In vivo electrotransfer of the cardiotrophin-1 gene into skeletal muscle slows down progression of motor neuron degeneration in pmn mice

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Among all vectors designed for gene therapy purposes, adenovirus appears to be the most efficient in vivo vehicle to transduce the broadest spectrum of cellular targets. However, the deleterious immunogenicity of this viral vector impedes its use in chronic diseases. Non-viral vectors, such as naked DNA, are attractive alternatives for safety and technical issues, such as scale-up production. Naked DNA injection, greatly improved when combined with electroporation, showed great potential in adult animals, especially when directed to the muscle. We have recently proven the therapeutic effect of a neonatal single intramuscular injection of a cardiotrophin-1 (CT-1)-encoding adenovirus in a hereditary disease mouse model of human motor neuron disease, the progressive motor neuronopathy (pmn) mutant. We now demonstrate that a single injection/electroporation of a CT-1-encoding plasmid in neonate pmn mice is almost as efficient as adenovirus-mediated gene transfer with respect to survival, muscular function and neuroprotection of the animals. Treated mice gain global weight, their mean lifespan is extended by 25%, all their electromyographic parameters are improved and myelinated axons of their phrenic nerves are protected. Moreover, we show that re-injection/electroporation leads to improvements in this neuroprotection. We therefore demonstrate for the first time the therapeutic efficacy of neonatal intramuscular DNA injection/electroporation in a murine model of a human hereditary disorder.

INTRODUCTION

Human motor neuron diseases (MND) such as amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA) are progressive fatal disorders. Despite the identification of some of the genes involved in these pathologies, namely the sod1 and smn genes (1,2), specific pathways leading to the selective destruction of motor neurons in these disorders have not yet been elucidated.

The progressive motor neuronopathy (pmn) mouse mutant (3), an animal model of human MND, has been used to test new therapeutic approaches, including gene therapy. Homozygous pmn mice are characterized by prominent axonal degeneration that starts at the endplates of motor nerves and leads to muscular atrophy and impaired motor performance and to premature death at around 6 weeks of age. The pmn mutation has been localized to a genetic interval of 0.3 cM on mouse chromosome 13 that contains five candidate genes (4). While awaiting the identification of the pmn gene, several neurotrophic factors have been shown to be effective in slowing down the progression of neurodegeneration in pmn mice (5–9). In this line, we have previously shown that adenoviral vectors encoding neurotrophin-3 (Ad–NT-3), ciliary neurotrophic factor (Ad–CNTF) and cardiotrophin-1 (Ad–CT-1) were effective in transducing newborn pmn skeletal muscle in vivo, conferring neuroprotection of motor neurons and prolonging the survival of pmn mice (7,8,10). Comparing the physiological effects of Ad–NT-3 and Ad–CT-1 in pmn mice, we demonstrated that the neuroprotective effects of CT-1 were higher than those of NT-3 using lower adenovirus titres (7,10).

The safety of adenoviral vector administration has, however, been questioned in human clinical trials (11). Moreover, adenovirus vector re-administration, required for transgene delivery in chronic diseases, is hampered by the immune
response triggered by this virus. In order to circumvent these limitations, we questioned whether non-viral DNA delivery could efficiently replace an adenoviral strategy. The purpose of this study was therefore to assess the therapeutic efficiency of CT-1 delivered by *in vivo* electroporation of plasmid DNA into skeletal muscle.

Electroporation consists of the transfer of a chosen molecule—DNA, protein or another small molecule—into cells by controlled electric pulses. *In vivo* electroporation of plasmid DNA has emerged as a promising gene delivery method because of its low cost, its safety in production and utilization, and the lack of risk of insertional mutagenesis (12). Plasmid DNA electroporation has been tested in a broad range of target tissues and organs, including liver (13), muscle (14–16), bladder (17) and cornea (18), and also in whole embryos (19). When used under appropriate conditions, DNA electroporation of skeletal muscle fibres offers many particular advantages, such as a very efficient cellular uptake of DNA and long-term transgene expression. The major advantage of the electroporated muscle, however, lies in its potential for the production and secretion of bioactive proteins into the bloodstream, allowing the local production of a recombinant protein that exerts its effects on remote targets. Based on this strategy, circulating factors such as interleukin-5 (14), erythropoietin (16,20), fibroblast growth factor 1 (15), interferon-α (21) and human factor IX (22) have been produced by electroporated muscle.

DNA electroporation has been very recently tested in adult models of renal anaemia (23), diabetes (24) and cisplatin-induced neuropathy (25), but has never been assessed in hereditary early-onset disorders. Since the onset of motor neuron degeneration in *pmn* mice is around two weeks of age, an early administration of neurotrophic factors is a prerequisite for their efficiency in this model. In the present study, we have therefore, in a first step, established parameters for safe and efficient muscle electroporation in young (6- to 7-day-old) mice using a β-galactosidase-encoding plasmid, and, in a second step, applied these conditions to test the therapeutic potential of electroporation-mediated CT-1 gene transfer in *pmn* mice.

RESULTS

Establishment of efficient electric-pulse parameters in 1-week-old mice

The *pmn* mice display first pathological symptom at around day 14 after birth and die from respiratory insufficiency at around 40 days of age. We previously demonstrated the therapeutic benefit of a CT-1–adenovirus when this vector was intramuscularly injected into *pmn* aged 5–6 days (10). In order to compare the efficacy of electroporation with our previous protocol, we first established the optimal electric-pulse conditions for electroporation at this stage using normal C57BL/6 mice and the control plasmid pJT–β-gal. According to parameters previously established by Mir et al. (26), we used eight long (20 ms) electric pulses applied at a 1 Hz frequency, and compared the influence of the electric field intensity and of the injected DNA quantity on the gene transfer efficacy.

Mice were electrooporated at day 6 or 7 into both gastrocnemius muscles, and β-galactosidase activity was assessed at day 13 by X-Gal histochemistry and by chemiluminescent dosing. A week after injection of 20 μg of pJT–β-gal and electroporation at 350 V/cm, β-galactosidase-positive muscle fibres were almost undetectable. After electroporation at 500 V/cm, between 20% and 40% of muscle fibres expressed β-galactosidase without histological clues of damage. In contrast, electroporation with electric field intensities above 750 V/cm led to clues of muscle injury, such as necrosis as exemplified by the presence of centronucleated fibres (Fig. 1 and data not shown). We therefore conducted all subsequent experiments using eight pulses of 20 ms, with an electric field of 500 V/cm and interval of 1 s. Under these conditions, a DNA dose-dependent effect was observed by chemiluminescent β-galactosidase dosing in muscle homogenates (data not shown).

Construction of a functional CT-1-encoding plasmid: pJT–CT-1

A plasmid (pJT–CT-1) encoding the murine CT-1 cDNA sequence driven by the cytomegalovirus promoter/enhancer (CMV) was constructed in a reduced backbone (pJT), since limiting CpG sequences have been proven to be deleterious for sustained transgene expression (27). The mouse βNGF leader sequence was added upstream of the CT-1 cDNA to facilitate secretion of the recombinant protein. To assess the activity of this vector, NIH 3T3 cells were transfected with pJT–CT-1 and their conditioned media tested in an ELISA assay developed for this purpose (see Materials and Methods). Conditioned media (CM) from cells transfected with a β-galactosidase-encoding plasmid harbouring the same backbone (pJT–β-gal) were used as a control. CT-1 protein levels were 178.1 ± 3.5 ng/ml in CM from pJT–CT-1-transfected NIH 3T3 cells versus 0.027 ± 0.009 ng/ml in CM from pJT–β-gal-transfected cells (mean ± SEM, n = 6, P < 0.0001; Fig. 2A). The biological activity of CT-1 protein in these CM was then confirmed using a chicken ciliary ganglion neuron survival assay. In this assay, the CM from pJT–CT-1-transfected NIH 3T3 contained a significantly higher neuronal survival activity than CM from control cultures (Fig. 2B). By comparing the neuronal survival activities obtained with the CM with those of recombinant CT-1 standards (inset in Fig. 2B), we were able to conclude that the CT-1 protein released from pJT–CT-1-transfected cells was fully biological active.

CT-1 expression in pJT–CT-1-electroporated gastrocnemius of *pmn* mice

Neonatal *pmn* mice were electroporated with pJT–CT-1 using the defined electric-pulse parameters (eight pulses of 20 ms, at 1 Hz, with a 500 V/cm electric field). Cardiotrophin-1 gene expression was evaluated by RT–PCR in the gastrocnemius of electroporated *pmn* mice at 25 days of age. The RT–PCR signal was clearly observed after pJT–CT-1 DNA injection followed by electroporation, hardly detectable in injected but non-electroporated muscle but undetectable in non-injected muscle (Fig. 3).

CT-1 expression in gastrocnemius muscles after pJT–CT-1 electroporation was further quantified using the CT-1 immuno-detection ELISA assay. We determined that CT-1 production
was maximal around day 13 (i.e. 7 days after electroporation), reaching a mean value of $180.6 \pm 10 \text{ng/g (mean \ SEM)}$ and then declining (Fig. 4). At day 25, CT-1 levels in electroporated gastrocnemius muscles were no longer significantly different from those measured in non-injected pmn muscles ($72.5 \pm 23.2 \text{ng/g versus } 55.1 \pm 9.9 \text{ng/g}$).

Dose-dependant survival of pJT–CT-1 electroporated pmn mice

In a preliminary set of experiments, we determined the therapeutic range of pJT–CT-1 in pmn mice by varying the dose of injected plasmid DNA from 5 to 40 \mu g per gastrocnemius. While the lowest dose (5 \mu g) was well tolerated, it did not significantly prolong the mean lifespan of the animals above that of non-injected pmn mice (data not shown). The highest dose of pJT–CT-1 (40 \mu g) induced growth retardation and led to the death of all treated pmn mice before day 35, in line with the known deleterious effects of high CT-1 levels on hepatic and cardiac functions (28). Twenty micrograms was a well-tolerated dose, but did not improve the lifespan of pmn mice ($40.3 \pm 2.0$ days; $n = 6$, $P = 0.53$). In contrast, the electroporation of 10 \mu g of pJT–CT-1 was well tolerated and significantly improved the survival of pmn mice. The mean survival of treated pJT–CT-1 pmn mice was $47.1 \pm 2.4$ days (mean $\pm$ SEM, $n = 16$) versus $37.4 \pm 1.9$ days ($n = 28$) in non-treated pmn mice ($P = 0.0066$; Fig. 5). At day 45, 55% of the treated pmn mice were still alive, in comparison with 20% of non-treated animals. Some pJT–CT-1 treated pmn mice survived for up to 74 days, while the maximum lifespan of control pmn mice was 47 days. Electroporation of the empty
vector (pJT) did not affect the mean survival of pmn mice (37.4 ± 1.9 days; n = 12).

Effects of pJT–CT-1 electroporation on neurodegeneration in pmn mice

In order to analyse the therapeutic benefits of CT-1 expression on neuromuscular function and motor neuron degeneration, we performed electrophysiological and histomorphological studies. At day 25, the weight of gastrocnemius muscles from pJT–CT-1 treated pmn mice was increased by 46% in comparison with that of muscles from untreated pmn mice (Table 1), suggesting that pJT–CT-1 electroporation prevented severe gastrocnemius atrophy.

We next performed an electromyographic (EMG) analysis by recording motor latency, compound muscle action potential (CMAP) amplitude and duration after supramaximal electrical stimulation of the sciatic nerve of the gastrocnemius muscle (Figure 2). Biological activity of the same CM was tested in a chicken ciliary ganglion neuron survival assay. Neuronal survival (in %) was significantly higher in the presence of pJT–CT-1 CM (52.7%) than in the presence of control CM (20.0%; P < 0.02). Residual survival in the control CM probably reflects the release of fibroblast growth factors by NIH 3T3 cells. The inset shows the neuronal survival as a function of different concentrations of recombinant CT-1 protein. Error bars in the histograms represent SEM.

Figure 2. Analysis of CT-1 protein in conditioned media (CM) of transfected NIH 3T3 cells. (A) ELISA analysis demonstrates that CT-1 immunoreactivity (CT-1 IR) in CM from pJT–CT-1-transfected NIH 3T3 cultures (n = 6) was more than 6000-fold higher than in CM from pJT–β-gal-transfected cells (P < 0.0001). (B) Biological activity of the same CM was tested in a chicken ciliary ganglion neuron survival assay. Neuronal survival (in %) was significantly higher in the presence of pJT–CT-1 CM (52.7%) than in the presence of control CM (20.0%; P < 0.02). Residual survival in the control CM probably reflects the release of fibroblast growth factors by NIH 3T3 cells. The inset shows the neuronal survival as a function of different concentrations of recombinant CT-1 protein. Error bars in the histograms represent SEM.

Figure 3. CT-1 gene expression in pmn mice. The right gastrocnemius muscles were analysed in pJT–CT-1-injected mice (lines 1–4). pJT–CT-1 transcripts were detected by RT–PCR in all gastrocnemius muscles of pJT–CT-1-injected mice at day 25, with a higher yield when combined with electroporation (lines 2–4). C+, cDNA from pJT–CT-1-transfected NIH 3T3 cells; C−, cDNA from gastrocnemius muscle of pJT-electroporated pmn; −, without RT; +, with RT. MT, 100 bp ladder weight marker. β-Actin transcripts were used as control.

Figure 4. CT-1 immunoreactivity in gastrocnemius muscles of pmn mice. The concentration of CT-1 immunoreactive protein (expressed in ng/g) in muscle homogenates was determined using ELISA analysis. At day 13 (i.e. 7 days after electroporation), CT-1 levels were 180.6 ± 10 pg/g (mean ± SEM, n = 5) after electroporation, in comparison with baseline levels of 57.0 ± 14.1 pg/g (n = 5; *P < 0.0001). At day 25, CT-1 levels had decreased to 72.5 ± 23.2 pg/g in muscles of pJT–CT-1-electroporated pmn mice (n = 5), which was not significantly higher than in untreated pmn mice (55.1 ± 9.9; n = 5). A re-injection of pJT–CT-1 at day 17 allowed the restoration of CT-1 levels in muscles at day 25 to 140.8 ± 15.1 pg/g, i.e. to values non-significantly different from those at day 13 (n = 7).
muscle in 10 mice of each treatment group. At age 25 days, untreated pmn mice showed a drastic reduction of CMAP amplitude (14.9 ± 1.6 mV versus 67.8 ± 3.5 mV, respectively) and an enhanced motor latency (1281 ± 65 ms versus 826 ± 58 ms) as compared with normal mice. pJT–CT-1 electroporation significantly improved these parameters: mean CMAP amplitude was increased to 31.7 ± 3 mV (P = 0.0002), motor latency was shortened to 1096 ± 54 ms (P = 0.041) and CMAP duration was decreased from 5.2 ± 0.4 s (untreated) to 3.8 ± 0.2 s (P = 0.0112) (Fig. 6A–C). Typical electromyograms of healthy littermates, non-treated and treated pmn mice are shown in Figure 6D.

A non-specific esterase staining on cross-sections of pmn gastrocnemius (Fig. 7) from each group confirmed that pJT–CT-1-electroporated pmn muscles contained less denervated fibres than untreated or pJT-electroporated pmn muscles, confirming the neuroprotection illustrated by the EMG.

The number of myelinated phrenic nerve fibres in pJT–CT-1-electroporated mice at day 25 was 140 ± 5, versus 110 ± 7 in untreated pmn mice and 242 ± 5 in controls (Table 2, Fig. 8). This result provides evidence that the improved survival and EMG of treated pmn mice were associated with an approximately 30% increase in the number of myelinated fibres.

Effects of re-electroporation on neurodegeneration in pmn mice

Because of the transient expression of the pJT–CT-1 plasmid after electroporation into newborn muscle, we tried to improve our results further by a second electrotreatment of 10 μg pJT–CT-1 into both gastrocnemius muscles at day 17, using eight pulses of 20 ms at 1 Hz and an electric field of 400 V/cm. At day 25, CT-1 transcripts were detected in all re-electroporated muscles by RT–PCR (data not shown), and CT-1 protein levels detected by ELISA were 140.6 ± 16.8 pg/g (mean ± SEM, n = 5), which is significantly higher than in muscles from non-injected pmn mice or from pmn mice submitted to a single electroporation (see Fig. 4).

We next analysed the effects of re-electroporation on functional and histological parameters. The results observed after one or two pJT–CT-1 plasmid electrotreatments were strikingly different at day 25. The second electroporation of pJT–CT-1 had a spectacular effect on some electrophysiological and histological parameters: while latency and CMAP duration were not improved in re-injected pmn mice, the amplitude of the CMAP became close to normal (55.7 ± 6.9 mV versus 66.2 ± 4.0 mV in healthy littermates; P = 0.177). Moreover, non-specific esterase immunohistochemistry revealed a staining pattern close to normal in muscles from re-electroporated pmn mice (Fig. 7). The number of myelinated phrenic nerve fibres also tended to be higher after pJT–CT-1 re-injection than after a single injection (153 ± 5; n = 8, P = 0.099 with respect to single-injected pmn, and n = 9, P = 0.0003 with respect to untreated pmn). In marked contrast to these improvements, however, the mean survival of pJT–CT-1-re-electroporated pmn mice was only 29 days and did not exceed 35 days. Moreover, the weight of the gastrocnemius was reduced to 30.7 ± 2.7 mg (mean ± SEM, n = 6), which is even lower than in untreated pmn mice (P = 0.15).

DISCUSSION

We have previously demonstrated that CT-1 is a very potent neurotrophic factor in two murine models of motor neuron diseases: the pmn (10) and the SOD1G93A mice (29). Neuroprotection was obtained in both models after a single intramuscular injection of an adenovirus encoding CT-1 at a very early stage of life (postnatal day 5–6).

In order to compare adenoviral injection and plasmid DNA electrotransfer, a single intramuscular injection of CT-1-encoding plasmid followed by electroporation was performed in pmn mice at the same age. We demonstrate in this work that this non-viral approach is as effective as an adenoviral therapy in the hereditary lethal pmn mouse.
In a first step, we optimized two parameters of electroporation: (i) the plasmid backbone (pJT) was improved by reducing the number of CpG sequences that have been shown to impede sustained transgene expression (27); and (ii) different electric field strength conditions were compared for efficiency of transfection of the gastrocnemius muscle in newborn mice. With a field of 500 V/cm, based on electric parameters reported by Mir et al. (15) (eight pulses of 20 ms at 1 Hz frequency), we obtained efficient muscle transfection without major side-effects. Protein expression reached its maximum level at day 13 and declined to baseline 3 weeks after injection. At this time (day 25), CT-1 transcripts were still present, rendering unlikely the hypothesis that CMV-promotor driven transgene expression was shut down. Moreover, we confirmed other published data reporting a stable gene expression from CMV-containing plasmids that had been electroporated in adult normal mice (15,16,30) (data not shown). We therefore assume that the expression profile obtained in young pmn mice was specific either to the muscle properties at this age (growth of the muscles leading to dilution of plasmid in muscular fibres and

Figure 6. Electromyography (EMG) in 25-day-old electroporated pmn mice. pJT–CT-1 electroporation significantly improved all EMG parameters, while pJT–CT-1 re-electroporation has only a major effect on CMAP amplitude. (A) Mean CMAP amplitude (B) latency and (C) time of response in the calf muscles of normal mice (n = 10), untreated pmn mice (n = 9) or after a single electroporation of pJT (n = 10) or pJT–CT-1 (n = 9) or a double injection–electroporation of pJT–CT-1 (n = 6) in pmn mice. (D) Representative EMG recordings from (a) normal mice, (b) untreated pmn mice, and (c) pJT-electroporated, (d) pJT–CT-1-electroporated and (e) re-injected pJT–CT-1-electroporated pmn mice (20 mV/div in vertical scale, 1 ms/div in horizontal scale).
protein in bloodstream, myotube fusion, etc.) or to the pathological muscle changes encountered in this particular mouse model (denervation-induced muscular necrosis and myotube degeneration).

In a second step, we treated neonate pmn mice with a CT-1-encoding plasmid injected in both gastrocnemius muscle, and electroporated the muscles according to the parameters defined previously. We observed a 25% enhancement of mean lifespan in pJT–CT-1-electroporated pmn mice as compared with pmn control animals. This significant increase in lifespan was associated with a global weight gain of electroporated muscles and improved electromyographic (EMG) parameters, reflecting the status of the sciatic nerve–gastrocnemius couple. Notably, our EMG analysis showed that the CMAP amplitude of the pJT–CT-1-treated pmn mouse response was twice that observed in untreated pmn, reaching about half the amplitude of normal littermates. Motor latency and CMAP duration, which are increased in untreated pmn mice, were shortened by 15% and 26%, respectively, after treatment. Latency, CMAP amplitude and CMAP duration reflect, respectively, rate of nerve fibre conduction, number of functional nerve fibres recruited by electrical stimulus and muscular fibre velocity. All of these EMG parameters were significantly improved in pJT–CT-1 electroporated mice, suggesting that a single injection/electroporation partially prevented muscle denervation and protected nerves from degeneration. Confirming this hypothesis, at the age of 25 days, phrenic nerves of pJT–CT-1-treated pmn mice displayed 31% more myelinated fibres, and gastrocnemius muscles of treated pmn mice showed less denervated fibres than those of untreated pmn littermates.

All of these therapeutic effects were almost comparable to those obtained with the adenovirus-mediated gene transfer approach, and therefore confirm the efficient protective function of CT-1 on motor neurons. Moreover, they demonstrate that electroporation is a powerful gene transfer method even in very young animals. Among all parameters evaluated, only the number of phrenic myelinated nerve fibres was improved to a lower extent after pJT–CT-1 electroporation than

<table>
<thead>
<tr>
<th>Group</th>
<th>Phrenic axons (day 25)</th>
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<tr>
<td>Normal mouse</td>
<td>242 ± 5 (n = 6)</td>
</tr>
<tr>
<td>Untreated pmn mice</td>
<td>110 ± 6 (n = 6)</td>
</tr>
<tr>
<td>pJT-electroporated pmn mice</td>
<td>107 ± 2 (n = 9), NS</td>
</tr>
<tr>
<td>pJT–CT-1-electroporated pmn mice</td>
<td>140 ± 5 (n = 14)*</td>
</tr>
<tr>
<td>pJT–CT-1-re-electroporated pmn mice</td>
<td>153 ± 5 (n = 8)</td>
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*pJT–CT-1-treated mice have significantly more myelinated axons than untreated pmn mice [140 ± 5 (n = 14) versus 110 ± 6 (n = 6)].

NS: not significant.

*Student’s t-test: P < 0.0001.

Table 2. Number of phrenic myelinated axons in pmn mice after pJT–CT-1 electroporation (mean± SEM)
after adenovirus injection [140 ± 5 versus 197 ± 12; \( n = 12 \) in Bordet et al. (10)]. Three hypotheses can be proposed to explain this slight difference:

(i) the protocol of injection – in the adenovirus protocol, three groups of muscles were unilaterally injected (gastrocnemius, triceps brachii and dorsal muscles of the thoracic trunk), whereas in the electroporation protocol, only gastrocnemius muscles were chosen as targets because of their size and accessibility;

(ii) adenoviruses are transported retrogradely from the injected muscle through the neuromuscular junction, along the axons to the cellular body of motor neurons, where they can still produce their trophic activity – this special uptake is not likely to happen for naked DNA;

(iii) the use of different promotors – CMV for electroporation and RSV for adenovirus protocols.

We favour the idea that alternative vector design and injection protocols account for this difference rather than an inherent superiority of the adenovirus vector.

After a single pJT–CT-1 injection/electroporation into newborn pmm mice, CT-1 immunoreactivity in the gastrocnemius muscle peaked at day 13, but decreased to low levels at day 25. We therefore decided to test whether a second injection and electroporation of pJT–CT-1 might result in more sustained CT-1 expression in the muscle (and hence further increase therapeutic benefits). Indeed, a second injection at day 17 with a lower electric field (400 V/cm) helped to restore a significant quantity of CT-1 in the gastrocnemius muscle of pmm mice at day 25. However, the general behaviour of these mice was affected: re-electroporated pmm mice suffered from a massive weight loss and their mean survival rate decreased, since many of them did not recover from the anaesthesia at day 25 (performed for the EMG) and all others died around day 30. Meanwhile, control pJT-re-electroporated pmm did not suffer from such impairments: they displayed exactly the same weight loss and motodegeneration course as control single-pJT-electroporated pmm mice. Despite these adverse effects on the survival rate of re-injected animals, the CMAP amplitude measured in calf muscle was restored to normal. In contrast, motor latency and duration of the CMAP were enhanced. This apparent discrepancy in EMG parameters, and particularly this striking CMAP amplitude, suggests that a sustained CT-1 expression allows for an efficient protection of nerves from complete degeneration. Nevertheless, pathological axons are heterogeneous, some being more slender than others, leading to desynchronization of the response and to prolongation of CMAP duration and latency. This correlates well with the
non-specific esterase staining observed in double-injected *pmn* mice, in which no clue of denervation was observed. The apparent discrepancy between EMG and histological parameters and survival might be explained by previously described CT-1 pleiotropic side-effects. Indeed, several studies have reported cardiotoxic and hepatotoxic effects of CT-1 (10,28). Therefore, the muscle loss is directly due to CT-1 toxicity peaking at high levels after re-electroporation, and not to re-electroporation itself. To bypass these side-effects, CT-1 might in the future be fused to neurotrophic carriers. We have recently demonstrated an efficient targeting of CT-1 to neurons, including motor neurons, *in vitro* by a genetic fusion between this cytokine and TTC, the non-toxic C fragment of tetanus toxin (31). Studies are currently being performed to test the *in vivo* efficacy of this approach in terms of neuroprotection and side-effects.

Recently, electroporation has proven to be efficient in several adult rodent models of human disorders (23–25). This strategy has many advantages over viral methods, particularly simplicity of production, safety, reduced costs and possibilities of targeting. However, no example of therapeutic efficacy has until now been reported in neonates. To the best of our knowledge, our study constitutes the first example of a therapeutic benefit in a hereditary lethal mouse disease using plasmid electroporation early after birth. Under our experimental conditions, a single injection/electroporation was as efficient as a single adenoviral injection in terms of muscle protection and increase in lifespan, and almost as efficient in terms of neuroprotection. Moreover, our results demonstrate that re-electroporation is possible and may further enhance neuroprotection in this model. This study in a hereditary lethal disorder therefore constitutes a ‘proof of principle’ demonstrating electroporation as an interesting therapeutic alternative to virus-mediated gene delivery.

**MATERIALS AND METHODS**

**DNA constructs**

Plasmid pJT was constructed by inserting the *XbaI–NspI* 255 bp of pcDNA3 (Invitrogen) containing the human β-globin poly(A) in the *XbaI–NspI* 3011 bp of pCI (Promega) encompassing the cytomegalovirus (CMV) promoter/enhancer, followed by a chimeric intron. In this pJT backbone, either the lacZ coding region of pCMVβ (Clonetech), or the murine CT-1 cDNA fitted with the 60 bp preNGF leader sequence (10) were inserted, giving respectively pJT–β-gal and pJT–CT-1.

These plasmids were prepared using Qiagen or Jetstar (Appligen) endofree kits.

**Cell culture methods and ELISA assay**

NIH 3T3 cells were grown to a density of $6 \times 10^5$ cells per 25 cm$^2$ dish, and transfected with either pJT–β-gal or pJT–CT-1 using FuGENE 6 transfection reagent (Roche). Conditioned media (2 ml) were sampled from 24–48 hours after transfection.

For ELISA analysis, 96-well plates were coated with anti-mouse cardiotoxophilin-1-specific goat IgG (R&D Systems) as primary antibody at 1 ng/ml final dilution, in a carbonate coating buffer (0.025 M sodium bicarbonate and 0.025 M sodium carbonate, pH 7.7) for 20 h at 4°C. After a wash and 1 h of blocking (BSB buffer, PROMEGA), CT-1 standards (from 0 to 1500 pg/ml, recombinant mouse CT-1, R&D Systems) and samples were incubated for 6 h at room temperature with shaking (500 r.p.m.). After five washes, biotinylated anti-mouse cardiotoxophilin-1-specific goat IgG (R&D Systems) was added at 100 ng/ml final dilution of 20 h at 4°C. After five washes, streptavidin HRP (Amersham Pharmacia Biotech, 0.1% dilution in fresh BSB buffer) was incubated for 2 h at room temperature with shaking (500 r.p.m.). Colour was revealed with TMB one solution (Promega, France) within 15 min. Reaction was stopped with 1 M phosphoric acid, and the absorbance was read at 450 nm. This ELISA allowed us to determine CT-1 level in both conditioned media from NIH 3T3 cells or in muscle homogenates.

**Biological activity assay**

Biological activity of CT-1 was assessed in a chicken ciliary ganglion neuron survival assay. Ciliary neurons were purified from 8-day-old chicken embryos and cultured as described previously (8). Recombinant CT-1 protein standards or conditioned media were diluted in chemically defined medium (CDM) and pipetted in triplicate wells of polystyrene/laminin-treated 96-well plates. The neuronal cell suspension was then seeded and the number of surviving neurons determined 48 h later after MTT staining. Neuronal survival was expressed as a percentage of the number of neurons surviving in an optimal concentration of CT-1 (100%) or in CDM (set to 0%).

**DNA injection and electric-pulse delivery**

Plasmid DNA was resuspended in 30 μl of sterile 0.9% NaCl and injected into both gastrocnemius muscles of hypothermia-anasthetized mice at postnatal day 6 or 7. Two minutes after DNA injection, transcutaneous electric pulses were applied by two stainless-steel plate electrodes encompassing the mouse limb. The standard protocol consisted of 8 square-wave pulses of 20 ms length, at 1 Hz and 500 V/cm. In a second set of experiments, another electroporation was performed at day 17. Mice were anaesthetized with 30 μl of a mix of xylamine and ketamine (3.9% and 31.25%, respectively, in 0.9% NaCl), their limbs were shaved, and the electroporation field was switched to 400 V/cm. Electrical contact with the skin was ensured by applying a conductive gel. Electric pulses were generated by an ECM 830 Electro Cell Manipulator electropulsator (Qiobioengine, France). Animal experiments were performed in compliance with institutional guidelines.

**Analysis of CT-1 expression in tissues**

CT-1 expression in electroporated or control muscles was studied by RT–PCR. Gastrocnemius muscles were dissected and flash-frozen in liquid nitrogen. Total RNAs were isolated using RNA-B reagent (Bioprobe, France) and reverse-transcribed with M-MLV RT (GIBCO BRL, USA). pJT–CT-1 transcripts were amplified by RT–PCR (30 s at 95°C, 30 s at 55°C for 30 cycles, 72°C for 30 s, and 72°C for 10 min) using a set of primers (Forward: 5′-CTGGTATAGGGAGTTGGGTG-3′, Reverse: 5′-CCTGGAGACCTCTGTTGCT-3′). 

This expression was confirmed by Northern blot analysis using a 32P-labeled cDNA fragment as a probe.
56°C and 45 s at 72°C for 40 cycles) using a primer in the CMV promoter/enhancer region (5′-CAGTGCTTCTGACA-CAACAGTCTC-3′) and in the CT-1 cDNA (5′-GCACGT-ATTCTCCAGAAG-3′). RT–PCR controls were carried out using β-actin primers.

Proteins of electroporated muscle were extracted from flash-frozen gastrocnemius by homogenization and sonication in 100 mM 20% w/v potassium phosphate buffer (pH 7.8), 0.2% Triton X-100 and protease inhibitors, centrifugation for 30 min at 30 000g, and collection of supernatants. CT-1 expression was quantified with the CT-1 ELISA described above.

**Electrophysiological and histological examinations**

At day 25, *pnn* mice were anaesthetized with a mix of xylamine and ketamine as above. Electromyographies were performed with a Keypoint 2 portable apparatus (Medtronic, France). Compound muscle action potentials of the gastrocnemius muscle were elicited after a supramaximal stimulation of the sciatic nerve. Peak-to-peak amplitude, latency, duration of the potential and surfaces under the curves were analysed.

For histological examinations, gastrocnemius muscles were removed and frozen in cooled isopentane, and phrenic nerves were removed and frozen in cooled isopentane, and phrenic nerves were post-fixed with osmium tetroxide and embedded in epoxy resin (32). Myelinated fibres were counted in 1% toluidine blue-stained 1 μm cross-sections, starting at the same level from the diaphragm.

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