal or mutant mice lacking the CNTF gene. Despite both procedures generated sprouting in the normal mice, in animals lacking the CNTF gene, sprouting was undetectable, but in mutant mice that received exogenous CNTF, sprouting was induced by partial denervation. Therefore, CNTF appears to be necessary for sprouting to occur as it was not replaced by another molecule in this role. English postulates that CNTF has an indirect role in the process: after binding to CNTFR in muscle cells, it could trigger the release of a muscle-derived sprouting factor that would be the signal triggering the motoneuron response. This is proposed given that CNTF-induced sprouting can be blocked if muscle cells are damaged before being exposed to CNTF [29]. Despite this effect on the promotion of sprouting in adult animals, when CNTF was exogenously applied to newly born rats early during the period of synapse elimination (P7–P13), the number of multiple innervated EDL muscle fibers was significantly higher for CNTF treated than for control muscles; that is, at this developmental stage, CNTF most probably decreases synapse elimination but does not induce sprouting [51].

We have recently described that exogenous CNTF can induce spontaneous electrical activity in innervated muscles which in turn can trigger small spontaneous contractions. We think this effect is most probably mediated by the motoneurons and it could explain some of the side effects related to skeletal muscle, like cramps or cough, reported by patients after clinical trials [83].

Exogenous CNTF also participates in myotube differentiation induced by lesion in adult muscles. Marques and
Neto [62] tested its effect on myotube differentiation in the EDL of adult mice. They induced a denervating–devascularizing lesion and administered rCNTF (0.5 μg/ml) by an osmotic pump delivering the drug to the damaged muscle. From days 4 to 8 after the lesion, they counted the number of myotubes and the number of surviving and regenerating myofibers. They found that, compared to controls, CNTF significantly increased the number of regenerating myofibers without affecting the number of surviving ones up to day 6. After that, control and CNTF-treated muscles had similar numbers of myotubes. They concluded that CNTF accelerates myotube differentiation, a fact that could be of potential help in cases of myoblast transplantation for pathologies, like Duchenne muscular dystrophy. In a later study, Kami et al. evaluated the spatiotemporal expression pattern of the mRNA for gp130, LIFR, IL-6R and CNTFRα in regenerating gastrocnemius of adult rats. Regeneration was induced by mechanical contusion. For CNTFRα, they could only detect signals above background levels at day 7 in some of the newly formed myotubes. They propose therefore that myotubes are not direct targets for CNTF. They think that part of the CNTFRα expressed by the myotubes is released in its soluble form and complexes with the CNTF liberated by the damaged Schwann cells. The retrogradely transported sCNTFRα/CNTF complex could finally enhance myofiber reinnervation after inducing motoneuron sprouting [53].

3.5. CNTF and muscle strength

In an attempt to identify possible causal factors associated to the muscle atrophy, weakness and fatigue that are associated to old age, Guillet et al. [39] studied, in rats, the correlation between CNTF expression levels in the sciatic nerve, the mRNA expression levels of its specific receptor in several lower leg muscles, and mechanical performance during aging. They also tested the effect of exogenously provided rCNTF on the mechanical performance of the soleus from aged rats. They found a decrease in the CNTF content of the sciatic nerve in aged as compared to young–adult rats at the protein and mRNA level. This suggests a transcriptional regulation of CNTF during aging. They also quantified mRNA for CNTFRα in the soleus, EDL, gastrocnemius and tibialis posterior of rats from 3 to 24 months of age. They found that CNTFRα mRNA increases with age for the four muscles studied. However, the protein level of the other two components of the receptor, gp130 and LIFR, determined in the same muscles, did not change with age. When evaluating the changes in expression of CNTFRα at the mRNA level, one has to consider that the changes at the protein levels would not necessarily follow the same kinetics as the mRNA as was described by DiStefano et al. [25].

The swimming speed was faster in younger animals and a positive correlation was found between swimming speed and CNTF content of the corresponding sciatic nerves (r=0.8; p<0.0003). This suggested to them that muscular performance is controlled by the interaction of CNTF and its receptor complex in skeletal muscles. They delivered rCNTF locally to the soleus in one leg from old rats and found that the treatment not only increased the average cross-sectional area of the muscle fibers as described above, but also improved the twitch and tetanic tension of the soleus for the treated leg. This is a somewhat intriguing but potentially important observation to consider for the clinical use of this cytokine. They detected circulating CNTF (1370±1082 pg/ml) during the pump implantation period but the physiological effect was local. From these results, Guillets’ group proposes an association between sciatic nerve CNTF content and muscle strength that would be particularly important at older ages. They propose that endogenous as well as exogenous CNTF can modify skeletal muscle performance in rats.

The paper just described motivated the investigation of the possible relationship between CNTF null genotype and muscle strength in humans [89]. Roth et al. studied a total of 494 adult volunteers (age range: 20–90 years) among which the distribution of their genotype with respect to CNTF’s null mutation was: 389 individuals homozygous for the wild type allele (G/G), 95 heterozygous (G/A) and 10 homozygous for the null mutation (A/A). When tested for high-speed movements, the heterozygous individuals for CNTF’s null mutation (G/A) developed higher force than normal (G/G) or the null homozygous (A/A) individuals. Considering previous Guillet’s results, they expected that the individuals with higher force development would be the wild type (G/G), and the ones with lower force would be the homozygous null mutants (A/A). The greater muscle strength associated to the G/A genotype was unexpected and no possible mechanisms to explain it were suggested. Thus, the hypothesis that CNTF genotype is associated to muscular performance and that endogenous CNTF is somehow related to the loss of muscle power observed during aging has some experimental evidences but clearly needs to be tested under more stringent conditions. For experimental animals, the possible mechanism should be searched, and for humans, studies considering larger populations of G/A and A/A older individuals are needed. Different studies have reported frequencies of heterozygous individuals that vary from ~20% to 30%; for homozygous individuals, values are more homogeneous around 2%. In addition, polymorphic variations in the CNTFRα could contribute to the different muscle strength phenotypes [90].

4. Exogenous CNTF and CNTF null genotype: clinical trials

The base for the design of clinical trials with around 1000 ALS patients was its success in studies showing its
trophic efficacy over motor neurons in in vitro and in vivo models of motoneuron degeneration [10,69,95]. In addition, a correlation with lower CNTF levels in the spinal cord of ALS patients was considered [76] (see also Ref. [107]). CNTF was delivered systemically but toxic side effects that included severe body weight loss, fatigue, muscle ache and lack of efficacy led to suspension of these trials [6,18,68]. Tests designed to minimize these systemic side effects by intrathecal delivery of the cytokine have been attempted in a small number of patients. In these trials, nanogram levels of CNTF were found in the cerebrospinal fluid and only minor side effects, such as cramps, were reported [2,79]. A factor that may limit CNTF’s efficacy for chronic treatments is that most patients that received 15 or 30 μg/kg three times a week for 9 months developed anti-CNTF antibodies [5]. Positive and negative correlations of CNTF genotype as a modifier in ALS have been described [3,35]. This is not surprising because several mutations have been related to the origin of this pathology; therefore, the susceptibility to CNTF may be variable among ALS patients.

What is not clear now is if CNTF will continue to be used for these patients because there have been no reports of improvement of motor function [6,9].

Actually, CNTF is being considered for the treatment of Huntington’s disease (HD), given that in a primate model of this pathology, this cytokine has been shown to restore not only motor but also cognitive functions [71]. In the quinolinic acid rat model for HD, a neuroprotective effect of CNTF has been described [56]. Regulier et al. used the tetracycline-regulated, lentiviral-mediated CNTF production system to quantify the levels of CNTF that protect neurons from quinolinic acid effects. They found neuroprotection with 15.5±4.7 ng CNTF/mg prot. In the off state of this inducible system, the residual production of CNTF (0.54±0.02 ng/mg prot) was not enough to protect against quinolinic acid toxicity [84].

With respect to a possible role of CNTF on the age of onset of multiple sclerosis (MS), there are contradictory results that probably reflect the low number of cases studied so far. Giess et al. [36] and Hoffmann et al. [44] identified the carriers of the CNTF null mutation within groups of ~300 MS patients. The homozygous mutation was found in ~2.5% of the persons, either MS or healthy controls as in the original study for the Japanese population [106]. When studying the correlation between age of onset or severity of the disease and CNTF null genotype, Giess’ group found a significantly earlier age of disease onset in the carriers of the null mutation (17 vs. 27 years) whereas Hoffmann’s group found no correlation of these parameters. Both groups found that CNTF null mutation is not a risk factor for development of MS.

A role for CNTF in the recovery from spinal cord contusive injury has been reported for rats. Exogenous CNTF was delivered intrathecally during 10 days after an injury at the T10 segment of the spinal cord. Six weeks after the lesion, CNTF-treated animals showed enhanced functional recovery, higher amounts of tissue spared and less damaged neurons in the rubrospinal descending tract than control animals. Nevertheless, CNTF increased gliosis at the injury site. Because the effects of increased glial reactivity for the recovery from spinal cord injury are controversial, this CNTF-induced gliosis should be taken into account if CNTF would be used clinically for spinal cord injuries [118].

The possible relationship between CNTF’s null genotype and schizophrenia has been addressed in several reports [92,109–113]. It has been suggested that the CNTF’s null mutation may be relevant to the aetiopathogenesis of schizophrenia but only in some patients [110]. Because schizophrenic disorders are heterogeneous and multifactorial, it is not surprising that a correlation for the pathology and CNTF’s genotype was found only in a subpopulation of patients.

It is possible that endogenous CNTF participates alone or in conjunction with other cytokines in processes of nerve regeneration and repair [48]. In sural nerves from 22 patients with chronic inflammatory demyelinating polyneuropathy, the mRNA for CNTF was downregulated but mRNA for other cytokines was upregulated [117]. This suggests that coordination between different cytokines is necessary for the normal function of the nervous system.

The expression of the CNTFRα gene has been found elevated in muscle biopsies from a subgroup of myasthenia gravis patients; therefore, signaling through this receptor could contribute to the severity of this disease [81]. A possible involvement of CNTFR genotype in other neuropathies should be studied.

An important aspect to consider for interpreting results or when designing clinical trials is that CNTF’s efficacy depends on the route of delivery. Haase et al. [41] designed an adenoviral vector coding for a secretable form of CNTF to treat newly born mouse mutants for progressive motor neuronopathy (pnn), a model of motoneuronal degeneration. A single dose of the vector was given by either intravenous, intramuscular or intraventricular injection, and the animals’ survival, weight gain and degeneration of motor axons was evaluated with respect to control animals. The intramuscular or intravenously treated animals showed a 25% increase of their life span and a reduced degeneration of myelinated nerve fibers. Animals receiving CNTF intraventricularly showed no benefits from the treatment [41]. Similar results have been described for CNTF’s participation in models of Huntington’s disease [56].

For most of the experiments described so far, CNTF has been administered either by local or systemic injections or by constant delivery with osmotic pumps. It is probable that the signaling cascades triggered by CNTF could be affected in different ways by chronic versus transient application as suggested by the report of DiStefano et al. [25]. They found that exogenous CNTF (daily subcutaneous injections of hrCNTF from 0.1 to 1 mg/kg) caused downregulation of its receptor in normal or denervated muscles from adult rats.
When the immediate-early response to CNTF was evaluated by giving a predose of CNTF followed by test doses a few hours later, they found desensitization of this process. On the other hand, treating previously denervated rats with four doses of CNTF per day resulted in better protection from soleus muscle weight loss than treating animals once a day. Therefore, although exposure to CNTF causes desensitization of the signaling cascade, the system keeps responding to frequent injections of the cytokine. The half-life of injected CNTF is of a few hours [25]; nevertheless, with osmotic pumps, steady state circulating levels have been reported [39]. It is probable then that signaling triggered by the cytokine and its regulatory mechanisms, like the activation of the SOCS proteins, will not be identical with the different application modes.

5. CNTF’s role in weight control

In the initial clinical trials designed to test the efficacy of CNTF to prevent motoneuron degeneration, some patients suffered a substantial weight loss [5,6], suggesting that this cytokine may have a cachectic effect. A similar situation had been reported by Martin et al. [63] to occur in rats. They cytokine may have a cachectic effect. A similar situation osmotic pumps, steady state circulating levels have been injected CNTF is of a few hours [25]; nevertheless, with to frequent injections of the cytokine. The half-life of tion of the signaling cascade, the system keeps responding Therefore, although exposure to CNTF causes desensitiza- by giving a predose of CNTF followed by test doses a few the pattern of immediate-early genes affected by intravenous exposure to these agents. They find that both drugs affect different CNS sites. Doses of CNTF that produce weight loss do not induce proinflammatory responses, conditioned taste aversion or corticosterone release as IL-1, whereas the classical cachectic agent does [58]. On the other hand, CNTF mimics the ability of leptin to produce fat loss in mice that are obese because of a genetic deficiency of leptin. [7]. Leptin, an adipocyte-secreted cytokine, interacts with leptin receptors (ObR) located on the arcuate nucleus of the hypothalamus, reducing appetite and selectively reducing body fat. ObR and CNTF receptor complex are closely related; both CNTF and leptin activate the STAT3 tran- scription factor and ObR and CNTFR are located in hypotalamic nuclei involved in hunger control [17,33,37]. However, CNTF, besides its capacity to activate hypothala- mic leptin-like pathways in obese mice deficient in leptin (ob/ob mice), suppresses food intake without triggering hunger signals.

CNTF can also activate the hypothalamic pathways that are unresponsive to leptin in the diet-induced obesity model [37,58]. This model is more representative of human obesity, which is resistant to leptin. Thus, CNTF acting via a leptin-like pathway may cause selective loss of fat and not of lean mass in obese animals, because of its action as an appetite suppressor and not as a typical cachectic agent. In lean animals, in contrast, the fat-depleting action of the cytokine might lead to a nearly total loss of body fat which may cause protein loss as a secondary event [37]. However, because animals lacking CNTF are not obese, it either does not play a physiological role in weight control or the CCL/CLF complex can replace for it in this function. On the other hand, high doses of CNTF can induce other effects, as stress responses [58], and might therefore produce muscle loss.

In an attempt to find a drug to treat obesity, variants of CNTF (axokine) are currently under study in clinical trials [30,82]. Ettinger et al. found that obese patients treated with axokine presented a higher weight loss than placebo-treated ones. Axokine was generally well tolerated, but moderate injection site reactions were reported in 78% to 93% of the patients in a dose-related way [30].

Despite the fact that CNTF can produce weight loss in obese patients, there is no correlation between the early onset of obesity and carriers of the CNTF genetic variants that do not express the functional protein [74]. With respect to body mass index (BMI) or body weight and CNTF’s genotype, there are contradictory reports. O’Dell et al. [75] found a weight and BMI increase in men but not in women but Jacob et al. [50] were unable to detect a correlation between these parameters.

Besides CNTF’s actions on the regulation of energy homeostasis in the central nervous system, this cytokine may also affect this parameter in peripheral tissues. CNTF activates metabolic signaling cascades in cultured brown adipocytes [77] and in mice adipocytes [119].
6. Final comments

In this review, we have mainly referred to the role of CNTF on the neuromuscular system for in vivo experiments. CNTF is a pleiotropic and redundant cytokine and the human peptide can also signal through both CNTFRα and IL-6 receptors; therefore, the interpretation of in vivo experiments in not always straightforward. Some of the CNTF effects on the neuromuscular system can be indirect and multifactorial and can reflect differences in genetic background or the level of other interacting signaling cytokines. Motoneurons and skeletal muscles are closely interdependent cell types, with different trophic requirements that change from the embryonic to the adult state [11,23,98]; hence, results obtained for newborn animals would not necessarily be the same for adults. The specific subunit of the CNTF receptor (CNTFRα) is present in both cell types, an indication that this cytokine affects the motor neurons as well as the muscle cells. It seems clear that early during development, CLC and/or NP, but not CNTF, are the ligands that signal through CNTFRα. In addition, in adults, at least some of the pharmacological effects of CNTF on skeletal muscle seem to be mediated through the motoneurons.

Endogenous peripheral CNTF levels have been described as affecting muscle strength in rats and humans; nevertheless, the molecular mechanisms of this postulated effect have not been established. Exogenously added CNTF will affect some aspects of muscle performance differently if muscles are normal or altered by denervation, unweight or other pathological conditions. In addition, there are clear examples of differential effects of CNTF upon slow and fast muscle fiber types.

From the results obtained after exogenously adding CNTF to either experimental animals or human patients, it is absolutely clear that this cytokine can affect skeletal muscle performance but we think it is not really established if it could be considered as having a long-term effect in reducing the denervation-induced atrophy. The most often cited reference of CNTF’s myotrophic role is that of Helgren et al. although they explicitly mention that this role is just temporal.

With respect to clinical trials of CNTF or CNTF-derived molecules, most efforts are now focused in finding a molecule to treat obesity and related pathologies. No progress has been informed in the treatment of neurological diseases; current studies in this respect consider the use of CNTF combined with other cytokines and trophic factors.

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