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# Gene Therapy Based on Fragment C of Tetanus Toxin in ALS: A Promising Neuroprotective Strategy for the Bench to the Bedside Approach

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Ana C. Calvo, Pilar Zaragoza and Rosario Osta

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/52896>

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## 1. Introduction

Neurodegenerative diseases cover a wide range of neurogenetic disorders including Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease (AD), Huntington's disease (HD), the spinocerebellar ataxias, inherited prion diseases, the inherited neuropathies, and muscular dystrophies among others.

In particular, ALS belongs to the group of motor neuron diseases, involving the loss of cortex, brainstem, and spinal cord motor neurons that result in muscle paralysis [1]. Motor neurons, which are localized in the brain, brainstem and spinal cord, behave as a crucial links between the nervous system and the voluntary muscles of the body, as they let synaptic signals travel from upper motor neurons in the brain to lower motor neurons in the spinal cord and finally to muscles. In accordance with the revised El Escorial criteria [2], both the upper motor neurons and the lower motor neurons degenerate or die in ALS, and as a consequence the communication between neuron and muscle is lost, prompting the progressive muscle weakening and the appearance of fasciculations. In the later stages of the disease, patients become paralyzed although the disease usually does not impair a person's mind or intelligence.

Nowadays, the cause of ALS and its early manifestations still remain to be elucidated. The pathophysiological mechanisms that prompt the neurodegenerative process in both familial (FALS) and sporadic (SALS) ALS are unknown. However, there is growing evidence that the pathogenic process involved in ALS are multifactorial and include oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, axonal transport systems and dysfunction of glial cells, yielding the damage of critical proteins and organelles in the motor neuron triggering the neurodegeneration [3]. Due to the fact that FALS and SALS share clinical and

pathological signs, the understanding of the pathophysiological process in FALS would provide a better understanding of the neurodegenerative mechanisms in SALS.

FALS follows a predominantly autosomal dominant pattern, while in SALS genetic factors that take place sporadically contribute to its pathogenesis. The majority of ALS cases are sporadic and 5-10% of cases correspond to FALS. Although the ages of onset of FALS, which follow a normal Gaussian distribution, correspond to a decade earlier than for SALS cases which have an age dependent incidence, males and females are affected equally in FALS [4].

The most significant candidate genes for SALS include *VEGF* (vascular endothelial growth factor), *angiogenin* (*ALS9*), *paraoxonase*, *neurofilaments*, *peripherin* and *SMN* (spinal muscular atrophy). Although *ALS9*, *paraoxonase*, *neurofilaments*, *peripherin* and *SMN* mutations have been found in ALS patients, except for *VEGF* mutations, these genes may play a small role in the pathogenesis of ALS and previous studies are conflicting [5].

Regarding FALS candidate genes, the mutations in the copper/zinc superoxide-dismutase-1 gene (*SOD1*), Tar DNA-binding protein gene (*TARDBP*) and in the most recent discovered DNA/RNA-binding protein called *FUS* (fused in sarcoma) or *TLS* (translocation in liposarcoma) produce the typical adult onset ALS phenotype. Other candidate genes that have been described in genome association studies of FALS include *dynactin*, *senataxin* (*ALS4*) and *VAPB* (*ALS8*) (VAMP/synaptobrevin-associated membrane protein B gene) [5,6].

The pathophysiology of *SOD1* mutations is probably the most studied one. Many hypotheses have been suggested and reinforced in transgenic mouse models that overexpress the mutated *SOD1* gene and therefore develop an ALS-like syndrome. Among the proposed mechanisms that support these hypotheses are the toxic gain of function of the mutated *SOD1* enzyme, which mainly increases the production of hydroxyl and free radicals, yielding improper binding metal properties, oxidative stress and inflammation induced by upregulation of proinflammatory cytokines [7,8]. Alternative hypothesis also suggested a conformational instability and misfolding of the *SOD1* peptide, forming intracellular aggregates which have been reported in motor neuron and glial cells [9].

Neurotrophic factors have been initially identified as potential therapeutic agents in the treatment of ALS, opening the door to a new tool for the treatment of motor neuron diseases [10]. Based on previous studies ciliary and glial derived neurotrophic factors, insulin-like growth factor (IGF-1) and erythropoietin improved motor behaviour and reduce motor neuron loss, astrocyte and microglia activation in preclinical animal models [11], albeit clinical trials in ALS patients showed lack of therapeutic efficacy [12].

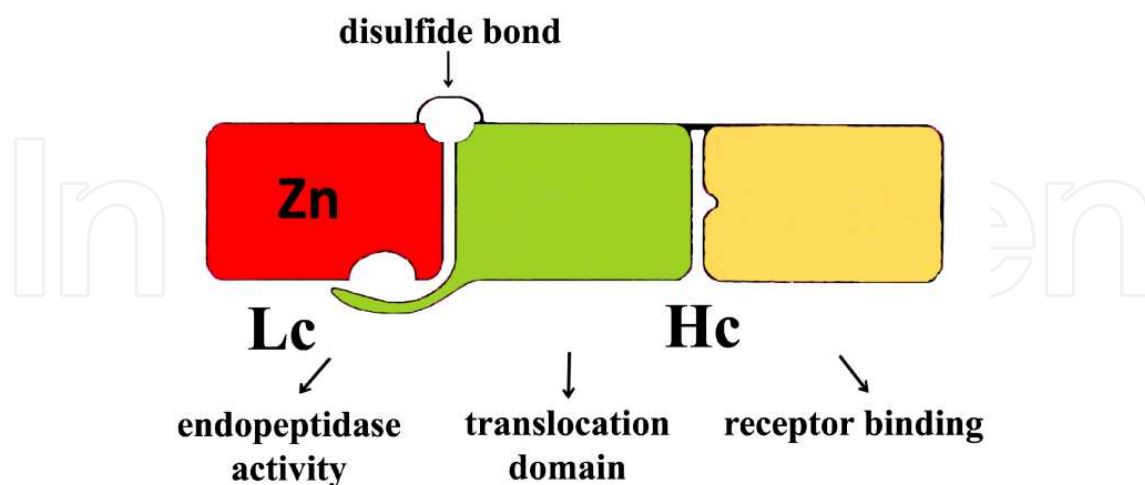
The failure of standard treatments in ALS could rely on the inappropriate route of administration and/or the poor bioavailability of molecules to the target cell [13]. The subcutaneous and intrathecal delivery of neurotrophic factors can cause adverse side effects such as weight loss, fever, cough, fatigue and behavioral changes [14], whereas viral gene therapy based on the use of an adeno-associated virus or lentivirus vectors is more efficient than the neurotrophic factor delivery but can induce several inherent hazards [15].

An alternative strategy that effectively reaches motor neurons, can exert neuroprotective properties and does not show such adverse side effects implies the use of the nontoxic fragment C

(TTC) of tetanus toxin. Tetanus toxin is a neurotoxin produced by *Clostridium tetani*, an anaerobic bacterium whose spores are commonly found in soil and animal waste. This toxin affects the nervous system and causes generalized muscle contractions, called titanic spasms [16, 17].

Tetanus toxin is a single peptide of approximately 150 kDa, which consists of 1315 amino-acid residues. The toxin forms a two-chain activated molecule composed of a heavy chain (HC) and a light chain (LC) linked by a disulfide bond. The catalytic domain of the toxin resides in the LC, while the translocation and receptor-binding domains are present in HC [18–21] (Figure 1). Tetanus and botulinum toxins are zinc metalloproteases that cleave SNARE (soluble NSF attachment receptor) proteins, which interfere with the fusion of synaptic vesicles to the plasma membrane and ultimately blocks neurotransmitter release in nerve cells [22].

The nature of the action of tetanus toxin has been widely described in different animal models [23–28], exploring its effect not only in the spinal cord but also in the cerebral cortex [29]. One of the unique characteristics of tetanus toxin is that it can be transported retrogradely to the central nervous system and shows remarkable affinity and specificity to neuronal terminals. The ganglioside-recognition domain in the C-terminal region of HC allows the toxin to be internalized into the neuron at the neuromuscular junction where it enters the axonal retrograde transport pathway and is subsequently transported to the neuronal soma in the CNS [30,31]. Once the toxin reaches the cytoplasm, it specifically cleaves neuronal proteins integral to vesicular trafficking and neurotransmitter release. In particular, the synaptic vesicle protein synaptobrevin (VAMP) is the target of tetanus toxin. This protein belongs to a family of proteins that facilitate exocytosis in neurons known as SNARE proteins. The other members of this family are syntaxin and SNAP-25, which are the main molecular targets of botulinum toxin. SNARE proteins are formed by coiled-coil interactions of the alpha-helices of its members, which is required for membrane fusion [32–35].



**Figure 1.** Diagram of the tetanus toxin molecule. The targeting and the translocation domains are located in the heavy-chain (HC), whereas the catalytic domain is located in the light-chain (LC) of the molecule. Its proteolytic activity is  $Zn^{2+}$ -dependent, and heavy-metal chelators generate inactive apo-neurotoxins. TTC is approximately 50 KDa and resides in the HC of the toxin. The ganglioside-recognition domain in TTC allows the toxin to be internalized into the neuron [35].

From the gene therapy point of view, the most interesting part of the toxin that must be outstanding is TTC. This fragment of the toxin is located in the HC of tetanus toxin molecule and it plays an important role in the neuronal internalization (Figure 1). In fact, TTC maintains transport properties of the native tetanus toxin without causing toxic effects, in such a way that in the absence of TTC, the toxin retains little ability to paralyze neuromuscular transmission [35,36].

The trans-synaptic transport of TTC was intensively studied in one of the best-characterized systems, the primary visual pathway [37, 38], confirming its capacity as a carrier once it was injected intramuscularly [39-41]. Furthermore, the possibility of constructing recombinant molecules with TTC has opened the door to an interesting research field, the discovery of neuro-anatomical tracers, whose main purpose is to map synaptic connections between neuronal cells.

One of the most well-known recombinant proteins that have been used for this purpose is the protein encoded by *lacZ*-TTC. This protein has been tested *in vitro* and *in vivo* to determine its activity in the hypoglossal system, and the detection of the labeled motor neurons was dependent on time post-injection [40-42]. Since neuronal integrity is crucial for TTC internalization, the transneuronal molecular pathway at neuromuscular junctions was intensively studied using this recombinant protein [43]. The protein was detected not only in the neuromuscular junction postsynaptic side but also the soma of the motor neuron, away from the active zones in large uncoated vesicles.

The advances in the understanding of these recombinant proteins have paved the way for new therapeutic approaches using TTC as a carrier of molecules to ameliorate the disease process of motor neuron diseases, neuropathies and pain. As an example, several proteins conjugated to TTC that have been used to study neuronal internalization *in vitro* and *in vivo* are horseradish peroxidase (HPRT), glucose oxidase (GO), green fluorescent protein (GFP),  $\beta$ -N-acetylhexosaminidase-A (HEXA), superoxide dismutase 1 (SOD1), survival motor neuron 1 (SMN1), cardiotrophin-1 (CT1), B-cell lymphoma-extra large (Bcl-xL), IGF-1, glial derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF) [17]. More recently, a novel multi-component nanoparticle system using polyethylene imine (PEI) has been evaluated to elicit the expression of BDNF in neuronal cell lines [44].

Apart from the carrier properties of TTC, the neuroprotective nature of TTC was one of the best kept properties to discover.

The neurotrophin family has been shown to regulate survival, development and functional aspects of neurons in the central and peripheral nervous systems through the activation of one or more of the three members of the receptor tyrosine kinases (TrkA, TrkB, and TrkC) in cooperation with p<sup>75NTR</sup> [45-48]. Nerve growth factor (NGF) can bind to the TrkA receptor or a complex of TrkA and p<sup>75NTR</sup> [45], BDNF and neurotrophin-4/5 can bind to TrkB, and neurotrophin-3 binds to TrkC. Interestingly, the retrograde pathway of TTC is shared by p<sup>75NTR</sup>, TrkB and BDNF, which is strongly dependent on the activities of the small GTPases Rab5 and Rab7 [49], therefore TTC alone might have a neuroprotective role and therefore it can be a valuable non-viral therapeutic agent in ALS.



## 2. Neuroprotective nature of TTC

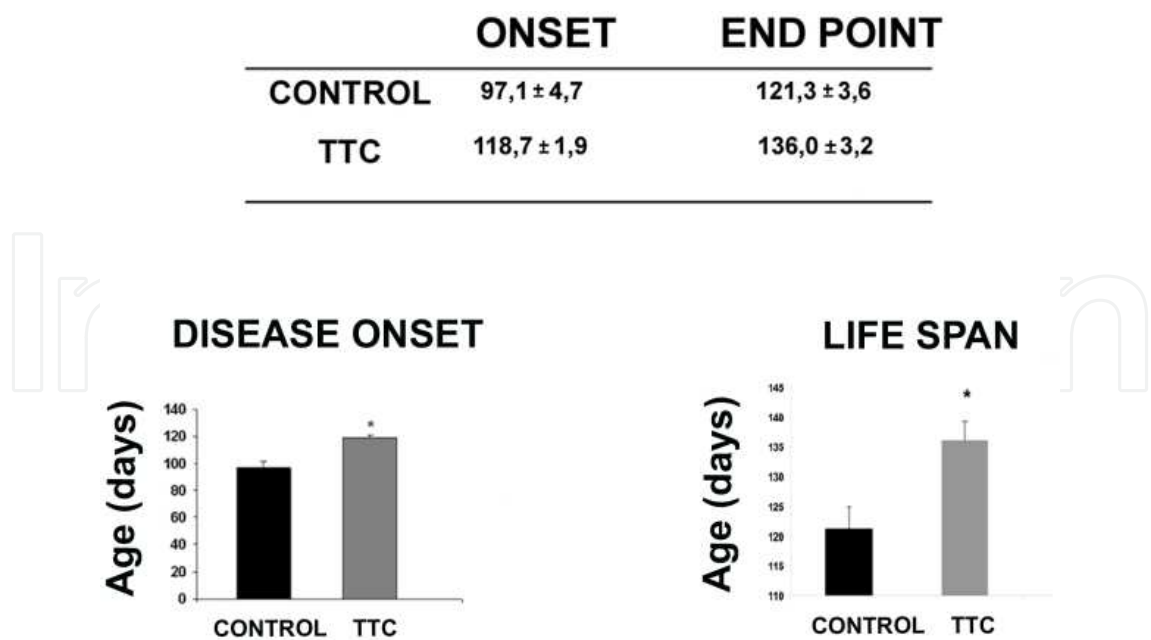
Many authors have suggested that the trans-synaptic transcytosis pathway used by tetanus toxin was most likely “designed” for the trafficking of trophic factors through a chain of connected neurons [50]. Furthermore, two trophic factors, GDNF and BDNF, have been reported to possess similar trans-synaptic transcytotic properties to those of tetanus toxin [51].

Tetanus toxin can induce an increase in serotonin synthesis in the central nervous system, suggesting that the toxin-affected serotonergic innervation in the perinatal rat brain can trigger the translocation of calcium phosphatidylserine-dependent protein kinase C (PKC) [52]. In particular, tetanus toxin is able to alter a component involving inositol phospholipid hydrolysis, which is associated with PKC activity translocation [53,54]. In addition to this translocation, an enhancement of the tyrosine phosphorylation of the tyrosine receptor TrkA, phospholipase C (PLC $\gamma$ -1) and ERK-1/2 can be also observed [55]. Due to the fact that TTC can stimulate the PLC-mediated hydrolysis of phosphoinositides in rat brain neurons, TTC seems to modulate some signaling pathways involving the transport of serotonin [56].

Moreover, the activation of intracellular pathways related to the PLC $\gamma$ -1 phosphorylation and activation of PKC isoforms and the kinases Akt (at Ser 473 and Thr 308) and ERK-1/2 (at Thr 202/Tyr 204) is induced by TTC in rat brain synaptosomes and cultured cortical neurons. This signal pathway activation is dependent on time and concentration, therefore TTC can exert neuroprotective effects, activating TrkA and TrkB receptors in a similar manner as do NGF and BDNF or neurotrophin-4/5 [57,58].

The neuroprotective role of TTC is also supported by the fact that it can also protect cerebellar granular cells against potassium deprivation-induced apoptotic death [59] and act as a neuroprotector in a model of 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>)-triggered apoptosis, enhancing the survival pathways in rats with a dopaminergic lesion and improving different motor behaviors. Particularly, TTC is able to induce Ser 112 and Ser 136 BAD phosphorylation, activate the transcription factor NF- $\kappa$ B, which prevents neuronal death, and induce a decrease in the release of cytochrome c and, consequently, a reduction in the activation of procaspase-3 and chromatin condensation [60,61].

More recently, the nature of TTC described by Longstreth and colleagues [62] and Larsen and colleagues [63], based on its stability to reach motor neurons specifically through the retrograde axonal transport system, has been reinforced as a potential neuroprotective agent in previous *in vivo* studies of gene and protein expression after injection of plasmid-DNA in transgenic SOD1<sup>G93A</sup> mice, which carries the mutation G93A in human superoxide dismutase 1 (SOD1) [64]. These studies suggested that intramuscular naked-DNA TTC gene therapy administered into neurodegenerative mouse model delayed the onset of symptoms (by approximately 5 days), prolonged survival (by approximately 13 days) and improved the motor function activity in TTC-treated mice throughout disease progression, by increasing numbers of surviving motor neurons (Figure 2).

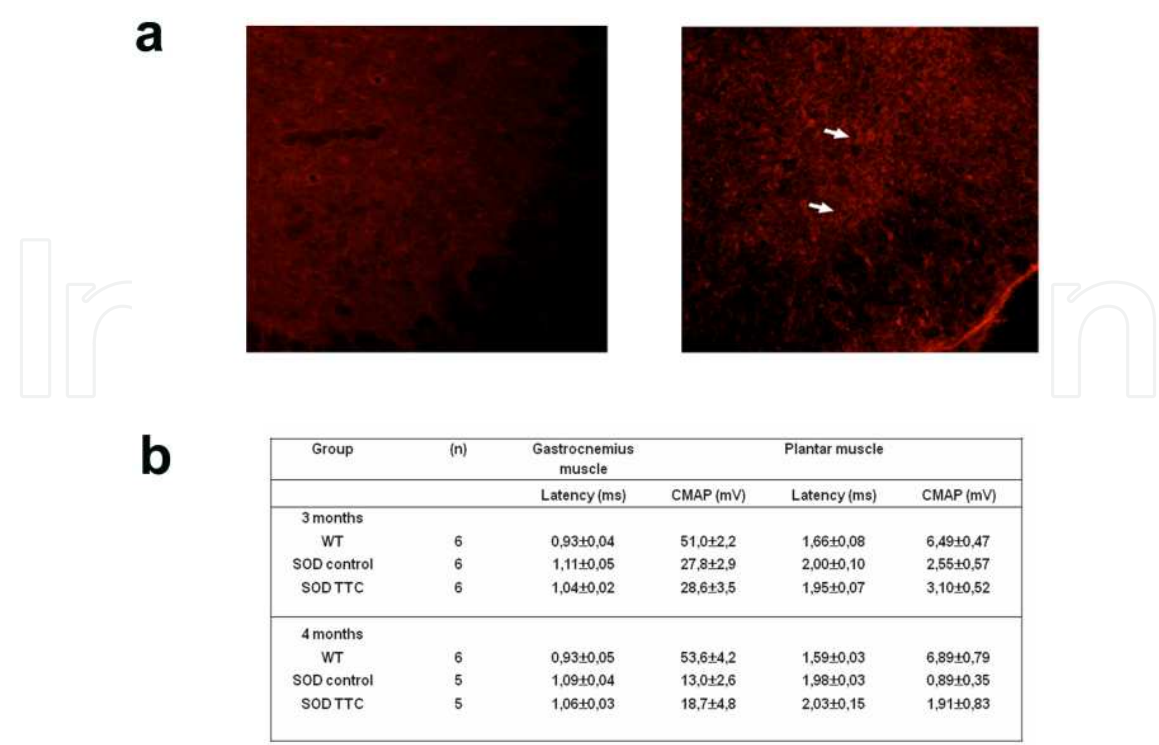


**Figure 2.** Functional and survival effect under TTC treatment. Intramuscular injection of TTC-encoding plasmid in SOD1<sup>G93A</sup> mice (grey bars) delays significantly disease onset and mortality compared to the control group (\*p<0,05, error bars indicate SEM) (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

Apart from functional and survival results obtained *in vivo* in transgenic SOD1<sup>G93A</sup> mice, the electrophysiological studies showed that, from three to four months of age, TTC treatment played a partial protective effect as demonstrated by the lower decline in amplitudes of the M waves, improvement in motor behavioral tests, and increased survival of motor neurons in the TTC-treated animals' lumbar spinal cord [64] (Figure 3).

Interestingly, TTC administration can also affect antiapoptotic pathways by means of calcium-related mechanisms [64]. The positive effects on motor neuron preservation, animal motor function, and survival were confirmed with studies of anti-apoptotic effects and survival signals in the spinal cords of treated animals. Transcriptional caspase-1 and caspase-3 levels were downregulated in the spinal cord of TTC-treated animals as well as significant variations in calcium-related gene expression were found [64]. Furthermore, a downregulation of the caspase-3 activation protein levels in the spinal cord of TTC-treated animals indicated that TTC might act through an anti-apoptotic pathway. Actually, Bax, Bcl2, phospho-Akt and phospho-ERK 1/2 protein expression levels in TTC-treated animals were statistically significant and close to those of wild-type animals, suggesting a decrease of apoptosis and a lower degree of motor neuron neurodegeneration due to TTC treatment [64].

Taking all these results obtained *in vitro* and *in vivo* as a whole, non-viral gene therapy treatment based on TTC could be a safe and promising neuroprotective strategy for neurodegenerative diseases, especially in ALS. However, the next question to be tackled is whether a recombinant molecule of TTC may have a synergistic effect and enhance the neuroprotective properties of TTC alone.



**Figure 3.** Motor neuron survival and neurophysiological study in gastrocnemius and plantar muscles in SOD1<sup>G93A</sup> mice. (a) Presence of TTC in the grey matter of the ventral horn of (a) positive control (SOD1<sup>G93A</sup> transgenic mice injected with empty plasmid) and (b) SOD1<sup>G93A</sup>-TTC treated mice. Arrows point to some of the neurons positively stained for TTC. Bar = 200 μm. (b) Electrophysiological study of compound muscle action potential (CMAP) in gastrocnemius and plantar muscles in wild-type mice (WT), control SOD1<sup>G93A</sup> mice, and SOD1<sup>G93A</sup> mice treated with naked DNA encoding for TTC. Values are the mean ± SEM. CMAP, compound muscle action potential; n, number of mice. \*P < 0.05 vs. WT group at the same age (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

3. Neuroprotective properties of recombinant molecules of TTC in a mouse model of ALS

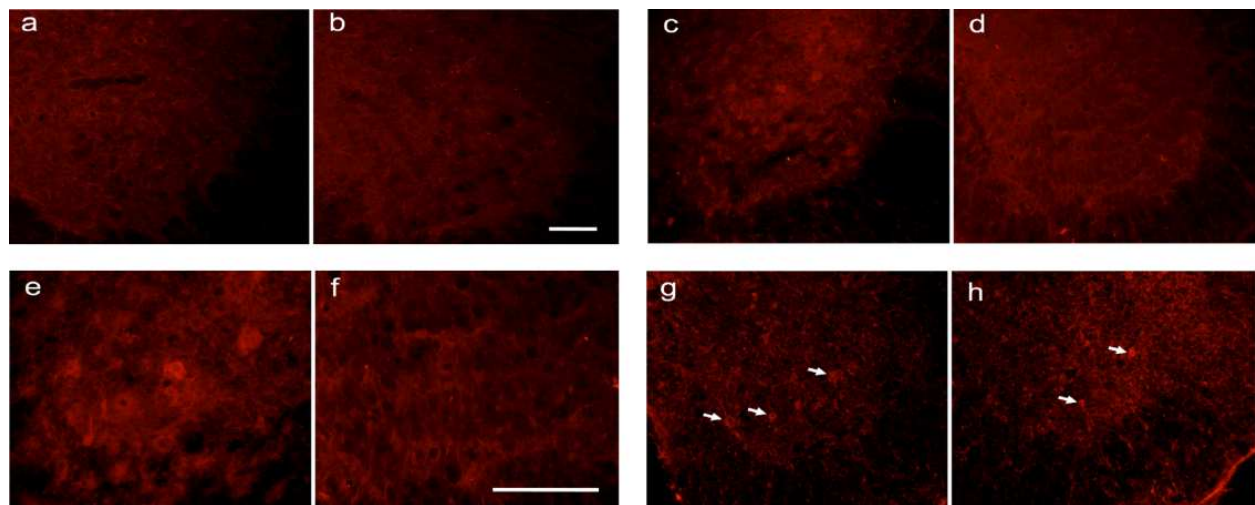
It has been very well described the specificity of a trophic factor for motoneurons and precisely this specificity could be increased by genetically fusing it to TTC, while the trophic factor could contribute to enhance the benefits observed for TTC. Therefore the next inevitable approach is to test naked-DNA gene delivery to encode for a chimeric molecule, to study the potential synergistic effect.

As previously mentioned, BDNF belongs to the family of neurotrophins and binds specifically to TrkB receptors to activate the intracellular signaling pathways that promote neuronal survival and the differentiation of neurons. The neurotrophic effects of BDNF on motoneuronal degeneration have been widely studied *in vitro* and *in vivo* [66,67]. This neurotrophin has also been proposed as a potential therapeutic agent for the treatment of human ALS [68], although no successful results have been achieved. This failure in the clinical

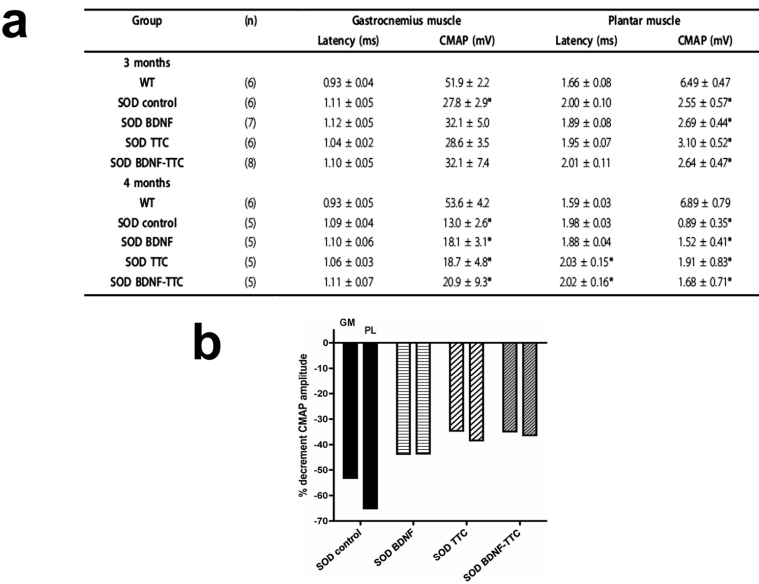


application of BDNF may be due to the low efficacy of targeting the neurotrophic factor to motoneurons. Alternatively, TTC possesses a high affinity for motoneurons [40], and the fusion of BDNF to the TTC protein might increase its accessibility. A previous study reported that some neurotrophic factors, in particular BDNF, facilitate the internalization of TTC recombinant molecules in motor nerve terminals [69]. In addition, TTC and the recombinant protein BDNF-TTC can inhibit apoptosis in cultured neurons, with the quimeric molecule being more effective than TTC alone [70]. Interestingly, BDNF may cause a relocation of membrane domains containing TTC receptors by activating Trk receptors, thereby facilitating the neuronal internalization of TTC. This observation is supported by other authors who state that TTC activates intracellular pathways involving Trk receptors [58]. Therefore, the hypothesis of a synergistic positive effect based on the fusion of the mature form of BDNF genes to TTC in a mouse model of ALS needs to be pointed out for the bench to the bedside approach.

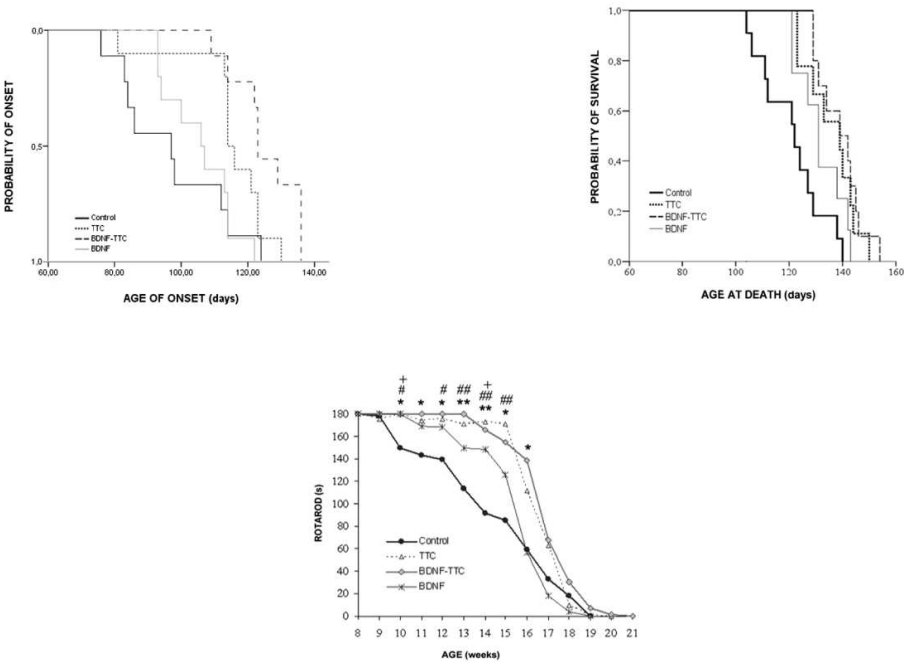
Similarly to the results observed in transgenic  $SOD1^{G93A}$  mice [64], an amelioration of the decline in hindlimb muscle innervation was observed in the animals that were injected with either naked DNA encoding TTC or naked DNA encoding the recombinant molecule TTC and BDNF (BDNF-TTC) [65] (Figures 4,5), in addition to a significant delay in the onset of symptoms and functional deficits (Figure 6), an improvement in the spinal motor neuron survival (Figure 7) (down-regulation of caspase-1 and caspase-3 levels and a significant phosphorylation of serine/threonine protein kinase Akt) (Figure 8) and a prolonged lifespan under both treatments [64,65].



**Figure 4.** Motoneuronal preservation in transgenic  $SOD1^{G93A}$  mice under TTC, BDNF and BDNF-TTC treatments. Immunohistochemical labeling for BDNF expression in the grey matter of the ventral horn of (a) positive control ( $SOD1^{G93A}$  transgenic mice injected with empty plasmid), (b)  $SOD1^{G93A}$ -BDNF and (c) L2 and (d) L4 spinal segments of  $SOD1^{G93A}$ -BDNF-TTC mice. (e, f) Detail of BDNF immunolabeling of the sections shown in c and d, at higher magnification. Presence of TTC in the grey matter of the ventral horn of (g)  $SOD1^{G93A}$ -BDNF-TTC and (h)  $SOD1^{G93A}$ -TTC treated mice. Arrows point to some of the neurons positively stained for TTC. Bar = 200  $\mu$ m in a, b, c, d, g and h; bar = 100  $\mu$ m in e and f (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

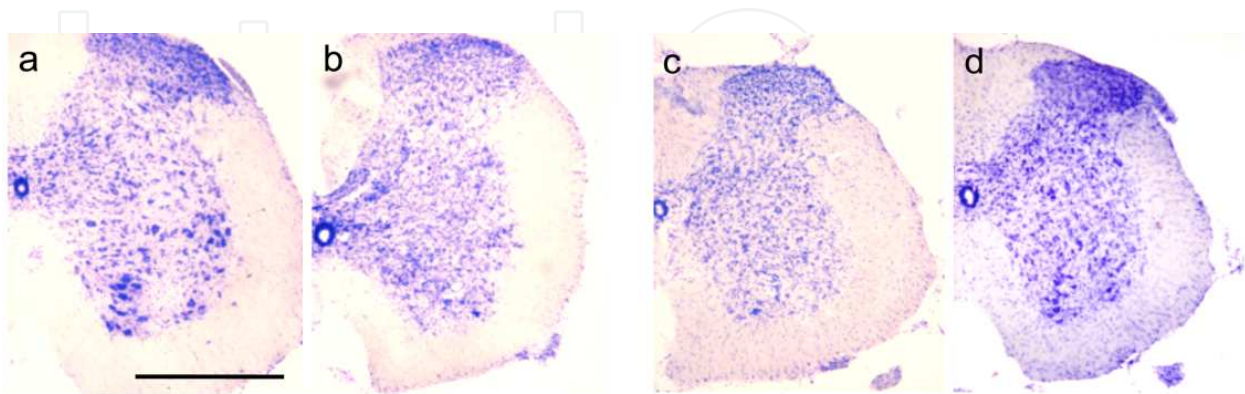


**Figure 5.** Neurophysiological study in gastrocnemius and plantar muscles. (a) Results of wild-type mice (WT), control SOD1<sup>G93A</sup> mice, and SOD1<sup>G93A</sup> mice treated with naked DNA encoding for BDNF, TTC, and BDNF-TTC are shown. Values are the mean ± SEM. CMAP, compound muscle action potential; n, number of mice. \*p < 0.05 vs. WT group at the same age. (b) Histogram representation of the decrement in the amplitude of the compound muscle action potential. CMAP was compared at 4 months with respect to values at 3 months of age in SOD1<sup>G93A</sup> mice, untreated and treated with naked DNA encoding for BDNF, TTC or BDNF-TTC. For each group, the left bar corresponds to the gastrocnemius muscle and the right bar to the plantar muscle (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

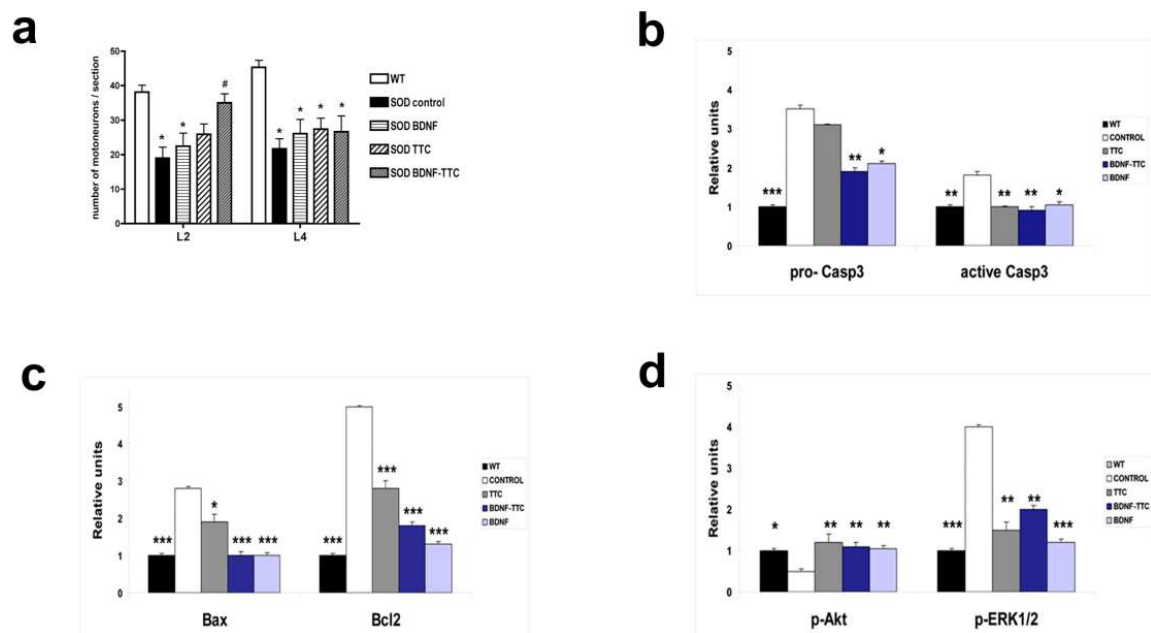


**Figure 6.** Improvement in disease clinical outcomes in transgenic SOD1<sup>G93A</sup> mice under TTC, BDNF and BDNF-TTC treatments. Cumulative probability of the onset of disease symptoms (hanging-wire test) and survival in SOD1<sup>G93A</sup> mice injected

at 60 days of age with TTC, BDNF-TTC, BDNF or empty (positive control) plasmids. Strength and motor function were tested using the rotarod at 15 rpm. Mice were given up to 180 s for the test performance and the time at which mice fell was recorded (\*, #, +,  $P < 0.05$ ; \*\*, ##,  $P < 0.01$ ; error bars indicate SEM); \* for BDNF-TTC vs. positive control comparisons; # for TTC vs. control comparisons; + for BDNF vs. positive control comparisons (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).



**Figure 7.** Spinal motor neuron survival of transgenic SOD1<sup>G93A</sup> mice under TTC, BDNF and BDNF-TTC treatments. Representative micrographs showing cross-sections of lumbar spinal cords stained with cresyl violet from wild-type, (a) SOD1<sup>G93A</sup> control (positive control), (b) BDNF-treated, (c) and BDNF-TTC-treated, (d) mice at 16 weeks of age. Bar = 500 μm (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

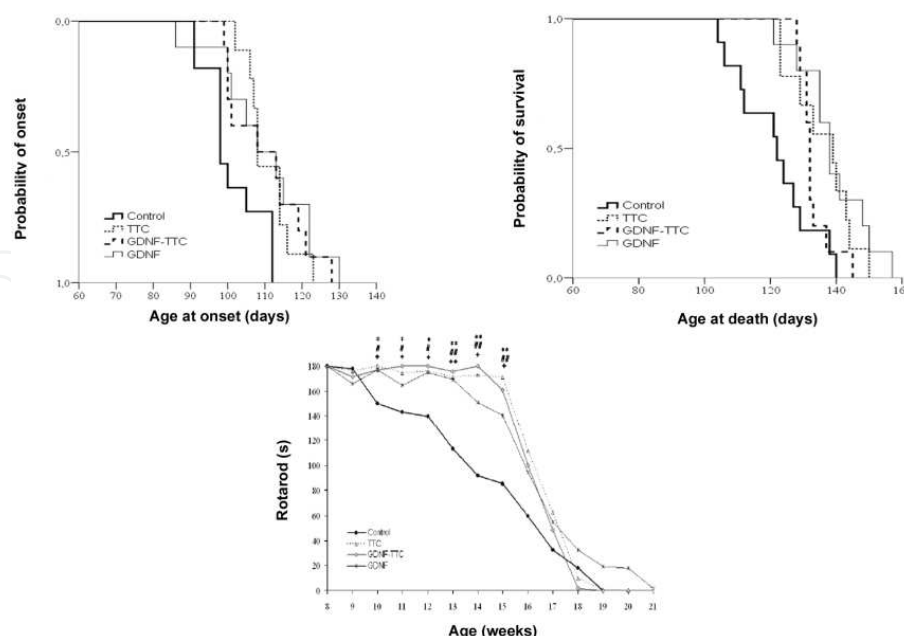


**Figure 8.** Apoptotic and survival pathways under TTC, BDNF and BDNF-TTC treatments. (a) Histogram representation of the average number of stained motoneurons per section in L2 and L4 spinal cord segments of wild-type littermates, control SOD1<sup>G93A</sup> and treated mice (n = 4-5 mice per group). \*  $p < 0.05$  vs. wild type; #  $p < 0.05$  vs. SOD<sup>G93A</sup> control mice. (b) Fold-changes in the expression of pro-Casp3 and active Casp3 proteins, (c) Bax and Bcl2 proteins and (d) phosphorylated states of Akt and ERK1/2 proteins in spinal cord lysates of control SOD1<sup>G93A</sup> animals (white) and ani-

mals treated with TTC (grey), BDNF-TTC (blue, BTTC) and BDNF (soft blue). Western blot quantities are shown as the ratios to b-tubulin and then related to age-matched wild-type (black) mice data (\* $P < 0.05$  and \*\* $P < 0.01$  vs. control SOD1<sup>G93A</sup> mice; \*\*\* $p < 0.001$ ; error bars indicate SEM) (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

Additionally, GDNF is another candidate neurotrophic factor for ALS therapy. This factor has been described to show potent trophic effects on proliferation, differentiation and survival of motor neurons *in vitro* and *in vivo* [63,71-76]. Furthermore, after the retrograde transport of GDNF to the cell bodies, a fraction of this trophic factor avoided degradation and was sorted to dendrites [51], similar to the known movement of the TTC [39]. It was also suggested that the transsynaptic and transcytotic pathway used by GDNF was similar to that of TTC, but not identical, and that GDNF protein degradation was lower than that of TTC protein. Furthermore, the combination of TTC and GDNF has been evaluated in a neonatal rat axotomy model [63] and in the ALS mouse model [77]. The combination of TTC with insulin growth factor (IGF-1) has also been assayed in transgenic SOD1<sup>G93A</sup> mice [78], although the effect of TTC alone has not been compared in any of these studies. When the effect of TTC was compared to the recombinant molecule *in vitro*, a significant increase in the survival capacity of neuronal cells was found [77]. However *in vivo*, no significant differences were observed, which is probably due to the possibility that the recombinant molecule might follow a GDNF route and not the TTC route under axotomy conditions [63].

When focusing the study *in vivo* in a mouse model of ALS, the recombinant molecule TTC and GDNF (GDNF-TTC), GDNF and TTC treatments prompted a delay in disease onset, an improvement in motor function and a longer lifespan in transgenic SOD1<sup>G93A</sup> mice, comparing to empty-plasmid injected control mice [79] (Figure 9).

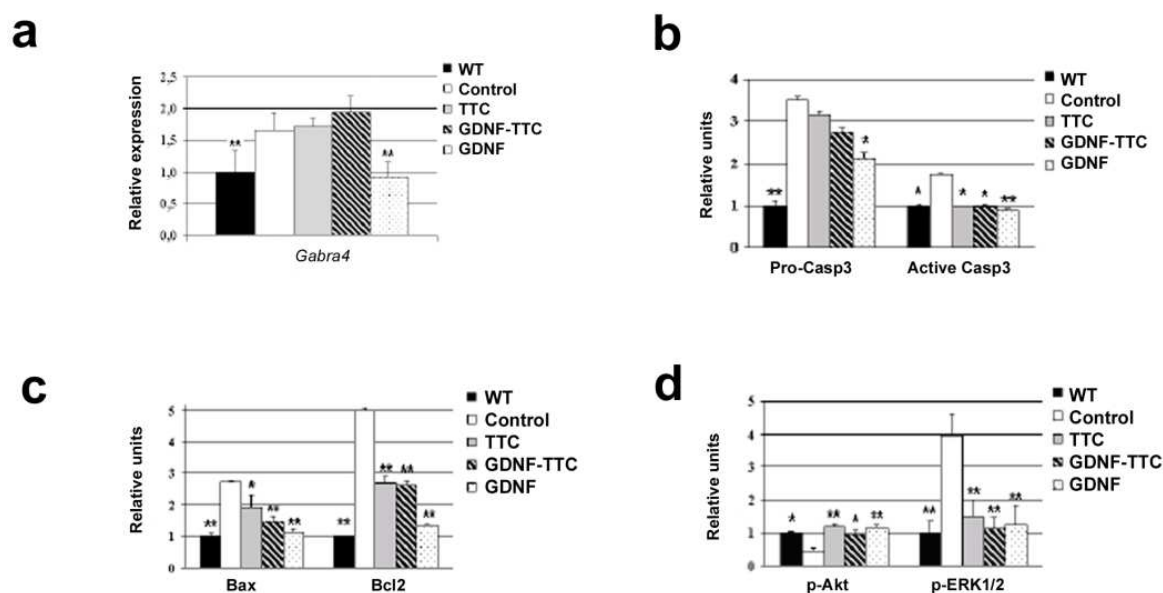


**Figure 9.** Improvement in disease clinical outcomes in transgenic SOD1<sup>G93A</sup> mice under TTC, GDNF and GDNF-TTC treatments. Cumulative probability of the onset of disease symptoms (hanging-wire test) and survival in SOD1<sup>G93A</sup> mice injected at 60 days of age with TTC, GDNF-TTC, GDNF or empty (positive control) plasmids. Strength and motor



function were tested using the rotarod at 15 rpm. Mice were given up to 180 s for the test performance and the time at which mice fell was recorded (\*, #, +,  $P < 0.05$ ; \*\*, ##,  $P < 0.01$ ; error bars indicate SEM); \*GDNF-TTC vs. control comparisons; # TTC vs. control comparisons; + GDNF vs. control comparisons (\*, #, +,  $P < 0.05$ ; \*\*, ##,  $P < 0.01$ ; error bars indicate SEM) (Reprinted from Restor. Neurol. Neurosci, 30, Moreno-Igoa M, Calvo AC, Ciriza J. et al. Non-viral gene delivery of the GDNF, either alone or fused to the C-fragment of tetanus toxin protein, prolongs survival in a mouse ALS model, p. 69-80, Copyright (2012), [79] with permission from IOS Press).

Moreover, the recombinant molecule GDNF-TTC and full-length GDNF inhibited apoptotic pathways in spinal cords of SOD1<sup>G93A</sup> mice by reducing the activation of caspase-3, as well as Bax and Bcl2 protein levels reached a profile expression similar than the one observed in wild type mice, highlighting the fact that treated mice biochemically resemble non-transgenic mice (Figure 10). In addition, all treatment molecules activated the PI3K survival pathway by phosphorylating Akt and ERK1/2, resembling again the wild type levels [79] (Figure 10).

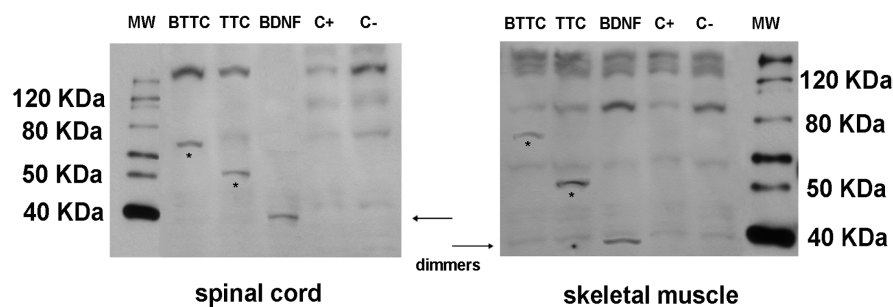


**Figure 10.** Apoptotic and survival pathways under TTC, GDNF and GDNF-TTC treatments. (a) Fold-changes in the expression of GABA(A) receptor subunit-4 (*Gabra4*) mRNA levels in total spinal cord of wild type, control transgenic mice and treated transgenic mice (n=5 per group). (\* $P < 0.05$ , \*\* $P < 0.01$ ; error bars indicate SEM). Fold changes in the expression of (b) pro-Casp3 and active Casp3 proteins, (c) Bax and Bcl2 proteins, and (d) phosphorylated states of Akt and ERK1/2 proteins. Western blots from spinal cord lysates of control animals (white) and treated with TTC (gray), GDNFTTC (hatched-columns) and GDNF (dotted-columns). Western blot quantities are shown as the ratio to  $\beta$ -tubulin and then related to age-matched wild type (black) mice data (Reprinted from Restor. Neurol. Neurosci, 30, Moreno-Igoa M, Calvo AC, Ciriza J. et al. Non-viral gene delivery of the GDNF, either alone or fused to the C-fragment of tetanus toxin protein, prolongs survival in a mouse ALS model, p. 69-80, Copyright (2012), [79] with permission from IOS Press).

Summarizing, albeit a significant improvement in behavioral assays together with an activation of anti-apoptotic and survival pathways under BDNF and GDNF treatments was observed in transgenic SOD1<sup>G93A</sup> mice, no synergistic effect was found neither using the BDNF-TTC nor GDNF-TTC recombinant molecules. Interestingly, recombinant plasmids BDNF-TTC and GDNF-TTC were detected in skeletal muscle and the corresponding recombinant protein reached the spinal cord tissue of transgenic SOD1<sup>G93A</sup> mice (Figure 11), reinforcing the carrier properties of TTC.



As a final point, the active state of the neurotrophic factors BDNF and GDNF in the recombinant molecule could suggest that either BDNF or GDNF could exert an autocrine and neuroprotective role together with TTC to a similar extent as TTC alone; however this effect could not be sufficient enough to prompt a synergistic effect. As a consequence, the recombinant molecules could mainly use the same pathway that mimics a neurotrophic secretion route, prompting survival signals in the spinal cord of transgenic SOD1<sup>G93A</sup> mice [65,79]. Despite all these contributions to the understanding of the neuroprotective properties of recombinant molecules, it is undoubtedly that TTC has open the door to an alternative therapeutic strategy for more neurodegenerative diseases although its molecular pathways is not yet well characterized.



**Figure 11.** TTC and BDNF detection in skeletal muscle and spinal cord of ALS transgenic SOD1<sup>G93A</sup> mice. Western blot detection of TTC in spinal cord and skeletal muscle tissues of wild-type (C-, negative control), SOD1<sup>G93A</sup> transgenic mice injected with empty plasmid (C+, positive control), TTC- and BDNFTTC (BTTC)-treated mice. In TTC and BDNF-TTC treated groups, the detected band was approximately of 50 and ~ 70 KDa respectively (\*), using both anti-TTC and anti-BDNF antibodies. In the BDNF group, the dimeric conformation, indicated by arrows, was observed at approximately 40 KDa (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

## 4. Conclusions

At present, gene and stem cell therapies are holding the hope for an efficient treatment in ALS. Regarding gene therapy, the possibility of delivering therapeutic molecules to damaged tissues crossing the blood-brain barrier has made possible the study of viral (adenovirus, adeno-associated and lentivirus) and non-viral (fragment C of tetanus toxin) vectors, which are retrogradely transported to motor neurons, in preclinical animal models showing promising neuroprotective effects.

Although therapeutic strategies, which tend to stop or slow down the progression of ALS, are one of the main goals in this field of research, the new property of TTC has opened the door to new non-viral therapeutic strategies in this disease. The fact that TTC as well as the recombinant molecules BDNF-TTC and GDNF-TTC can be transported through motoneurons to induce a later onset of symptoms, improve motoneuron survival and extend the survival of SOD1<sup>G93A</sup> mice support the fact that the naked DNA-mediated intramuscular

delivery of TTC and fusion molecules can promote neuroprotective effects in the SOD1<sup>G93A</sup> murine model of ALS. The active states of BDNF and GDNF in the recombinant molecules also confirm that these neurotrophic factors could exert an autocrine and neuroprotective role together with TTC to a similar extent as TTC alone, but this effect was not sufficient to enhance the survival signals observed under TTC treatment alone.

Definitively, the neuroprotective role of fragment C has shed light on the understanding of the disease neurodegeneration processes and the study of this promising property of TTC can be extended to other neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and Spinal Muscular Atrophy (SMN). Essentially, a better understanding of these neurodegenerative diseases will facilitate the translation from animal model to patients to find a definitive therapeutic approach.

## Acknowledgements

This work was supported by from Caja Navarra: "Tú eliges, tú decides"; PI10/0178 from the Fondo de Investigación Sanitaria of Spain; ALS association N° S54406 and Ministerio de Ciencia e Innovación INNPACTO IPT-2011-1091-900000.

## Author details

Ana C. Calvo, Pilar Zaragoza and Rosario Osta\*

\*Address all correspondence to: [osta@unizar.es](mailto:osta@unizar.es)

Laboratory of Genetics and Biochemistry (LAGENBIO-I3A), Aragón's Institute of Health Sciences (IACS), Faculty of Veterinary School, University of Zaragoza, Spain

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