Stroke

BASIC AND TRANSLATIONAL SCIENCES

Recombinant GDF11 Promotes Recovery in a Rat Permanent Ischemia Model of Subacute Stroke

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BACKGROUND: Stroke remains a leading cause of death and disability, underscoring the urgent need for treatments that enhance recovery. GDF11 (growth differentiation factor 11), a member of the TGF-β (transforming growth factor-β) superfamily, is a circulating protein involved in cellular development and tissue repair. GDF11 has gained attention for its potential regenerative properties in aging and disease contexts, making it a candidate for stroke recovery therapies.

METHODS: The therapeutic benefits of rGDF11 (recombinant GDF11) were evaluated using a rat ischemic stroke model, in which focal cerebral infarcts were induced in 8- to 10-week-old young adult male Sprague-Dawley rats by permanently occluding the proximal right middle cerebral artery. Rats received single or multiple doses of rGDF11 (0.1–4 mg/kg) or vehicle from 24 to 72 hours post-injury. Sensorimotor functions were evaluated, and brain and serum samples were examined to determine the mechanisms of action and identify biomarkers, using immunofluorescence, target-specific ELISAs, and an aptamer-based proteomics platform.

RESULTS: We confirmed rGDF11 activity in vitro and in established in vivo mouse models of cardiac hypertrophy and glucose metabolism and assessed the efficacy of rGDF11 treatment in 6 preclinical stroke studies using independent Contract Research Organizations, with all study animals and treatment groups blinded. All 6 studies revealed consistent improvement in sensorimotor outcomes with rGDF11. rGDF11-treated rats showed increased cortical vascularization and radial glia in the ventricular zone. Serum analysis revealed that rGDF11 caused dose-dependent decreases in CRP (C-reactive protein) and identified novel pharmacodynamic biomarkers and pathways associated with potential mechanisms of action of rGDF11.

CONCLUSIONS: These results demonstrate that systemically delivered rGDF11 enhances neovascularization, reduces inflammation, promotes neurogenesis, and improves sensorimotor function post-injury in a rat model of ischemic stroke. More importantly, these data define an optimized and clinically feasible rGDF11 dosing regimen for therapeutic development in ischemic stroke and identify a panel of candidate pharmacodynamic and mechanistic biomarkers to support clinical translation.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: aging ■ biomarkers ■ glucose ■ inflammation ■ stroke

There is currently an unmet need for therapeutics that can promote the recovery of neurological function and provide regenerative benefits beyond neuroprotection postischemic stroke. This is particularly important at ≥24 hours after the stroke, when patients are in the hospital and stabilized with baseline functions

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Nonstandard Abbreviations and Acronyms

Alad	aminolevulinate dehydratase
Aldob	aldolase B, fructose-bisphosphate
Angpt1	angiopoietin-1
Angpt2	angiopoietin-2
APP/PS1	amyloid precursor protein/presenilin 1
CRO	Contract Research Organization
CRP	C-reactive protein
Fam177a1	family with sequence similarity 177 member A1
Fgl1	fibrinogen-like 1
Fh	fumarate hydratase
GDF11	growth differentiation factor 11
Htra2	HtrA serine peptidase 2
Inhba	inhibin subunit β A
Isoc1	isochorismatase domain containing 1
Nog	Noggin
Nppb	natriuretic peptide B
Nrp1	neuropilin 1
Olfm2	olfactomedin 2
pMCAO	permanent middle cerebral artery occlusion
pSmad2/3	phospho-Smad2/3
rGDF11	recombinant growth differentiation factor 11
Rtn4rl1	reticulon 4 receptor-like 1
Sparcl1	SPARC-like 1
TGF-β	transforming growth factor-β
Thbs2	thrombospondin 2
tPA	tissue-type plasminogen activator
Ucn3	urocortin 3

assessed, but outside the treatment time windows for tPA (tissue-type plasminogen activator) and endovascular therapy.[1] A circulating blood factor that has triggered interest in the scientific community in recent years is GDF11 (growth differentiation factor 11), a member of the TGF-β (transforming growth factor-β) superfamily, which can stimulate regeneration in multiple tissues and organs and has beneficial effects in some aging disease models.[2,3] The specific impacts of GDF11 on tissue repair and aging appear to be both concentration- and context-dependent, emphasizing the importance of a comprehensive assessment of its effects across a broad range of doses and in biologically relevant aging-related disease models.

Studies in aged mice demonstrated that systemic administration of exogenous rGDF11 (recombinant GDF11) increases neovascularization broadly in the brain and enhances neurogenesis in both the subventricular zone and hippocampus.[4,5] In addition, in the APP/PS1 (amyloid precursor protein/presenilin 1) mouse model of Alzheimer disease, rGDF11 improves cognitive function and increases cerebrovascular blood flow.[6] rGDF11 administered once daily for 7 days promoted neurogenesis, increased neovascularization, and improved sensorimotor function in young mice after middle cerebral artery occlusion, a model of ischemic stroke.[7] Similarly, Hudobenko et al[8] demonstrated that rGDF11 supplementation increased neovascularization, improved sensorimotor function and white matter integrity, and reduced brain atrophy, gliosis, inflammation, and mortality after middle cerebral artery occlusion in aged mice.

Here, we describe the effects of rigorously characterized recombinant GDF11 on functional sensorimotor recovery following stroke. Optimization of the dosing regimen, including timing of dosing initiation post-injury, duration of dosing, and dose range, was performed to maximize efficacy in the rat permanent middle cerebral artery occlusion (pMCAO) model. This extensively validated rodent model allows for the assessment of 2 key functional outcomes: limb placement, which primarily evaluates sensorimotor cortical function, and body swing, which reflects subcortical function.[9,10] We also identified factors that promote recovery poststroke and circulating biomarkers whose levels were modified in response to systemic rGDF11 administration, providing a panel of candidate biomarkers for clinical validation.[11]

METHODS

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Protein Production

rGDF11 was isolated and purified from mammalian cell culture supernatants, either from transient expression in HEK Expi293 cells (Thermo Fisher Scientific, Waltham, MA) or from a CHO cell line (CHOZN GS−/− ZFN-modified; Millipore Sigma) engineered to stably express rGDF11 generated as described in the Supplemental Methods. Commercially available *E. coli*-expressed rGDF11 was purchased from Peprotech (Cranbury, NJ) for comparison with internally produced material.

Luciferase Assays

HEK 293 $(CAGA)_{12}$ cells containing a SMAD2/3-responsive luciferase reporter gene were seeded in 96-well plates, treated with serial dilutions of proteins, and incubated, as described in the Supplemental Methods. EC50 values were generated by analysis of luminescence data.[12]

Animal Welfare Statement

All animal studies were conducted in collaboration with NeuroVasc Preclinical Services (Billerica, MA) and NeoSome Life Sciences (Billerica, MA) in a facility managed and operated by NeoSome. Both NeuroVasc and NeoSome are Contract

Research Organizations (CROs) specializing in preclinical research. All experimental protocols involving animals were reviewed and approved by NeoSome's Institutional Animal Care and Use Committee in accordance with applicable guidelines and regulations. NeoSome's Institutional Animal Care and Use Committee ensured that animal welfare concerns were thoroughly addressed, with measures in place to minimize discomfort and distress. All procedures were performed following ethical principles, and comprehensive documentation was maintained throughout the study to ensure compliance. All animal studies adhered to the Animal Research: Reporting of in vivo Experiments Guidelines 2.0 to promote rigor, reproducibility, and transparency in animal research (Supplemental Materials).[11]

rGDF11 In Vivo Activity Study

An in vivo activity study assessed the exposure of internally produced HEK Expi293 cell-expressed rGDF11 protein in 12-week-old male C57BL/6J mice (stock 000664; The Jackson Laboratory, Bar Harbor, ME). Intraperitoneal injections were administered, and tissue and serum samples were collected for analysis. Proteins were extracted from tissue samples and analyzed using phospho-Smad2/3 (pSmad2/3) ELISA and GDF11 serum ELISA assays. Animal work for this study was conducted at an independent CRO (NeoSome), with animals randomized and investigators blinded to treatment assignment. Tissue and serum samples remained blinded for in-house processing and ELISA analysis.

Cardiac Hypertrophy, Glucose, and Insulin Tolerance

Male C57BL/6J mice (stock 000664; The Jackson Laboratory, Bar Harbor, ME) at 14 weeks old (young) or 84 weeks old (aged) received intraperitoneal injections of 1 mg/kg rGDF11 (HEK Expi293 cell-produced) or vehicle once daily for 15 days. Baseline measurements for the glucose tolerance test and insulin tolerance test were performed on days 7 and 3, respectively, to be compared with readings on treatment days 10 and 14. Cardiac hypertrophy was assessed by calculating the ratio of heart weight-to-tibial length. All harvested tissues were weighed, measured, and flash-frozen on day 16. This study was conducted at an independent CRO (NeoSome), with animals randomized and investigators blinded to treatment assignments throughout the study, including end point assessments.

Stroke Efficacy Studies

Ischemic stroke recovery studies evaluating rGDF11 treatment were conducted to assess sensorimotor behavioral changes in 8- to 10-week-old young adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) following focal cerebral infarcts induced by pMCAO via microbipolar coagulation.[13] Designs for all 6 independent stroke studies are described in Table S1 and the Supplemental Methods. Dosing for the intraperitoneal dose ranging (study 4), intravenous dose ranging (study 5), and intravenous dose and regimen optimization (study 6) studies was initiated 24 hours post-injury (pMCAO or Sham), employing daily, intermittent (every 48 hours), or single-day dosing regimens for various dosing durations (1, 5, or 7 days), with behavioral testing performed out to 14, 21, or 28 days and study termination as indicated in Figure 1.

Limb placement and body swing tests were performed, with treatment groups blinded to the operator, and rats randomized into different pMCAO or sham groups (Supplemental Methods). All ischemic stroke recovery efficacy studies were conducted by an independent CRO (NeuroVasc) employing CHO cell-produced rGDF11. Serum samples from these studies were either analyzed in-house (study 5: intravenous dose ranging) using CRP (C-reactive protein) and GDF11 ELISAs on day 7 (Supplemental Methods; R&D Systems, Minneapolis, MN) or used for SomaScan analysis on days −1, 2, 3, 5, and 7 (study 6: intravenous dose and regimen optimization; Tables S1 and S2; Supplemental Methods). In all studies, animals were either exposed to pMCAO or sham surgery on day 0. For all analyses, samples were randomized, and investigators were blinded to treatment assignments throughout the studies.

Immunohistochemistry

Immunofluorescence staining was performed on brain tissue sections using antibodies against specific markers to assess cellular changes following pMCAO. To ensure robustness and validity of results, all samples were randomized and blinded in these analyses, including end point assessments.

Proteomic Profiling

Serum proteome profiling was performed using the SomaScan Assay (SomaLogic, Inc, Boulder, CO), capable of measuring over 7000 proteins. Serum samples from different time points of the dose and regimen optimization study (study 6) were selected for analysis, and standard procedures were followed for sample preparation and data analysis.[14] This study was conducted at an independent CRO (SomaLogic, Inc, Boulder, CO), and investigators were blinded to treatment assignment, throughout the study, including end point assessments, as detailed in Figure 1, Tables S1 and S2, and the Supplemental Methods.

RESULTS

rGDF11 Activity In Vitro and In Vivo

A representative Coomassie Blue-stained protein gel of rGDF11 expressed and purified from CHO cells is presented in Figure S1A. To compare the potency of in-house-produced protein generated from transient expression in HEK Expi293 cells (denoted as GDF11-Elevian in Figure S1B) against commercial *E. coli*-expressed rGDF11 purchased from PeproTech (Cranbury, NJ; denoted as GDF11-Peprotech in Figure S1B), cell-based assays employing a SMAD2/3-responsive luciferase reporter gene were conducted.[12] As shown in Figure S1B, internally produced (GDF11-Elevian) and commercial (GDF11-Peprotech) rGDF11 had comparable activities, as demonstrated by their EC50 values of 0.18 and 0.23 nmol/L, respectively. All lots of rGDF11 (HEK Expi293- and CHO-expressed) used in the studies presented here were characterized

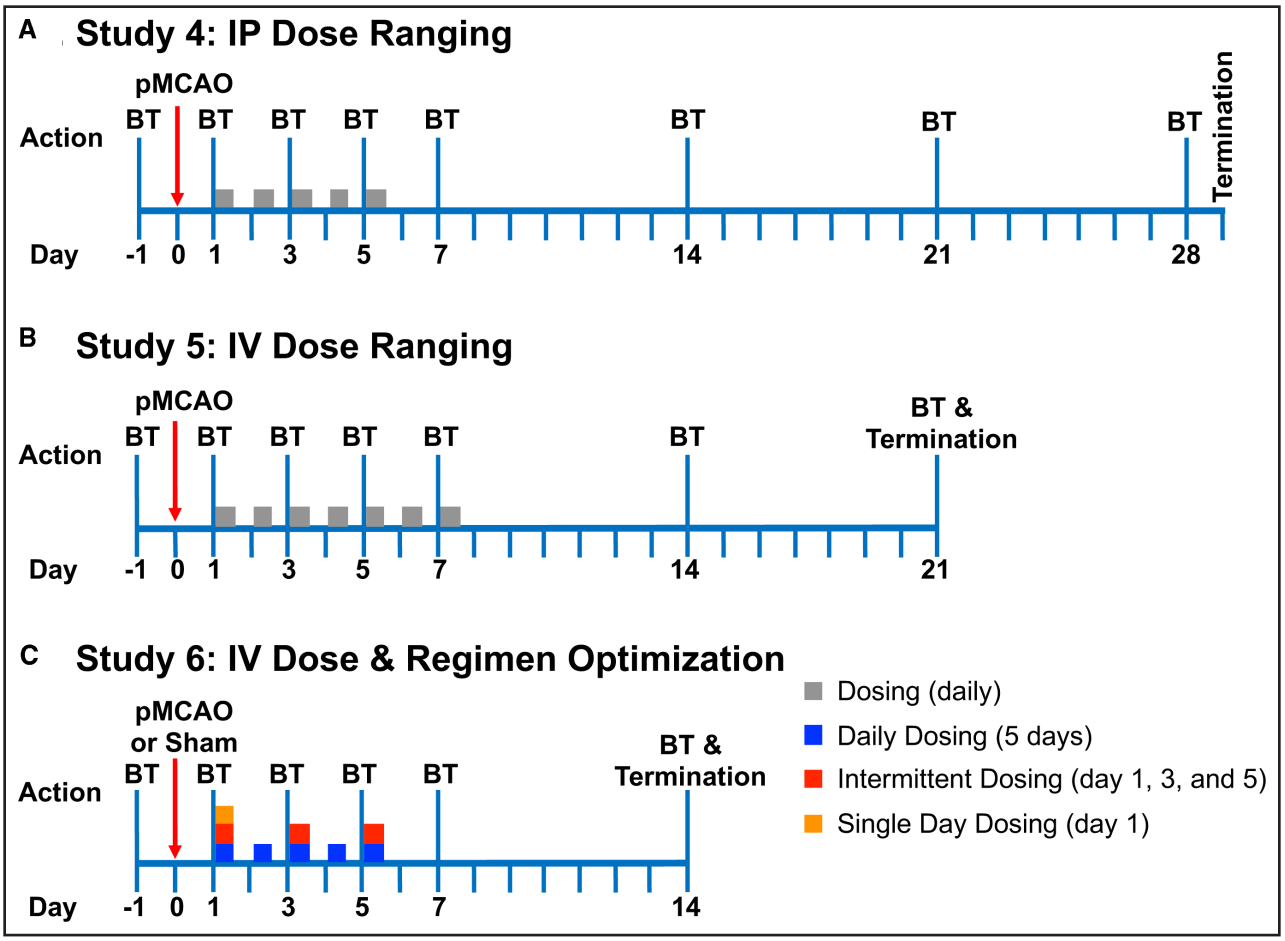

Figure 1. Study designs for evaluating the efficacy of rGDF11 (recombinant growth differentiation factor 11) in the permanent middle cerebral artery occlusion (pMCAO) rat model.

A through **C**, Experimental designs for studies 4, 5, and 6, detailing the timing of pMCAO or sham surgeries (indicated by red arrows), dosing initiation, dosing frequency, dosing duration, behavioral testing (BT), and study termination. **A**, Study 4: intraperitoneal dose ranging. Once-daily intraperitoneal dosing for 5 days. Dosing days are shown as gray squares. **B**, Study 5: intravenous dose ranging. Once-daily intravenous dosing for 7 days. Dosing days are shown as gray squares. **C**, Study 6: intravenous dose and regimen optimization. A single intravenous dose on day 1 is marked by an orange square; intermittent dosing on days 1, 3, and 5 is marked by red squares; and daily dosing from days 1 to 5 is marked by blue squares. For all studies, animals received only 1 dose per day on dosing days. On days when multiple activities were performed, behavioral tests were conducted first, followed by drug administration, and then blood draws.

and qualified before use and had comparable purity and EC50 values (data not shown).

To assess the in vivo function and biological activity of rGDF11, levels of pSmad2/3 were analyzed by ELISA in 4 different organs (spleen, liver, heart, and pancreas) of mice (Figure S1C through S1F) after a single intraperitoneal injection of 1 mg/kg purified protein. rGDF11 induced elevation of Smad2/3 phosphorylation in all organs tested. Phosphorylation levels were comparable in the spleen and liver and began to rise at or before 0.25 hours post-dosing, peaking at ≈1 hour, and returning to baseline at the 12-hour timepoint. rGDF11-mediated Smad2/3 phosphorylation in the heart was less pronounced than in the liver and spleen. In heart tissue, elevated pSmad2/3 levels returned to baseline by 6 hours post-dosing. Smad2/3 signaling in pancreatic tissue peaked at ≈1 hour, was sustained for at least 6 hours, and dropped back to baseline by 12 hours. Overall, the levels of pSmad2/3 were as follows: Spleen>Liver>Pancreas>Heart. Thus, the SMAD2/3-responsive luciferase reporter activity data, coupled with in vivo pSmad2/3 ELISA data, indicate that Expi293 cell-produced rGDF11 exhibits high purity and potent activity both in vitro and in vivo, with EC50 values comparable to commercially available *E. coli*-produced rGDF11 protein.

rGDF11 Decreased Cardiac Hypertrophy and Improved Glucose Tolerance in Aged Mice

To validate the activity and efficacy of internally produced rGDF11 in vivo, we evaluated its effects on cardiac hypertrophy (Figure S2A) and glucose metabolism (Figure S3) in aged mice, as these are well-established

experimental models in which rGDF11 effects have been studied previously.[15,16] Young (14 weeks) and old (84 weeks) mice were treated with either vehicle or 1 mg/kg rGDF11 once daily for 15 days via intraperitoneal injection. Before treatment, baseline measurements of body weight (Figure S2B and S2D), glucose tolerance (Figure S3A and S3C), and insulin tolerance (Figure S3B and S3D) were recorded. In response to rGDF11 treatment, aged mice exhibited a decrease in body weight, which was reduced by 15.5% on day 10 compared with the first day of treatment (day 1; Figure S2C). In contrast, young mice showed a maximum mean weight reduction of 10.1% on day 11. Percent body weight change was significantly decreased for rGDF11-treated versus vehicle-treated young mice starting on day 4 (Figure S2E). It is important to note that some changes in body weight were due to fasting, which began 16 hours before the glucose tolerance test on days 7 and 10 (Figure S3E).

We compared the heart weight-to-tibial length ratios between rGDF11- and vehicle-treated mice, as well as between aged and young mice (Figure S2A), after 15 days of treatment.[3,16] The heart weight-to-tibial length ratio was significantly higher in aged vehicle-treated mice compared with young vehicle-treated mice. Aged mice treated with rGDF11 had significantly lower heart weight-to-tibial length ratios compared with aged vehicle-treated mice, consistent with previously published data indicating that rGDF11 decreases age-related cardiac hypertrophy.[3,16] There were no differences in heart weight-to-tibial length ratios between rGDF11-treated aged mice and either vehicle-treated or rGDF11-treated young mice.

We then evaluated the in vivo effects of rGDF11 on glucose metabolism using glucose tolerance tests and insulin tolerance tests in these young and aged mice. Consistent with previous findings,[15] rGDF11 treatment improved glucose tolerance in both young and aged mice (Figure S3A and S3C). In aged mice, significant reductions in blood glucose were observed at 15 and 30 minutes after glucose injection in the rGDF11-treated group compared with the vehicle-treated group. In young mice, significant blood glucose reductions were observed at 30 and 60 minutes post-glucose injection in the rGDF11-treated group compared with the vehicle-treated group (Figure S3C). Insulin tolerance test results showed no significant effect of rGDF11 treatment on insulin tolerance in either age group (Figure S3B and S3D), except for a significant difference which was noted at 90 minutes for the rGDF11-treated aged group on day 14 as compared with rGDF11 baseline (Figure S3B). These data rigorously validate our rGDF11 in established in vitro and in vivo models, and thus we proceeded to study the effects of this protein in a validated rat model of ischemic stroke.

rGDF11 Dosing Regimen With Optimized Efficacy in Promoting Recovery Postischemic Stroke

To evaluate the benefits of rGDF11 and develop an optimized dosing regimen for promoting recovery poststroke, 6 independent preclinical ischemic stroke rat experiments were performed, all with blinding and randomization (Table S1).[13] Young adult male rats were selected for these studies because of their relatively stable hormonal and physiological profiles, which minimize variability in key outcome measures such as infarct volume, behavioral recovery, and drug pharmacokinetics.[17,18] In addition, age-related factors and hormonal cycles in females could introduce confounding variables,[19] complicating the interpretation of dose-response relationships and therapeutic effects during these studies designed to develop an optimized dosing regimen, characterize the mechanism of action, identify pharmacodynamic and mechanistic biomarkers, and define a safety and therapeutic window for rGDF11 in the pMCAO model. Results from 6 experiments showed improved sensorimotor function recovery in pMCAO rats treated with rGDF11 compared with vehicle-treated controls (Table S1). Functional behavioral results for 3 of the 6 studies are presented in Figures 2A through 2C, 3A through 3C, 4A through 4F, and Figure S4A through S4F. Although all 6 studies showed efficacy, we identified 1 dosing regimen that clearly outperformed others with respect to improving sensorimotor function (Figure 4A through 4F) and mitigating rGDF11-induced reductions in body weight (Figure S5A through S5D). In particular, we found that 3 intravenous doses of rGDF11 administered once every 48 hours over 5 days at a concentration of 1 mg/kg and initiated 24 hours following pMCAO performed best (Figure 4A through 4F) across the 6 studies.

rGDF11 Improved Sensorimotor Function, Increased Neural Stem Cell Numbers, and Enhanced Neovascularization (Study 4)

Daily doses of vehicle or rGDF11 (0.1, 0.5, 1, 2, and 4 mg/kg) were administered intraperitoneally once daily for 5 days to pMCAO rats, with dosing initiated 24 hours post-injury. Superior and sustained motor function recovery was observed for the 1, 2, and 4 mg/kg-treated rats compared with vehicle-treated animals in all behavioral assays, which included body swing, forelimb placement, and hindlimb placement over the 28-day post-pMCAO study period (Figure 2A through 2C). Superior sustained body swing recovery was also observed for the 0.5 mg/kg rGDF11 dose (Figure 2A). The effect of rGDF11 on neural stem cells poststroke was quantified using immunofluorescence analysis of the ventricular zone at study termination (28 days post-injury). In the ipsilateral ventricular zone, the numbers of Sox2-positive cells (a

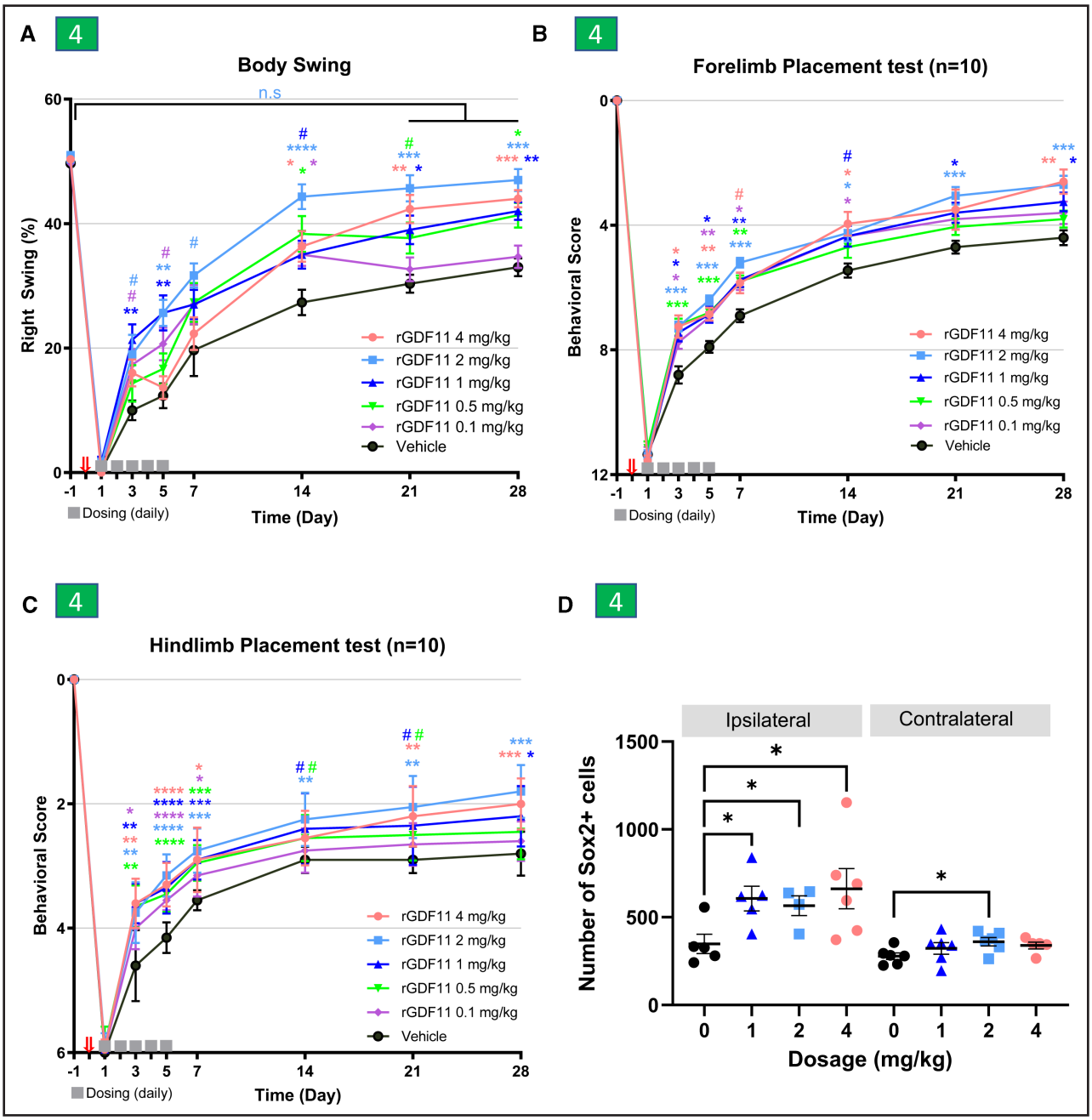

Figure 2. Intraperitoneal rGDF11 (recombinant growth differentiation factor 11) improves motor function and increases radial glial cell numbers (study 4: intraperitoneal dose ranging).

A through **C**, Motor function analysis for study 4 (green number): One daily dose for 5 days (days 1–5; gray squares represent dosing days) after stroke (red arrow) with n=10 rats per group. Dose groups were 0 (vehicle), 0.1, 0.5, 1, 2, and 4 mg/kg rGDF11. Graphs show rat body swing (**A**), forelimb placement (**B**), and hindlimb placement (**C**) with baseline measurements performed on day −1, stroke procedure performed on day 0, and poststroke measurements on days 1, 3, 5, 7, 14, 21, and 28. **D**, Graph showing the number of neuronal progenitor cells in rat ipsilateral and contralateral brain slides after 0 (vehicle), 1, 2, and 4 mg/kg rGDF11 treatment 28 days following permanent middle cerebral artery occlusion (pMCAO). Stats: **A** through **C**, Data plotted as mean±SEM. Two-way ANOVA repeated measures with multiple comparison (for each day compare doses to vehicle) and Dunnett post hoc correction. **A**, For 2 mg/kg, the body swing score at days 21 and 28 was comparable to prestroke measurements (day −1). For the latter, we ran a 2-way ANOVA repeated measures with multiple comparison (for each dose to compare days to day −1) and Dunnett post hoc correction. **D**, One-way ANOVA nonsignificant: $F(3,6)=2.682$ and $P=0.0818$ and parametric unpaired t test with *$P<0.05$. Data plotted as individual values with mean±SEM. For all graphs, *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, and #$P<0.1$.

marker for neural stem cells)[20] increased for the 1, 2, and 4 mg/kg doses, whereas in the contralateral ventricular zone significant increases in progenitor cell number were only observed for the 2 mg/kg dose (Figure 2D). This study also evaluated the impact of rGDF11 on vascularization in the peri-infarct region using CD31 staining.

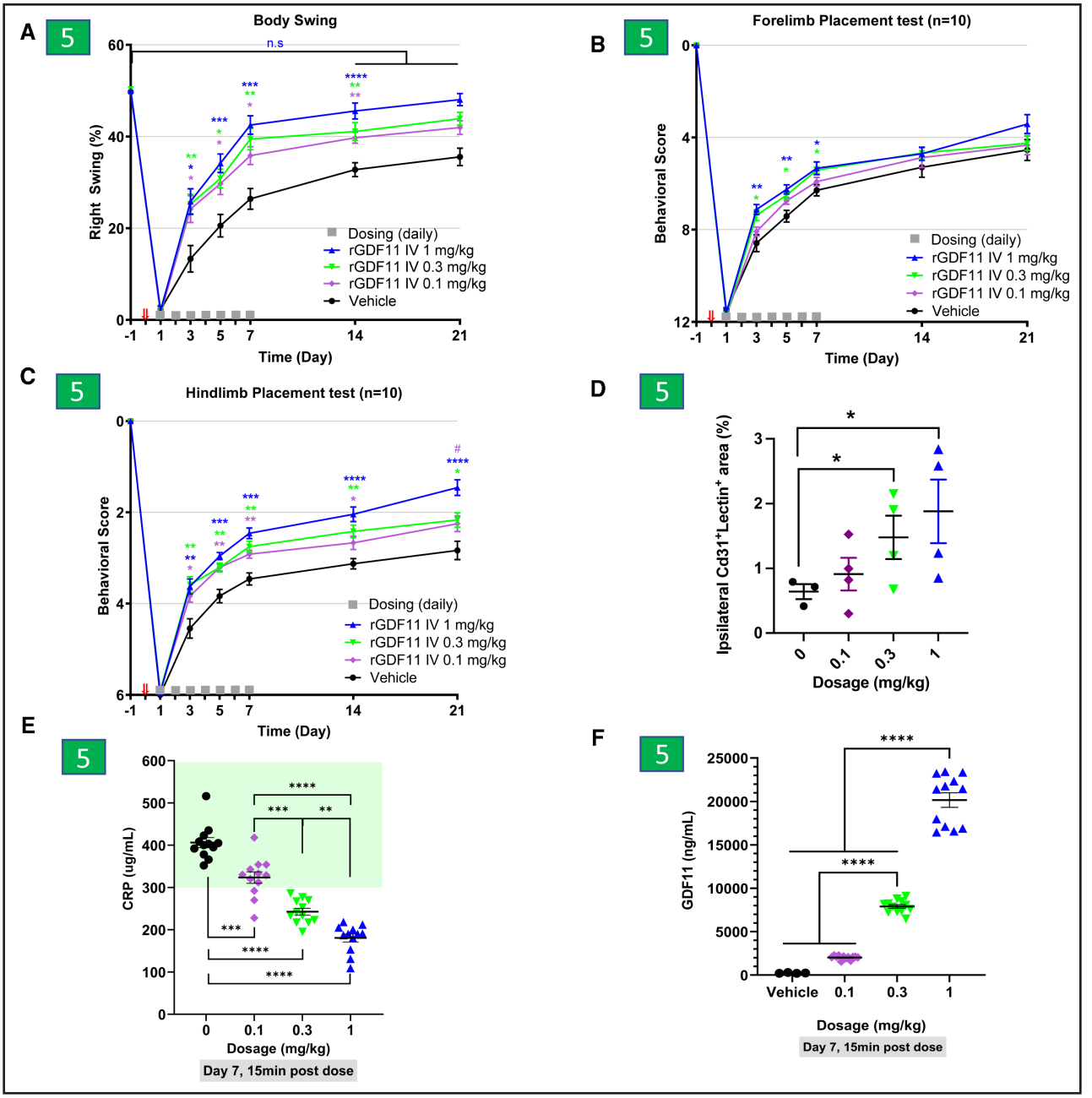

Figure 3. Intravenous rGDF11 (recombinant growth differentiation factor 11) improves motor function and increases vascularization (study 5: intravenous dose ranging).

A through **C**, Motor function analysis for Study 5 (green number): One dose daily for 7 days (days 1–7; gray squares represent dosing days) after stroke (red arrow) with n=10 rats per group. Dose groups were 0 (vehicle), 0.1, 0.3, and 1 mg/kg rGDF11. Graphs show rat body swing (**A**), forelimb placement (**B**) or hindlimb placement (**C**) with baseline measurement performed on day −1, stroke procedure performed on day 0 and poststroke measurements on days 1, 3, 5, 7, 14, and 21. **D**, Graph showing the percent area in rat ipsilateral brains with CD31+ Lectin+ endothelial progenitor cells after 0 (vehicle), 0.1, 0.3, and 1 mg/kg rGDF11 treatment 21 days following permanent middle cerebral artery occlusion (pMCAO). **E**, CRP (C-reactive protein) concentration measured in rat serum collected 15 minutes after 0 (vehicle), 0.1, 0.3, and 1 mg/kg rGDF11 treatment on day 7. The shaded green area represents the normal CRP concentration range in healthy rats.[22] **F**, GDF11 concentration measured via ELISA assay in rat serum on day 7 after stroke, 15 minutes after 0 (vehicle), 0.1, 0.3, and 1 mg/kg rGDF11 treatment. Stats: **A** through **C**, Data plotted as mean±SEM. Two-way ANOVA repeated measures with multiple comparison (for each day compare doses to vehicle) and Dunnett post hoc correction. **A**, For 1 mg/kg, we also show a body swing score at days 14 and 21 comparable to prestroke measurements (day −1). For the latter, we ran a 2-way ANOVA repeated measures with multiple comparison (for each dose compare days to day −1) and Dunnett post hoc correction. **D** through **E**, Data plotted as individual value with mean±SEM. **D**, One-way ANOVA nonsignificant: $F(3, 11)=2.429$ and $P=0.1203$ and parametric unpaired t test with $^{*}P<0.05$. **E** and **F**, Two-way ANOVA with multiple comparison (compare each dose with all other doses) with Tukey post hoc correction. n=12 per group for 0.1, 0.3, and 1 mg/kg doses and n=4 for the vehicle group. For all graphs, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, and $\#P<0.1$.

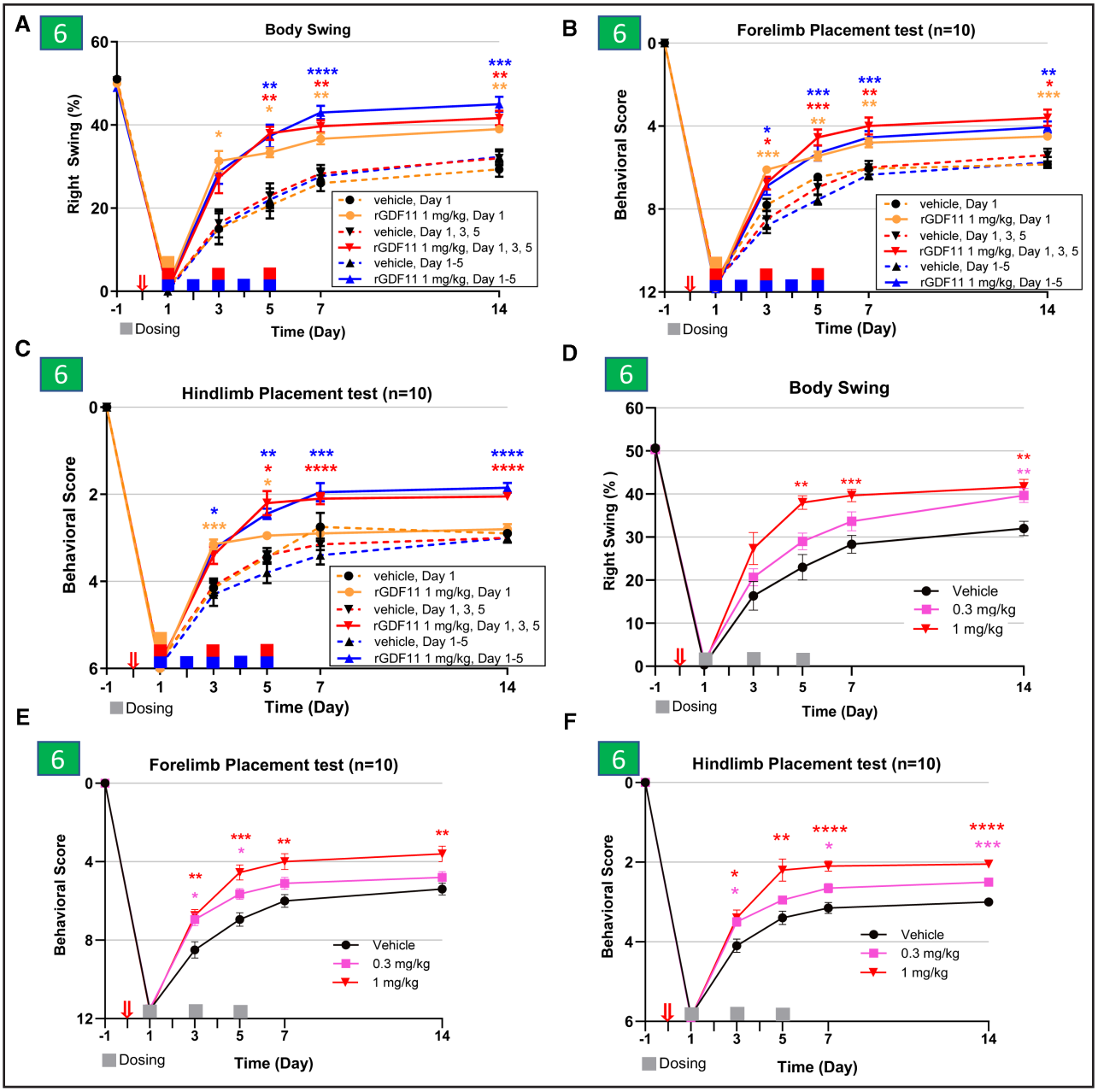

Figure 4. Intravenous rGDF11 (recombinant growth differentiation factor 11) dose regimen optimization and dose dependency (study 6: intravenous dose and regimen optimization).

A through **C**, Motor function analysis for study 6 (green number) encompassing all intravenous dosing regimens at 1 mg/kg rGDF11 treatment: (1) single dose on day 1 (orange), (2) intermittent dosing on days 1, 3, and 5 (red) and (3) daily dosing on days 1 to 5 (blue) after stroke (red arrow) for 1 mg/kg rGDF11 (continuous lines) or vehicle (dotted lines). The small squares show the dosing days for each group with their respective color. **D** and **E**, Motor function analysis for intermittent dosing only on days 1, 3, and 5 (gray squares represent dosing days) after stroke (red arrow). Dose groups were 0 (vehicle), 0.3, and 1 mg/kg rGDF11. Graphs show rat body swing (**D**), forelimb placement (**E**) or hindlimb placement (**F**) with baseline measurement performed on day −1, stroke procedure performed on day 0 and poststroke measurements on days 1, 3, 5, 7, and 14. Stats: Data plotted as mean±SEM with n=10 per group. **A** through **C**, Two-way ANOVA repeated measures with multiple comparison (for each dosing regimen, compare vehicle vs treatment) and Sidak post hoc correction. **D** through **F**, Two-way ANOVA repeated measures with multiple comparison (for each day compare doses to vehicle) and Dunnett post hoc correction. For all graphs, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and #P<0.1.

Vascular morphometric analysis demonstrated a significant increase in the percentage of vessel area and total vessel length in rats treated with 1 mg/kg of rGDF11 compared with vehicle-treated controls. In addition, a marginal increase in junction density and average vessel length was observed in the 1 mg/kg-treated group compared with the vehicle group (Figure S6A through S6D).[21] The peri-infarct region used for vascularization analysis is shown in Figure S7A, along with representative images in Figure S7B.

rGDF11 Improved Sensorimotor Function, Increased Neovascularization, and Decreased CRP Levels (Study 5)

A study of once-daily intravenous administration of either vehicle alone or 0.1, 0.3, or 1 mg/kg rGDF11 for 7 days was performed to determine an efficacious dose range in the rat pMCAO model. All 3 rGDF11 dose levels showed significant and sustained improvement in motor function in body swing (Figure 3A) and hindlimb placement tests (Figure 3C) compared with the vehicle control, with the 1 mg/kg dose showing the greatest efficacy. In these 2 tests, motor function was significantly improved as early as day 3 after rGDF11 administration and exhibited persistent improvement through day 21. The 1 mg/kg dose showed superior improvement over the 0.1 and 0.3 mg/kg doses. The 1 and 0.3 mg/kg doses showed significant improvement in the forelimb placement test from day 3 to day 7 (Figure 3B). The effect of rGDF11 on neovascularization 21 days post-pMCAO was assessed via transcardial perfusion of biotinylated tomato lectin before fixation. Immunofluorescent analysis of lectin- and CD31-double-positive cells indicated a rGDF11-mediated increase in functional vessels for the 0.3 and 1 mg/kg doses in the ipsilateral cortex (Figure 3D). Vascular morphometric analysis was also conducted to evaluate total vessel length, average vessel length, and junction density.[21] The analyses revealed a marginal increase in total vessel length in the 1 mg/kg dose group compared with the vehicle, with no significant changes observed in average vessel length (Figure S8A and S8B). A significant increase in junction density was identified in the 0.3 mg/kg dose group compared with the vehicle. In addition, both the 1 and 0.3 mg/kg dose groups showed a marginal increase in junction density compared with the 0.1 mg/kg dose group (Figure S8C). The peri-infarct region used for vascularization analysis is shown in Figure S9A, along with representative images in Figure S9B.

Serum levels of CRP were also measured to evaluate the potential impact of rGDF11 on inflammation and to evaluate CRP as a candidate mechanistic biomarker. Serum samples collected 15 minutes after the final rGDF11 administration on day 7 were analyzed by CRP and rGDF11 ELISAs. Results showed a significant, dose-dependent decrease in CRP levels (Figure 3E) with increasing rGDF11 treatment doses. rGDF11 ELISA of serum samples confirmed increased rGDF11 exposure in the serum for each dose (Figure 3F). Pearson correlation showed a negative correlation trend (r=−0.9190; P=0.0810) between rGDF11 levels and CRP levels in the serum (data not shown). Once-daily intravenous dosing with 1 mg/kg rGDF11 had the largest effect on sensorimotor recovery, neovascularization, and CRP post-pMCAO injury.

Improved Motor Function Recovery With Acute Intermittent Dosing of rGDF11 (Study 6)

We further optimized the frequency of rGDF11 administration by treating pMCAO-injured rats with either a single dose (day 1), 3 intermittent doses (days 1, 3, and 5), or 5 repeated daily doses (days 1–5) of 1 mg/kg rGDF11. Body swing (Figure 4A), forelimb placement (Figure 4B), and hindlimb placement (Figure 4C) behavioral tests were performed to assess motor function recovery poststroke (Table S2). Interestingly, intermittent dosing post-pMCAO showed comparable improvements in functional sensorimotor recovery to those observed for 5 daily doses as compared with vehicle controls in all functional outcome measures (Figure 4A through 4C). Furthermore, intermittent and daily dosing of 0.3 and 1 mg/kg showed dose-dependent behavioral improvements when compared with their respective vehicle-treated animals (Figure 4D through 4F; Figure S4A through S4C). In addition, dose-dependent sensorimotor function recovery was also observed after a single intravenous administration of rGDF11 (0.3 and 1 mg/kg; Figure S4D through S4F), although to a lesser extent than intermittent and repeated daily dosing.

Because rGDF11 treatment is known to moderate food consumption and reduce body weight in rodent models,[15] we systematically studied body weight when considering an appropriate dose regimen. On days 3 through 7 poststroke, 5 daily doses of rGDF11 showed a greater effect in reducing body weight than intermittent doses on days 1, 3, and 5, when compared with their respective vehicle controls (Figure S5). All dose regimens showed a recovery in body weight by day 14 poststroke. Taken together, intermittent dosing showed a comparable efficacy and smaller body weight loss than daily dosing, which supported the design of safety and toxicology studies (Table S3) in both male and female rats using the intermittent dosing approach. No adverse effects were observed in rat (Sprague-Dawley) safety and toxicology studies for either sex employing an intermittent dosing regimen with 3 doses over a 5-day period at a concentration of up to 10 mg/kg, suggesting a safety window of at least 10-fold above the optimized efficacious dose in rats (Table S3).

To extend our findings demonstrating the effect of rGDF11 treatment on circulating stroke-associated inflammatory biomarkers, we performed CRP analysis on longitudinally collected serum from animals treated at 1 mg/kg for each dosing regimen. For the single-dose, intermittent-dose, and daily-dose groups, ELISA analyses were performed on serum samples from pre-stroke (day −1) and on days 2, 5, 7, and 14. All dosing regimens showed a decrease in circulating CRP levels in response to 1 mg/kg rGDF11 treatment compared with vehicle controls, beginning on day 2 (1 day

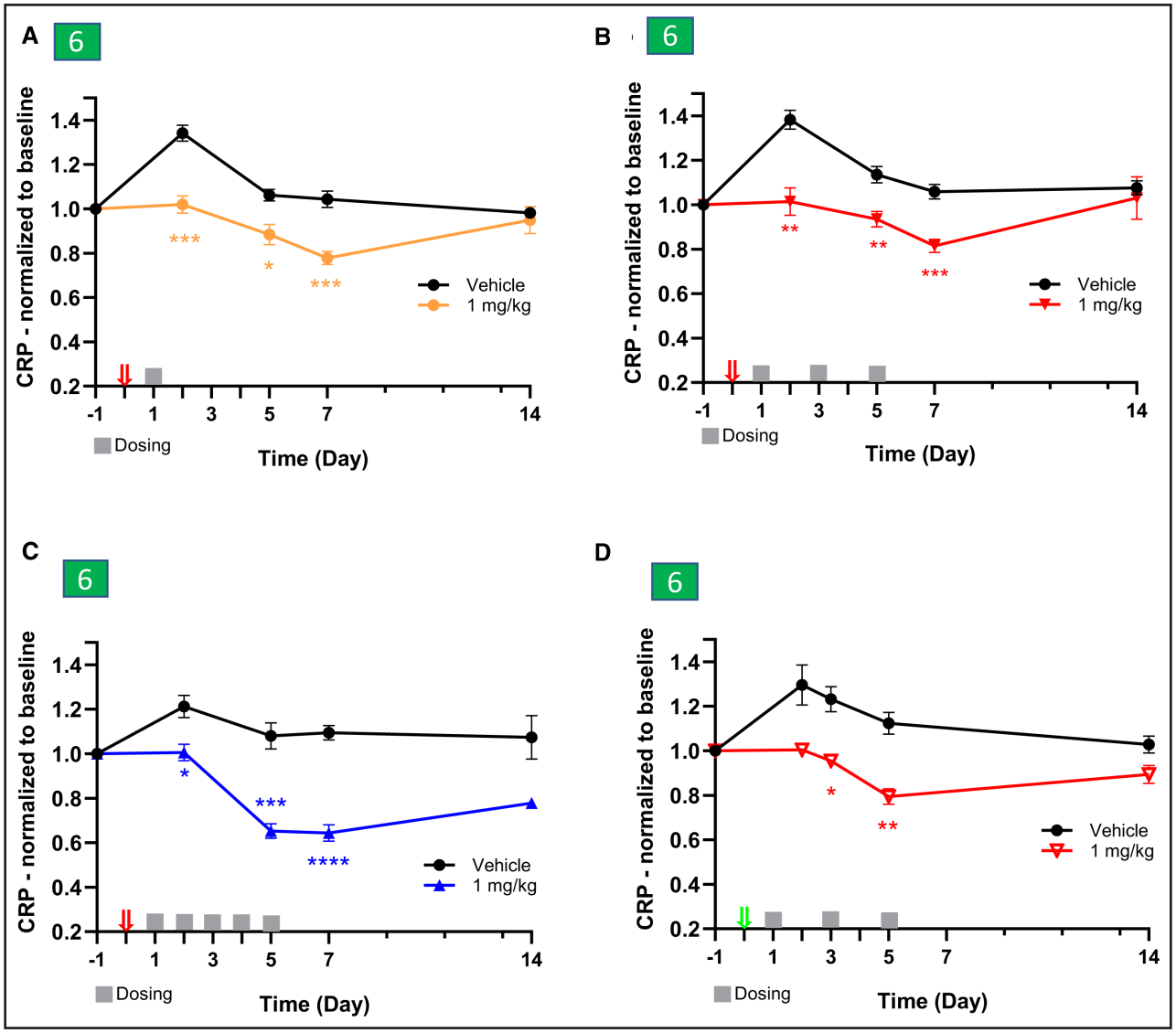

Figure 5. Intravenous rGDF11 (recombinant growth differentiation factor 11) treatment decreases serum CRP (C-reactive protein) concentrations post-injury (study 6: intravenous dose and regimen optimization).
CRP levels normalized to baseline for study 6 (green number) encompassing all dosing regimens at 1 mg/kg rGDF11 treatment: (**A**) single dose on day 1, (**B**) intermittent dosing on days 1, 3, and 5 and (**C**) daily dosing on days 1 to 5 after stroke (red arrow) and (**D**) intermittent dosing on days 1, 3, and 5 after sham (green arrow). The gray squares show the dosing regimen for each graph. Stats: All graphs were analyzed for 2-way ANOVA repeated measures with multiple comparison (each treatment compared with vehicle) and Sidak post hoc correction. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

following the first treatment) through day 7 (Figure 5A through 5D).

Serum Analysis Reveals a Candidate Panel of Stroke-Specific Biomarkers (Study 6)

Exogenous rGDF11 triggers signaling pathways that promote spatiotemporal gene expression, protein translation, and secretion.[23] A SomaScan aptamer-based analysis was performed on longitudinally collected serum samples from the daily 1 mg/kg rGDF11 intravenous and vehicle treatment animal groups. We also included samples from the 1 mg/kg intermittent dosing group to identify any overlapping candidates. In addition, serum samples from sham animals exposed to 1 mg/kg rGDF11 and vehicle were analyzed to distinguish the stroke-associated candidate biomarkers (see Table S2 for the full study design and selected samples). The number of proteins that surpassed our significance threshold for the pathway analysis (P<0.01 and fold change >1.5) was the lowest on day 2 and the highest on day 5 when comparing rGDF11 treatment to vehicle control. Furthermore, data from the intermittent dosing group and sham groups showed a limited number of significant changes compared with the day 5 daily dose group (Table S4), supporting the decision to limit pathway analysis to the daily dose animals.

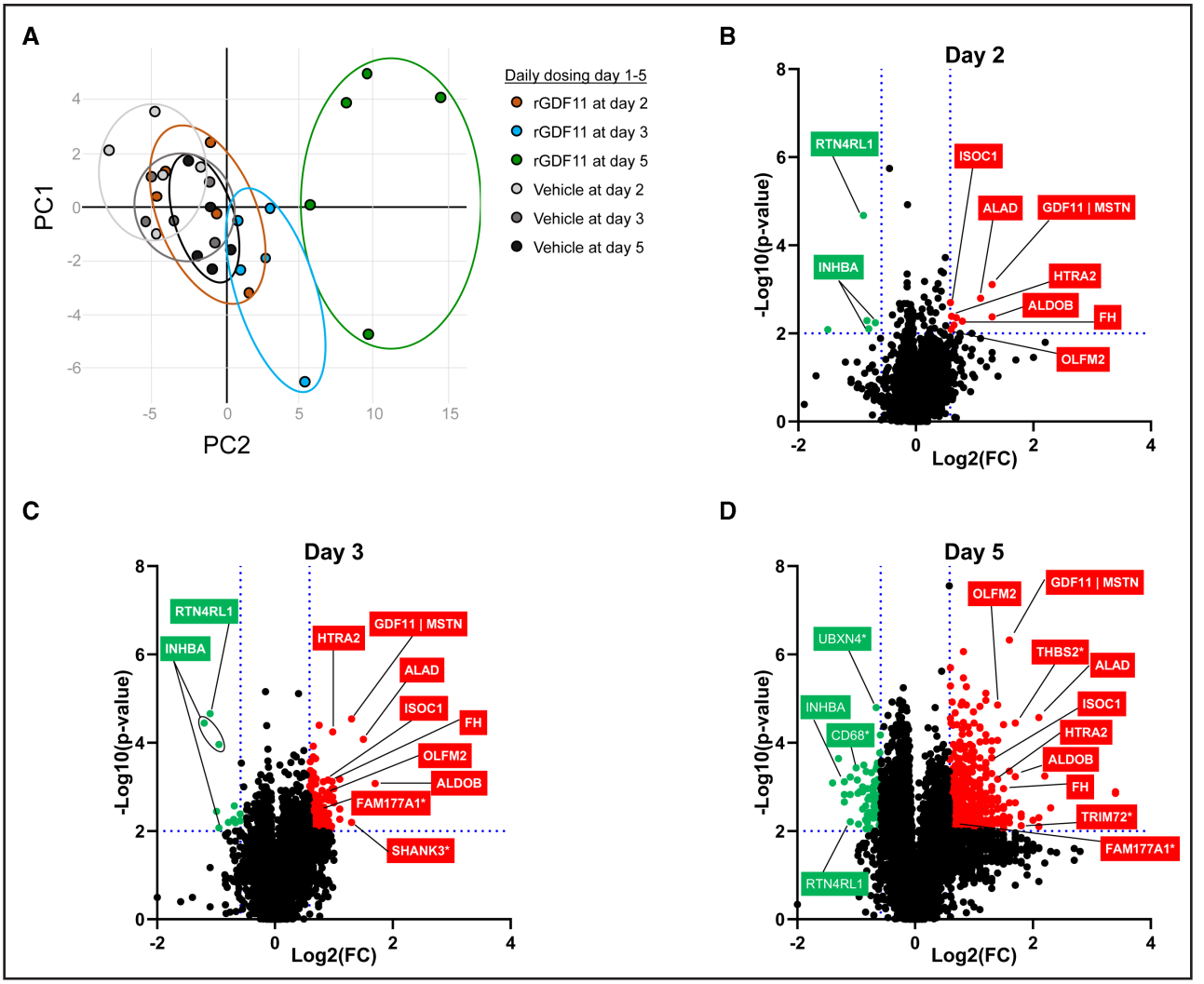

Figure 6. Biomarker analysis (study 6: intravenous dose and regimen optimization).
A, Principal component analysis (PCA) analysis of biomarkers discovered using SomaScan analysis. Circles represent individual rats exposed to daily dosing on days 1 to 5 with either 0 mg/kg (vehicle) or 1 mg/kg rGDF11 (recombinant growth differentiation factor 11). SomaScan analysis was run on rat serum on days 2, 3, and 5 after stroke. The different colors correspond to rats treated with either rGDF11 or vehicle and from which serum was harvested on days 2, 3, and 5, respectively as indicated in the associated legend. **B** through **D**, Volcano plot showing the distribution of SomaScan biomarkers for day 2 (**B**), day 3 (**C**) or day 5 (**D**) after stroke with the specific names of proteins related to stroke. Biomarkers with a fold change >1.5 and $P<0.01$ when compared with vehicle control, are highlighted in green (decreased in circulation) or red (increased in circulation). Biomarkers marked with a * are specific to each day (**B**, day 2; **C**, day 3; and **D**, day 5) except for FAM177A1 which is increased on both days 3 and 5. All other highlighted biomarkers green or red are days 2, 3, and 5.

Principal component analysis was performed on samples collected on days 2, 3, and 5 from both vehicle- and 1 mg/kg-treated animals in the daily dosing regimen. All samples from vehicle-treated animals clustered together with no apparent separation, regardless of the number of days of treatment. On the other hand, samples from the 1 mg/kg rGDF11 treated animals showed clustering per day of treatment with increasing separation for increasing days of treatment (Figure 6A). The increased variance in the day 5 daily dosing group that was seen in the principal component analysis was further explored by comparing the volcano plots of rGDF11 versus vehicle on days 2, 3, and 5 (Figure 6B through 6D). The effect of rGDF11 treatment on the circulating proteome increased with the number of treatment days, with more proteins showing significantly altered abundance on day 5 compared with days 2 or 3 of dosing. In addition to the expected dose-related increase in serum GDF11, several proteins showed consistent changes across all 3 days: 7 were increased (GDF11, Alad [aminolevulinate dehydratase], Aldob [aldolase B, fructose-bisphosphate], Fh [fumarate hydratase], Olfm2 [olfactomedin 2], Htra2 [HtrA serine peptidase 2], and Isoc1 [isochorismatase domain containing 1]), while 2 were decreased (Inhba [inhibin subunit β A] and Rtn4rl1 [reticulon 4 receptor-like 1]) in the serum on days 2, 3, and 5 in response to rGDF11 treatment (Figure 6B through 6D).

We also performed pathway analysis to compare vehicle- versus rGDF11-treated animals for each day, as well as pre-pMCAO versus post-pMCAO animals. To maximize analytical power, we focused on data from serum collected on days 2, 3, and 5 in the daily dosing group. Ingenuity Pathway Analysis (IPA) software identified differentially activated signaling pathways, many of which are associated with the hypothesized mechanism of stroke repair, including synaptogenesis, NGF (nerve growth factor) signaling, ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) signaling, and IGF-1 (insulin-like growth factor-1) signaling (Figure S10). Under the tested comparison analysis thresholds ($P<0.0001$ and Z score >4), day 2 showed no activated pathways in the paired comparison of vehicle versus rGDF11 nor treatment versus pre-pMCAO. Activated pathways on day 3 were observed when comparing rGDF11-treated animals with pre-pMCAO animals and vehicle controls. Comparison of vehicle control with pre-pMCAO animals at day 3 did not show a proteomic signature that was associated with any activated pathways. In fact, vehicle-treated animals on days 2 and 3, when compared with pre-pMCAO animals, showed a trend toward inhibition of the synaptogenesis signaling pathway.

To further explore the mechanisms by which rGDF11-responsive proteins may facilitate stroke recovery, we analyzed proteins that showed Bonferroni-corrected significance for the 1 mg/kg rGDF11 daily dosing group compared with control. Path Explorer analysis of these proteins from days 2, 3, and 5 predicted activation of functions related to angiogenesis, neurogenesis, axongenesis, and developmental process of synapses, together with inhibition of nervous system inflammation. While some of these proteins showed a direct path to the predicted functions, others may have an indirect role in pathway activation or inhibition (Figure S11).

We evaluated individual proteins that showed statistical significance, a fold change >1.5 between rGDF11 treatment and control, and known biological activity that is potentially relevant to GDF11 function and stroke repair. We also incorporated data from the intermittent dosing group to identify proteins that show similar trends and included the data from the sham groups to determine whether changes were dependent on pMCAO. Candidate biomarkers that showed a consistent decrease in response to rGDF11 treatment compared with the vehicle-treated group were Rtn4rl1, Cd68, Inhba, Fgl1 (fibrinogen-like 1), and Nppb ([natriuretic peptide B]; Figure S12). Candidate biomarkers that were upregulated in response to rGDF11 treatment included Thbs2 (thrombospondin 2), Nrp1 (neuropilin 1), Ucn3 (urocortin 3), Sparcl1 (SPARC-like 1), Nog (Noggin), and Fam177a1 ([family with sequence similarity 177 member A1]; Figure S13).

To further substantiate our earlier findings of enhanced neovascularization in response to rGDF11, we examined circulating vascular modulators that may contribute to vascular repair mechanisms. On day 5, we observed a significant increase in Angpt1 (angiopoietin-1) levels in the rGDF11 daily dosing group compared with the vehicle control (Figure S14A). In addition, circulating levels of Angpt2 (angiopoietin-2) showed an upward trend on day 5; however, this increase was only marginal after adjustment for multiple testing (Figure S14B).

DISCUSSION

Here, we show that in vivo supplementation with rigorously validated recombinant GDF11 promotes recovery and regenerative processes in the brain poststroke. We initially confirmed that rGDF11 reversed cardiac hypertrophy and improved glucose metabolism in aged mice and demonstrated that both of these beneficial effects were observed using the same dosing regimen. We then completed an extensive investigation into the therapeutic potential of rGDF11 to promote recovery and regeneration poststroke. This investigation interrogated dosing initiation, frequency, duration, and range, as well as the route of administration with rGDF11 and demonstrated that a short-term acute intermittent dosing paradigm initiated 24 to 72 hours post-injury is sufficient to improve sensorimotor function, promote neurogenesis and neovascularization in the brain, and reduce inflammation post-injury in the rat pMCAO model.

CRP, a sensitive marker for inflammation,[24] was found to decrease dose-dependently in response to rGDF11 (Figure 3E). Elevated CRP increases secondary brain damage in animal models of focal cerebral ischemia[25] and elevated CRP levels are found in up to 75% of patients with ischemic stroke.[26] An unexpected and striking finding in our experiments was the rapid effect of rGDF11, with differences apparent within the first week after ischemic stroke. It is possible that multiple mechanisms are responsible for the observed effects on inflammation, neurogenesis, vascularization, and functional recovery. Additional experiments will be required to better understand the mechanism of the early sensorimotor benefits of rGDF11 in ischemic stroke, as these may not be explained by the analyses at later time points.

Proteomics analysis identified several canonical pathways activated by rGDF11 treatment, particularly on days 3 and 5 poststroke. Analysis of protein levels revealed consistent upregulation (Alad, Aldob, Fh, Olfm2, Htra2, and Isoc1) and downregulation (Inhba and Rtn4rl1) of specific proteins. These persistent changes in protein levels provide a wide temporal window for biomarker measurements. The serum proteomics analysis focused on daily dosing study arms, with fewer significant changes observed in the intermittent dosing group, possibly due to the longer intervals between dosing and measurements. These findings highlight the need for further studies to investigate the role of these

biomarkers in stroke recovery and validate them using orthogonal assays. We also believe that additional biomarker and molecular studies will be needed to understand the reproducible and surprising early benefit of rGDF11 during stroke recovery.

In this study, we utilized young adult male rats to establish a controlled and reproducible model for investigating the effects of rGDF11 post-pMCAO. Young adult males were chosen to minimize biological variability, as age- and sex-related differences in stroke pathology and recovery have been well documented.[19,27] Aged male and female rats exhibit distinct cerebrovascular physiology, hormonal influences, and immune responses that could introduce additional variables, complicating the interpretation of experimental outcomes.[28–30] By focusing on a homogeneous group, we aimed to ensure consistency and reliability in our findings, providing a robust foundation for future translational and clinical studies. We recognize the importance of developing stroke recovery therapeutics to address the diverse population of affected individuals particularly older patients and those with comorbidities such as hypertension, diabetes, and hypercholesterolemia. Thus, we suggest that future experiments on exogenous GDF11 treatment in experimental stroke should address the important variables of sex, age, and associated comorbidities, as this will provide greater confidence in the translational potential of rGDF11 as a therapeutic to promote recovery poststroke.[31] Significantly, the data presented here demonstrate that systemically delivered rGDF11 enhances neovascularization, reduces inflammation, promotes neurogenesis, and improves sensorimotor function post-injury in a rat model of ischemic stroke. The surprisingly rapid onset of sensorimotor benefits and the dose-dependent reduction in CRP underscore rGDF11's potential to address key pathological processes in stroke recovery.[22] Coupled with the identification of an optimized and clinically feasible dosing regimen, a panel of candidate pharmacodynamic and mechanistic biomarkers, and a broad therapeutic and safety window, our data provide an important foundational basis to support the clinical translation of rGDF11 as a stroke recovery therapeutic.

ARTICLE INFORMATION

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Supplemental Material

Supplemental Methods
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