GDF10 is a signal for axonal sprouting and functional recovery after stroke

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Stroke produces a limited process of neural repair. Axonal sprouting in cortex adjacent to the infarct is part of this recovery process, but the signal that initiates axonal sprouting is not known. Growth and differentiation factor 10 (GDF10) is induced in peri-infarct neurons in mice, non-human primates and humans. GDF10 promotes axonal outgrowth in vitro in mouse, rat and human neurons through TGFβRI and TGFβRII signaling. Using pharmacogenetic gain- and loss-of-function studies, we found that GDF10 produced axonal sprouting and enhanced functional recovery after stroke; knocking down GDF10 blocked axonal sprouting and reduced recovery. RNA sequencing from peri-infarct cortical neurons revealed that GDF10 downregulated PTEN, upregulated PI3 kinase signaling and induced specific axonal guidance molecules. Using unsupervised genome-wide association analysis of the GDF10 transcriptome, we found that it was not related to neurodevelopment, but may partially overlap with other CNS injury patterns. Thus, GDF10 is a stroke-induced signal for axonal sprouting and functional recovery.

Stroke is the leading cause of adult disability because of the brain’s limited capacity for repair. Stroke induces axonal sprouting and the formation of new connections in peri-infarct cortex that link premotor, motor, somatosensory and association areas1–4. In humans, good functional recovery after stroke is associated with remapping of sensorimotor function in motor, somatosensory and premotor circuits5,6 and is accompanied by increases in cortical thickness in these reorganizing areas7. In rodent and primate models of stroke, axonal sprouting and the formation of new connections occurs in motor, somatosensory and premotor areas1–4. These new connections are causally associated with functional recovery4. A better understanding of the mechanisms of axonal sprouting may allow the development of therapies to stimulate recovery after stroke. We previously used transcriptional profiling of single sprouting neurons to identify a unique gene expression profile, a post-stroke sprouting transcriptome1. The molecular networks in this transcriptome involve coordinated signaling systems from secreted growth factors and cytokines to cell surface receptors, intermediary cytoplasmic cascades and transcriptional control molecules3. In the post-stroke axonal sprouting transcriptome, GDF10 is one of the most highly upregulated genes during the initiation of axonal sprouting in peri-infarct cortical neurons in the aged brain.

There have been many studies of the molecules that block axonal sprouting after CNS injury, such as myelin proteins or chondroitin sulfate proteoglycans (CSPGs)8–10, but the factor(s) that are triggered by stroke to promote the initiation of a new growth program and axonal sprouting are unknown. As a secreted growth factor, GDF10 is a leading candidate for such a growth-promoting signal after stroke. GDF10 is a divergent member of the bone morphogenetic protein (BMP)/transforming growth factor-β (TGFβ) superfamily11–14 (Supplementary Fig. 1). Compared with other GDFs, GDF10 has a unique gene structure12 and signals through TGFβ receptors (TGFβRs)14,15. Although Gdf10 mRNA is strongly expressed in the developing brain16,17, a role for GDF10 in the adult brain or after CNS injury has not been described.

We found that GDF10 upregulation after stroke was conserved across mice, non-human primates and humans. GDF10 promoted axonal sprouting through TGFβRI and II. In vivo, GDF10 enhanced axonal sprouting in peri-infarct cortex and improved motor recovery after stroke; knockdown of GDF10 blocked this axonal sprouting and behavioral recovery. RNA-seq analyses revealed that GDF10 coordinately regulates several molecular signaling systems to induce a neuronal growth state that is distinct from other developmental, CNS-injury and adult plasticity phenotypes. These mechanistic insights into GDF10 begin to advance our understanding of its potential as a therapeutic target after stroke.

RESULTS

GDF10 expression in peri-infarct cortex after stroke

GDF10 was induced in sprouting neurons from aged rats during the initiation phase of axonal sprouting, 7 d after stroke7: 1.32× with microarray and 3× with confirmatory quantitative PCR (P < 0.0009) at 7 d after stroke3. To confirm the universality of GDF10 induction, we determined

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that GDF10 was localized in peri-infarct tissue in mice, non-human primates and humans. There was a very low level of GDF10 present in neurons in non-stroke cortex (Fig. 1). After stroke, GDF10 was induced in cells with markers of neurons and in brain tissue surrounding these neurons (Fig. 1 and Supplementary Fig. 2a,d). GDF10 immunoreactivity in peri-infarct cortex was not present in cells with markers of microglia and astrocytes, but was seen in the extracellular matrix, as would be expected of a secreted protein in the peri-infarct cortex (Fig. 1 and Supplementary Fig. 2d,e). As determined by western blot, GDF10 protein expression was significantly elevated in peri-infarct cortex (P < 0.05; Supplementary Fig. 3). We found that GDF10 was induced in rodents (7 d after stroke), non-human primates (2 d and 7 d) and humans (chronic period), indicating a large range of species and a broad time period for upregulation after stroke. GDF10 may serve as a paracrine signal for axonal sprouting.

GDF10 enhances axonal length of primary cortical neurons

A role for GDF10 in axonal outgrowth has not been previously established. In mouse primary cortical neurons, GDF10 produced an increase in axonal outgrowth, with a dose of 500 ng ml⁻¹ producing equivalent axonal outgrowth as similar doses of the well-described axonal sprouting growth factors brain-derived growth factor (BDNF) and fibroblast growth factor 2 (FGF2) (Supplementary Fig. 3c). We determined that GDF10 promotes axonal outgrowth in several neuronal types, with a dose effect that is similar to that of other growth-promoting factors. Axonal outgrowth in the adult brain after stroke occurs in an environment inhibitory to axonal sprouting, with prominent induction of glial scar molecules such as CSPGs. CSPGs inhibit axonal outgrowth of postnatal day 4 (P4) cortical neurons in culture medium alone or with a protein control (Supplementary Fig. 3b). GDF10 has a dose-dependent promotion of outgrowth in the presence of CSPGs (Fig. 2c).

GDF10 promotes axonal outgrowth in vitro via TGFβRs

Unlike other GDFs, GDF10 signals through TGFβRI and TGFβRII and the downstream transcription factors Smad2 and Smad3, and not BMPRI, BMPRII, Smad1 or Smad5 (Supplementary Fig. 1). To identify the molecular signaling systems for GDF10’s axonal outgrowth effect, we treated neurons with the TGFβRI antagonist SB431542 or TGFβRII or TGFβRII siRNA. All siRNAs knocked down their respective protein target (Supplementary Fig. 3c,d) in this culture model system in which GDF10 is expressed in neurons that have undergone the plating process (Supplementary Fig. 4). The axonal growth-promoting effect of GDF10 was significantly reduced either with blockade of TGFβRI or knockdown of TGFβRII (P < 0.05; Fig. 3a). No statistical difference was noted between the groups treated with TGFβRII siRNA + GDF10 and scrambled siRNA control + GDF10 (P > 0.05; Fig. 3a). Knockdown of Smad2 or Smad3 significantly inhibited the axonal outgrowth effect of GDF10 on primary neurons (P < 0.05; Fig. 3b). No significant difference in axonal
Figure 2  GDF10 enhances axonal outgrowth in primary neurons in vitro. (a-c) Axonal outgrowth in P4 mouse primary cortical neurons. Axon length was measured after 3 d in culture. Cyto C = cytochrome C, a protein control for the addition of growth factor, used in the in vivo studies (Fig. 4a). In c, wells were plated with CSPG before cell growth. In all graphs, boxes extend from the 25th–75th percentiles and the lines in the boxes are the mean. The whiskers show the minimum and maximum values. (d) P4 cortical neurons stained with SMI-312 after 2 additional days culture in medium alone or medium + GDF10 (500 ng ml⁻¹). Scale bar represents 20 μm. (e) Rat adult RGCs cultured in the presence of GDF10, forskolin or mannitol. n = 7 in culture medium only; n = 8 for the other three groups. Two independent cultures per condition and, in each culture, four wells repeating the condition. *P < 0.05, **P < 0.01 compared with medium only. #P < 0.05, ##P < 0.01 compared with scrambled + GDF10; *$P < 0.05$ compared with scrambled siRNA. All conditions were tested in quadruplicate, in two separate experiments. a, F(6, 105) = 7.220; b, F(6, 105) = 8.384; c, F(6, 105) = 22.44; e, medium versus GDF10: t = 2.852, df = 13; fosk/mann versus fosk/mann GDF10: t = 2.371, df = 14. All observations were normalized to the number of NeuN⁺ cells in each sample (Supplementary Fig. 13). Statistical testing was repeated-measures ANOVA followed by Tukey-Kramer’s post hoc test (a–c) or one-tail unpaired t test (e).

These findings raise the question of whether TGFβ itself will promote axonal outgrowth from cortical neurons. This has been described with other primary neuronal

length was noted between the groups consisting of the scrambled siRNA + GDF10 and Smad1 or Smad5 siRNA treated with GDF10 (P > 0.05; Fig. 3b). Furthermore, in vivo pharmacological blockade of TGFβRI and TGFβRII using SB431542 and losartan¹⁹,²⁰ decreased Smad2 and Smad3 signaling in the peri-infarct tissue (Supplementary Fig. 5) during the period of GDF10 activity after stroke. In total, our results indicate that TGFβRI, TGFβRII, Smad2 and Smad3 mediate the effects of GDF10 in enhancing axonal outgrowth.

Figure 3  GDF10 enhances axonal outgrowth in human neurons via TGFβ signaling. (a,b) P4 mouse cortical neuron culture with TGFβRI and TGFβRII and Smad blockade. SB431542 is a TGFβRI antagonist, added at initial plating. In all graphs, boxes extend from the 25th–75th percentiles and the lines in the boxes are the mean. The whiskers show the minimum and maximum values. (c,d) Human IPS-neurons cultured in the presence of GDF10, SB431542, or TGFβRII, Smad2 and Smad3 siRNA. Each condition consisted of 2–4 observations in 2–3 independent experiments. (e,f) IPS-NPCs in culture with GDF10 for 2 d, stained with SMI-312 for axons. Scale bar represents 20 μm. (g) TGFβ1 and Smad2 enhanced axonal outgrowth of P4 primary cortical neurons. Axon length with treatment of TGFβ1 at ascending concentrations is shown. n = 3 for each experiment. (h) Axonal outgrowth with transfection of Smad2 expression plasmid. Data are presented as in g. *P < 0.05, **P < 0.01, ***P < 0.005 compared with medium only; P < 0.05 compared with medium + GDF10. $#P = 0.05$ compared with scrambled + GDF10. All conditions were tested in quadruplicate in two separate experiments. a, F(5, 186) = 10.28; b, F(2, 93) = 6.138; c, F(4, 155) = 10.23; d, F(4, 155) = 11.49; g, F(2, 93) = 4.435; h, t test, two-tailed t = 3.073, df = 62. All observations were normalized to the number of NeuN⁺ cells in each sample (Supplementary Fig. 13). Statistical testing was repeated-measures ANOVA followed by Tukey-Kramer’s post hoc test (a–d,g) or one-tail unpaired t test (h).
Figure 4 GDF10 promotes axonal connections in peri-infarct cortex after stroke. (a) Quantitative cortical mapping of connections in layers I/II of the flattened mouse cortical hemisphere ipsilateral to the forelimb motor cortex in stroke with protein control (Cyto C) (blue, n = 8), GDF10 + stroke (red, n = 8) and areas of dense overlap of these two conditions (dark blue). x and y axes are distances in millimeters from the center of the BDA tracer injection (empty circle). P value is Hotelling’s T^2. (b) Polar plot of connections of forelimb motor cortex projections relative to the tracer injection in forelimb motor cortex as the origin. Filled polygons represent the 70th percentile of the distances of all BDA-labeled connections from the injection site in each segment of the graph. Weighted polar vectors represent the median vector multiplied by the median of the normal distribution of the number of points in a given segment of the graph. P value is Watson’s nonparametric two-sample U test. Inset shows schematic lateral view of mouse brain. The horizontal line shows the position in which neuronal label was quantified c. (c) Projections from forelimb motor cortex after stroke with GDF10 delivery (red) and protein control (Cyto C) (cyan) taken from counts along the line in (a). *P < 0.05, **P < 0.01. Inset shows schematic mouse brain with the location of the BDA injection (black dot) and the linear quantification construct (line). In c and f, boxes extend from the 25th–75th percentiles and the lines in the boxes are the mean. The whiskers show the minimum and maximum values. (d) Quantitative cortical mapping of GDF10 knockdown in stroke. Data are presented as in a. (e) Polar plots of GDF10 siRNA and scrambled siRNA after stroke. Data are presented as in b. (f) Linear quantification of neuronal connections in treatment groups of GDF10 siRNA+Stroke and scrambled siRNA+Stroke. Data are presented as in c, f(1, 10) = 12.03; f, f(1, 10) = 20.24; b, U^2 = 647.176, df = 9039, df2 = 180911; e, U^2 = 78.616, df = 38554, df2 = 5906. The circle in a and d indicates the center of the stroke site.

GDF10 stimulates axonal outgrowth of hiPS neurons

Human and rodent cells may respond differently to TGFβ/BMP signaling2,3. We examined whether GDF10 has axonal growth-promoting effects on human neurons using human induced pluripotent stem cell–derived neurons (hiPS neurons)26,27. GDF10 induced significant axonal outgrowth of hiPS neurons (P < 0.01; Fig. 3c, e, f) and blockade of TGFβRI or knockdown of TGFβRII in the presence of GDF10 prevented GDF10-induced axonal outgrowth (Fig. 3c). In addition, the growth-promoting effect of GDF10 was blocked by knockdown of Smad2 and Smad3 (Fig. 3d). This data indicates that GDF10 has a common signaling pathway for axonal outgrowth in human and rodent neurons.

GDF10 increases axonal connections in peri-infarct cortex

A candidate signal for axonal sprouting in peri-infarct cortex after stroke would be induced at the stroke site and promote the formation of new connections. To test this hypothesis, we produced GDF10 gain and loss of function directly from the stroke through sustained release via biopolymer hydrogel, which produces release over 2–3 weeks from the stroke site3,4,28,29 without altering local inflammation, gliosis, neuronal or vascular structure4. As previously shown in other gene systems3,4, nucleotide-modified siRNA delivered directly into the infarct core produces protein knockdown over at least 2 weeks after stroke with GDF10 (Supplementary Fig. 3a).

Mice received a stroke in forelimb motor cortex, followed 7 d later by injection of GDF10 and hydrogel or GDF10 siRNA. This 7-d period is when GDF10 is induced in peri-infarct cortex in the rodent1 (Fig. 1). 21 d later the neuronal tracer biotinylated dextran amine (BDA) was microinjected into the intact forelimb motor cortex anterior to the stroke site. New connections in peri-infarct cortex can be detected after stroke in this timeframe3,4,28,29. The BDA-labeled connections of the forelimb motor cortex were quantitatively mapped and registered to functional cortical areas using the somatosensory body map28,29 and to the location of corticospinal neurons in motor, premotor and somatosensory areas retrogradely labeled from the spinal cord (P < 0.05; Supplementary Fig. 6a). There was no significant difference in BDA injection location and volumes, or in infarct volumes across all experimental conditions (P > 0.05; Supplementary Fig. 7a, b). As reported previously3,4,28,29, stroke alone, but significantly, induced axonal sprouting in the motor cortex adjacent to the stroke site compared to normal motor system connections (P = 0.039, Supplementary Fig. 6a; P < 0.05, Supplementary Fig. 6b). This post-stroke axonal sprouting occurs from motor cortex, posteriorly toward immediate peri-infarct motor and somatosensory areas.
Two control conditions were used for GDF10 protein and siRNA delivery: stroke + scrambled siRNA and stroke + protein control (cytochrome C) (Fig. 4). These produced the same pattern of connections as stroke alone (n = 8–9 per group; Supplementary Figs. 8 and 9). Quantitative connectional mapping revealed that stroke + GDF10 induced a statistically significant change in cortical connections compared with stroke alone and stroke + protein control, with a robust projection from motor cortex anteriorly to premotor and prefrontal cortex (n = 8–9 per group; P = 0.041, Fig. 4a; P < 0.05, Fig. 4b; P < 0.01; Fig. 4c). This is a non-isotropic increase in projections, with a unique frontally projecting axonal connection with GDF10 delivery after stroke. To further quantify the location of individual neuronal projections, we counted the number of labeled axons in a linear distribution in each tangential section through the injection site, extending across the cortical hemisphere (Fig. 4c). This revealed a significant increase in neuronal projections with GDF10 delivery, particularly anterior to the motor cortex (P < 0.05 and P < 0.01). Furthermore, colocalization analysis of the pre- and postsynaptic markers VGLUT2 and Homer1 showed that BDA-labeled sprouting neurons contain the presynaptic glutamatergic marker in tight association with the postsynaptic marker Homer1 (Supplementary Fig. 10 and Supplementary Video 1). siRNA knockdown of GDF10 significantly inhibited cortical axonal sprouting after stroke (P = 0.041, Fig. 4d; P < 0.05, Fig. 4c; P < 0.01; Fig. 4f), producing a pattern of motor system axonal connections that resembles the non-stroke, normal brain (Fig. 4d and Supplementary Figs. 8 and 11). These data show that GDF10 induces substantial axonal sprouting and synapse formation after stroke into premotor and prefrontal areas, and that blocking GDF10 eliminates the normal pattern of axonal sprouting seen in stroke to produce a pattern of motor system connections that is not statistically different from that of the normal brain.

GDF10 increases angiogenesis and gliosis after stroke

TGFβ family members stimulate astrocyte responses and modify angiogenesis and neuroinflammation\(^{30,31}\). To determine what tissue changes occur concomitantly with enhanced axonal outgrowth with GDF10, we analyzed markers for these processes following GDF10 delivery or knockdown in stroke at the time of increased axonal sprouting. As previously reported\(^4\), stroke alone produced increased levels of GFAP, CD31/PECAM and IBA-1, markers for astrocytes, endothelial cells and microglia/macrophages, respectively (Fig. 5). GDF10 significantly increased astrocytosis, as indicated by an increase in GFAP+ endothelial cell area (P < 0.05 compared with stroke + protein control; Fig. 5e) and in blood vessel area, as indicated by an increase in PECAM+ endothelial cell area (P < 0.05 compared with stroke + protein control; Fig. 5e), above that seen in stroke, with no significant effect on microglial staining (P > 0.05 compared with stroke + protein control; Fig. 5e). GDF10 knockdown decreased vascular, astrocyte and microglial staining compared with stroke alone. The delivery of control protein or scrambled siRNA had no effect on these processes. These data indicate that GDF10 delivery not only enhances axonal sprouting, but also induces vascular remodeling.
Figure 6 GDF10 improves behavioral recovery after stroke. (a) Cylinder test of forelimb symmetry in exploratory testing (n = 7 conditions in behavioral testing). (b) Error bars represent s.e.m. (c) Pasta handling task after stroke. (d) Timeline of behavioral studies and schematics of GDF10 delivery and behavioral testing procedures. a, F(1,958, 11,75) = 22.07; b, F(1,866, 11.21) = 10.70; c, F(2,101, 12.61) = 3.982. Error bars represent s.e.m.

and astrocytosis. The GDF10 knockdown effect indicates that, in the normal process of post-stroke tissue reorganization, GDF10 is partially responsible for post-stroke astrocyte, blood vessel and microglial changes.

GDF10 improves functional recovery after stroke

To determine whether GDF10-induced axonal sprouting promotes functional recovery, we tested mice on forelimb motor tasks using the same forelimb motor cortex stroke in which axonal sprouting was measured. The grid walking, cylinder and pasta-handling tasks measure gait, exploratory forelimb use and skilled control of thin pasta pieces. GDF10 and GDF10 siRNA were delivered as in the axonal sprouting studies (Fig. 4). Stroke produced impairments in forelimb motor control for an extended period: at least 11 weeks (pasta handling) or 15 weeks after stroke (cylinder, gridwalking) (Fig. 6). Delivery of GDF10 starting 1 week after stroke enhanced recovery beginning from 3 weeks post-stroke (Fig. 6). With GDF10 delivery, mice performed at the level of normal control 5 weeks after the infarct. Notably, GDF10 siRNA produced a significant reduction in the normal process of recovery after stroke, beginning 1–3 weeks after delivery (P < 0.05; Fig. 6a–c). No significant changes in motor performance were observed among animals treated with protein or siRNA delivery controls (P > 0.05; Fig. 6a–c). Thus, administration of GDF10 protein improved motor recovery after stroke and knocking down GDF10 levels substantially decreased functional recovery. These behavioral data indicate that endogenous GDF10 is important for normal recovery after stroke and that the neuronal sprouting mapped in vivo may represent formation of neural circuits that cause behavioral improvement.

Systems biology of GDF10 in the CNS

To understand the molecular program induced by GDF10 during axonal sprouting and functional recovery after stroke, we FACS-isolated cortical neurons (based on NCAM) (Fig. 7a) from GDF10 + stroke, stroke alone, normal control and age P4, a time at which cortical neurons are forming new connections during development. The number of reads ranged from 43,087,682 to 85,407,243. The differentially expressed genes were analyzed after a cutoff for a false discovery rate (FDR) of <0.1.
The greatest difference in the transcriptomes among conditions was between adult cortical neurons, from control, stroke and stroke + GDF10, compared with the developmental age of P4 (Fig. 7b). This indicates that the biggest alteration in the neuronal transcriptome is between adult and developmental states, rather than with the presence of a stroke or with GDF10 delivery. Unsupervised cluster analysis of these transcriptomes confirmed that the adult versus neonatal gene expression changes clustered together (Fig. 7c). After this distinction, the most unique transcriptome was stroke + GDF10 versus stroke. Thus, GDF10 delivery in stroke regulates a unique transcriptome that consists of a smaller set of genes from that seen during the initial process of axonal sprouting in neurodevelopment. An open question in the field of neural repair is whether the molecular program stimulated by tissue regeneration resembles that seen in neurodevelopment, that is, whether ‘regeneration recapitulates development’. These results indicate that GDF10 delivery in stroke, which promotes axonal sprouting in the adult, regulates a markedly different and smaller set of genes from that seen during the initial process of axonal sprouting in neurodevelopment and in the adult state, GDF10 induces a unique transcriptome after stroke.

Stroke + GDF10 significantly regulated specific canonical pathways in post-stroke neurons (−log(Fisher’s exact P value calculation with Benjamini Hochberg correction for multiple comparisons) < 0.183; Supplementary Table 1). Among the most highly regulated were axonal guidance, PTEN and PI3K signaling (Fig. 8a). PTEN canonical signaling (eight genes in class) was downregulated and PI3K canonical signaling (12 genes in class) was upregulated with GDF10 in stroke (Supplementary Tables 2–5). Axonal guidance molecules that were differentially regulated by GDF10 in stroke include Ephrin A3, Tctn1/ubulin, βII tubulin, VEGF-d, neuropilin 1, SOCS3, and downstream molecules in the Rho and Rac pathways (Supplementary Tables 6 and 7). PTEN and SOCS3 inhibition induce axonal sprouting in multiple adult CNS systems, including optic nerve and spinal cord injury.1,2,6 The differential control of PTEN, PI3K and SOCS3 signaling by GDF10 represents a potential mechanism of action in axonal sprouting after stroke.

Axonal sprouting after stroke shares similarities with the neuronal morphology changes in learning and memory, optic nerve and spinal cord regeneration, and neurodevelopment.1,2,6 We used unsupervised genome-wide association analysis to compare our data from GDF10 and stroke with transcriptomes from cortical neuronal outgrowth during development, the cortical critical period, mouse growth cones, P7–28 mouse neurons, motor cortex after spinal cord injury, retina after optic nerve crush, contralateral cortex after stroke, and learning and memory procedures in hippocampus and medial prefrontal cortex (Supplementary Table 8). The data from 180 microarray or RNA-seq studies were analyzed from original array files, normalized, controlled for batch effect (Supplementary Fig. 12) and statistically compared with our data sets for cortical neurons from stroke, stroke + GDF10, control and P4. There was a variance in statistical spread across transcriptomes, even from the same cell population in the same experiment (Fig. 8c), which likely has to do with the fact that these transcriptional profiles are mostly from specific cell types, rather than from large in vitro preparations or tissue-level sources. For example, in layer V/VI from motor cortex after spinal cord injury, transcriptomes from the same condition can vary across 30 units in statistical space; similar variance is seen with cortical efferent neurons, such as callosal neurons at P4 and in adult optic nerve injury. In the variance in gene expression in these conditions of axonal sprouting, injury or, learning and memory, stroke and stroke + GDF10 transcriptomes were separated by the largest difference from cortical P4 neurons, confirming the cluster analysis in this data set (Fig. 8c). Stroke and stroke + GDF10 transcriptomes clustered closely together with a relationship to retina 12 h after optic nerve crush. Mouse growth cone, mouse cortical critical period, and corticospinal and callosal postnatal neuronal transcriptomes clustered together, but not near stroke + GDF10 or stroke alone. These data indicate that GDF10 induces a unique transcriptional profile after stroke that is more closely related to stroke alone than to the transcriptomes seen in several contexts of neuronal development, CNS injury, or learning and memory.

**DISCUSSION**

Axonal sprouting in the adult occurs in a limited manner after stroke, optic nerve lesions, spinal cord injury and in models of neurodegenerative diseases.1,2,6 After stroke, axonal sprouting has been...
Figure 8  GDF10 canonical signaling pathways and genome-wide associations. (a) Top canonical pathways significantly regulated in stroke + GDF10 versus stroke. The y-axis is the inverse log of the P value corrected for multiple comparisons in Benjamini-Hochberg (B-H) test. Significance was set to a B-H P < 0.05 = −log(B-H P) of 1.3. Red represents net upregulation of this gene in this pathway and green represents net downregulation. Gray represents mixed up or downregulation in pathway genes such that there was no net trend. (b) Genome-wide associations of stroke + GDF10 transcriptome to learning and memory, neurodevelopmental and CNS injury transcriptomes. Statistical testing was Fisher’s exact P value, Benjamini-Hochberg correction for multiple comparisons (a) and principle component analysis of 180 transcriptomes (Supplementary Fig. 12).

demonstrated in mice, rats and non-human primates1–4,10,37. In humans, the initial cloning studies of GAP43, a neuronal growth cone marker, revealed that it is induced in peri-infarct cortex after stroke38 and increases cortical thickness in regions of cortical map plasticity during recovery37. The induction of axonal sprouting in adult cortex after stroke and the formation of new patterns of connections suggests that neurons have been placed into a growth state. Much scientific study has focused on the molecular components of the injury response that block axonal sprouting, such as the glial growth inhibitors NogoA and the chondroitin sulfate proteoglycan8–10. However, there has been comparatively less research on the molecular signals for a neuronal growth program after stroke. Our data indicate that GDF10 is induced after stroke in a wide range of species, promotes axonal outgrowth in vitro in human, mouse and rat neurons, and induces axonal sprouting and functional recovery after stroke in vivo. Knockdown of endogenous GDF10 after stroke reduces axonal sprouting and recovery. GDF10 controls a unique transcriptional profile with differential regulation of PTEN, PI3K and axonal outgrowth molecules. These findings suggest that GDF10 is a post-stroke axonal sprouting signal that induces functional recovery.

Stroke normally produces a limited pattern of axonal sprouting from motor or somatosensory cortex posteriorly to areas adjacent to or caudal to the stroke2,4,38,39. GDF10 delivery induces a unique connectional pattern: substantial projections anterior from motor cortex for mouse primary somatosensory areas. This anterior projection from motor cortex to premotor cortex is causally associated with functional recovery in this model of stroke5. The magnitude of post-stroke axonal sprouting induced by GDF10 exceeds that seen with the blockade of several axonal growth inhibitory systems, such as ephrin-A5 (ref. 4) and Nogo/NgR1 (ref. 3). Furthermore, knockdown of GDF10 after stroke abolishes the normal process of post-stroke axonal sprouting and reduces functional recovery. These results indicate that the normal release of stroke-induced GDF10 participates in the limited motor recovery that occurs naturally in stroke.

GDF10 signals through TGFβ receptors. In other in vitro systems, TGFβ promotes axonal outgrowth of injured neurons21,22,38,39 and blockade of TGFβRII alters neuronal polarity and leads to shorter axons40. However, activation of Smad2 signaling has also been reported to occur with myelin axonal growth inhibition and direct activation of Smad2 reduces axonal outgrowth in some preparations22,24. These discrepant findings of both axonal sprouting and axonal growth inhibition with TGFβ may stem from non-canonical signaling or context-specific signaling. Although TGFβ and GDF10 both signal through TGFβRI and TGFβRII12–14, we found that their signaling levels were not equivalent. These growth factors may produce non-canonical signaling outside of the Smad transcription factors, such as through the MAPK pathway39,41.

The action of TGFβRI and TGFβRII signaling in axonal outgrowth may be context specific in terms of the local tissue environment and differ between local axonal sprouting in peri-infarct cortex and distant axonal sprouting from corticospinal neurons after spinal cord injury. We found that the transcriptomes for these two sprouting conditions were very different (Fig. 8b). Such context-specific signaling is a hallmark of TGFβ in its role in cancer, where it switches from the inhibition of tumor growth and metastasis to the promotion of endothelial to mesenchymal transition and metastasis as the local tumor environment evolves14,42.

GDF10 activates a coordinated upregulation of genes in the PI3K pathway, downregulation of genes in the PTEN pathway and control of specific axonal guidance molecules, including the downregulation
of SOCS3. PTEN inhibition potentiates axonal sprouting in optic nerve injury and spinal cord injury through induction of PI3K signaling and activation of mTOR. Inhibition of SOCS3 further promotes axonal sprouting on top of PTEN inhibition.\(^8\)\(^,\)\(^7\) Detailed analysis of the GDF10 transcriptome in stroke will provide further insights into mechanisms for the GDF10 trigger for post-stroke axonal sprouting. In all, GDF10 is an important regulator of axonal outgrowth and plasticity for the GDF10 trigger for post-stroke axonal sprouting. The GDF10 transcriptome in stroke will provide further insights into mechanisms for the GDF10 trigger for post-stroke axonal sprouting.

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ONLINE METHODS

Mouse model of stroke. Animal procedures were performed in accordance with the US National Institutes of Health Animal Protection Guidelines and the University of California Los Angeles Chancellor’s Animal Research Committee. Focal cortical strokes on adult C57BL/6 male mice weighing 20–25 g (2–4 months old, Charles River Laboratories) were produced by photothermalism at surgery as previously described. Mice were experimentally naive before studies. Briefly, under isoflurane anesthesia, mice were placed in a stereotactic apparatus with the skull exposed through a midline incision, cleared of connective tissue, and dried. A cold light source (KL1500 LCD; Carl Zeiss MicroImaging) attached to a 40× objective, giving a 2-mm diameter illumination, was positioned 1.5 mm lateral from the bregma used to produce a 2-mm diameter focal stroke upon light illumination. Rose Bengal is administered (10g/l), with typically 0.2 ml per 25 g mouse. After 5 min, the brain was illuminated through the intact skull for 15 min. The mice were then sutured along the scalp, removed from the stereotactic frame (Model 900, David Kopf Instruments), and allowed to recover. Body temperature was maintained at 37.0 °C with a heating pad throughout the operation. Control animals received no stroke. Mice were housed in a 12:12 h light:dark cycle at 4 mice per cage.

Non-human primate model of stroke. Non-human primate tissues are taken from a previously reported stroke neuroprotection study in which stroke animals did not receive study drug with saline treatment (n = 3, stroke n = 2). Adult, male rhesus macaques (M. mulatta) were single-housed indoors in double cages on a 12:12 h light/dark cycle, with lights-on from 0700 to 1900h, and at a constant temperature of 24 °C ± 2 °C. Laboratory diet was provided biaidaly (Lab Diet 5047, PMI Nutrition International) and supplemented with fresh fruits and vegetables. Drinking water was provided ad libitum. The animal care program is compliant with federal and local regulations, regarding the care and use of research animals and is Association for Assessment and Accreditation of Laboratory Animal Care accredited. All experiments were approved by the Institutional Animal Care and Use Committee.

The right middle cerebral artery (distal to the orbitofrontal branch) and both anterior cerebral arteries were exposed and occluded with vascular clips for blood pressure monitoring throughout the surgery and to maintain a mean arterial blood pressure of 60–80 mmHg. End-tidal CO2 and arterial blood gases were continuously monitored to titrate ventilation to achieve a goal CO2 pressure of 35–40 mm Hg. Postoperative analgesia consisted of intramuscular hydromorphone HCl and buprenorphine. Animals were killed 2 d after stroke and tissue fixed in formaldehyde. 100× photomicrographs were taken in four coverslips and 32 images for each condition were analyzed. Axonal length was quantified (NeuriteTracer). Total axonal length per image was calculated for each treatment and normalized for total neuronal cell number in each culture plate (stained for NeuN). Rat RGCs cultures were prepared from dissociated retinas of mature Fisher rats. In brief, rats were anesthetized with a mixture of ketamine-xylazine, the superior colliculi were exposed bilaterally, and retinal ganglion cells (RGCs) were retrogradely labeled by injecting Fluorogold (FG, Fluorochrome) into the superior colliculi. After allowing 7 d for retrograde transport of the dye to RGCs, rats were terminally anesthetized and retinas were rapidly dissected, enzymatically dissociated with gentle trituration, and the dissociated retinal after removing larger tissue fragments, cells were cultured in defined, serum-free medium for 3 d. All conditions were tested in quadruplicate, in two separate experiments. Results were quantified as the percentage of FG-labeled cells, that is, RGCs, extending an axon ≥ 2 cell diameters (approximately 30 µm) averaged across the four wells in each experiment and the two experiments. The observer was blinded to each of the treatment conditions.

Pharmaceutical blockade of TGFβ in vivo. Animals received photothermal stroke and were treated with two different TGFβ antagonists, SB431542 (R&D Systems) and Losartan (Sigma) at 10 mg per kg and 100 mg per kg based on published intraperitoneal (IP) dosages. Each animal (n = 3 per group) received daily IP injections for 5 treatment days after stroke. Brains were post-fixed for 4 h in 4% paraformaldehyde (wt/vol), cryosectioned at 40 µm and immunostained with pSmad2/3 (1:200, Cell Signaling, 8828S). 100× photomicrographs were taken in four per 25 g mouse. After 5 min, the brain was illuminated through the intact skull for 15 min. The mice were then sutured along the scalp, removed from the stereotactic frame (Model 900, David Kopf Instruments), and allowed to recover. Body temperature was maintained at 37.0 °C with a heating pad throughout the operation. Control animals received no stroke. Mice were housed in a 12:12 h light:dark cycle at 4 mice per cage.

Cultures of primary cortical neurons, iPSC-NPCs and RGCs and quantification of axonal outgrowth. Primary cortical neurons were prepared from P4 CD1 mice (Charles River Laboratories). Briefly, mice were killed, brains removed, and the cerebral cortex dissected, stripped of meninges, and dissociated by a combination of calcium and magnesium free HBSS containing 0.2% papain suspension (wt/vol, Worthington Biochemical) and digested for 12 min at 37 °C. The triturated cells were passed through a 70-µm strainer and counted. The cells were plated in laminin and poly-D-lysine–coated coverslips (cat. no. 354086, BD Biosciences) at a density of 5 x 104 cells per ml (in 24-well plates) in culture medium (NbActiv4, BrainBits). Experiments were carried out after 24 h of seeding. Primary neurons were incubated with drugs or vehicle in new medium for an additional 48 h. For CSGP studies, coverslips were coated with CSGP (25 µg ml−1, cat. no. CC117, Millipore) before cell plating. Human induced pluripotent stem cells (iPSCs) (W. Lowry, University of California Los Angeles) were plated on 12-mm polyornithine/laminin coated coverslips at 5 x 104 per well (24-well plates) in NbActiv4 (BrainBits) containing 100 units per ml penicillin, 100 µg ml−1 streptomycin, fibroblast growth factor 2 (FGF2) (20 ng ml−1) and epidermal growth factor (EGF) (20 ng ml−1) (Invitrogen), iPSCs were cultured for 7 d with every other daily media change. For neural differentiation, media was supplemented with brain-derived neurotrophic factor (BDNF) (20 ng ml−1) and (neurotrophin 3) NT3 (20 ng ml−1) (R&D Systems) and changed every other day for 21 d. Experiments were carried out on day 22. This LPS line has been extensively characterized. Expression profiles were compared with NPCs derived under standard conditions from hESCs and hiPSCs grown on murine feeder cells. Clustering and Pearson analysis demonstrate that this iPS-NPCs is highly similar to other iPS-NPCs and ES-NPCs derived under standard conditions (Pearson: 0.926–0.959) and by immunostaining are relatively homogenous. With simple growth factor withdrawal for three weeks, most of these iPS-NPCs develop into neurons expressing Tuji or MAP2 (ref. 49). BDNF and NT3 treatment were used to promote further neuronal differentiation. This approach generates neurons with markers of maturity by transcript profiling, immunohistochemical staining and action potential generation (see Figure 1 of ref. 27).

TGFβRII antagonist SB431542 (Sigma), TGFβRII siRNA and GDF10 siRNA were applied to cultures during GDF10 incubation for 2 d before fixation and axonal staining, TGFβRII, Smad2, Smad3 and GDF10 siRNA protein knockdown was evaluated with western blotting (Supplementary Fig. 3c,d). Axons of primary cortical neurons and iPS-NPCs were stained with anti-SM1-312 monoclonal antibody. 16 images were taken from each coverslip at 40× objective lens using unbiased sampling (Stereoinvestigator, MBF Biosciences). At least two coverslips and 32 images for each condition were analyzed. Axonal length was quantified (NeuriteTracer). Total axonal length per image was calculated for each treatment and normalized for total neuronal cell number in each culture plate (stained for NeuN). Rat RGCs cultures were prepared from dissociated retinas of mature Fisher rats. In brief, rats were anesthetized with a mixture of ketamine-xylazine, the superior colliculi were exposed bilaterally, and retinal ganglion cells (RGCs) were retrogradely labeled by injecting Fluorogold (FG, Fluorochrome) into the superior colliculi. After allowing 7 d for retrograde transport of the dye to RGCs, rats were terminally anesthetized and retinas were rapidly dissected, enzymatically dissociated with gentle trituration, and the dissociated retinal after removing larger tissue fragments, cells were cultured in defined, serum-free medium for 3 d. All conditions were tested in quadruplicate, in two separate experiments. Results were quantified as the percentage of FG-labeled cells, that is, RGCs, extending an axon ≥ 2 cell diameters (approximately 30 µm) averaged across the four wells in each experiment and the two experiments. The observer was blinded to each of the treatment conditions.

GDF10 protein, GDF10 siRNA administration and BDA injection. GDF10 protein was delivered from the stroke cavity with a hyaluronan plus heparin sulfate hydrogel (Extracel-HP, Glycosan BioSystems) injected 7 d after stroke (A/P, 0.0 mm; M/L, 1.5 mm; D/V, 1.0 mm). This biopolymer hydrogel releases both small and large molecules over a 3-week period from the infarct core3,4,29. 6 µl of hydrogel impregnated with recombinant GDF10 (1.33 mg ml−1, n = 8) (C2506-50MG, Sigma) was implanted into the stroke core. Three GDF10 siRNA duplex (cat. no. MSS236596, MSS236597, MSS236598; Invitrogen) and negative control siRNA (12935-200, Invitrogen) were used to determine protein knockdown in vitro using P4 mouse cortical primary neurons. The combination of MSS236597 and MSS236598 siRNA produced the greatest
knockdown in GDF10 protein expression (Supplementary Fig. 3c). This GDF10 siRNA (n = 8) or scrambled control siRNA (n = 8) duplex with RNAiMAX (cat. no. 13778-075, Invitrogen) (6 μl) was introduced at 150 nM directly into the stroke cavity (A/P, 0.0 mm; M/L, 1.5 mm; D/V, 1.0 mm) 7 days after stroke. At day 21 after stroke or 14 d after GDF10 protein, GDF10 siRNA or Cyto C injection, each mouse received an injection of 300 nl of 10% BDA (wt/vol, Sigma) into the forelimb motor cortex (A/P, 1.5 mm; M/L, 1.75 mm; D/V, 0.75 mm)24. At 28 d after stroke, mice were perfused with paraformaldehyde and the cortex removed, flattened and sliced to 40 μm tangentially3,28,29.

Quantitative cortical mapping of axonal sprouting. Axonal sprouting was quantified by digitally marking each BDA positive process in the cortex with a digitizing microscope system (Leica Microsystems, Ludl Electronic Products) and analysis program (Stereoinvestigator, MBF Biosciences). A BDA-labeled process was plotted irrespective of its cellular position as an axon shaft, pre-terminal or terminal axon field. This allowed each tangential map of axonal connections to contain an unbiased picture of the entire projection zone and trajectory of projections from forelimb motor cortex in each case. BDA-positive processes were marked in x,y coordinates relative to the center of the injection site by an observer blind to the treatment conditions. This process generates an x,y plot of the location of all labeled axons in each brain section. The x,y axonal plots of each brain from each experimental group were registered to the injection site and co-registered with functionally relevant anatomical regions, produced by the staining of the mouse somatosensory body map in cytochrome oxidase and the retrograde labeling of the corticospinal neurons (Supplementary Fig. 6a) to generate a composite axonal map for each treatment condition. Custom software3,24 produces quantitative connectional maps that consist of pixels, with the number of axons in each pixel mapped in register with anatomical brain structures. Polar plots were constructed with the x,y position of each BDA-labeled element plotted in relation to the tracer injection in forelimb motor cortex as the origin. This polar mapping shows both location and direction of axonal label. Surface maps and polar maps analyzed for statistically significant differences in connectional profiles between treatment groups (see below). In a separate quantification method, the number of axons within the ipsilateral hemisphere was counted a linear construct from the center of the injection site through the ipsilateral cortical hemisphere. Stroke infarct volume was calculated by multiplying lesion area by the thickness of each section plus the distance between sections and then added with these measurements from each section through the tangential flattened cortical tissue. BDA injection volume was measured by calculating the average injection core volume for each treatment group. The average BDA injection area in each section, determined by outlining the limit of extracellular tracer deposition, was multiplied by the sum of the thickness of the section and then summed for all sections in the series. Anterior-posterior and medial-lateral BDA injection location was analyzed by measuring the distance from the center of the injection site to the rostral edge of the tissue and the midline of the cortex, respectively. The size and location of each BDA injection and stroke size did not vary significantly across individuals or by treatment condition (P > 0.05, Supplementary Fig. 7).

Immunohistochemistry. Fluorescence immunohistochemistry with floating frozen sections was performed as described34. Primary antibodies are given in Supplementary Table 9. In all experiments, no-primary and no-secondary antibody controls were run in parallel. There was no specific staining with these controls. For the quantification of GFAP, PECAM and IBA-1 immunoreactive areas, 3 fields (650 × 450 μm) in peri-infarct cortex were precisely taken from three independent tangential sections of each animal using a 20× objective with confocal microscopy (Nikon C2). The parameters for scanning were kept constant across treatment conditions. Single images were analyzed using ImageJ macro software to quantify the area stained for each marker. A level of 400× was used to ensure that all labeled neurons were counted accurately.

A novel quantitative method of analyzing synapse morphology and connectivity was performed on confocal images using custom software34. Briefly, single synapses were manually identified and traced from each image, and the size and shape of each synapse was captured using ImageJ software. This method allowed for the detailed analysis of synapse morphology and connectivity, providing a comprehensive understanding of the synaptic network. Additionally, the software allowed for the generation of connectivity maps and the identification of synaptic connectivity patterns.

The experimental setup involved the use of a combination of confocal microscopy and ImageJ software to analyze the morphology and connectivity of synapses. The method was validated through the analysis of a set of known control samples, ensuring the accuracy and reliability of the results. The data was presented in a clear and concise manner, making it easier for the reader to understand the findings and implications of the study.
kit for paired-end 2 × 69 RNA-sequencing (HiSeq2000, UCLA ICNN core). After library preparation (Encore NGS Library System I, Nugen) amplified double-stranded cDNA was fragmented into 300 bp (Covaris-S2, Woburn, MA). DNA fragments (200 ng) were end-repaired to generate blunt ends with 5’-phosphates and 5’-hydroxyls and adapters ligated. The purified cDNA library products were evaluated using the Agilent Bioanalyzer and diluted to 10 nm for cluster generation in situ on the HiSeq paired-end flow cell using the CBot automated cluster generation system. Three samples at a time, all samples were multiplexed into single pools and run in nine lanes total of Paired-End 2 × 100 bp flow cells in HiSeq 2000 (Illumina).

Six Libraries for RNA-seq were prepared using the NuGEN Ovation UltraLow library preparation protocol (NuGEN Technologies) and sequenced using an Illumina HiSeq 2500 sequencer across ten lanes of 100-bp paired-end sequencing, corresponding to three samples per HiSeq 2500 lane. After demultiplexing, we obtained between 50 and 79 million reads per sample. Quality control was performed on base qualities and nucleotide composition of sequences. Alignment to the mouse genome. Total counts of read-fragments aligned to candidate gene regions were derived using HTSeq program (http://www.huber.embl.de/users/anders/HTSeq/doc/overview.html) and used as a basis for the quantification of gene expression. Only uniquely mapped reads were used for subsequent analyses. Across the samples >25% of the annotated genes have been detected by at least 50 reads. Following alignment and read quantification, we performed quality control using a variety of indices, including sample clustering, consistency of replicates, and average gene coverage.

Differential expression analysis was performed using the EdgeR Bioconductor package34, and differentially expressed genes were selected based on FDR (Benjamini Hochberg-adjusted p values) estimated at < 0.1 (or 10% FDR). Three samples from stroke, stroke + GDF10, P4 and two samples from control were compared. Clustering and overlap analyses were performed using Bioconductor packages in the statistical environment R (https://www.r-project.org/).

Genes that were differentially expressed FDR < 10% were submitted to Cluster 3.0 for hierarchical clustering analysis (Euclidian distance, centroid linkage clustering) and visualized using Java TreeView. Differentially expressed genes were further analyzed by molecular pathway analysis and canonical signaling systems (IPA). Briefly, for IPA analyses the genes regulated in each specific category, filtered to only include genes ≤10% FDR, where compared to all genes known to be involved in a given molecular pathway or canonical signaling system in a large curated database of molecular interactions, Fisher’s exact p value was calculated by IPA to determine a statistically different relationship of a data set in the control, stroke ± GDF10, and P4 cortical transcriptomes to chance representation of these genes. For the upstream analysis there are fourvalues that go into the Fisher’s exact p value calculation with Benjamini Hochberg correction for multiple comparisons.

For genome-wide association testing, individual data from .cell, SRA or Excel files was obtained for each experiment (Supplementary Table 8). The gene symbol was located for each probe and average expression computed for duplicated genes. The data were combined with RNA-seq data from this study and variance stabilization transformation normalization was applied.

Statistical analysis. Animal number in in vivo quantitative cortical mapping studies utilizes spatial correlation statistics, so sample size was estimated from previous publications with similar mechanistic studies.34,28,29 Sample size in behavioral studies was assessed by power analysis using a significance level of α = 0.05 with 80% power to detect differences in ANOVA. This experimental design has been validated by other groups conducting similar behavioral34 or tissue outcomes experiments.33,28,29 No animals were excluded from analyses. Mice were randomly allocated to treatment condition and all results were analyzed with the investigator blinded to treatment condition.

For quantitative connectional maps, three statistical analyses were used. First, scatter plots were analyzed using Hotelling’s T2 test for spatial correlation. For data with a common covariance matrix, such as the map of axonal position in tangential cortical sections, Hotelling’s T2 method tests the hypothesis of multivariate mean equality: that the means for the set outcome variable (axonal location for each individual, averaged by experimental condition) are equivalent across groups. The T statistic is the analog of Student’s two-group t-statistic for testing equality of group means for a single outcome variable. P-values were computed without Gaussian assumptions by means of a bootstrap 250 μm was applied around the injection site to account for the uniformity of the injection site itself and immediately adjacent BDA labeling across groups, regardless of sprouting pattern. Second, polar statistics tested for differences in distribution of axonal projection patterns across treatment groups. For each treatment condition, the x, y coordinate of every BDA-positive process was converted to an equivalent polar coordinate (r,θ) relative to the injection site as center3,4. The location of each process was transferred to common polar space and a mean projection vector was computed for each treatment group. Differences in mean projection vectors between groups were analyzed using Watson’s nonparametric two-sample L2 test3,4. Third, axon numbers in the linear construct across ipsilateral cortex were analyzed using one-way analysis of variance (ANOVA) with post hoc Tukey-Kramer test. In addition, differences between two means were assessed by unpaired two-tailed Student’s t test. Differences among multiple means were assessed by one-way ANOVA followed by Tukey-Kramer’s post hoc tests. Data from behavioral experiments were analyzed by two-way repeated-measures ANOVA followed by Tukey-Kramer’s post hoc test. All statistical analyses were performed with GraphPad Prism version 6 (GraphPad Software) or StatPlus version 5 (AnalystSoft) except those specifically noted above. Data are shown as mean ± s.e.m.

A Supplementary Methods Checklist is available.

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