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Regulatable Transgene Expression for Prevention of Chemotherapy-Induced Peripheral Neuropathy

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Chemotherapy-induced peripheral neuropathy (CIPN) is a debilitating complication associated with drug treatment of cancer for which there are no effective strategies of prevention or treatment. In this study, we examined the effect of intermittent expression of neurotophin-3 (NT-3) or interleukin-10 (IL-10) from replication-defective herpes simplex virus (HSV)-based regulatable vectors delivered by subcutaneous inoculation to the dorsal root ganglion (DRG) on the development of paclitaxel-induced peripheral neuropathy. We constructed two different tetracycline (tet)-on-based regulatable HSV vectors, one expressing NT-3 and the other expressing IL-10, in which the transactivator expression in the tet-on system was under the control of HSV latency-associated promoter 2 (LAP-2), and expression of the transgene was controlled by doxycycline (DOX). We examined the therapeutic effect of intermittent expression of the transgene in animals with paclitaxel-induced peripheral neuropathy modeled by intraperitoneal injection of paclitaxel (16 mg/kg) once a week for 5 weeks. Intermittent expression of either NT-3 or IL-10 3 days before and 1 day after paclitaxel administration protected animals against paclitaxel-induced peripheral neuropathy over the course of 5 weeks. These results suggest the potential of regulatable vectors for prevention of chemotherapy-induced peripheral neuropathy.

INTRODUCTION

Paclitaxel, a diterpene originally isolated from the bark of the Pacific yew tree, is widely used to treat lung, ovarian, breast, and head and neck cancers and advanced forms of Kaposi's sarcoma.[1–4] Paclitaxel has two adverse effects on the peripheral nervous system. Beginning very soon after the first dose, patients develop a painful condition, most commonly characterized by cold-induced pain (cold allodynia),[5] a condition that is not directly related to nerve damage.[6,7] As treatment continues, many patients develop structure damage to the peripheral nerves, a chemotherapy-induced peripheral neuropathy[5] that negatively affects the quality of life and often requires alteration of the treatment schedule or termination of treatment.[8,9] Paclitaxel-induced peripheral neuropathy is characterized by loss of thermal sensation,[10] numbness in a glove and stocking distribution,[5] and impaired sensorimotor coordination.[11] Not infrequently, the symptoms fail to resolve following the end of the drug treatment, persisting for months or years.[5] There are no effective treatments to

prevent the development of neuropathy or to reverse it when established.

We have previously demonstrated that herpes simplex virus (HSV)-based gene transfer to the dorsal root ganglion (DRG) of neuroprotective factors effectively prevents the progression of neuropathy caused by pyridoxine, diabetes, or the administration of cisplatin[12–16] and that intermittent expression of erythropoietin (EPO) from a regulatable vector is effective in preventing diabetic neuropathy in rodents.[17] Because the half-life of paclitaxel in vivo is short,[18,19] and the drug is administered repetitively in cycles, intermittent expression of neuroprotective peptides achieved by gene transfer could appear to be a very favorable treatment strategy. We now report that intermittent expression of the neuroprotective peptide neurotrophin 3 (NT-3) or interleukin-10 (IL-10) from tetracycline (tet)-on-based regulatable HSV vectors prevents the development of paclitaxel-induced peripheral neuropathy. These results suggest that HSV-based regulatable vectors expressing neuroprotective peptides may be useful clinically for prevention of paclitaxel-induced peripheral neuropathy.

RESULTS

Prolonged Regulatable NT-3- and IL-10-Expressing Constructs

The HSV-based regulatable vectors utilize a modified tet-on-based platform we previously constructed and characterized[17] in which transgene expression is induced by doxycycline (DOX) (Figure 1A) and the transactivator expression is under the control of HSV latency-associated promoter 2 (LAP-2). Vector vL2rtNT-3 expressing NT-3 and vector vL2rtIL-10 encoding for IL-10 were generated by homologous recombination between the endpoint plasmid and the non-replicating HSV vector[20] (Figure 1B). Each construct contains two copies of the transgene, inserted into the two ICP4 loci of the HSV genome.

Expression of IL-10 or NT-3 from the Vectors Is Strictly Controlled by DOX In Vitro

Regulated expression of NT-3 from vL2rtNT-3 and IL-10 from vL2rtIL-10 in response to exposure to DOX was tested in

CrossMark

A

transcription of GOI

| TRE | K | GOI | pA |

| pLAP2 | K | rtTA | pA |

B

| TRE | K | NT-3 | pA | | pLAP2 | k | rtTA | pA |

| TRE | K | IL-10 | pA | | pLAP2 | k | rtTA | pA |

expression cassette →

Δ146207-151814

Δ113322-114517   Δ126413-131930
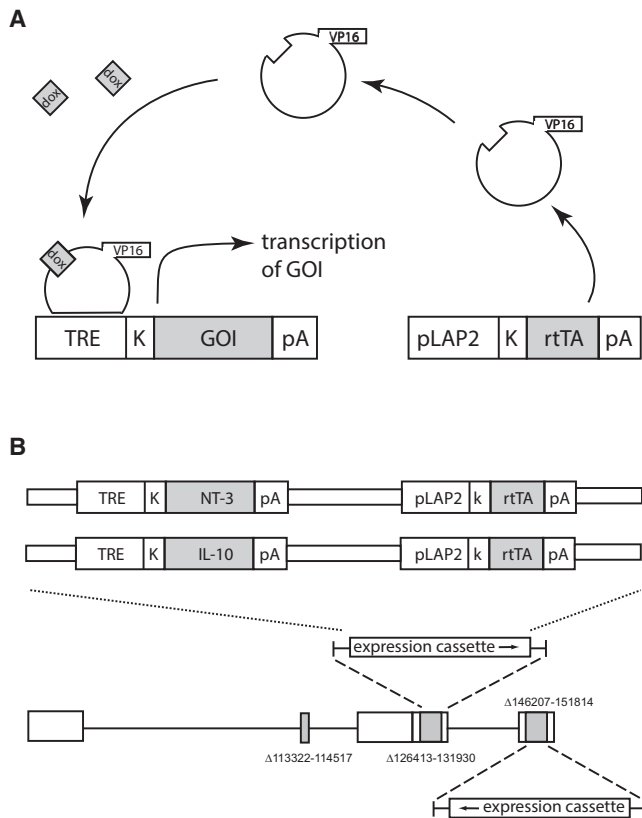
← expression cassette

Figure 1. Prolonged Regulated HSV Vector

Prolonged regulated gene expression was achieved using a modified tet-on system we previously developed in which the expression of the transactivator was under the control of HSV latency-associated promoter 2 (LAP2). (A) The transactivator in the tet-on system is constitutively expressed and binds to the tetracycline response element (TRE)-minimal human cytomegalovirus immediate early promoter (HCMV IEp) of the inducible transgene expression element in the presence of DOX, thus resulting in the expression of the transgene. (B) In the vectors, two copies of the regulatable transgene expression units were inserted into the ICP4 loci of the replication-deficient parental HSV virus. DOX, doxycycline; NT-3, neurotrophin 3; IL-10, interleukin 10; rtTA, reverse tet-controlled transactivator; K, kozak sequence; GOI, gene of interest.

complementing 7b cells. 7b cells were infected with vL2rtNT-3 or vL2rtIL-10 at an MOI of 0.5; DOX at different concentrations was added 1 hr after infection, and NT-3 or IL-10 concentration in the medium was assessed by ELISA after 2 days of DOX treatment. To establish the turn-on and turn-off kinetics of NT-3 or IL-10 expression from the vectors, we examined the amount of NT-3 or IL-10 released into the medium from infected cells using DOX on–off and off–on paradigms. 7b cells were infected with vL2rtNT-3 or vL2rtIL-10 at an MOI of 0.01, and infected cells were either treated with 1 μg/mL DOX for 2 days after infection followed by culturing in normal medium or cultured in normal medium for 2 days and subsequently exposed to 1 μg/mL DOX for 4 days. A small aliquot of the medium was collected from each treatment schedule every 2 days, and NT-3 or IL-10 concentration in the medium was measured by ELISA.

A substantial release of NT-3 into the medium was observed when vL2rtNT-3-infected cells were exposed to DOX at 1 or 10 μg/mL for 2 days, whereas NT-3 in the medium from vL2rtNT-3-infected cells without DOX treatment was below the level of detection (Figure 2A). Removal of DOX resulted in a rapid decrease in NT-3 in the medium from vL2rtNT-3-infected cells, and by day 4 after DOX removal, no NT-3 was detectable in the medium (Figure 2B). Cells infected with vL2rtNT-3 and continuously exposed to DOX starting 2 days after infection showed a substantial and statistically significant increase in NT-3 released into the medium, measured on days 4 and 6 (Figure 2C). Similar turn-on and turn-off kinetics of IL-10 expression from vL2rtIL-10 were seen in vL2rtIL-10-infected cells. 1 μg/mL DOX induced robust release of IL-10 from vL2rtIL-10-infected cells into the medium after 2 days of treatment (Figure 2D); removal of DOX for 4 days resulted in IL-10 not being detectable in the medium (Figure 2E), and addition of DOX 2 days after infection restored a substantial release of IL-10 from vL2rtIL-10-infected cells (Figure 2F).

Regulated Expression of IL-10 and NT-3 in DRGs from the Vectors Is Obtained In Vivo

To test inducible expression of the transgenes from the vectors in vivo, rats were inoculated subcutaneously into both hindfeet with vL2rtNT-3 or vL2rtIL-10 (3 × 10^8 plaque-forming units in 30 μL PBS). Two weeks after vector inoculation, normal food was replaced by DOX-containing chow for 1, 3, or 7 days to induce transgene expression from the vector. To examine shut-off of transgene expression from the vector in the absence of DOX, animals were fed with DOX-containing chow for 3 days, followed by normal chow for 4 days. When each DOX treatment paradigm was completed, the animals were euthanized, and the lumbar 4-6 dorsal root ganglions (L4-6 DRGs) were dissected for determination of NT-3 or IL-10 mRNA and protein levels by semiquantitative PCR and western blot. Basal expression of NT-3 mRNA and protein was observed in L4-6 DRGs of control animals, and similar NT-3 mRNA (Figure 3A) and protein (Figure 3B) levels were observed in animals receiving the vector but not fed DOX-containing chow (day 0). Increased NT-3 mRNA and protein were detected in vL2rtNT-3-infected animals fed DOX-containing chow compared with control animals, and abundant expression of NT-3 mRNA and protein was observed in animals receiving 3 days of DOX treatment, which persisted through 7 days of DOX induction. Removal of DOX for 4 days after 3 days of DOX induction rendered NT-3 mRNA (Figure 3A) and protein (Figure 3B) in DRGs of animals inoculated with vector vL2NT-3 to drop to levels similar to those observed in control animals. Also, similar results for DOX-induced expression of IL-10 from vector vL2rtIL-10 were seen in vL2rtIL10-infected animals. 3 days of DOX induction gave rise to high levels of expression of IL-10 mRNA and protein in vL2rtIL-10-inoculated animals; feeding vL2rtIL-10-inoculated animals normal chow for 4 days after 3 days of DOX induction resulted in IL-10 mRNA (Figure 3C) and protein (Figure 3D) expression not significantly different from that observed in control animals. Taken together, those results indicated that expression of NT-3 and IL-10 from the vectors in vivo was strictly controlled by DOX.
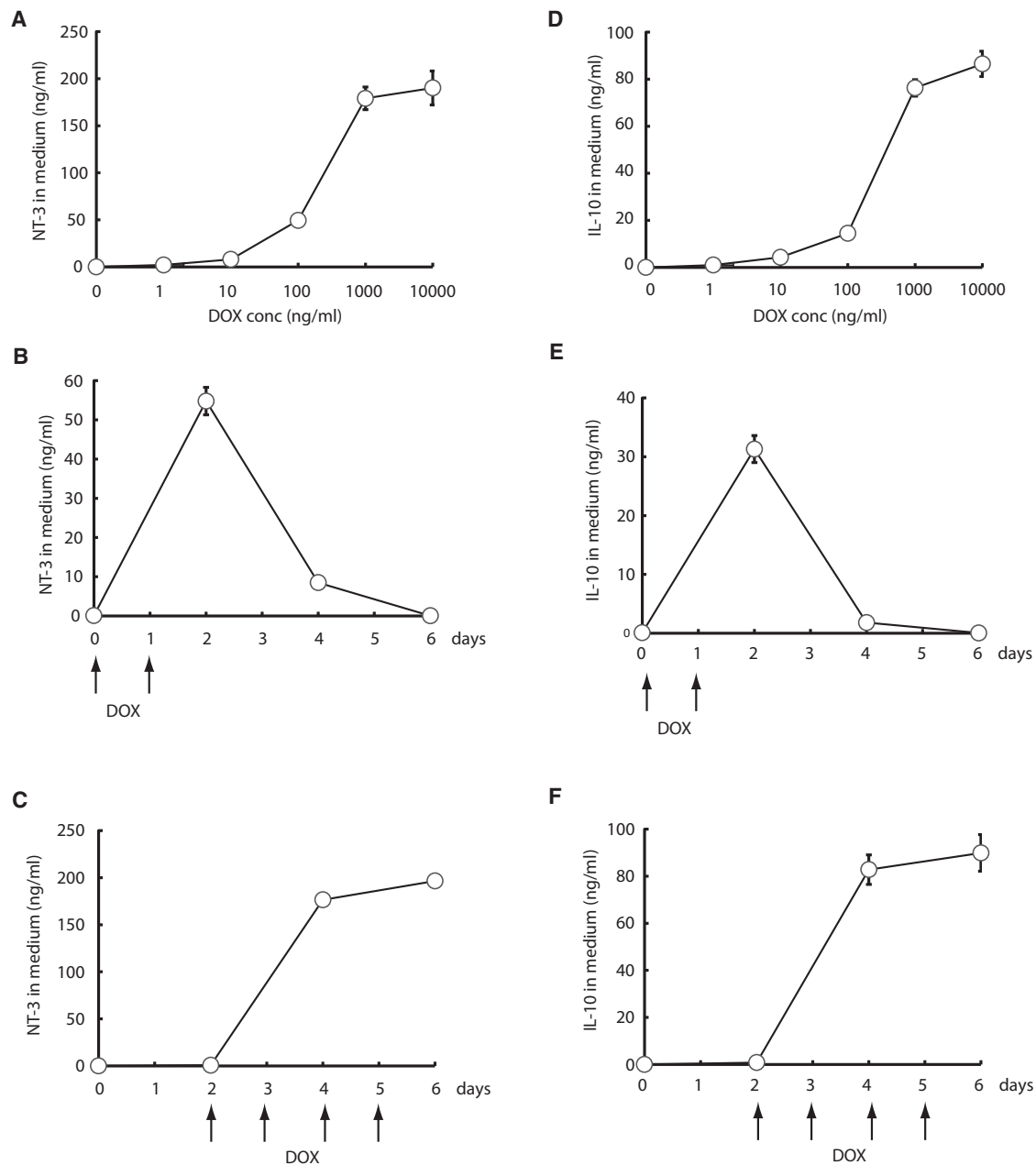
Figure 2. Regulated Expression of NT-3 and IL-10 from the Vectors by DOX In Vitro

Complementing 7b cells were infected by vL2rtNT-3 or vL2rtIL-10 with an MOI of 0.5 and exposed to DOX at different concentrations 1 hr after infection. (A and D) NT-3 or IL-10 concentration in the medium (A, NT-3; D, IL-10) was measured by NT-3 or IL-10 ELISA kit after 2 days of DOX treatment. (B, C, E, and F). To test the kinetics of the turn-on and turn-off of NT-3 or IL-10 expression from the vectors, 7b cells were infected with either vL2rtNT-3 or vL2rtIL-10 at an MOI of 0.01 and cultured either in 1 μg/mL DOX-containing medium for 2 days and subsequently in normal medium for 4 days (B, NT-3; E, IL-10) or cultured in normal medium for the first 2 days after vector infection, followed by 4-day culturing with 1 μg/ml /mL DOX-containing medium (C, NT-3; F, IL-10). Under each culture condition, 50 μL of medium was collected every 2 days, and NT-3 or IL-10 concentration in the medium was measured by ELISA.

Paclitaxel Treatment Impairs Sensory Nerve Electrophysiological Function and Sensorimotor Coordination

We modeled paclitaxel-induced peripheral neuropathy by intraperitoneal (i.p.) injection of paclitaxel (16 mg/kg) into animals once a week for 5 weeks.[10] Paclitaxel treatment resulted in impaired sensorimotor coordination in animals after 2 weeks of treatment, as evidenced by shorter stay times on a rotarod spinning bin observed in paclitaxel-treated animals compared with vehicle-treated animals,
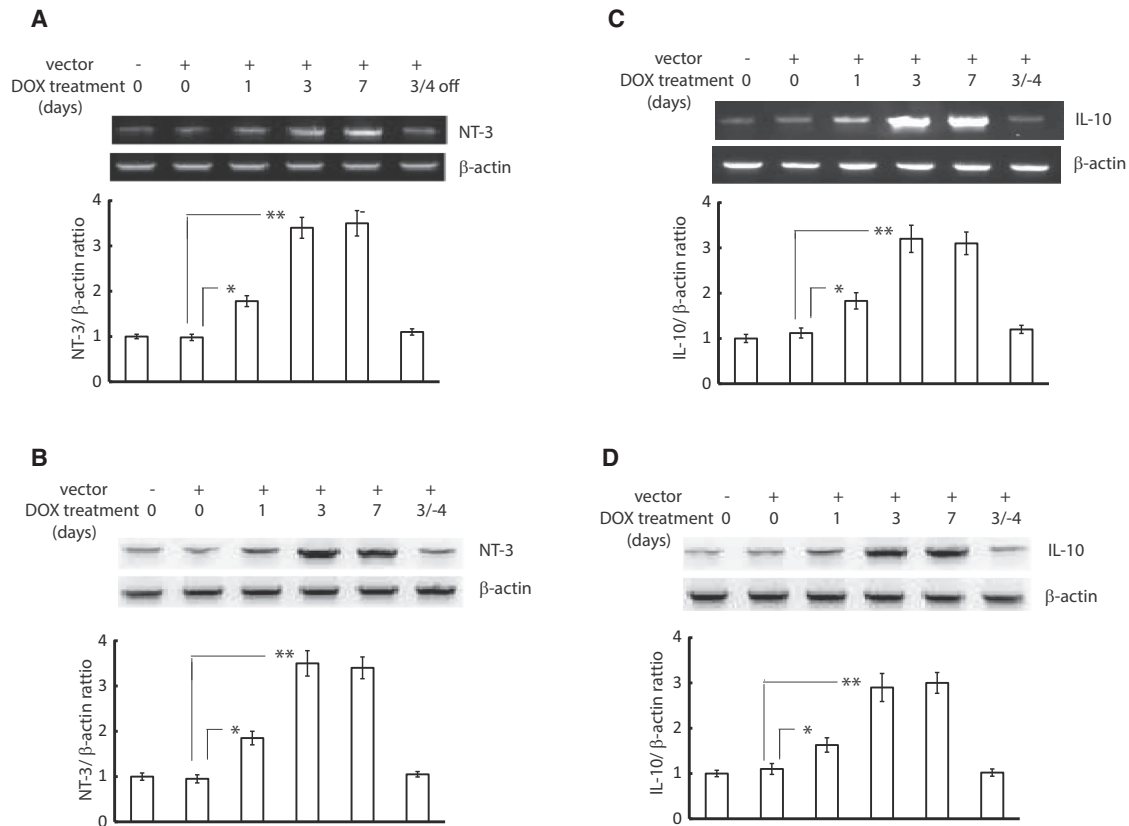
Figure 3. Induction of NT-3 and IL-10 Expression from the Vector by DOX in Animals

Animals were inoculated subcutaneously into the skin of both hindfeet with either vL2rtNT-3 or vL2rtIL-10 and fed DOX-containing chow for 1, 3, and 7 days to examine the induced expression of the transgenes from the vectors, or animals receiving the vectors were fed DOX-containing chow for 3 days, followed by normal food for 4 days, to test the shut-off of the expression of the transgenes from the vectors in the absence of DOX. After each DOX schedule was completed, L4-6 DRGs of both sides were dissected, and NT-3 or IL-10 mRNA and proteins in the DRGs were analyzed by semiquantitative PCR and western blot. β-Actin served as a loading control. (A) Semiquantitative NT-3 PCR and NT-3 mRNA relative amount. 3/4 off, 3 days with DOX treatment/4 days without DOX treatment. (B) NT-3 western blot and NT-3 protein relative amount. (C) Semiquantitative IL-10 PCR and IL-10 mRNA relative amount. (D) IL-10 western blot and IL-10 protein relative amount. *p < 0.05; **p < 0.01; #, 3 days of DOX treatment followed by 4 days of DOX removal.

and this effect persisted through 5 weeks of treatment (Figure 4A). Paclitaxel treatment did not significantly alter sensory nerve electrophysiological function after 2 weeks of treatment, as demonstrated by similar sensory nerve amplitude (Figure 4B) and conduction velocities (Figure 4C) observed in both paclitaxel- and vehicle-treated animals. However, by 5 weeks of paclitaxel treatment, impairment in sensory nerve electrophysiological function was apparent, as demonstrated by reduced sensory nerve amplitude (Figure 4B) and slowed conduction velocities (Figure 4C) in paclitaxel-treated animals compared with vehicle-treated animals.

Intermittent Expression of IL-10 or NT-3 Prevents the Development of Paclitaxel-Induced Peripheral Neuropathy

To determine whether intermittent expression of NT-3 or IL-10 from the vectors prevents paclitaxel-induced neuropathy, rats were inoculated subcutaneously into the skin of both hindfeet either with vector vL2rtNT-3 or vL2rtIL-10. 2 weeks after vector injection, during which

the vector established a latent state in DRG neurons, thus allowing the transgene to express from a vector robustly in the presence of DOX,[17] animals inoculated with the vector were repeatedly injected with paclitaxel once a week for 5 weeks to model paclitaxel-induced neuropathy.[10] Naive animals served as the negative control, whereas animals receiving paclitaxel only served as the peripheral neuropathy-positive control, and vL2rtGFP expressing GFP was used as the control vector. Animals receiving the test vector were divided into two subgroups 3 days before the first paclitaxel treatment: one cohort of animals was continuously fed with DOX-containing chow to induce continuous transgene expression from the vector, whereas the other animals were fed with DOX-containing chow 4 days per week, with feeding of DOX-containing chow starting 3 days before each paclitaxel treatment and ending 1 day after each paclitaxel treatment to achieve intermittent expression of the transgene from the vector (Figure 5A) so that a high level of expression of the transgene from the vector would be expected on the day of each paclitaxel
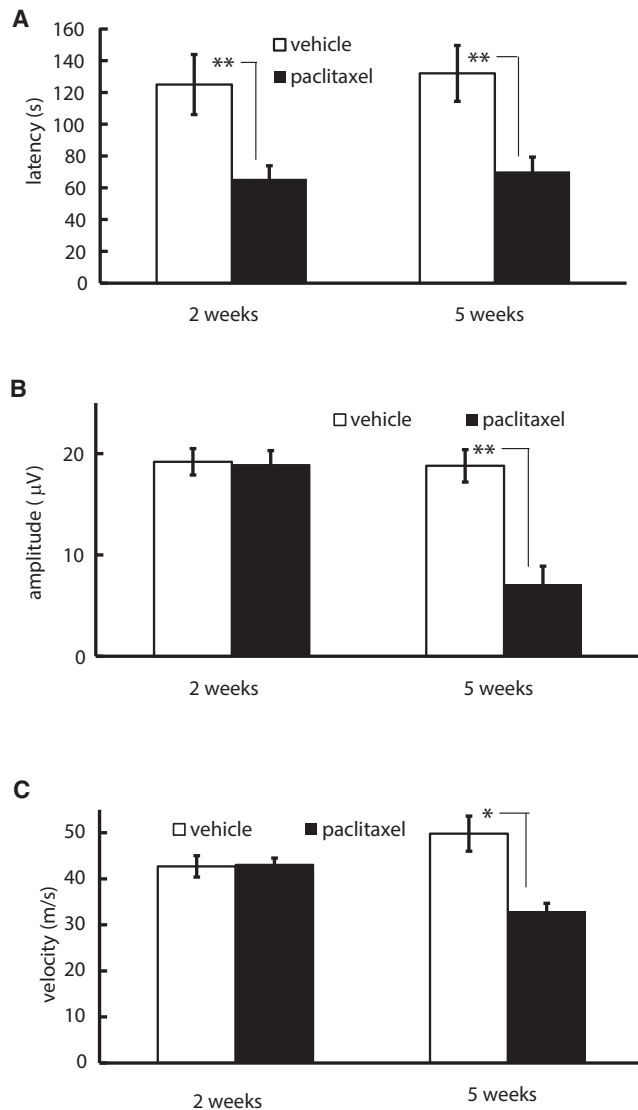
Figure 4. Impaired Sensory Nerve Electrophysiological Function and Sensorimotor Coordination in Animals with Paclitaxel-Induced Peripheral Neuropathy

Paclitaxel-induced peripheral neuropathy was modeled by i.p. injection of paclitaxel (16 mg/kg) once a week for 5 weeks. Sensorimotor co-ordination and sensory nerve electrophysiological function (amplitude and conduction velocity) were evaluated 2 and 5 weeks after the initiation of paclitaxel treatment. (A) Sensorimotor coordination. (B) Amplitude. (C) Conduction velocity. *p < 0.05, **p < 0.01.

treatment. Animals receiving a control vector and treated with paclitaxel were constantly fed with DOX-containing chow. Sensorimotor coordination and sensory nerve electrophysiological function were measured 1 week after the last paclitaxel treatment. Paclitaxel-induced deficits in both sensorimotor coordination and sensory nerve electrophysiological function were observed both in animals receiving paclitaxel only and in paclitaxel-treated animals inoculated with the control vector and fed DOX-containing chow continuously; however,

such deficits were not seen in paclitaxel-treated animals receiving vL2rtNT-3 or vL2rtIL-10 and fed DOX-containing chow either continuously or 4 days per week, as evidenced by similar sensorimotor coordination, similar sensory amplitude, and conduction velocities as observed in control animals and paclitaxel-treated animals receiving vL2rtNT-3 (Figure 5B) or vL2rtIL-10 (Figure 5C) with the transgene expressed continuously or 4 days per week. Those results indicate that intermittent expression of NT-3 or IL-10 from the vectors surrounding paclitaxel treatments is sufficient to prevent the onset of paclitaxel-induced peripheral neuropathy.

Repeated Paclitaxel Treatments Do Not Interfere with Regulated Expression of the Transgene from the Vector in Animals

To determine whether repeated paclitaxel treatments interfere with regulated expression of the transgene from the vectors, we examined the induced expression of NT-3 or IL-10 mRNA and protein from the vectors in the DRGs of paclitaxel-treated and vector-inoculated animals by feeding the animals for an additional 4 days after sensorimotor coordination and sensory nerve electrophysiological function in animals had been evaluated, which was 5 weeks after the initiation of paclitaxel treatment, as mentioned above. Naive and paclitaxel-treated animals receiving a vector and fed normal chow served as the control. Basal NT-3 or IL-10 mRNA (Figures 6A and 6C) and protein (Figures 6B and 6D) levels were detectable in naive and paclitaxel-treated animals fed normal chow, whereas significantly increased NT-3 or IL-10 mRNA (Figures 6A and 6C) and protein (Figures 6B and 6D) levels were observed in paclitaxel-treated and vector-inoculated animals after 4 days of DOX induction, no matter whether the animals were previously fed DOX-containing chow continuously or 4 days per week compared with both naive and paclitaxel-treated animals fed normal chow. These results indicate that repeated paclitaxel treatments do not affect the inducible expression of a transgene from the vectors.

DISCUSSION

In this proof-of-principle study, we have demonstrated that expression of NT-3 or IL-10 from the regulatable vectors is strictly controlled by DOX in vitro and in vivo; that repeated administration of paclitaxel does not interfere with the robust, prolonged, and regulated expression of the transgenes from the vectors in animals; and that intermittent expression of NT-3 or IL-10 from the vectors 4 days per week with peak transgene expression reached at the day of each paclitaxel treatment is sufficient to protect animals against paclitaxel-induced peripheral neuropathy over a time course of 5 weeks.

Neuroprotective peptides are small proteins that prevent programmed neuronal cell death during development and protect neurons against a wide variety of toxic insults. Neuroprotective peptides consist of the classical members of the nerve growth factor family, including nerve growth factor, brain-derived neurotrophic factor, and NT-3,[21,22] together with many other peptides, such as erythropoietin,[22–25] vascular endothelial growth factor,[24] and immune-modulatory peptides like IL-10, which were originally identified by functions other than neuronal survival and subsequently have been shown to
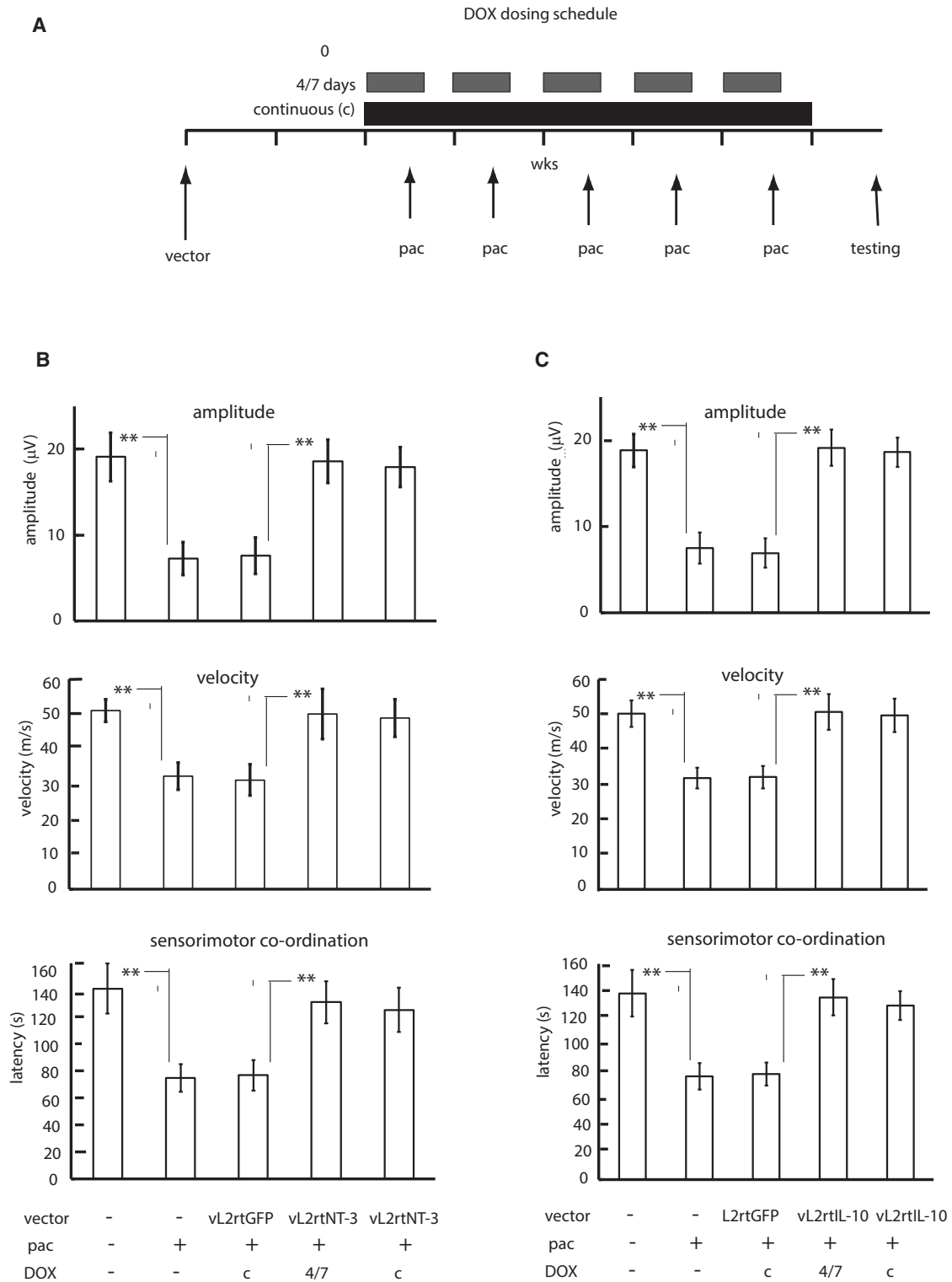
Figure 5. Protective Effect of Intermittent Expression of NT-3 or IL-10 from the Vectors on the Development of Paclitaxel-Induced Peripheral Neuropathy

Animals were subcutaneously inoculated with either vector vL2rtNT-3 or vL2rtIL-10 or control vector vL2rtGFP and injected i.p. with paclitaxel (16 mg/kg) once a week for 5 weeks to mimic paclitaxel-induced peripheral neuropathy. Naïve animals and animals receiving paclitaxel only served as the symptom-negative and -positive controls.
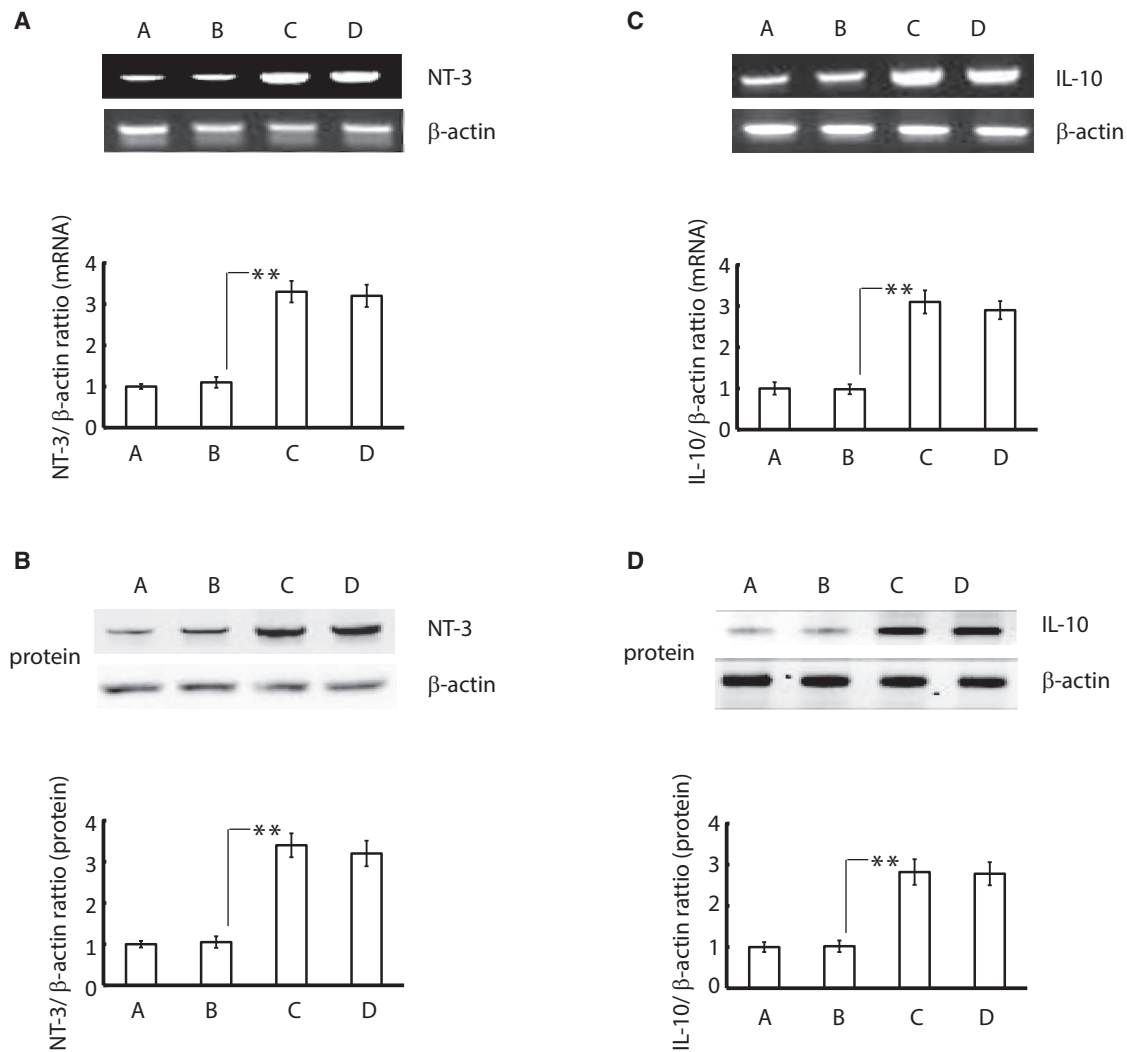
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Figure 6. No Effect of Repeated Paclitaxel Treatments on Induced Expression of the Transgenes from the Vectors

To analyze the effect of repeated paclitaxel treatments on the induced expression of the transgenes from the vectors, naive animals and animals receiving paclitaxel alone were fed normal chow for 4 more days, whereas vector-infected animals were fed DOX-containing chow for 4 additional days 1 week after the fifth injection in the repeated paclitaxel treatment schedule. L4-6 DRGs of both hindfeet were dissected for determination of NT-3 or IL-10 mRNA and protein, measured by semiquantitative PCR and western blot. (A) Semiquantitative NT-3 PCR and NT-3 mRNA relative amount. (B) NT-3 western blot and NT-3 protein relative amount. (C) Semiquantitative IL-10 PCR and IL-10 mRNA relative amount. (D) IL-10 western blot and IL-10 protein relative amount. A, naive animals fed normal chow; B, paclitaxel-treated animals fed normal chow; C, vector-inoculated, paclitaxel-treated animals previously fed DOX-containing chow 4 days per week fed DOX-containing chow for 4 additional days; D, vector-inoculated, paclitaxel-treated animals previously fed DOX-containing chow continuously fed DOX-containing chow for 4 additional days.

also provide substantial neuroprotective effects in vitro and in vivo.[26,27] Pre-clinical studies indicated that systemic administration of neuroprotective peptides prevents the emergence or progression of neuropathy resulting from diabetes, HIV infection, pyridoxine, acrylamide, or chemotherapeutic agents, including cisplatin, oxaliplatin, vincristine, thalidomide, and paclitaxel.[26–31]

However, the findings of pre-clinical studies have failed to translate into clinical therapies because of the major issue of dosing, among many reasons for this failure. Nerve growth factor (NGF), for example, administered intraperitoneally at 3–5 μg/kg, prevents neuropathy in rodents,[25,32,33] but clinical trials indicated that NGF administered at doses much lower than 3 μg/kg causes off-target

Sensorimotor coordination and sensory nerve electrophysiological function (amplitude and conduction velocity) were evaluated 5 weeks after the initiation of paclitaxel treatment. (A) Schematic of the treatment protocol. (B) Effect of intermittent expression of NT-3 (top, sensory nerve amplitude; center, sensory nerve conduction velocity; bottom, sensorimotor coordination). (C) Effect of intermittent expression of IL-10 (top, sensory nerve amplitude; center, sensory nerve conduction velocity; bottom, sensorimotor coordination). *p < 0.05, **p < 0.01.

effects, whereas NGF administered at reduced doses does not produce therapeutic effects at all.[34]

The off-target side effects associated with systemic application of neuroprotective peptides for treatment of neuropathy can be overcome by gene transfer using virus-based vectors from which short-lived peptides are continuously expressed only in the targeted cells and released locally. Among all available gene transfer vectors, a replication-deficient HSV-based vector is particularly well suited for prevention of peripheral neuropathy because peripheral neuropathy is mainly sensory, and an HSV-based vector selectively targets DRG neurons after subcutaneous injection into foot skin.[35] In preclinical studies in models of neuropathy resulting from diabetes or caused by treatment with cisplatin or by overdose of pyridoxine, published studies demonstrate that gene transfer to the DRGs of neurotrophic peptides, achieved by subcutaneous inoculation of HSV vectors, prevents the progression of various forms of neuropathy.[12–16]

Neuroprotective peptides are neuroprotective, but prolonged continuous expression of these molecules might cause local unwanted side effects. Thus, it would be preferable to be able to put the expression of neuroprotective peptides in an inducible system so that gene expression can be turned on and off as needed to maximize the clinical applications of neuroprotective molecules for treatment of chronic conditions, such as chemotherapy-induced peripheral neuropathy (CIPN). Therefore, we developed modified tet-on based regulatable HSV vectors and examined the effect of intermittent expression of EPO from such a regulatable vector on sensory nerve electrophysiological function in diabetic animals. We found that expression of EPO from the regulatable vector 3 days per week preserves sensory nerve function for 3 month (the end of experiments) in diabetic animals.[17] These results suggest that the modified tet-on system can give rise to prolonged regulated transgene expression and that intermittent expression of a neuroprotective peptide produces therapeutic benefits.

CIPN is an important clinical problem and also represents an excellent model to test the regulatable vectors for therapeutic benefits because chemotherapies are administered in cycles, and the half-lives of chemotherapies are short ranging from half an hour to several hours.[18,19,36,37] Thus, intermittent expression of a neuroprotective peptide from a regulatable vector surrounding each cycle of chemotherapy treatment should be sufficient to protect against the development of CIPN. As expected, intermittent expression of NT-3 or IL-10 preserves sensorimotor coordination and sensory nerve electrophysiological function in repeatedly paclitaxel-treated animals.

Paclitaxel induces peripheral neuropathy by causing large-fiber degeneration in the sciatic nerve.[10] It was originally thought that paclitaxel causes nerve fiber degeneration by binding and stabilizing β-tubulin, the same mechanism through which paclitaxel kills tumor cells because, in early pre-clinical studies in which paclitaxel was injected into the sciatic nerve, impaired axonoplasmic transport resulting from paclitaxel binding to β-tubulin was observed in animals.[38–41] However, recent animal studies demonstrated that paclitaxel treat-

ment via either intravenous or intraperitoneal injection, which is more clinically relevant, induces impairment in nerve function long before β-tubulin accumulation is seen,[6] suggesting that mechanisms other than β-tubulin stabilization might be responsible for paclitaxel-induced neuropathy. Nevertheless, we believe that NT-3 protects animals against paclitaxel-induced peripheral neuropathy, most likely by binding to its cognate receptor TrkC to activate survival pathway phosphatidylinositol 3-kinase (PI3K)/AKT signaling, the same mechanism through which NT-3 prevents the development of various toxic insult-induced neuropathies, because no other NT-3 neuroprotective mechanisms have been reported.[42,43]

IL-10 can function as a neuroprotective peptide by engaging its receptor, IL-10R, to activate both Jak-Stat3 and PI3K/AKT to enhance the expression of the anti-apoptotic protein,[44] whereas IL-10 has been shown to be an anti-inflammatory cytokine by inhibiting the production of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α).[45,46] Because our unpublished work strongly suggests that paclitaxel induces neuropathy by binding to Toll-like receptor 4 to upregulate the expression of TNF-α, these results suggest that IL-10 prevents paclitaxel-induced neuropathy either through blockade of pro-inflammatory cytokine production or via the blockade together with its neuroprotective property.

In this study, our work indicates that intermittent expression of IL-10 or NT-3 for 4 days of 7 days (per week), with peak expression reached on the day of each paclitaxel treatment, prevents paclitaxel-induced peripheral neuropathy. However, a shorter time period of IL-10 or NT-3 expression surrounding each paclitaxel treatment while maintaining the therapeutic benefit could be possible. Therefore, experiments need to be conducted to optimize the DOX treatment schedule to determine the minimal DOX treatment needed for protection.

We chose to develop therapies for the prevention of paclitaxel-induced peripheral neuropathy rather than treatment because we believe prevention is a reasonable goal: the development of paclitaxel-induced peripheral neuropathy is dose-dependent and highly predictable, the onset is gradual but subacute, and strategies to recover after axonal degeneration has occurred are likely to be more complex and less successful.

In short, our work demonstrates that intermittent expression of neuroprotective factors surrounding each paclitaxel treatment protects animals against paclitaxel-induced peripheral neuropathy. Because the inducer needed for induction of transgene expression from the vector, DOX, is an approved drug used in the clinic, these results warrant a clinic trial to test the vectors for prevention of paclitaxel-induced peripheral neuropathy in patients.

MATERIALS AND METHODS
Cells and Viruses
7b cells (provided by Joseph Glorioso, University of Pittsburgh), derivatives of Vero cells that express HSV ICP27 and ICP4, were maintained and grown in DMEM supplemented with 10% fetal bovine

serum (Atlanta Biologics), 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, 0.03% glutamine, and 0.375% sodium bicarbonate (Invitrogen) in a 5% CO_2 atmosphere. HSV vector UL41E1G6 (provided by Joseph Glorioso, University of Pittsburgh) (an HSV-null mutant deleted for the essential gene ICP27 with both copies of the IE gene ICP4 replaced by GFP that was used as the parental vector for generating the regulatable vectors used in this study) and vectors QL2HNT3[47] and QHIL10,[46] which contain a rat NT-3- or IL-10-encoding region in the viral genome, respectively, and were used as the template for amplifying the two genes for cloning, were propagated in 7b cells. Virus titer was determined by plaque assay.[48]

Construction of the Regulatable NT-3- and IL-10-Expressing Plasmids pSASB3-L2rtNT-3 and pSASB3-L2rtIL-10

To generate the two prolonged regulatable transgene expressing plasmids, full-length NT-3 and IL-10 were amplified from infected cellular DNA isolated from 7b cells infected with either vector QL2HNT3[47] or QHIL10[46] by PCR, with the forward primer containing a BamH1 site at the 5′ end and the reverse primer possessing a HindIII site at the 5′ end, respectively. The PCR product, after being cleaved by BamH1 and HindIII, was ligated into the BamH1 and HindIII sites of plasmid pTRE-Tight (Clontech Laboratories), which contains a DOX-responsive inducible promoter, resulting in plasmids pTRE-Tight-NT-3 and pTRE-Tight-IL-10. The inducible NT-3 and IL-10 expression cassettes were released from plasmids pTRE-Tight-NT-3 and pTRE-Tight-IL-10 by Xho1 cleavage and ligated into the Xho1 site of plasmid pSP72-link-L2-tet-on, which expresses the transactivator in the tet-on system under the control of HSV LAP-2, to achieve prolonged regulatable expression of the transgene from the tet-on system.[17] The entire NT-3- and IL-10-inducible expression units, including the inducible NT-3 or IL-10 expression cassette and the transactivator expression element, were released from the resulting plasmids pTRE-Tight-NT-3-L2-tet-on and TRE-Tight-IL-10-L2-tet-on by BglII cleavage and cloned into the BamH1 site of the endpoint plasmid pSASB3 (Joseph Glorioso, University of Pittsburgh) to facilitate the construction of HSV-based NT-3 and IL-10 expression vectors.[17] The resulting plasmids, pSASB3-L2rtNT-3 and pSASB3-L2rtIL-10, were used for generation of the corresponding recombinant vector.

Construction of Long-Term Regulatable NT-3 and IL-10 Expression Vectors

The recombinant HSV vectors vL2rtNT-3 and vL2rtIL-10, which express NT-3 and IL-10, respectively, were generated based on homologous recombination between plasmid pSASB3-L2rtNT-3 or pSASB3-L2rtIL-10 and the parental replication-deficient HSV genome DNA (UL41E1G6) in complementing 7b cells, and two copies of the regulatable NT-3 or IL-10 expression unit were inserted into the ICP4 loci of the HSV genome because the regulatable gene expression unit in the endpoint plasmid pSASB3 was flanked by sequences corresponding to the up- and downstream sequences of the ICP4 open reading frame in the HSV genome, which served as the basis for homologous recombination between the plasmid DNA and the viral genome. Transfection and infection for generating the recombinant NT-3 or IL-10 expression vector and the screening, purification, and confirmation of the recombinant virus were conducted using methods described previously.[49] Briefly, recombinant viruses were obtained by screening under florescence microscope via green-black selection because GFP was inserted into the ICP4 loci of the parental viral genome and confirmed by PCR amplification using the primers used for NT-3 or IL-10 cloning mentioned above and DNA sequencing.

Rat Model of Paclitaxel-Induced Peripheral Neuropathy

Paclitaxel (6 mg/mL in 50:50 cremophor EL/ethanol, Bristol-Myers Squibb) was diluted to 2 mg/mL with saline just before use. Male Sprague-Dawley rats weighing 200–250 g were injected i.p. with paclitaxel (16 mg/kg) once a week for 5 weeks to model paclitaxel-induced peripheral neuropathy.[10] 50:50 cremophor EL/ethanol diluted 3-fold with saline was used as the vehicle control.

Vector Injection

Animals were anesthetized by isoflurane and laid on one side on a heating pad. Approximately 3×10^8 plaque-forming units (PFUs) of vector in 30 μL PBS were inoculated subcutaneously in both hindfeet using a 50 μL microinjector.[17,20] vL2rtNT-3 and vL2rtIL-10 were used as the test vectors, whereas the regulatable vector vLrtGFP expressing GFP served as the control vector. All animal procedures in this study were performed in compliance with approved institutional animal care and use protocols.

Sensorimotor Coordination

Sensorimotor coordination was assessed by rotarod test.[50] Rats were transferred to the laboratory 30 min prior to commencement of the experiment and gently placed on the rotating beam of the rotarod test device, which was pre-programmed at 5 rpm for 30 s, followed by increasing speeds with an acceleration of 0.1 rpm/s with the maximal speed set at 22 rpm, which was reached within 200 s. The length of time the rats remained on the beam before falling was recorded. Testing was repeated three times.

Electrophysiological Testing

Sensory nerve recordings were performed on the right leg using a Nicolet Viking III electromyography (EMG) device (Nicolet Biomedical) as described previously.[20] Rats were anesthetized with isoflurane for testing, and subcutaneous temperature was maintained at 36°C–37°C. The hindlimbs were secured at an angle of 30° relative to the body, and a ground electrode was inserted into the tail. The recording electrode was inserted into the sciatic notch, the stimulating electrode was placed at the ankle, and the reference electrode was positioned at the first digit.

RNA Isolation and Reverse Transcription

Tissues were homogenized and isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA quality was monitored by agarose electrophoresis, and concentration was measured using a UV spectrophotometer. Reverse transcription was conducted using the Superscript Reverse Transcriptase II kit from Invitrogen.

The RNA input was 2 μg, and poly-T was used as the primer for reverse transcription. The synthesized complementary DNA was used for quantification of mRNA levels by semiquantitative PCR.

PCR and Semiquantitative PCR

PCR amplification was carried out in 50 μL using a standard protocol with an initial denaturing step at 94°C for 5 min, followed by either 40 cycles (DNA amplification for cloning and vector confirmation) or 30 cycles (semiquantitative PCR for quantifying mRNA levels) at 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min. The primers used for PCRs were as follows: for rat NT-3 PCR, forward primer 5′-GGG GGA TCC CGC CAC CAT GTC CAT CTT GTT TTA TGF G-3′and reverse primer 5′-GGG AAG CTT TCA TGT TCT TCC GAT TTT TCT TGA C-3′; for rat IL-10 PCR, forward primer 5′-GGG GGA TCC CGC CAC CAT GCC TGG CTC AGC ACT G-3′ and reverse primer 5′-GGG AAG CTT TCA ATT TTT CAT TTT GAG TGT C-3′; for β-actin PCR, forward primer 5′-CAG TTC GCC ATG GAT GAC GAT ATC-3′ and reverse primer 5′-CAC GCT CGG TCA GGA TCT TCA TG-3′.

Western Blot

The western blot was performed as described previously.[17,20] Lysates were prepared from L4-6 DRGs dissected from animals in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing protease inhibitor cocktail (Sigma) by homogenization, sonication, and centrifugation. Protein concentration was determined using the BCA protein assay kit (Thermo Scientific). 100 μg of proteins was separated on 4%–20% gradient SDS–PAGE gels (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Thermo Scientific). Immunoblots were incubated with the primary antibodies anti-NT-3 (1:1,000, Abcam), anti-IL-10 (1:1,000, Sigma), or anti-β-actin (1:1,000, Sigma) for 2 hr at RT followed by incubation with an HRP-conjugated secondary antibody (1;2000, Santa Cruz Biotechnology, Dallas, Texas) 1 hr at room temperature (RT) after being washed by 0.05% Tween 20-containing PBS (PBST) three times. Protein bands were visualized using an enhanced chemiluminescent substrate (Thermo Scientific) after membranes were washed by PBST three times, and the amount of protein was quantitated using ChemiDoc (Bio-Rad). Protein levels for each sample were normalized to β-actin and compared between the treated and control groups.

ELISA for IL-10 and NT-3 in Medium

To evaluate NT-3 or IL-10 concentration in medium, medium was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was collected. IL-10 or NT-3 concentration in the supernatants was measured using IL-10- or NT-3-specific ELISA kits (Thermo Scientific).

Data Analysis

The significance of the difference between treatment and control was determined using multivariate analysis of variance or the Kruskal-Wallis test for nonparametric measures. Single comparisons were performed with Student's t test, with a $p < 0.05$ considered significant. All data are expressed as means ± SEM.

AUTHOR CONTRIBUTIONS

D.K. conducted the research and reviewed and edited the manuscript. Z.W. designed the experiments, conducted the research, and wrote the manuscript.

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