An Optimized Dosing Regimen of Cimaglermin (Neuregulin 1β3, Glial Growth Factor 2) Enhances Molecular Markers of Neuroplasticity and Functional Recovery After Permanent Ischemic Stroke in Rats

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Cimaglermin (neuregulin 1β3, glial growth factor 2) is a neuregulin growth factor family member in clinical development for chronic heart failure. Previously, in a permanent middle cerebral artery occlusion (pMCAO) rat stroke model, systemic cimaglermin treatment initiated up to 7 days after ischemia onset promoted recovery without reduced lesion volume. Presented here to extend the evidence are two studies that use a rat stroke model to evaluate the effects of cimaglermin dose level and dose frequency initiated 24 hr after pMCAO. Forelimb- and hindlimb-placing scores (proprioceptive behavioral tests), body-swing symmetry, and infarct volume were compared between treatment groups (n = 12/group). Possible mechanisms underlying cimaglermin-mediated neurologic recovery were examined through axonal growth and synapse formation histological markers. Cimaglermin was evaluated over a wider dose range (0.02, 0.1, or 1.0 mg/kg) than doses previously shown to be effective but used the same dosing regimen (2 weeks of daily intravenous administration, then 1 week without treatment). The dose-frequency study used the dose-ranging study's most effective dose (1.0 mg/kg) to compare daily, once per week, and twice per week dosing for 3 weeks (then 1 week without treatment). Dose- and frequency-dependent functional improvements were observed with cimaglermin without reduced lesion volume. Cimaglermin treatment significantly increased growth-associated protein 43 expression in both hemispheres (particularly somatosensory and motor cortices) and also increased synaptophysin expression. These data indicate that cimaglermin enhances recovery after stroke. Immunohistochemical changes were consistent with axonal sprouting and synapse formation but not acute neuroprotection. Cimaglermin represents a potential clinical development candidate for ischemic stroke treatment. © 2015 The Authors.

Approximately 800,000 people each year suffer a stroke in the United States (Go et al., 2014). Eighty percent of stroke victims survive (Lloyd-Jones et al., 2009) but often have permanent neurologic deficits and significant disability, leading to an enormous healthcare burden. There is a lack of approved interventions beyond acute

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SIGNIFICANCE:

Early results with cimaglermin, a neuregulin isoform, demonstrated functional improvements when initiated up to 7 days after middle cerebral artery occlusion in preclinical animal models of stroke. The studies presented here extend these observations to show that functional improvements in a rat stroke model using cimaglermin initiated within a 24-hr poststroke window are dose and frequency dependent, with 1 mg/kg cimaglermin significantly improving all functional measures vs. vehicle. Results from markers of new neuronal axon growth and contact formation were consistent with promoting neurologic recovery.

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treatment with tissue plasminogen activator and/or intra-arterial thrombectomy, when appropriate, and physical therapy to address persistent functional deficits that affect quality of life and independence.

Many strategies in preclinical studies that have demonstrated robust acute neuroprotection when treatment is initiated in the first few hours after injury have not successfully translated to the clinical setting (Sutherland et al., 2012). More recent efforts have focused on enhancing and promoting neurorecovery during the subacute and chronic setting after stroke, which have the promise of a much wider therapeutic window than neuroprotective strategies (Chen et al., 2014). Neuregulins have been explored in middle cerebral artery occlusion (MCAO) preclinical models of stroke over the past decade, but work has largely examined and characterized neuroprotective and anti-inflammatory effects during the acute and subacute period after the ischemia (Xu et al., 2005; Guo et al., 2006; Li et al., 2007). For example, in a model of transient stroke in which ischemia was followed by reperfusion, neuregulin treatment up to 12 hr after reoxygenation demonstrated preservation of tissue when examined 24 hr after the initial MCAO (Xu et al., 2006). Furthermore, in a model of permanent MCAO (pMCAO) with neuregulin pretreatment, some protection of brain tissue was observed 24 hr after occlusion, although it was not as robust as when reperfusion of the tissue was allowed (Li et al., 2007).

The neuregulin protein growth factor family is derived from splice variants expressed from four genes, with neuregulin 1 and its isoforms being the most often studied (Falls, 2003). Neuregulins bind and signal through ErbB receptor activation across a variety of cell types (Britsch, 2007). Recent studies with the neuregulin 1 iso-form cimaglermin in a pMCAO model demonstrated functional improvements weeks after ischemia without lesion volume reduction, suggesting that more than an acute neuroprotective mechanism of action is involved (Xu et al., 2006; Iaci et al., 2010). These improvements were observed even when treatment was initiated up to 7 days after the ischemic event, suggesting an enhancement of the endogenous recovery process (neurorestorative) and possible stimulation of neural plasticity in surviving pathways.

The studies presented here extend the evaluation of cimaglermin as a potential treatment for stroke by assessing the effects of dose range and dose frequency on functional improvements in the pMCAO model in rats. In addition, histological analysis showed treatment-related increases in markers of neural plasticity, growth-associated protein 43 (GAP43), and synaptophysin (SYP) across areas of the brain both contralateral and ipsilateral to the infarcted area. Areas of enhanced GAP43 and SYP included cortex and basal ganglia. These data are consistent with induction of plasticity to enhance remodeling and promote synapse formation, which may contribute to improved function. Cimaglermin represents an attractive candidate as a stroke therapy given the relatively long time window for effective intervention and the fact that it is currently in clinical development for another indication, heart failure (Acorda Therapeutics, n.d.).

MATERIALS AND METHODS

Test Compounds

Cimaglermin (USAN, cimaglermin alfa, also known in the literature as *neuregulin1β3* or *glial growth factor 2*) was produced, purified, and characterized for bioactivity at Acorda Therapeutics (Ardsley, NY) as described previously (Iaci et al., 2010). Cimaglermin is a full-length splice variant of the neuregulin-1 gene and is produced recombinantly as a 52.6-kDa glycoprotein, with a half-life of 1–2 hr according to unpublished rat GLP studies. The vehicle consisted of 20 mM histidine, 100 mM sodium sulfate, 100 mM arginine, and 1% mannitol. Cimaglermin is soluble at 100 mg/ml and was tested in 3-month stability studies at room temperature in the vehicle. No aggregation or precipitation was observed. Dosing solutions were prepared and coded at Acorda Therapeutics before being shipped to Biotrofix. Therefore, investigators were blinded to the solutions for use in the studies.

pMCAO Model

All animal procedures were reviewed and approved by the institutional animal care and use committee at Biotrofix, and the study was conducted with the goals of minimizing pain and distress and reducing the number of animals required. Adult male Sprague Dawley rats (RRID:RGD_734476; Charles River Laboratories, Wilmington, MA) were housed and handled for behavioral assessment and acclimation for 7 days before surgery. Focal cerebral infarctions were made by permanent occlusion of the proximal right middle cerebral artery with a modification of the method of Tamura et al. (1986). Rats (300–400 g at the time of surgery) were anesthetized with 1–3% isoflurane in a 2:1 mixture of $N_2O:O_2$ and were maintained with 1–1.5% isoflurane in a 2:1 mixture of $N_2O:O_2$. The right temporalis muscle was bisected and reflected through an incision made midway between the eye and the external auditory meatus. The proximal middle cerebral artery was exposed through a subtemporal craniectomy without removing the zygomatic arch and without transecting the facial nerve. The artery was then occluded by microbipolar coagulation from just proximal to the olfactory tract to the inferior cerebral vein and was transected. Body temperature was maintained at $37°C ± 1°C$ throughout the entire procedure; brain temperature was not measured. Intraperitoneal cefazolin (40 mg/kg; Baxter, Marion, NC) was given 30 min before pMCAO to prevent infections. Subcutaneous buprenorphine (NDC 12496-0757; 0.05–0.1 mg/kg) was given before the pMCAO surgery as analgesia.

Dose–Response Study

Starting 24 hr after pMCAO, rats were randomly assigned (http://www.graphpad.com/quickcalcs/randomize1.cfm) to one of four groups (n = 12/group) receiving cimaglermin intravenously at doses of 0.02, 0.1, or 1.0 mg/kg or an equivalent amount of vehicle (1 ml/kg) daily for 14 days. Animals were sacrificed on day 21 after pMCAO, 1 week after cessation of treatment.

Dose–Frequency Study

In a separate experiment, equal numbers of animals were assigned to one of four groups (cimaglermin administered daily, twice per week, and once per week and vehicle only) based on a rotating order for each surgical day. Groups of animals (n = 12) received 1.0 mg/kg cimaglermin intravenously starting 24 hr after pMCAO for 3 weeks; the daily group received 21 total doses, the twice/week group received six total doses, and the once/week group received three total doses. Vehicle-treated animals received 21 daily intravenous injections. Animals were sacrificed on day 28 after pMCAO, 1 week after treatment cessation.

Behavioral Testing

All behavioral evaluations were performed by investigators blinded to treatment assignment. The tests described below were performed 1 day before surgery, 1 day after surgery, and at 3, 7, 14, and 21 days after pMCAO for both the dose-response and the dose-frequency studies and also on day 28 for the dose-frequency study. On days that coincided with drug administration, animals were tested before drug administration. Time points are designated with the day of surgery (day 0) as a reference. The forelimb- and hindlimb-placing tests largely reflect recovery of function of the sensorimotor cortex (De Ryck et al., 1992), whereas the body-swing test is thought to reflect recovery of striatal function (Borlongan and Sanberg, 1995).

For the forelimb-placing test, the examiner held the rat close to a tabletop and scored the rat's ability to place the forelimb on the tabletop in response to whisker, visual, tactile, or proprioceptive stimulation. Similarly, for the hindlimb-placing test, the examiner assessed the rat's ability to place the hindlimb on the tabletop in response to tactile and proprioceptive stimulation. Separate subscores (half-point designations possible) were obtained for each mode of sensory input and added to give total scores (forelimb-placing test, 0 = normal and 12 = maximally impaired; hindlimb-placing test, 0 = normal and 6 = maximally impaired).

For the body-swing test, the rat was held approximately 1 inch from the base of its tail. It was elevated to 1 inch above the surface of a table. The rat was held in the vertical axis, defined as no more than 10° to either the left or the right side. The rat had to return to the vertical position for the next swing to be counted. Thirty total swings were counted. A normal rat typically has an equal number of swings to either side. After focal ischemia, the rat tends to swing to the contralateral (left) side.

Histology

After behavioral evaluations at the study endpoints (21 days after pMCAO in the dose-response study and 28 days after pMCAO in the dose-frequency study), rats were anesthetized and perfused transcardially with normal saline followed by 4% paraformaldehyde, and brains were removed. Fixed brains were embedded in paraffin, and 5-μm coronal sections were cut with a microtome. Seven sections (+4.7, +2.7, +0.7, −1.3, −3.3, −5.3, and −7.3 compared with bregma) were cut and stained as described below.

Infarct volumes were assessed from both the dose-response and the dose-frequency studies. Sections were stained with hematoxylin and eosin with standard methods, and all seven sections from each brain were photographed with a Canon EOS Rebel digital camera (Canon USA, Melville, NY). The infarcted area on each slice was determined in Image J by the "indirect method" (area of the intact contralateral [left] hemisphere − area of intact regions of the ipsilateral [right] hemisphere). Infarct areas were then summed among slices and multiplied by slice thickness to give total infarct volume, which was expressed as a percentage of intact contralateral hemispheric volume.

Brain sections from the vehicle (n = 6) and 1 mg/kg cimaglermin-treated (n = 12) groups of the dose-response study were processed for further immunohistochemical analysis of GAP43 and SYP. Half of the vehicle-treated animals, representing those closest to the mean by the forelimb-placing data, were selected for histological analysis. All 12 animals were included from the drug-treated group because their recovery was affected by treatment; how this would impact the histological variability was not known. A standard indirect immunohistochemical staining method was used with avidin–biotin complex/horseradish peroxidase and detected by colorimetric assay (diaminobenzidine; Hsu et al., 1981). In brief, brain sections mounted on slides were deparaffinized, hydrated, and subjected to heated citrate buffer antigen retrieval. Tissues were stained on a Dako (Carpinteria, CA) autostainer at room temperature. Autostaining consisted of the following steps (separated by rinses with wash buffer): peroxidase block 5 min; two separate protein blocks, normal goat serum 10 min and CAS block (casein solution [Dako]) 15 min; rabbit primary antibody (see below) 60 min; Envision[+] rabbit/horseradish peroxidase (Dako; see below) 30 min; diaminobenzidine 3 min. The sections were counterstained with hematoxylin, dehydrated, and coverslipped by standard methodologies.

Immunohistochemical staining intensity was first scored semiquantitatively by a board-certified veterinary pathologist with the industry standard severity system of 0 = normal, 1 = minimal or trace, 2 = mild, 3 = moderate, 4 = severe. Intensity of staining and presence of tissue on all sections through the brain were used to determine the areas selected for further morphometric analysis.

Morphometric analysis from digitized images was performed in MicroSuite Five (v. 1235; Olympus, Tokyo, Japan). Briefly, three predefined perilesional regions of a single SYP- or GAP43-immunostained section were photographed at ×20 on both the ipsilateral side and the contralateral side for each brain section. Analysis was performed by an observer blinded to the treatment groups. The software calculated the total positively stained area (pixels) and compared this with total pixel area of the tissue image for each region of interest to calculate the percentage of positive expression for GAP43 and SYP.

For detection of GAP43 and SYP with fluorescence-based immunohistochemistry, tissue sections (on slides) were deparaffinized, hydrated, and then incubated in Tris-buffered saline (0.1 M Tris-HCl, pH 7.4, and 0.9% w/v NaCl) for 10 min and subjected to heated sodium citrate buffer antigen retrieval. After cooling, sections were incubated in blocking buffer (10% fetal bovine serum and 0.2% Triton X-100 in Tris-buffered saline) for 2 hr. Incubation with the primary antibodies was performed overnight at 4°C. Rabbit polyclonal

TABLE I. Antibody Characterization

Antibody	Immunogen	Source	IHC dilution
Anti-GAP43	Complete sequence of the recombinant rat GAP43	Millipore, catalog No. AB5220, lot No. NG1897856, RRID:AB_2107282, rabbit, polyclonal	1:100 (indirect) 1:400 (fluorescence)
Anti-SYP	Synthetic peptide conjugated to KLH derived from within residues 1–100 of human synaptophysin	Abcam, catalog No. ab23754, lot No. 918197, RRID:AB_778203, rabbit, polyclonal	1:100 (indirect) 1:400 (fluorescence)
Envision[+] rabbit/ horseradish peroxidase	Immunoglobulins, mainly IgG, isolated from rabbit serum	Dako, catalog No. K4011, RRID:AB_2313609, goat, polyclonal	None (indirect)
Anti-NeuN	Purified nuclei from mouse brain cells	Millipore, catalog No. MAB377, RRID:AB_2298772, mouse, monoclonal	1:400 (fluorescence)
Anti-MAP2	Cow brain MAP2 purified from the material that pellets with GTP[a] polymerized tubulin	Abcam, catalog No. ab5392, RRID:AB_2138153, chicken, polyclonal	1:3,000 (fluorescence)
Anti-rabbit 488	IgG (H+L) secondary antibody, Alexa-Fluor 488 conjugate	Life Technologies, catalog No. A11034, RRID:AB_10562715, goat, polyclonal	1:1,000 (fluorescence)
Anti-mouse 568	IgG (H+L) secondary antibody, Alexa-Fluor 568 conjugate	Life Technologies, catalog No. A11031, RRID:AB_10562420, goat, polyclonal	1:1,000 (fluorescence)
Anti-chicken 647	IgY (H+L) secondary antibody, Alexa-Fluor 647 conjugate	Life Technologies, catalog No. A21449, RRID:AB_10374876, goat, polyclonal	1:1,000 (fluorescence)

★Guanosine triphosphate.

anti-GAP43 antibody (see below) and rabbit monoclonal anti-SYP (see below) were used at 1:400 dilution. Anti-NeuN (see below) and anti-MAP2 (see below) were used as neuronal markers. After four rinses (30 min each) in Tris-buffered saline and Tween 20 (0.2% Triton X-100 in Tris-buffered saline), sections were incubated for 1 hr at room temperature with the appropriate secondary antibodies conjugated with Alexa dyes (see below) and then washed and coverslipped with Fluorsave (Roche, Indianapolis, IN) for subsequent observation with a Zeiss (Oberkochen, Germany) LSM 700 confocal microscope. Two animals per group and three serial sections per animal (+3.24 mm and −1.32 mm from bregma, for GAP43 and SYP detection, respectively) were observed via confocal microscopy.

Antibody Characterization

Primary antibodies used were rabbit anti-GAP43 (catalog No. AB5220; lot No. NG1897856; RRID:AB_2107282; Millipore, Billerica, MA) and rabbit anti-SYP (catalog No. ab23754; lot No. 918197; RRID:AB_778203; Abcam, Cambridge, MA; Table I). The anti-GAP43 antibody has been previously shown to stain a band at 43 kDa on Western blots (Hannila and Kawaja, 2005) and more recently was characterized in the brain, in which the antibody stained neural cells (Kawaja et al., 2011). The anti–SYP antibody has been shown to stain a band at 38 kDa on Western blots from human brain lysates and to be detected by immunohistochemistry in human brain cortex, with patterns characteristic of proteins in small neurosecretory vesicles, including presynaptic vesicles (Abcam, n.d.).

The Envision[+] rabbit/horseradish peroxidase (catalog No. K4011; RRID:AB_2313609; Dako) consists of a goat anti-rabbit secondary antibody complexed to horseradish peroxidase (Table I). This antibody is supplied with the Envision[+] kit and has been verified by the supplier (Dako, n.d.). Anti-NeuN (mouse monoclonal; 1:400; catalog No. MAB377; RRID:AB_2298772; Millipore) and anti-MAP2 (chicken polyclonal; 1:3,000; catalog No. ab5392; RRID:AB_2138153; Abcam) were used as neuronal markers (Table I). The details and specificity of anti-NeuN and anti-MAP2 have been described previously (An et al., 2012; Foxworthy et al., 2013). The secondary Alexa dyes (goat anti-rabbit 488 [catalog No. A11034; RRID:AB_10562715; Life Technologies, Grand Island, NY], goat anti-mouse 568 [catalog No. A11031; RRID:AB_10562420; Life Technologies], and goat anti-chicken 647 [catalog No. A21449; RRID:AB_10374876; Life Technologies]) were used at 1:1,000 dilution.

Statistical Analysis

All behavioral data are expressed as group mean (±SEM). Behavioral and body weight means were analyzed by repeated-measures ANOVA (treatment over time) and Bonferroni post-tests, compared with a common control (vehicle) unless otherwise specified. Positive F values for an overall ANOVA, including all groups, permitted pairwise ANOVA between groups. Infarct volume group mean data were compared by one-way ANOVA (post hoc analysis was not conducted because ANOVA did not detect a significant difference within the data set). ANOVA and post hoc tests were conducted in Prism v.5.0 (GraphPad Software, La Jolla, CA). For GAP43 and SYP immunohistochemical analysis, the mean percentage of the area expressing GAP43 or SYP immunopositivity, as a function of total area for the two treatment groups, was compared statistically by Student's t-test (two-tailed, heteroscedastic) in Excel 2007 (Microsoft, Redmond, WA). An $\alpha = 0.05$ was assumed for each statistical test.

RESULTS

Cimaglermin was produced recombinantly, and activity of purified cimaglermin was confirmed with established bioassays, demonstrating dose-dependent activation of the

phosphoinositide 3-kinase pathway and competitive binding for erbB receptors (Iaci et al., 2010). In the dose-response study, one animal was excluded on day 1 before dosing because of lack of expected deficit in forelimb placing; this animal was replaced. No other animals died or were sacrificed before the study endpoint. No animals were excluded from the dose-frequency study.

Cimaglermin Significantly Improves Functional Recovery in a Dose-Dependent Manner

All animals had limb-placing scores of 0 (no deficit) on day −1, prior to pMCAO. In agreement with previous studies (Iaci et al., 2010), 1 day after pMCAO but before treatment, animals in all groups had a forelimb-placing score (FPS) of ≥ 11, indicating a near absence of placing activity (Fig. 1A). In general, animals partially recovered forelimb-placing function over approximately 3 weeks after pMCAO. Animals that received daily doses of 0.1 and 1.0 mg/kg cimaglermin for 14 days had significantly improved recovery in forelimb function (FPS 3.3 and 3.0, respectively, $P < 0.0005$) compared with those that received vehicle (FPS 4.9; Fig. 1A). The 0.02 mg/kg cimaglermin group was not improved compared with the vehicle group.

Similarly, and in agreement with previous studies, 1 day after pMCAO but before treatment, animals in all groups had a hindlimb-placing score (HPS) of ≥ 5, indicating a near absence of placing activity (Fig. 1B). Animals partially recovered hindlimb-placing function over approximately 3 weeks after pMCAO. Animals in all groups that received cimaglermin for 14 days had significantly improved recovery in hindlimb function (HPS 2.1 for 0.02 mg/kg dose, $P < 0.005$; HPS 1.6 for 0.1 mg/kg dose, $P < 0.0005$; HPS 1.2 for 1.0-mg/kg dose, $P < 0.0005$) compared with the vehicle group (HPS 2.5); the greatest recovery was observed in the higher-dose groups (Fig. 1B).

In agreement with previous studies, 1 day after pMCAO but before treatment, animals in all groups had a near complete loss of body swing to the ipsilateral (right) side (Fig. 1C). Animals partially recovered over approximately 3 weeks after pMCAO. Animals that received 1.0 mg/kg cimaglermin for 14 days had significantly improved scores compared with those that received vehicle (body swing 36.1 and 25.3, respectively, $P < 0.0005$; Fig. 1C). Groups treated with 0.1 and 0.02 mg/kg showed trends toward normalization but were not significantly different from vehicle.

Cimaglermin Significantly Improves Functional Recovery When Delivered More Frequently

All animals had limb-placing scores of 0 (no deficit) on day −1, prior to MCA occlusion. Animals had deficits in FPS, HPS, and body-swing scores 1 day after pMCAO but before treatment and partially recovered in all functional tests over 3 weeks (Fig. 2A–C). Animals that received daily doses of 1.0 mg/kg cimaglermin for 21 days demonstrated significant improvements in both forelimb-
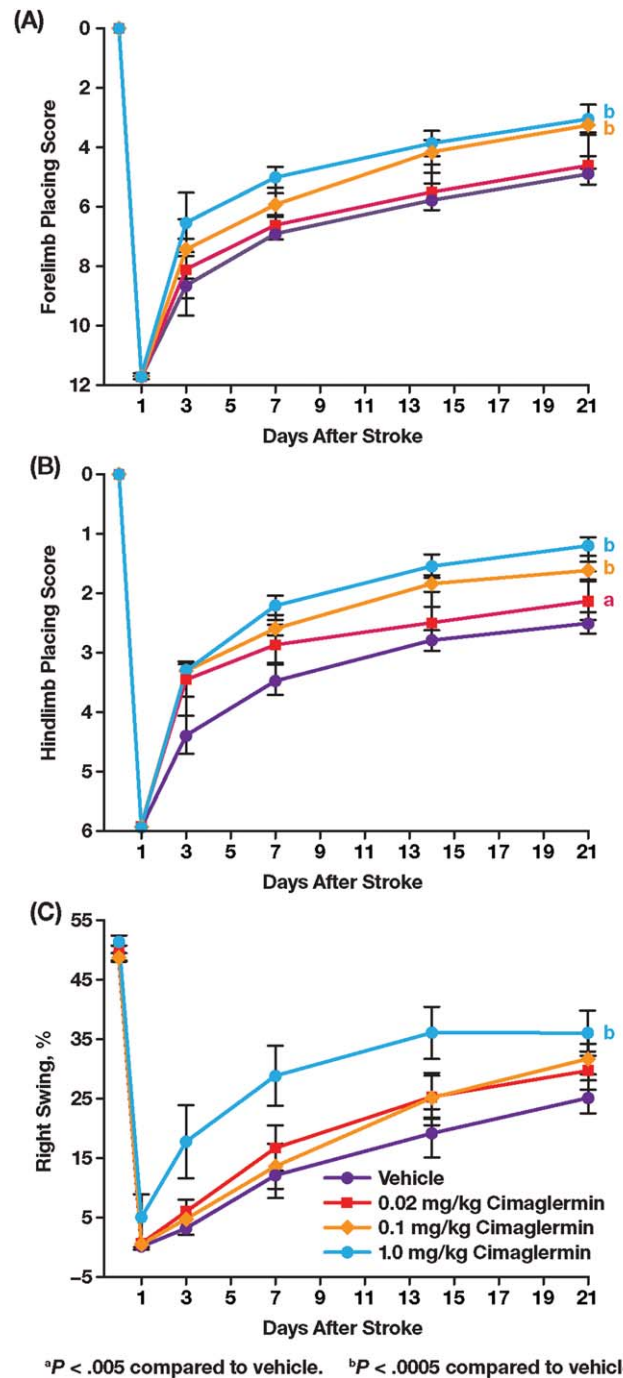

Fig. 1. Dose-dependent behavioral improvements with cimaglermin treatment. **A**: Cimaglermin at doses of 0.1 and 1.0 mg/kg daily but not at a dose of 0.02 mg/kg daily for 14 days significantly improved recovery in sensorimotor function of forelimb compared with vehicle. **B**: Cimaglermin at doses of 0.02, 0.1, and 1.0 mg/kg daily for 14 days significantly improved recovery in hindlimb function, with the greatest recovery seen in the higher-dose groups of animals. **C**: Cimaglermin at a dose of 1.0 mg/kg daily for 14 days significantly improved body-swing score compared with vehicle; n = 12 per group.
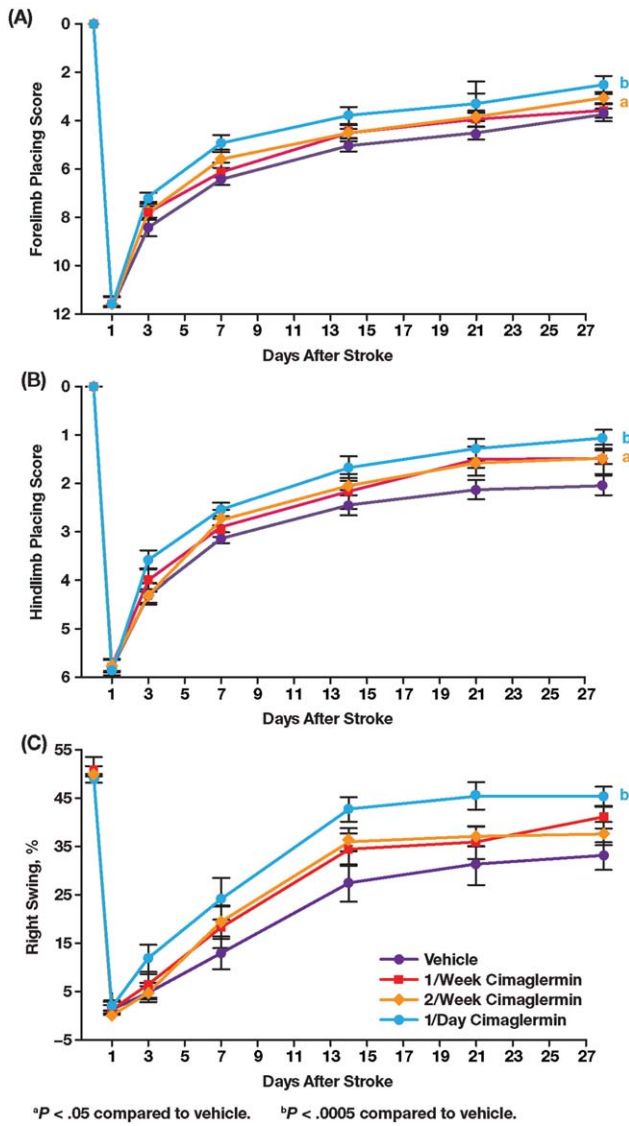
Fig. 2. Dose-frequency-dependent behavioral improvements with cimaglermin treatment. Compared with rats treated with vehicle, animals treated with daily doses of 1.0 mg/kg cimaglermin for 21 days demonstrated significant improvements in forelimb-placing (**A**) and hindlimb-placing (**B**) tests and in body-swing (**C**) assessment. Animals that received cimaglermin twice/week at 1.0 mg/kg for 21 days also demonstrated significantly improved forelimb- and hindlimb-placing responses but not to the extent seen with daily dosing. Rats treated with cimaglermin once/week at 1.0 mg/kg for 21 days did not show improvement in any of these tests; n = 12 per group.

and hindlimb-placing tests and body-swing assessment compared with vehicle-treated animals (FPS 2.5 vs. vehicle 3.8, HPS 1.1 vs. vehicle 2.0, body swing 45.6 vs. vehicle 33.1, $P < 0.0005$ for all; Fig. 2A–C). Animals that received cimaglermin twice/week at 1.0 mg/kg for 21 days demonstrated significantly improved forelimb- and hindlimb-placing responses compared with vehicle-treated animals (FPS 3.1 vs. vehicle 3.8, HPS 1.5 vs. vehi-

cle 2.0, $P < 0.05$ for both) but not to the extent seen with daily dosing (Fig. 2A,B). Once/week dosing at 1.0 mg/kg was not effective in restoring function in any of the behavioral tests (Fig. 2A–C).

Cimaglermin Does Not Reduce Infarct Volume Following pMCAO

In agreement with previous data (Iaci et al., 2010), there were no differences in infarct volume between treated and untreated animals. In the dose-response study, infarct volumes were $28.8 \pm 2.1\%$, $32.8 \pm 2.4\%$, $33.5 \pm 2.4\%$, and $35.9 \pm 5.5\%$ for the vehicle-treated and 0.02, 0.1, and 1.0 mg/kg cimaglermin-treated groups, respectively. Similar infarct volumes were observed with no differences between treated and controls in the dose-frequency study (Table II).

Cimaglermin Increases Expression of GAP43 and SYP in Ipsilateral and Contralateral Hemispheres Following pMCAO

Both GAP43, as a marker of neuronal regeneration, and SYP, as a marker of synapse formation, were increased with cimaglermin treatment compared with vehicle treatment in defined brain regions (Fig. 3) that included peri-infarct tissue on the ipsilateral hemisphere. Table III describes structures present in brain regions at specific coronal levels.

Quantitative digital image analysis from multiple brain regions for GAP43 and SYP on the infarcted and noninfarcted sides of each brain section revealed a significant increase in expression in cimaglermin-treated animals. Significant increases in expression of GAP43 were observed mainly in the primary and secondary motor cortices, frontal cortex, visual cortex, hippocampus, caudate-putamen, and corpus callosum of both hemispheres in animals receiving cimaglermin treatment (Fig. 4). Analysis of SYP expression revealed a significant increase in the cimaglermin-treated animals, most evident in the primary and secondary motor cortices, frontal cortex, and visual

TABLE II. Cimaglermin Treatment Does Not Reduce Infarct Volume Following pMCAO

Dosing regimen	Total doses	Infarct volume as percentage of contralateral hemisphere (SEM)[*]
Dose-ranging study		
Daily 0.0 mg/kg (vehicle)	14	28.8 (2.1)
Daily 0.02 mg/kg	14	32.8 (2.4)
Daily 0.1 mg/kg	14	33.5 (2.4)
Daily 1.0 mg/kg	14	35.9 (5.5)
Dose-frequency study		
Daily 0.0 mg/kg (vehicle)	21	30.9 (2.6)
1/week 1.0 mg/kg	3	30.7 (2.3)
2/week 1.0 mg/kg	6	29.1 (2.6)
Daily 1.0 mg/kg	21	27.3 (2.7)

[*]Data are group mean, analyzed by one-way ANOVA, with no statistically significant difference observed among groups; n = 12 per group.

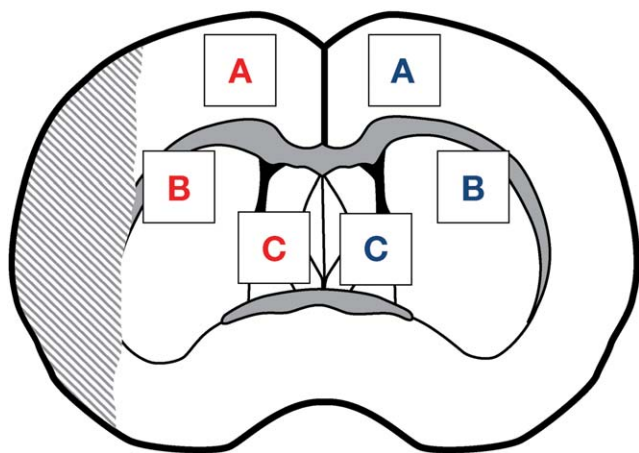| Letter Designation	Brain Area Measured
A	Cortex adjacent to midline
B	Regions dorso-lateral to infarct zone
C	Regions ventromedial to infarct zone

Fig. 3. Brain regions for analysis of GAP43 and SYP expression. These regions represent some peri-infarct areas in the ipsilateral hemisphere and their matched areas in the contralateral hemisphere. The hatched area represents the approximate infarct area at a coronal section of Bregma +0.7.

cortex (Fig. 5). Expression was much more intense on the infarcted side compared with the noninfarcted side. Ventromedial increases of SYP expression in the thalamic nuclei, dentate gyrus, tectal nuclei, and superior colliculus were observed primarily in the noninfarcted hemisphere of animals receiving cimaglermin treatment (Fig. 5, area C).

These results were confirmed by fluorescence-based coimmunostaining against SYP or GAP43 and the neuronal markers NeuN and MAP2. These analyses focused on the motor cortex, one of the brain areas in which the most evident changes of SYP and GAP43 were observed. Consistent with the diaminobenzidine staining, SYP and GAP43 were increased in the brains of cimaglermin-

treated rats, and this increase was higher in the infarcted side compared with the noninfarcted hemisphere of these animals (Figs. 6, 7).

These results indicate that cimaglermin treatment produced a significant increase in both GAP43 and SYP labeling in surviving neuropil and peri-infarcted brain regions following pMCAO compared with vehicle treatment. Expression of both markers was increased with cerebral infarction compared with the contralateral tissue from the same section, which is consistent with promotion of neuroplastic changes in surviving brain tissue following ischemic stroke.

DISCUSSION

The studies presented here show functional improvements in a rat stroke model using cimaglermin initiated at 24-hr poststroke, with 1 mg/kg cimaglermin significantly improving all functional measures vs. vehicle. A stroke occurs in the United States every 40 seconds, and, although many individuals survive, they frequently have functional impairments that affect quality of life (Go et al., 2014). The approved therapies require patients to present with a stroke within 3–6 hr of confirmed onset (Furlan et al., 2003; Prince et al., 2013), which is not easily attained (Adeoye et al., 2011). Physical therapy has been the only intervention that improves patient function after endogenous recovery has plateaued.

Previous results have demonstrated that cimaglermin promotes a neurorestorative environment in animals poststroke in which a 10-day daily intravenous dosing regimen resulted in functional improvements even when treatment was delayed for up to 1 week postocclusion without evidence of neuroprotection (Iaci et al., 2010). The underlying mechanisms explaining these functional improvements were not elucidated in earlier studies. Therefore, the work presented here seeks to continue exploring cimaglermin as a therapeutic option as well as to identify potential mechanisms by which it exerts beneficial effects poststroke in animals.

TABLE III. Brain Regions Corresponding to Histomorphometry Sites at Coronal Levels Through the Brain

| Bregma	Area A	Area B	Area C
+4.7	Frontal association cortex	Frontal association cortex and dorsolateral orbital cortex	Prelimbic cortex
+2.7	Secondary motor cortex	Primary motor cortex	Prelimbic and cingulate (A1) cortex
+0.7	Primary and secondary motor cortex	Caudate-putamen and corpus callosum	Lateral septal nuclei and caudate-putamen
−1.3	Primary and secondary motor and hindlimb region somatosensory cortex	Globus pallidus, caudate-putamen, corpus callosum	Thalamic nuclei
−3.3	Retrosplenial, primary, and secondary motor cortex	Caudate–putamen and internal capsule, fimbria of hippocampus, medial aspects of somatosensory (auditory) cortex	Thalamic nuclei and ventral hippocampus (dentate gyrus)
−5.3	Retrosplenial and visual cortex	Hippocampus	Tectal nuclei, superior colliculus, nucleus of posterior commissure
−7.4	Visual cortex	Secondary visual cortex, forceps major of corpus callosum, hippocampal subiculum	Superior colliculus, superior area of periaqueductal gray
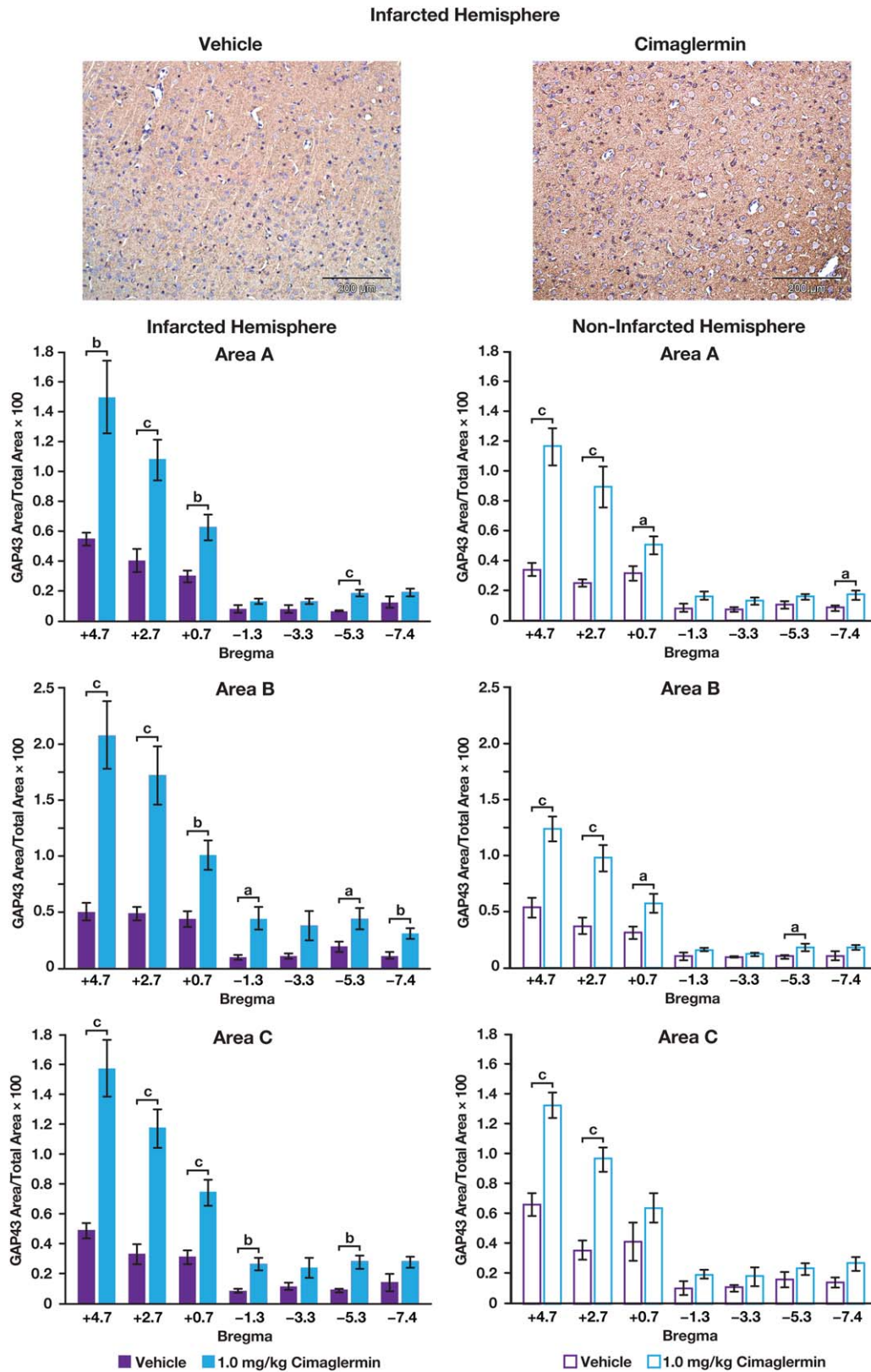
Fig. 4. Quantitation of GAP43 expression in brain regions of interest from contralateral and ipsilateral hemispheres. Cimaglermin treatment (n = 12) significantly increased the expression of GAP43 in areas associated with the primary and secondary motor cortices in both hemispheres compared with vehicle (n = 6). Infarcted hemispheres from area A of vehicle- and cimaglermin-treated animals are shown. $^aP < 0.05$, $^bP < 0.005$, $^cP < 0.001$.
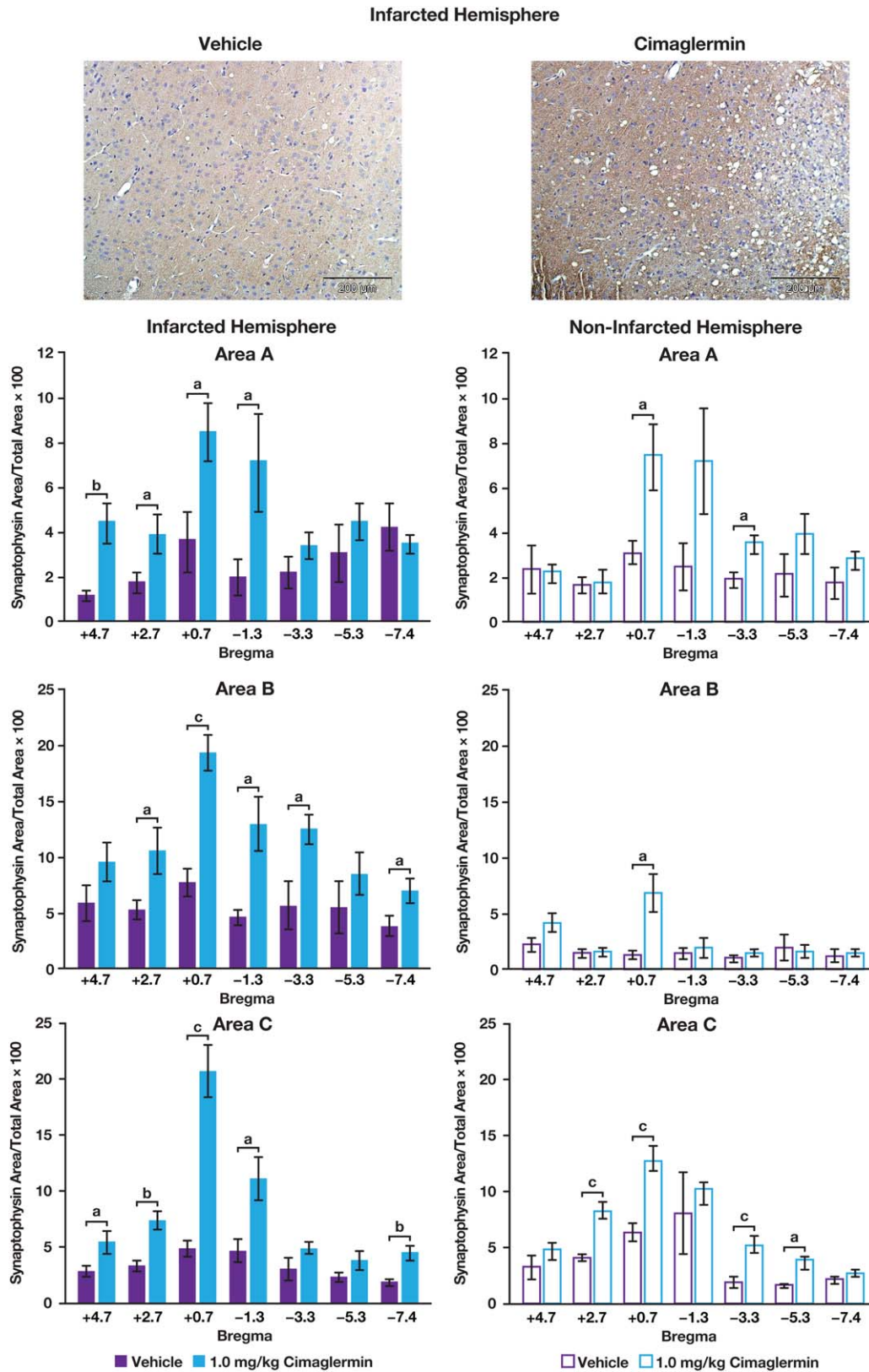
Fig. 5. Quantitation of SYP expression in brain regions of interest from contralateral and ipsilateral hemispheres. Cimaglermin treatment (n = 12) significantly increased the expression of SYP in areas associated with the primary and secondary motor cortices compared with the vehicle group (n = 6) in both hemispheres. Infarcted hemispheres from area A of vehicle- and cimaglermin-treated animals are shown. [a]$P < 0.05$, [b]$P < 0.005$, [c]$P < 0.001$.
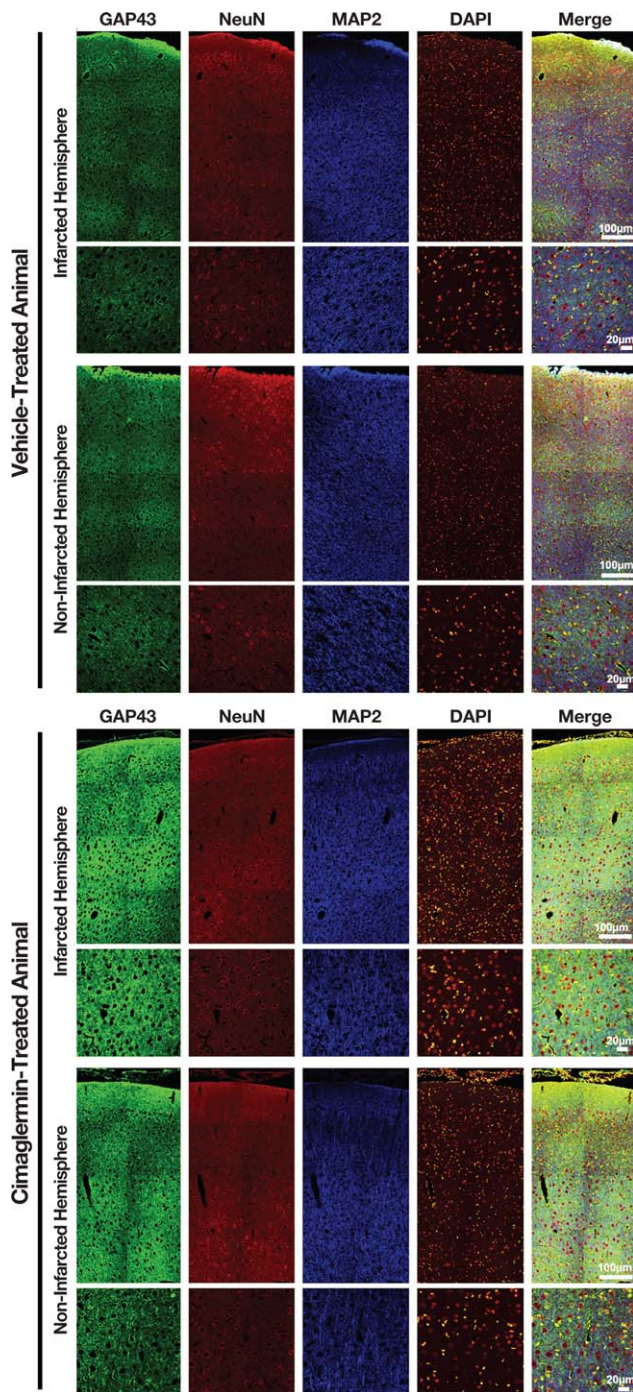
Fig. 6. Representative confocal optical images for GAP43 (green) in the motor cortex of coronal brain sections of vehicle- and cimaglermin-treated rats. The infarcted and noninfarcted hemispheres of the same animal for each treatment are shown. There is an increase of GAP43 in the cimaglermin-treated rats that is pronounced in the infarcted hemisphere. NeuN (red) and MAP2 (blue) were used as neuronal markers and indicators of neural network integrity. DAPI (pseudocolor) was used for nuclei counterstain. Optical images were a stack of z-series (5-μm thickness) from brain sections (+3.24 mm from bregma; Paxinos Rat Brain Atlas (Paxinos and Watson, 2013)).

Dose response and frequency of administration with cimaglermin were evaluated after pMCAO in rats with the goal of determining minimum exposure levels that still result in functional improvements. Initially, three dose levels of cimaglermin (0.02, 0.1, and 1.0 mg/kg) were compared with vehicle, with dosing initiated 24 hr after pMCAO surgery and continued for 14 days. It should be noted that, in the context of neuroprotective strategies that have been evaluated for stroke, a 24-hr treatment window is easily achieved clinically and is substantially wider than that for tissue plasminogen activator. Doses of 1.0 and 0.1 mg/kg cimaglermin resulted in forelimb- and hindlimb-placing improvements that were significant compared with vehicle at 21 days post pMCAO. The 0.02 mg/kg dose level did show some improvements in the HPS, but these did not reach the same level of improvement as seen in the two higher-dose groups. Although all doses showed modest improvement in trunk stability (body-swing test), only the 1 mg/kg dose resulted in significant improvements compared with vehicle. However, no reduction in infarct volumes was observed with cimaglermin treatment. In addition, there were no observable effects from the perioperative antibiotics because all animals, including controls, were treated, and immunomodulatory effects of cefazolin in rats have not been described. No hypersensitivity reactions were observed in any rats that were treated. Therefore, treatment-related changes were deemed cimaglermin specific; these data confirm previous reports that cimaglermin at a daily dose of 1 mg/kg for 2 weeks is efficacious in this model and suggest that cimaglermin promotes recovery rather than providing neuroprotection.

Given the more robust functional responses seen in the cimaglermin 1.0 mg/kg daily intravenous dosing group, this dose level was selected for the dose-frequency study, in which daily dosing was compared with twice/week and once/week dosing. Less frequent dosing was explored because efficacy has been observed in other neurologic and nonneurologic models with infrequent dosing (Cannella et al., 1998; Hill et al., 2013). The present results suggest that daily and twice/weekly dosing are effective for promoting neurorecovery of forelimb and hindlimb function in this model, but reduced frequency of dosing is associated with reduced efficacy in all measures used.

Overall, these results indicate that greater improvements in neurobehavioral function following pMCAO are achieved at higher doses and with more frequent dosing. The body-swing test was most sensitive to reduced exposure, possibly because of its greater intrinsic variability. Future studies will determine whether this effect on trunk stability is related to differential erbB receptor expression within the basal ganglia (Fox and Kornblum, 2005) or whether different plasticity-related responses to treatment are brain region specific.

The permanent occlusion and initiation of treatment at 24 hr following pMCAO do not provide much opportunity for neuroprotection; the at-risk neurons in the penumbra are likely dead or undergoing apoptosis. This
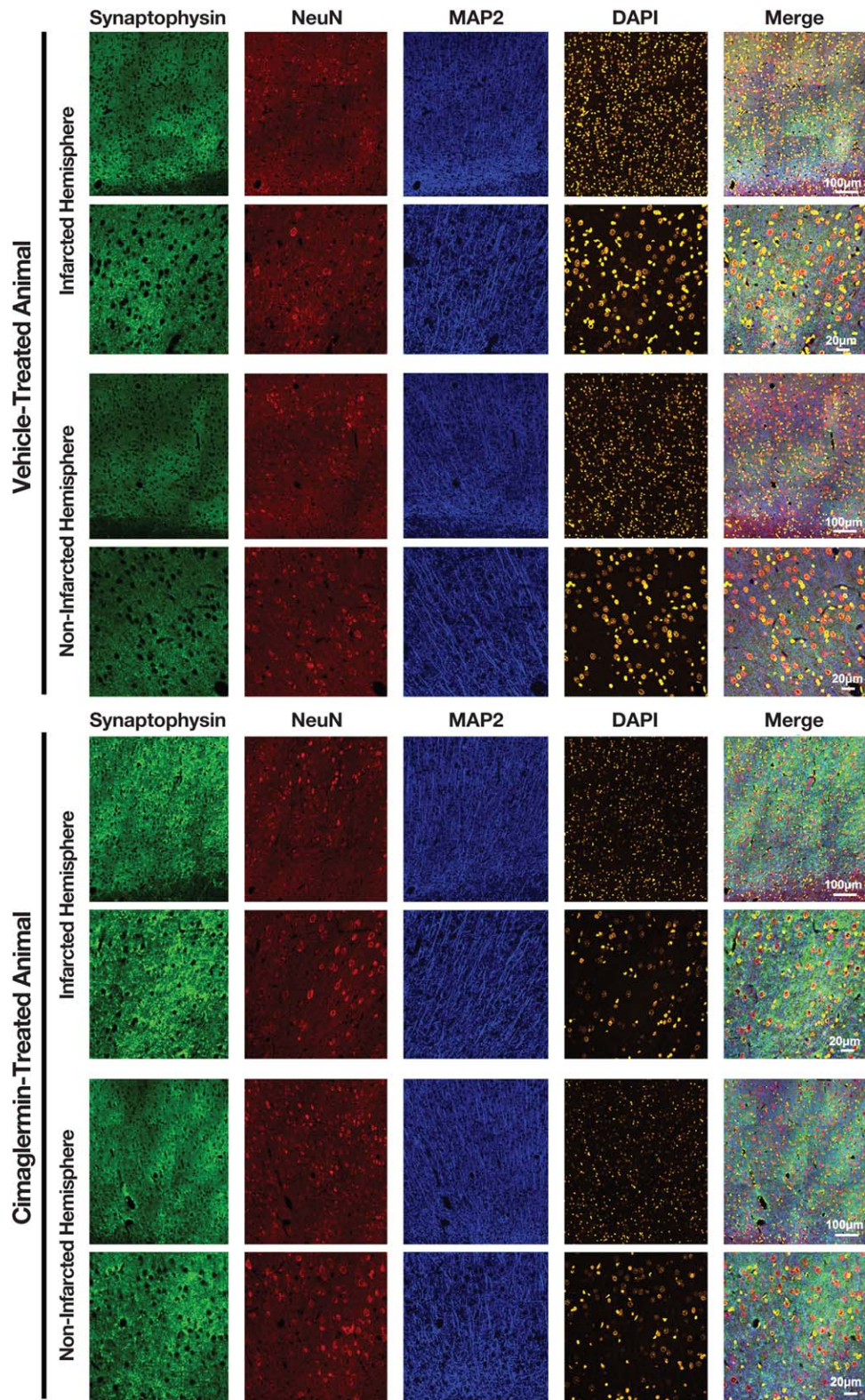
Fig. 7. Representative confocal optical images for synaptophysin (green) in the motor cortex of coronal brain sections of vehicle- and cimaglermin-treated rats. The infarcted and noninfarcted hemispheres of the same animal for each treatment are shown. There is an increase of synaptophysin in the cimaglermin-treated rats that is pronounced in the infarcted hemisphere. NeuN (red) and MAP2 (blue) were used as neuronal markers and indicators of neural network integrity. DAPI (pseudocolor) was used for nuclei counterstain. Optical images were a stack of z-series (10-μm thickness) from brain sections (−1.32 mm from bregma; Paxinos Rat Brain Atlas (Paxinos and Watson, 2013)).

study confirms a lack of neuroprotection at the level of infarct volume. The functional improvements, therefore, may be attributed to a remodeling or plasticity effect or perhaps to activation of latent pathways as opposed to a protective effect in the penumbra.

Brain reorganization has been correlated with functional recovery poststroke with recent advances in neuroimaging techniques in the context of patient recovery (Calautti and Baron, 2003; Ward et al., 2003; Chollet, 2013; Lazaridou et al., 2013). It has been shown that GAP43 and SYP are increased following stroke and are associated with recovery (Carmichael et al., 2005; Granziera et al., 2007; Machado et al., 2013; Zhao et al., 2013; Mizutani et al., 2014). It is possible that therapeutic interventions such as growth factors can maximize the innate ability of the nervous system to utilize alternate circuits to restore function postinjury (Carmel and Martin, 2014). To evaluate indirectly whether plasticity or sprouting could be involved in the recovery response, we assessed expression changes in GAP43 and SYP after cimaglermin treatment. The GAP43 and SYP immunohistochemical results in vehicle-treated animals were in agreement with previous reports of their activity in axonal growth and synapse formation (Benowitz et al., 1987; Skene, 1989; Pfenninger et al., 1991) and were significantly increased in animals treated with cimaglermin. Although there were increases in expression that were significant in specific brain regions, it should be noted that cimaglermin treatment enhanced expression of both markers across all regions of the brain that were analyzed. This suggests that neuroplastic mechanisms involved in endogenous recovery following stroke are further enhanced with cimaglermin treatment. The increased expression in both the ipsilateral and the contralateral hemispheres, with the greatest increased expression seen in the primary and secondary motor and frontal association cortices as well as in the caudate–putamen, indicates that intact and compensatory circuits may be altered in an effort to restore function. Combined with the functional improvements also observed, these data indicate that neuroplastic changes potentially drive improved neurologic function poststroke. Animals did not appear to have any unusual responses to normal handling compared with vehicle-treated animals, but, given that the data are consistent with an increase in sensorimotor cortex–related axonal growth and synapse formation, it will be critical to assess allodynia and hyperalgesia (Brown and Weaver, 2012) in a more focused manner to ensure that inappropriate or negative connections are not also increased with cimaglermin treatment.

Additional studies should evaluate the permanence of the effect with a washout period after dosing as well as demonstrate efficacy in a nonhuman primate stroke model. In future studies, taking into account the complexity of a larger brain and the impact of cimaglermin on fine motor function will be critical for ascertaining the level of benefit that might be possible in patients.

CONCLUSIONS

The present study extends earlier observations that cimaglermin improves sensorimotor function in a rat model and demonstrates the potential for cimaglermin as a therapeutic intervention for stroke recovery. Furthermore, these studies show that more frequent dosing starting at a clinically relevant 24 hr poststroke could provide the greatest recovery. This information, combined with knowledge gained from cimaglermin in the clinical setting, in which it is being evaluated for another indication, can inform future pharmacology and toxicology studies that may be required to allow for short-term daily dosing poststroke.

CONFLICT OF INTEREST STATEMENT

J.F. Iaci, T.J. Parry, Z. Huang, E. Pavlopoulos, and A. Caggiano are employees and stockholders of Acorda Therapeutics, Inc. S.P. Finklestein is an acting consultant for Acorda Therapeutics, Inc. The other authors have no conflicts to report.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JFI, TJP, ZH, JR, AC. Acquisition of data: JR, EP. Analysis and interpretation of data: JFI, TJP, ZH, EP, JR, AC. Drafting of the manuscript: JFI, TJP, ZH, EP. Critical revision of the article for important intellectual content: SPF, AC. Statistical analysis: JR, JFI. Administrative, technical, and material support: JFI, ZH, EP, JR. Study supervision: JFI, TJP, AC.

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