Intranasal Administration of PEGylated Transforming Growth Factor-α Improves Behavioral Deficits in a Chronic Stroke Model

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We previously demonstrated that infusion of transforming growth factor (TGF)-α after chronic middle cerebral artery occlusion (MCAO) stimulates stem and progenitor cell proliferation, migration, and neuronal differentiation associated with the amelioration of neurologic impairment. But the use of TGF-α in humans is impeded by impracticality of intracranial infusion and the inability of intravenous TGF-α to cross the blood-brain barrier. Here we investigated whether intranasal delivery of PEGylated TGF-α (PEG-TGF-α) is a viable alternative. We found that intranasal PEG-TGF-α can also induce the proliferation of neural progenitors and their migration to the damaged striatum, and that this is associated with significant behavioral improvement in the MCAO model. This nonsurgical approach represents a potential therapeutic strategy for human patients. **Key Words:** Adult stem cell—injury—proliferation—intranasal—neurotrophic factor—PEGylation.

Transforming growth factor (TGF)-α is a mitogenic polypetide expressed in the developing and adult central nervous system (CNS),[1-3] with pleiotropic functions mediated through activation of the epidermal growth factor receptor.[4] In the adult brain, TGF-α is reportedly necessary for regulating the proliferation of progenitor cells in the subventricular zone.[5,6] TGF-α also has been reported to stimulate neuronal differentiation in vitro, with 90% of cells acquiring a photoreceptor phenotype after TGF-α exposure.[7] In contrast, transgenic adult mice lacking TGF-α exhibited a 50% decrease in the number of dopaminergic neurons of the substantia nigra and a 20% decrease in the volume of the dorsal striatum.[8] We previously reported the efficacy of TGF-α in stimulating neurogenesis in a Parkinson disease model and a long-term stroke model in rats, accompanied by behavioral improvement.[9-11] Consequently, TGF-α appears to be an important candidate for the treatment of early- and late-onset neurologic disorders in humans.

One obstacle impeding the use of TGF-α in human therapy is that this growth factor, like many other large molecules, is not readily transported across the blood-brain barrier (BBB). TGF-α proteins range in size from 5 to 35 kDa[12-14] and reportedly accumulate in the cerebral vasculature after intravenous bolus injection.[15] Another obstacle is that the protein can be degraded by proteinase activity and sequestered by cells responding to immunogenicity. Existing approaches to bypassing the BBB they typically involve invasive neurosurgeries, which restrict clinical application to only the most severe cases. Noninvasive techniques that are capable of delivering growth factors to the CNS would represent a therapeutic alternative to surgery. Since it was first demonstrated that intranasally administered neurotrophic growth factor (NGF) can bypass the BBB, the prevalence of intranasal administration for CNS treatment has grown considerably.[16-21] In this mode of administration, therapeutic molecules traverse the BBB through the olfactory pathway and the less-studied trigeminal neural pathway.[16,22] An important advantage of intranasal administration is that factors are delivered directly into the brain, thereby avoiding adverse systemic effects.

The stability of therapeutic proteins under physiological conditions can be enhanced by covalent binding to

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polyethylene glycol (PEG), a process also known as PEGylation.[23] PEGylation has been used to improve drug delivery into the brain with brain-derived neurotrophic factor, where it reduces systemic clearance and enhances neuroprotection.[24,25] In the present study, we assessed the effect of intranasally administered PEG-TGF-α on cellular proliferation and on animal behavior in a chronic- phase model of stroke. We found that intranasal administration of PEG-TGF-α induced significant cellular proliferation (a prerequisite for restorative neurogenesis), and also improved behavioral deficits. We conclude that intranasally administered PEG-TGF-α is a viable therapeutic strategy for treating chronic stroke injury.

Materials and Methods

PEGylation of TGF-α

The PEG-TGF-α used was manufactured by American Peptide Co. First, 4 mg of TGF-α protein (R&D Systems) was incubated with 75 mg of a 20-kDa polyethylene glycol-N-hydroxy succinimide ester in dimethyl sulfoxide overnight. The 50-amino acid sequence of the human TGF-α protein is as follows: Val-Val-Ser-His-Phe-Asn-Asp-Cys-Pro-Asp-Ser-His-Thr-Gln-Phe-Cys-Phe-His-Gly-Thr-Cys-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-Pro-Ala-Cys-Val-Cys-His-Ser-Gly-Tyr-Val-Gly-Ala-Arg-Cys-Glu-His-Ala-Asp-Leu-Leu-Ala. The resulting product was precipitated out of cold ether, washed with cold ether 3 times, and then dried under high vacuum. Dialysis was performed to remove small-molecule impurities. The lyophilized final product was diluted in phosphate-buffered saline (PBS) at 0.67 mg/mL.

Experimental Groups

The animals used in the study were categorized as follows: middle cerebral artery occlusion (MCAO)-PBS, n = 7; MCAO-PEG-TGF-α, n = 7; sham-PBS, n = 3; sham-PEG-TGF-α, n = 3; no procedure-PBS, n = 6; and no procedure-TGF-α, n = 6.

Transient Middle Cerebral Artery Occlusion

The MCAO procedure used has been described in detail previously.[11] In brief, 3-month- old adult Sprague-Dawley rats underwent an ischemic procedure using an intraluminal filament technique. The animals were anesthetized with xylazine (8 mg/kg) and ketamine (100 mg/kg) (Western Medical Supply, Arcadia, CA). The left common carotid artery and external and internal carotid arteries were exposed, and the external carotid, left occipital, and left pterygopalatine arteries were cauterized. A 4-0 nylon monofilament suture with silicon (Doccol, Redlands, CA) was gently introduced via the left external carotid artery stump into the lumen of the internal carotid artery until it reached and occluded the middle cerebral artery. A surgical clip (Fine Science Tools, Foster City, CA) was placed into the lower part of the internal carotid artery during the time of the occlusion. After 90 minutes of occlusion, the suture was withdrawn from the internal carotid artery, and the wound was sutured. Only rats with clear neurologic deficits were infused with growth factors or control solutions. For sham animals, the same surgical procedure was performed, except that a suture was not inserted into the vessel. A femoral artery catheter was placed for the continuous monitoring of mean arterial blood pressure and the measurement of arterial blood gases before, during, and after ischemia.

Intranasal Administration of TGF-α

One month after the MCAO surgery, 20 μg of PEG-TGF-α, 20 μg of un-PEGylated TGF-α, or vehicle (PBS) was given in nose drops. The drops were given over a 20-minute period, alternating every 2 minutes between the left and right nares. The mouth and the opposite naris were closed during administration to promote inhalation of the drops into the upper nasal cavity. One dose per week was given to each animal for 4 weeks.

Administration of BrdU

At the commencement of the intranasal administration, 50 mg/kg of 5′-Bromo-2-deoxyuridine (BrdU; Boehringer Mannheim, Indianapolis, IN) was administered intraperitoneally once per day for 4 weeks.

Behavioral Testing

Animals were assessed for both the severity of the MCAO stroke and functional improvement resulting from TGF-α infusions with 2 highly sensitive tests, the corner test and the cylinder test. Both tests were performed once a week. The purpose of this schedule was to record a pattern of behavior before and after the delivery of the growth factor, including baseline measurements from before the ischemic injury. An average of the 3 trials for each week was compiled. Trends were noted over the timeline of the experiment, and the significance of differences between experimental groups was assessed using the Student *t*-test and analysis of variance (ANOVA).

Corner Test Methodology

In this test, the edges of two $30 \times 20 \times 0.5$ cm^3 Plexiglas boards (Interstate Plastics, Sacramento, CA) were placed together to form a 30-degree angle in front of the adult rat. The 2 boards were then moved slowly toward the rat until both sides of the rat's vibrissae were stimulated simultaneously. This stimulation, as well as the clarity of the Plexiglas, encourages the animal to rear and turn to exit at the open end. Each test comprised 10 trials, in which the total number of left and right turns was recorded. Nonlesioned rats turned randomly in either

direction, while ischemic rats preferentially turned toward the nonimpaired side. Our results are reported as the percentage of ipsilateral (injury site, left side) versus contralateral turns. The test was conducted a total of 3 times per day to achieve statistical significance.

Cylinder Test Procedure

Three trials of the cylinder test were conducted to evaluate forelimb use asymmetry for weight shifting during vertical exploration. The test was given to all groups once a week in a room controlled for light and sound. Each rat was placed in a transparent cylinder 20 cm in diameter and 30 cm in height with an open top (Interstate Plastics). It was counted whether the rat used its right forepaw, left forepaw, or both paws at the same time for support for 3 trials of 10 paw touches, for a total of 30 noted paw touches per weekly test. For data analysis, the percentage of left (ipsilateral to the MCAO lesion) paw use was determined for each trial using the following equation:

100 (ipsilateral paw touches + 0.5 (both paw touches))/ (ipsilateral + contralateral + both paw touches).

Cardiac Perfusion

Two months after stroke, the rats were sacrificed by intracardiac perfusion, using methods consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The rat was anesthetized using intraperitoneal ketamine (100 mg/kg) and xylazine (8 mg/kg). In the deeply anesthetized animal, a thoracic incision was made and the pericardium was opened. A 0.9% saline solution was perfused intracardially, through the left ventricle, and released via an incision in the right atrium. This was followed by perfusion with a 4% paraformaldehyde solution. The brain was extracted and postfixed at 4°C in 4% paraformaldehyde and cryoprotected in 30% sucrose. Finally, the extracted brain was frozen in methylbutane (Sigma) at −20°C.

Histological Studies

The brain was cut in the coronal plane at 40 μm in a cryostat at −20°C. For the colocalization of BrdU with nestin, immunohistochemical double-staining was performed on free-floating sections. The tissue slices were treated with 50% formamide and 50% 2 × standard saline citrate (SSC) at 65°C for 1 hour to denature the DNA. This was followed by 3 washes in 2 × SSC at room temperature, denaturation with 2 M HCl in H_2O at 37°C for 30 minutes, neutralization with 0.1 M borate buffer (pH 8.5) for 10 minutes, and finally a rinse in PBS (pH 7.4) for 5 minutes. Sections were then blocked in 3% horse serum with 0.3% Triton X-100 in PBS for 1 hour at room temperature, and then probed with a rat monoclonal anti-BrdU (1:100; Accurate Chemical & Scientific, Westbury, NY) and mouse anti-nestin monoclonal antibody (1:100; Chemicon, Temecula, CA). The tissues were incubated overnight at room temperature and subsequently rinsed in PBS. Alexa Fluor secondary fluorescent antibodies (Invitrogen, Carlsbad, CA) were incubated for 1 hour at room temperature, and the prepared sections were washed in PBS and mounted. Epifluorescence microscopy was performed with a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY).

Quantitative Analysis

Cells labeled by BrdU and nestin were counted from 3 consecutive 40-μm sections in each animal. The fields were randomly selected in the infarcted striatum starting at +1.2 mm from the bregma (anteromedial lateral ventricle/striatum). Three animals were counted in each of 2 treatment groups, MCAO-PEG-TGF-α and MCAO-PBS, for a total of 6 animals and 9 fields per treatment group (3 sections per animal). The counts were performed on an Olympus DSU Spinning Disk Confocal Microscope (Olympus, Williston, VT) at 63 × magnification, and a microcator attached to the microscope stage was used to facilitate identification of colocalized signals.

Statistical Analysis

The data are expressed as mean ± standard deviation. Statistical analysis between groups was performed using the unpaired Student *t*-test and a post hoc Bonferroni test. The data passed the Shapiro-Wilk test and Anderson-Darling normality tests included in an ANOVA analysis. The *P* value for the comparisons is included in the results and/or figures.

Results

Intranasal PEG-TGF-α Stimulates Proliferation and Migration of Progenitors

We used our recently described rodent model of chronic stroke[11] to assess the effectiveness of intranasally administered PEG-TGF-α. We considered the stroke lesion sizes induced by MCAO to be homogenous based on both the gross anatomy and the full extent of behavioral loss observed across all of the MCAO-treated groups, because similar behavioral deficits have been shown to be indicative of similarly sized lesions.[26,27] One month after the MCAO procedure, we began intranasal administration of PEG-TGF-α at the rate of one 20-μg dose per week. When BrdU was given every day beginning at the onset of PEG-TGF-α administration, we found a substantial increase in the number of BrdU-labeled cells compared with controls in the lateral wall of the forebrain lateral ventricle on the injured side (Fig 1D and E). Expression of the neural stem cell marker nestin[28,29] also was increased compared with controls, but this appeared to be limited to the infarct site (Fig. 1 G and H). The number of cells double-labeled by BrdU and nestin increased

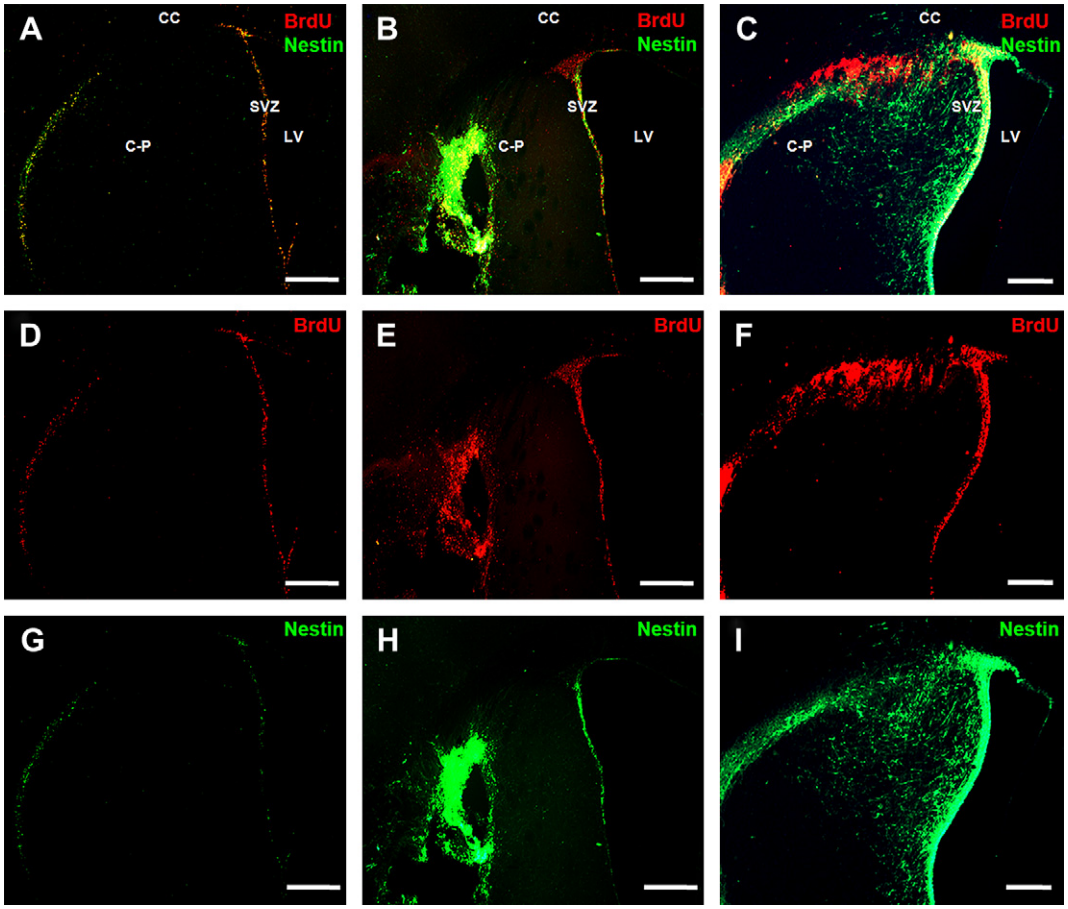

Figure 1. *Proliferation and migration of neural stem cells induced by intranasal administration of PEG-TGFα. Immunofluorescent double-label images of coronal brain sections 2 months post-MCAO in adult rats. (A, D, and G) A control animal that received only PBS after the MCAO. The hemisphere ipsilateral to the MCAO infarct is depicted and stained for both BrdU incorporation and nestin. (B, E, and H) The ipsilateral hemisphere of an animal that received 1 weekly intranasal treatment of 20 μg of PEG-TGFα for 4 weeks, stained for both BrdU and nestin. (C, F, and I) The same hemisphere in an animal that received direct intracranial infusion of 20 μg of TGFα over a 4-week period. In (I), the lesion site lies outside the frame of the image. (Scale bar: 500 μm.) LV, lateral ventricle; C-P, caudate putamen; CC, corpus callosum; S, septum; SVZ, subventricular zone.*

as well (Fig. 1A and B). Quantification of this increase showed nearly twice as many BrdU-labeled cells in the PEG-TGF-α- treated animals compared with the PBS controls (459.33 ± 31.50 cells vs 234.66 ± 10.11 cells). The percentage of cells double-labeled by BrdU and nestin was approximately 3-1/2-fold greater in the treated animals than in the PBS controls (326.67 ± 41.68 cells vs 86 ± 17.34 cells); however, these increases were less dramatic than the results obtained when un-PEGylated TGF-α was infused intracranially (Fig 1C, F, and I). Increased labeling was not significant in the contralateral hemisphere of MCAO/PEG-TGF-α. No increase in labeling was observed in either hemisphere of animals treated with PEG-TGF-α only. Tests of un-PEGylated TGF-α also produced no effect.

Intranasal PEG-TGF-α Promotes Behavioral Recovery

Two behavioral tests were used to assess the functional recovery of MCAO rats treated intranasally with PEG-TGF-α: the cylinder test and the corner test.[11,26,27] We found that before the development of ischemic lesions, there was constant symmetry of approximately 50% in forelimb use and turning behavior, signifying no behavioral deficit. The same results were obtained in animals treated with un-PEGylated TGF-α or PBS (Fig 2A). After the MCAO lesion, ANOVA analyses revealed a significant modification of forelimb use in all groups, with a 90%+ increase in left forelimb (unaffected) placements at days 14 and 21 ($P < .001$) compared with baseline, reflecting near-total loss of function of the affected (right) limb. However, over the 4-week treatment period, the PEG-TGF-α-treated animals gradually recovered the use of their injured forelimb. At day 71, the improvement was 57% in the PEG-TGF-α animals, compared with a right forelimb use of 26% in the PBS group ($P < .01$ by ANOVA) (Fig 2A). This recovery of function was substantiated by data from the corner test showing a significant (53%0 decrease in the number of left turns by day 71 in the PEG-TGF-α-treated animals ($P < .01$) (Fig. 2B).

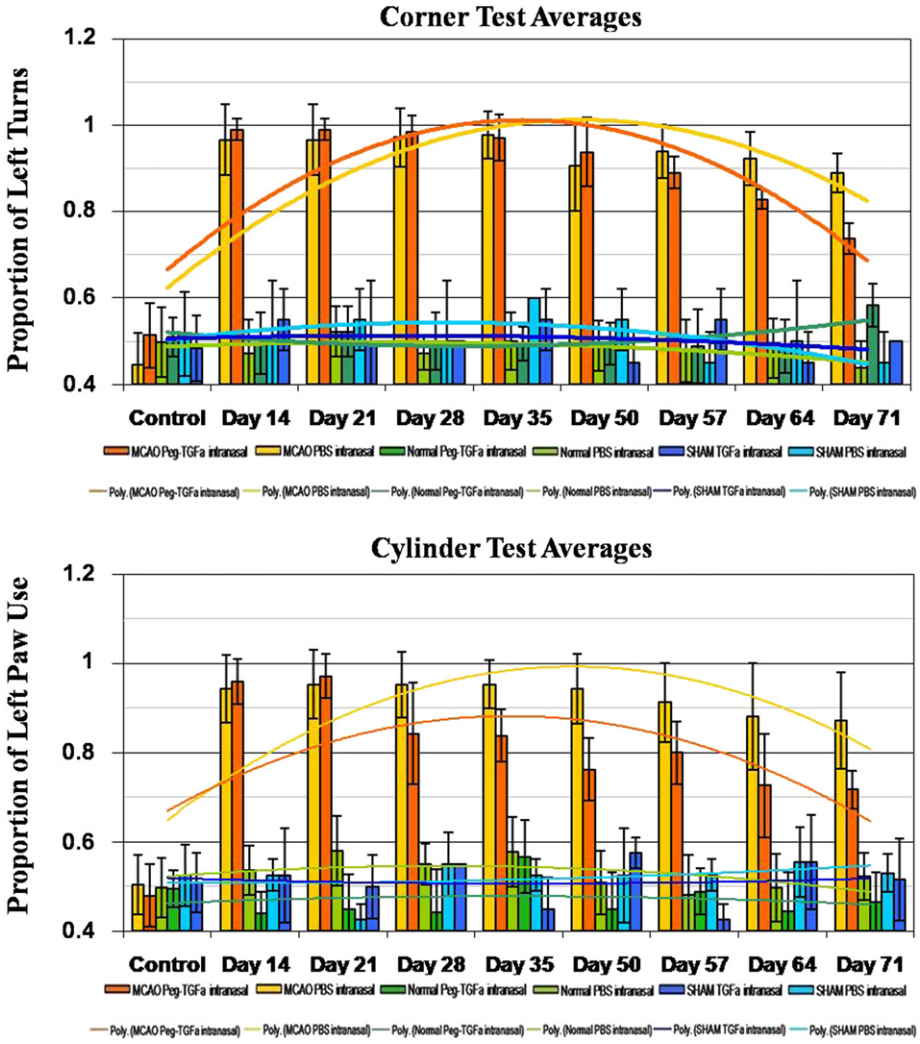

Figure 2. *Behavioral recovery. All animals were tested on 3 consecutive days before ischemia set in to obtain a baseline of side or paw preference and then once per week during the subsequent 2 months. The results are presented as left (impaired) paw use as percentage of the total paw contacts with the wall of the cylinder, or as the percentage of turns toward the injured side of the brain in the corner test. (A) In the Corner Test, the MCAO-injured animals treated with PBS, showed only marginal improvement in asymmetrical turning behavior over the testing period, whereas the PEG-TGF-a treated animals showed a statistically significant increase in turning toward the damaged side at the end of the treatment period (*P ,.05 by ANOVA). (B) Similarly, in the Cylinder Test injured animals treated with PBS demonstrated strong bias in favor of left paw use, showing only marginal improvement at the end of the study. In contrast, the PEG-TEFa-treated group exhibited a partial, but significant, recovery in right paw use at 8 weeks after administration. Data are mean6DEM. *P,.001 significantly different from all other groups (1-way ANOVA).*

Discussion

We previously reported that in a rat chronic stroke model, intracranial administration of TGF-α induced neural stem cells in the ependymal layer and their presumptive progeny in the subventricular zone to proliferate, migrate to the infarct, and differentiate into context-appropriate neurons.[11] We now report that a similar response can be induced when PEGylated-TGF-α is delivered intranasally to chronic stroke model rats. The intermediate filament marker nestin has been commonly used to identify neural progenitor and stem cells in the mammalian brain,[28,29] and brain injury is known to upregulate nestin expression.[30] We found a significant increase in the nestin-positive population in MCAO-injured animals treated with PEG-TGF-α, and the fact that nestin was found colocalized with many BrdU-labeled cells indicates that it may be directly involved in cellular proliferation. Our results suggest that PEG-TGF-α may trigger the proliferation of neuronal progenitors, and, given the significantly improved behavioral response, it is likely that these progenitor cells participate in the generation of new neurons.

That un-PEGylated TGF-α given intranasally did not produce a similar effect suggests that it is susceptible to degradation and probably has a short half-life in the nasal environment. More study is needed to explore the specific pathway by which PEG-TGF-α enters the brain and the points along that pathway at which degradation might occur. It has been proposed that intranasally administered NGF is transported either via intercellular clefts in the olfactory epithelium or by an extracellular pathway along neuronal processes,[16,31] and we presume that PEG-TGF-α also might utilize these routes to gain entry into the brain.

Although intranasally administered PEG-TGF-α did produce a significant increase in BrdU incorporation and nestin expression in the stroke-injured side of the brain, the response was weaker compared to that produced when TGF-α was infused intracranially. This histological finding mirrors the behavioral results, which demonstrated significant improvement, but less recovery, with PEG-TGF-α intranasal administration compared with direct intracranial infusion of un-PEGylated TGF-α.[11] These differences might be attributable to the fact

that intranasal administration is a less direct route, even with PEG-TGF-α, compared with intracranial infusion. Considering that in this experiment, a total of 80 μg of PEG-TGF-α was given over the 4-week period, compared with only 20 μg given over the same period in our earlier intracranial tests, there appears to be a loss of TGF-α mitogenic activity resulting from intranasal administration.

Nevertheless, the histological and behavioral results reported here mirror those found with intracranial administration[11] and strongly indicate that intranasal delivery of PEG-TGF-α holds great therapeutic potential. These findings also affirm the results of several previous studies documenting the potential of TGF-α for treating neurologic disorders.[9-11,32,33] The facility with which PEGylated TGF-α can be administered makes it an outstanding strategy, and the targeted effect of the drug through this route compared with other, more systemic means of administration makes it a strong candidate for drug development.

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