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TITLE: Translational Pharmacologic Efficacy Studies of Glial Growth Factor 2 (GGF2) in Spinal Cord Injury Models and in the Veterinary Clinical Setting

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14. ABSTRACT The goals of our studies in year 2 were to (a) use the most effective dosages and route of administration of GGF2 on the proliferation of NG2-expressing glia cells after SCI that we identified in year 1 in long term studies of effects on functional recovery and chronic histopathology and (b) subject spinal cords from some of these rats to MRI and Diffusion Tensor Imaging (DTI) of the spinal cord to develop MRI imaging method that can detect treatment effects that improve survival and myelination of axons after SCI for potential use in future dog studies.					
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Introduction

Whittaker (2012) and Zai (2005) showed that GGF2 administration increases NG2-expressing glial progenitor cell (GPC) proliferation in surviving white matter when treatment is initiated 24 hours after spinal cord injury (SCI) in rats and mice, an effect associated with long-term beneficial effects on functional recovery following SCI (Whittaker 2012). In a recent study, subcutaneous administration of GGF2 for 1 week after SCI beginning 24 h after injury has been shown to increase the percentage of NG2-positive GPC's in residual ventral medial white matter in rats (Annual Report 22 Oct 2011 to 21 Oct 2012 for SC100266P1). Since these studies showed that GGF2 increases the number of GPC's in surviving white matter, we evaluated the effects of GGF2 using the same dosing regimen and route as well as a twice weekly maintenance dose on locomotor and bladder function recovery as well as temporal volume changes of hind limb skeletal muscle in rats following compression injury of the thoracic spinal cord. A follow-up study with a similar design with and without twice weekly maintenance dosing was recently completed.

Methods

Study 1

Eighty female Long Evans rats weighing (200-250 g) were enrolled in this study. Animals were pair housed in species-appropriate cages with enrichment items. Animals were assigned to various surgical groups based on their body weights to minimize group variance and achieve similar group body weight means. Rats were subjected to surgically-induced forceps compression-induced SCI at two severity levels (cord compressed to 0.9 mm with a rostral-caudal extent of 2.5 [moderate injury] or 4 mm [severe injury]) at the level of T9-T10 under isoflurane anesthesia. A separate group was subjected to sham-surgery.

Treatment groups:

Treatment groups were randomly assigned as follows:

Group	Animal No.	Surgical Procedure	Treatment	Dose Level (mg/kg)	Route	Regimen
1	12	0.9 X 4 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
2	11	0.9 X 4 mm	GGF2	0.24	SC	Daily for 7 days and twice weekly for 7 weeks
3	12	0.9 X 4 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
4	10	0.9 X 2.5 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
5	12	0.9 X 2.5 mm	GGF2	0.24	SC	Daily for 7 days and twice weekly for 7 weeks
6	12	0.9 X 2.5 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
7	8	Laminectomy	NA	NA	NA	NA

Endpoints:

The effects of GGF2 on open-field locomotion bladder function and changes in skeletal muscle volume were evaluated. Animals were gently handled to adapt them to behavioral testing conditions prior to surgery. Locomotor function was assessed using the 21-point open-field locomotion score (BBB) developed by Basso, Beattie and Bresnahan (Basso et al., 1995) at 2, 7 and 10 days post SCI and then weekly for 8 weeks. Urinary bladder function was evaluated daily by measuring residual urine volume (RUV) during manual bladder expression throughout the study.

Skeletal muscle volume was quantified using 3-dimensional ultrasonographic imaging of the gastrocnemius and soleus muscles on the right side at 0, 4 and 8 weeks post SCI (Vevo 2100 High Resolution Ultrasound System (Visualsonics, Toronto). Stacked images were analyzed for gastrocnemius and soleus dimensions using segmental analysis algorithms (Visualsonics, Toronto).

At the conclusion of the study, animals were euthanized under deep anesthesia by transcardial perfusion of phosphate-buffered saline followed by 4% paraformaldehyde. Spinal cord segments (area of injury and lumbar enlargement) and the medial aspect of the right lateral gastrocnemius were harvested and placed in 4% paraformaldehyde. Twenty-four hours later, muscle was transferred to 70% ethanol and the spinal cord segments were transferred to a sodium azide solution. Spinal cord segments will be subsequently paraffin embedded and sectioned using a cryomicrotome. Spinal cord tissue sections will be stained for myelin with Eriochrome cyanine in order to assess white matter sparing. Separate spinal cord sections will be evaluated for markers of neuronal plasticity (GAP43, synaptophysin and other markers). Skeletal muscle sections were stained with hematoxylin/eosin and trichrome to evaluate both the degree of skeletal muscle fibrosis (degree of collagen staining) as well as myofiber size (area) distribution.

All endpoints were assessed in a manner blinded to treatment.

Mean BBB scores, RUVs, and skeletal muscle volume changes between various groups at each of the severity levels were compared using analysis of variance (ANOVA) as well as repeated measures analysis of variance over the course of the study ($\alpha = 0.05$). When ANOVA values were significant ($p < 0.05$), group means from the treatment groups were compared to the respective vehicle control as well as other treatment groups using Dunnett's and Tukey post hoc tests, respectively ($\alpha = 0.05$).

Study 2

The methods in Study 2 were identical to those in Study 1 for surgical procedures and evaluation of the various endpoints. The difference in the study design is highlighted in the table below.

Treatment groups:

Treatment groups were randomly assigned as follows:

Group	Animal No.	Surgical Procedure	Treatment	Dose Level (mg/kg)	Route	Regimen
1	12	0.9 X 4 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
2	11	0.9 X 4 mm	GGF2	0.8	SC	Daily for 7 days
3	12	0.9 X 4 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
4	10	0.9 X 2.5 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
5	12	0.9 X 2.5 mm	GGF2	0.8	SC	Daily for 7 days
6	12	0.9 X 2.5 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
7	8	Laminectomy	NA	NA	NA	NA

Key Research Accomplishments

Study 1

Results from Study 1 demonstrated that subcutaneous administration of GGF2 24 h post-injury produced significant improvements in locomotor function as assessed by the BBB locomotor rating scale following severe and moderate compression SCI (see Figures 1 A and B, respectively).

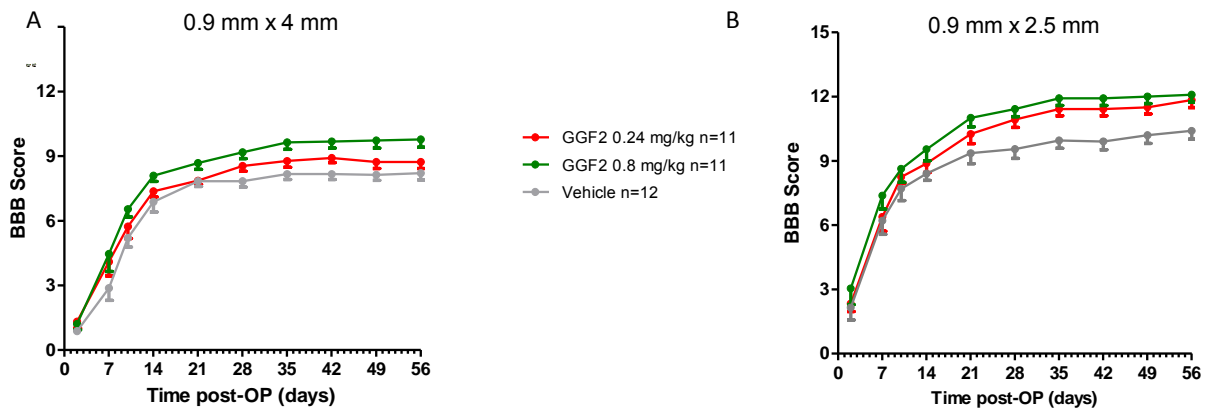


Figure 1. Effects of GGF2 on overground locomotion following severe (A) and moderate (B) compression SCI. Data are presented as mean \pm SEM. A and B. $p < 0.05$ GGF2 at both doses vs vehicle (repeated measures ANOVA).

Locomotor function was not only statistically improved by GGF2, but the changes observed in open-field locomotion were considered biologically meaningful in that at both severities of injury, the GGF2 treatment led to functionally relevant improvements on the BBB scale. Specifically, GGF2-treatment improved coordination of hind limbs during locomotion compared to vehicle treatment in moderate SCI. In severe SCI, GGF2 promoted plantar stepping compared to vehicle treated animals. Further, GGF2 treatment also improved bladder function as assessed by residual urine volumes (RUVs, Figure 2). In this case, significant reductions in residual urine volumes suggesting improvements in spontaneous voiding over time were observed at both injury severities with GGF2 treatment relative to vehicle (both dose levels in the case of moderate injury, high dose only in the case of severe injury). Skeletal muscle volume did not change significantly post-SCI for either the gastrocnemius or soleus muscles over time compared to laminectomy control animals (moderate injury subjects analyzed only, data not shown). Although GGF2 appeared to improve soleus muscle volume at a dose of 0.8 mg/kg compared to vehicle controls in the moderate injury cohorts, the effects on skeletal muscle volumes were not statistically significant at any dose tested (Figure 3). Muscle volume changes from the severe injury group are being analyzed.

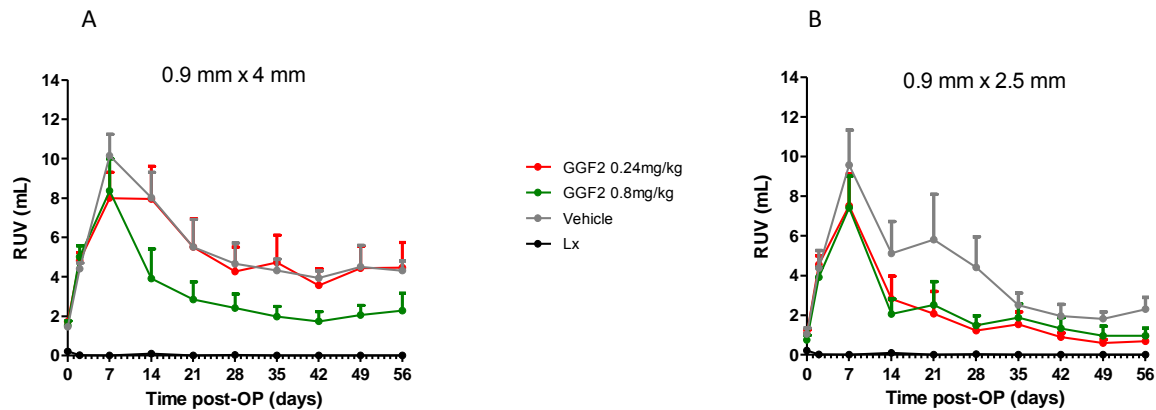


Figure 2. Effects of GGF2 on bladder function (RUVs) following severe (A) and moderate (B) SCI. Data are presented as mean \pm SEM. A. $p < 0.05$ GGF2 0.8 mg/mL vs vehicle. B. $p < 0.05$ GGF2 at both doses vs vehicle (repeated measures ANOVA).

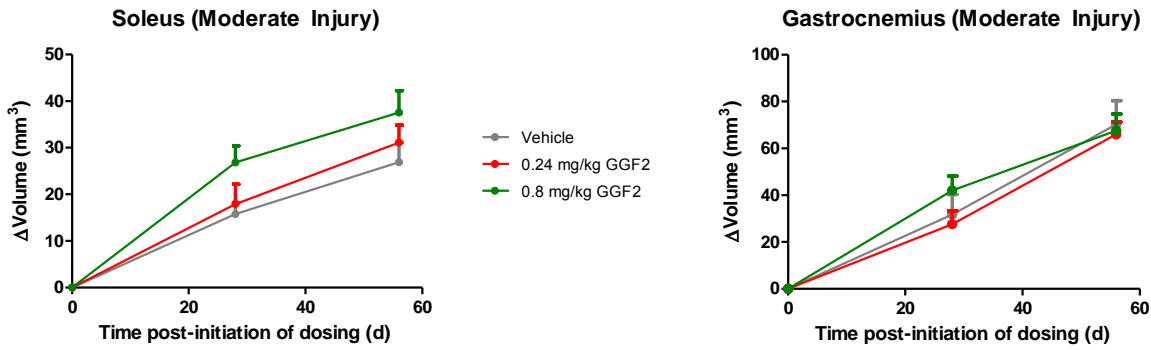


Figure 3. Effects of GGF2 on changes in gastrocnemius and soleus muscle volume (mm³) over time following moderate SCI. Data are presented as mean ± SEM.

Study 2

Results from Study 2 demonstrated that subcutaneous administration of GGF2 24 h post-injury produced only modest improvements in locomotor function as assessed by the BBB locomotor rating scale following severe and moderate (see Figures 5 a and b, respectively) SCI. The magnitude of the effect observed in the daily dosing plus maintenance arm of this study was substantially less than that observed in the corresponding arm of maintenance Study 1. The difference in BBB scores in the moderate injury group with maintenance dosing in Study 2 was not sustained at the last two observation time points.

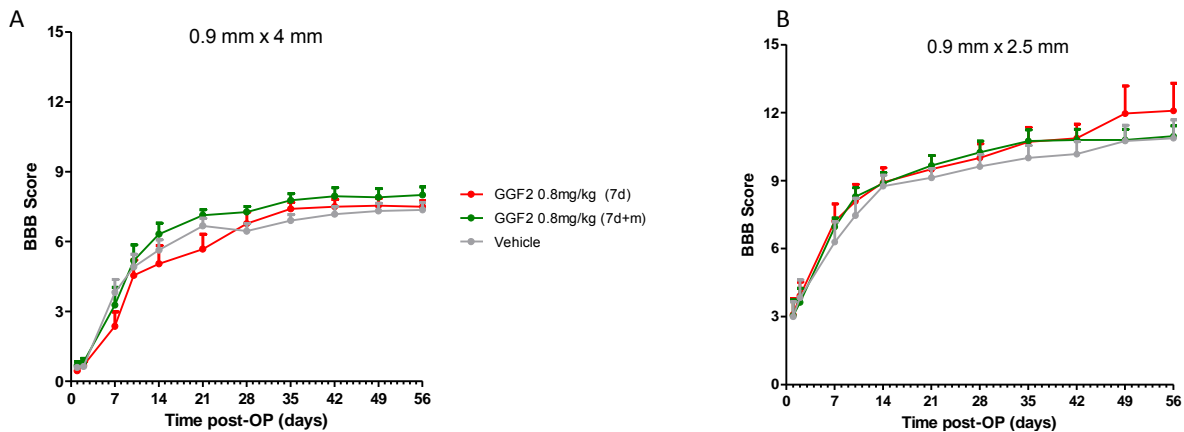


Figure 5. Effects of GGF2 on overground locomotion following severe (A) and moderate (B) SCI. Data are presented as mean ± SEM. . A. $p < 0.05$ GGF2 0.8 mg/kg with maintenance dosing vs vehicle. B. $p < 0.05$ GGF2 both dosing regimens vs vehicle (repeated measures ANOVA).

GGF2 treatment had no significant effect on RUV with either dosing regimen in the severe injury group but significantly improved RUV in the 7 day treatment group without maintenance regimen compared to vehicle treatment in the moderate injury group (see Figure 6 A and B). Gastrocnemius and soleus muscle volumes are not reported since image analysis is ongoing.

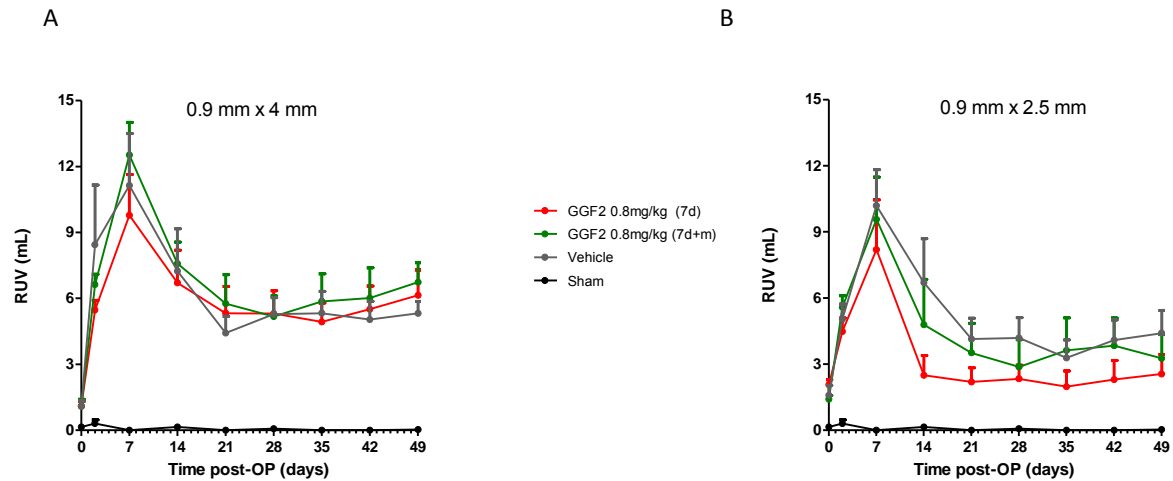


Figure 6. Effects of GGF2 on bladder function (RUVs) following severe (A) and moderate (B) SCI. Data are presented as mean \pm SEM. A. No significant differences. B. $p < 0.05$ GGF2 0.8 mg/kg (repeated measures ANOVA).

Reportable Outcomes

Animal models of SCI should bear clinical relevance for understanding disease pathophysiologic changes and developing therapies. SCI not only impacts locomotor function, but also produces skeletal muscle atrophy and autonomic dysfunction at and distal to the level of injury. These pathophysiologic elements are reproduced to a certain extent in the forceps compression model of rat spinal cord contusion injury used in these studies. In addition to capturing the features of human spinal cord injury, the forceps-compression model is also sensitive to drug treatment. Specifically, Caggiano and coworkers (1995) showed that a bacterial enzyme improved locomotor and bladder function in this model. The present studies employed this model to evaluate the effects GGF2 on locomotor and bladder function as well as skeletal muscle volume following SCI.

Locomotor function

Subcutaneous administration of GGF2 24 h post-injury produced improvements in locomotor function as assessed by the BBB locomotor rating scale following severe and moderate SCI. In Study 1, locomotor function was not only statistically improved by GGF2 vs vehicle treatment, but the changes observed in open-field locomotion were biologically meaningful (e.g. improved coordination and promoted plantar stepping in moderate and severe injury, respectively) in that at both severities of injury the GGF2 treatment led to functionally relevant endpoints on the BBB scale. In contrast, only modest improvements in locomotor function were observed with GGF2 treatment compared to vehicle controls in Study 2. While some of the effects in Study 2 were statistically significant, the changes observed in

recovery of locomotion following GGF2 treatment were deemed to be consistent but modest and in some cases not to be functionally relevant or not sustained by the final time point .

Bladder function

SCI above sacral levels leads to significant autonomic dysfunction as evidenced by an impaired micturition in humans and in rats following SCI. In rats, RUV is used to assess urinary bladder function (Caggiano, 2005; Mure, 2004; Kruse 1993). The results from Study 1 suggest that GGF2 produces a significant reduction in RUV, implying improved efficiency in bladder emptying. In Study 2, the effects of GGF2 on bladder function were less pronounced than in Study 1.

Skeletal muscle changes

The reduction in skeletal muscle mass typically observed following spinal cord injury is related to a reduction in both type I and II skeletal muscle fiber size and occurs rapidly (within 10 days) in humans (Dupont-Versteegden, 1998). It is important to preserve as much skeletal muscle as possible in order to maintain muscle strength for rehabilitation and avoid metabolic syndromes associated with muscle loss. Although SCI typically produces skeletal muscle atrophy at levels below the injury, no significant reductions in target hind limb muscle volumes following SCI were noted in Study 1 (moderate injury). Analysis is ongoing for the severe injury group in Study 1 and all groups in Study 2.

Conclusion

In conclusion, the results presented here provide evidence that GGF2 improves locomotor function following moderate and severe compression injury when treatment is initiated 24 hours after forceps compression spinal cord injury. Although improvements in locomotor function produced by GGF2 in both studies were statistically significant, the magnitude of the effects on locomotion varied greatly from study to study ranging from what is considered biologically meaningful in study 1 to much smaller changes in study 2. Study 1 provided preliminary evidence that GGF2 treatment improves bladder function following thoracic SCI; however, bladder function in Study 2 was not improved. Although the effects of GGF2 treatment on skeletal muscle changes were not significant (Study 1), histological analysis is ongoing to determine the effects of GGF2 on microscopic changes in the muscle. Additional work is required to determine the reproducibility of these findings, the mechanisms of GGF2 action, optimal treatment regimens and potential side effects.

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