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Hydrogel-delivered brain-derived neurotrophic factor promotes tissue repair and recovery after stroke

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Abstract

Stroke is the leading cause of adult disability. Systemic delivery of candidate neural repair therapies is limited by the blood–brain barrier and off-target effects. We tested a bioengineering approach for local depot release of BDNF from the infarct cavity for neural repair in chronic periods after stroke. The brain release levels of a hyaluronic acid hydrogel + BDNF were tested in several stroke models in mouse (strains C57Bl/6, DBA) and non-human primate (*Macaca fascicularis*) and tracked with MRI. The behavioral recovery effects of hydrogel + BDNF and the effects on tissue repair outcomes were determined. Hydrogel-delivered BDNF diffuses from the stroke cavity into peri-infarct tissue over 3 weeks in two mouse stroke models, compared with 1 week for direct BDNF injection. Hydrogel delivery of BDNF promotes recovery of motor function. Mapping of motor system connections indicates that hydrogel-BDNF induces axonal sprouting within existing cortical and cortico-striatal systems. Pharmacogenetic studies show that hydrogel-BDNF induces the initial migration of immature neurons into the peri-infarct cortex and their long-term survival. In chronic stroke in the non-human primate, hydrogel-released BDNF can be detected up to 2 cm from the infarct, a distance relevant to human functional recovery in stroke. The hydrogel can be tracked by MRI in mouse and primate.

Keywords

Axonal sprouting, bioengineering, hyaluronan, neurogenesis, neural repair

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Introduction

Stroke is the leading cause of adult disability.[1] There are no therapies that directly stimulate repair and recovery in this disease. Stroke triggers enhanced plasticity in the brain in the generation of new local and long distance connections[2–6] and the production of new neurons[7] within brain tissue adjacent to the stroke site – peri-infarct tissue. Growth factor signaling in this region of brain after stroke plays a role in recovery.[3,4] For example, positive allosteric modulators of AMPA receptor signaling enhance motor recovery after stroke through induction of local BDNF in peri-infarct tissue. Surprisingly, although these AMPA receptor modulators are given systemically, their activation of the BDNF receptor (TrkB) occurs only locally in the cortex adjacent to the stroke.[8] This suggests that stroke induces a zone of enhanced plasticity in peri-infarct cortex that is susceptible to modulation of BDNF signaling in the promotion of functional recovery.

BDNF promotes recovery of function in several preclinical models in stroke.[9,10] However, BDNF does not penetrate the blood–brain barrier well, making systemic injection impractical[11] and BDNF has a short tissue distribution time after direct delivery into the brain.[12,13] Small

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molecule agonists have been developed to directly bind to TrkB and these promote recovery of function in stroke in pre-clinical models.[13,14] However, this approach activates BDNF signaling systemically throughout the body in addition to the brain and may produce off-target effects outside of the brain. Systemic activation of BDNF is associated with the development of chronic pain, obesity, and altered liver glucose regulation.[16–18] Many of the growth factors or cytokines that stimulate neural repair in pre-clinical models have widespread effects in other body tissues, such as erythropoietin, fibroblast growth factor, and G-CSF. In pre-clinical studies, these molecules stimulated axonal sprouting, neurogenesis, and other aspects of neural repair.[19] However, in clinical trials, their off-CNS effects limited their use, with renal, hemodynamic, bone marrow, and thrombogenic complications.[20–23]

An alternative approach is to deliver BDNF via a depot that can be injected into the stroke cavity and then will release into the adjacent surrounding area of heightened plasticity and known sensitivity to BDNF. The infarct cavity is the center of the stroke site and is indeed a cavity[24] that can be targeted for depot BDNF release. We tested the hypotheses that sustained depot delivery of BDNF from a biopolymer hydrogel in the stroke cavity will enhance tissue repair and recovery, and achieve relevant diffusional distances in the brain for human stroke. Delivery of a single dose level of BDNF from depot release in the stroke cavity was recently reported in a mouse model of stroke to produce a modest behavioral recovery.[25] The present studies characterize the behavioral and mechanistic effects of sustained BDNF release from a brain-compatible biopolymer hydrogel in the stroke cavity using varied doses of BDNF, determining brain biodistribution in two rodent stroke models and in stroke in the non-human primate, and testing whether this therapeutic approach could be tracked *in vivo* with MRI. We find that a hyaluronan (HA) hydrogel-BDNF combination achieves BDNF release over weeks and promotes motor recovery and axonal sprouting within the motor system, enhances the initial migration of immature neurons to peri-infarct cortex and supports their long-term survival. The hydrogel-BDNF therapeutic combination is visible over time on MRI in the living mouse and in the non-human primate, and promotes BDNF diffusion in functionally relevant distances not just in mouse but also in the gyrencephalic non-human primate brain that more closely mimics the anatomy of the human brain.

Materials and methods

Surgical procedures

All animal studies were performed in accordance with National Institutes of Health animal protection guidelines and were approved by the UCLA Chancellor's Animal Research Committee (mice) or Queen's University Animal Care Committee (monkeys). Adult male (2–4 month old) C57Bl6 (Jackson Labs) and pDCX-DSRed2 mice (DBA background) were used. Focal stroke was produced in forelimb motor cortex.[3–5,8] Four nice were housed to the cage. pDCX-DsRed2 mice were bred in a UCLA colony. Striatal stroke was produced with local injection of the vasoconstrictor N5-(1-iminoethyl)-L-ornithine, dihydrochloride (l-NIO) into the striatum or photothrombotic stroke in the motor cortex with Rose Bengal dye.[3–5] After 7 d, BDNF or hydrogel/BDNF was injected into the infarct core. A 25 µl microsyringe (30 gauge needle, Hamilton Company) was placed 0.8 mm below the cortical surface in cortical stroke and 3.0 mm below the cortical surface in striatal stroke. Injections were made at 1 µl/min. In all experiments, stroke surgeries, hydrogel, or BDNF injections, behavioral testing, MRI, or euthanasia were carried out between 9 a.m. and 4 p.m. Mice were kept on a reverse dark cycle, with darkness from 12 p.m. to 12 a.m., so that behavioral testing was accomplished during their active phase.

BDNF dosing and delivery

Within hydrogel, BDNF (R&D Systems) was delivered in a dose of the maximal aqueous solubility of the molecule (1 µg/µl in sterile saline). The final concentration of BDNF in hydrogel is 0.167 µg/µl and is termed hydrogel/med BDNF. A high dose of BDNF in hydrogel was prepared using the total volume of the aqueous hydrogel to estimate volume for solubility (0.83 µg/µl), termed hydrogel/highBDNF. A hyaluronan-based hydrogel that is cross-linked with poly(ethylene)-glycol-diacrylate (Hystem C, Biotime, Inc.) was used to deliver BDNF from the stroke cavity. For the two groups with hydrogels impregnated with BDNF, 1 µl of BDNF in sterile saline with two different concentrations, medium (final concentration: 0.167 µg/µl) and high (0.83 µg/µl), were added to the hydrogel mixture to assess dose-dependent effects. For the hydrogel control group, 1 µl of sterile saline was added instead of BDNF. For the soluble BDNF-only group (no hydrogel), 1 µl of the medium dose BDNF, with the highest aqueous solubility, was mixed with 5 µl of sterile saline. The final injection volume for all groups was 6 µl.

ELISA

Mice were sacrificed ($n = 4$–5 per group) and brains were retrieved at 1 week after stroke, and 1, 3, 6, and 11 weeks after hydrogel or BDNF injection. Three naïve brains were collected to measure basal BDNF levels. The peri-infarct area was dissected and frozen.

ELISA was performed following the protocol of the manufacture (AbD Serotec). Samples were run in triplicate and all studies were performed blinded to treatment condition.

Behavioral assessment

Recovery of forelimb motor function was assessed using two well-characterized behavioral measures in the photothrombotic stroke model.[4,5,8] Animals ($n = 9$–11 per group) were tested once on both the grid-walking and cylinder tasks 1 week before surgery to establish baseline performance levels and were then tested weekly out to 10 weeks post-stroke (9 weeks post hydrogel or BDNF injection). Behaviors were scored by observers who were blinded to the treatment group of animals in the study.

Tissue processing

Mice were perfused with 4% paraformaldehyde and tissue processed and immunohistochemically stained.[3–5,7] Four series of 50 μm thick sections, each spaced 250 μm apart, were cut in a cryostat and stained for microglia/macrophages (IBA-1, Wako Chemicals), astrocytes (GFAP, Zymed), neurons (NeuN, Millipore), and blood vessels (PECAM/CD31).[3–5,7,27] All sections were processed in parallel. All processing and analysis procedures were performed blind to experimental condition. Fluorescence microscopy imaging was used to measure GFAP, IBA-1, and vessel density.[4,5] For infarct size measurement, four sections spanning the location of the infarct in a series separated by 250 μM were chosen and infarct size or cortical tissue atrophy was measured by calculating the ratio of stroke cortex (ipsi) to contra cortex[4,5] (StereoInvestigator, MBF Bioscience).[5]

BDA quantification of axonal connections

Mice received photothrombotic stroke and stroke + hydrogel/BDNF procedures as above ($n = 6$–7 per group). Groups were control (no stroke), stroke-alone, stroke + hydrogel (no BDNF), stroke + hydrogel+ medBDNF, stroke + hydrogel + highBDNF, and stroke + BDNF (soluble BDNF). At 4 weeks after BDNF or hydrogel delivery or 5 weeks in stroke-alone, mice received a microinjection of the axonal tracer BDA in forelimb motor cortex rostral to the stroke site.[3–5] Mice survived 1 week after tracer injection and tissue processed for densitometric analysis of axonal label in coronal tissue sections using fluorescence measurement methods.[28] In a separate study, mice in groups of control, stroke-alone, and stroke + hydrogel + medBDNF ($n = 5$–6 per group) received the same stroke and BDA injection. After euthanasia, the tissue was processed for tangential sections through cortex using a quantitative cortical mapping procedure.[3–5,29]

BrdU and doublecortin studies

Two cohorts were studied. In the long-term cohort, five groups of mice (Naïve $n = 3$; stroke-only, stroke+ hydrogel, stroke + hydrogel/ + medBDNF, and stroke + medium BDNF, $n = 5$–6) were given a stroke and then injected with BDNF or hydrogel 7 d later. 5-Bromo-2′-deoxyuridine (50 mg/kg) was injected i.p. twice daily from day 7 to day 14 after stroke. Mice were euthanized 5 weeks after stroke and tissue processed for BrdU and NeuN.[7] In the short-term cohort, stroke-only and stroke + hydrogel + BDNF ($n = 5$ each group) were given a stroke and then injected with BDNF or hydrogel 7 d later. Mice were euthanized at 3 weeks after stroke. In the long-term cohort, BrdU/NeuN double labeling was determined from confocal Z plane stacks (C2, Nikon), overlaid with a 350×250 μm grid and images were collected from 20 to 30 grid boxes per section in peri-infarct cortex at a step size of 1 μm increments. Double-labeled cells were counted by absolute co-localization of the BrdU and NeuN signals.

pDXC-DsRed2 mice ($n = 5$ for each group) received stroke or stroke + hydrogel/medBDNF 7 d later and then were euthanized 3 weeks after stroke. Serial coronal sections were processed for doublecortin immunohistochemistry[7] (Santa Cruz Biotechnology) or DsRed2 staining (Abcam).

MRI

Mice received photothrombotic stroke and hydrogel injection as above, and a group was an experimentally naïve control ($n = 4$ per group). Mice were anesthetized and placed in a 7T small animal MRI (Bruker Biospin) on days 7, 10, 14, 22, 28, and 67 after stroke. Respiratory rate was monitored throughout the procedure and the body temperature was maintained at $37 \pm 0.5\,^{\circ}C$. A T2-weighted image set was acquired: rapid acquisition relaxation enhancement factor 8, repetition time 5300 ms, and echo time 15.00 ms with an in-plane resolution of 0.039_0.039_0.50 mm with 13 contiguous slices.

Non-human primate studies

The hydrogel/BDNF studies were performed in adult male *Macaca fascicularis* monkeys 60 d after completion of an experimental study of a PSD95 inhibitor (male, captive bred of Chinese origin imported by Alpha Genesis Inc., average weight $3.99\,kg \pm 0.31$; placebo group $= 3.97 \pm 0.44$). BDNF group $= 4.00\,kg \pm 0.16$).

Middle cerebral artery stroke and MRI imaging were performed.[30] Five 2 ml boluses of hydrogel alone or hydrogel + medBDNF (0.167 mg/ml, the same dose as effective in recovery in mouse) were administered under ultrasound guidance into the stroke cavity ($n = 5$ for each group). Soluble BDNF was delivered into a BDNF group ($n = 2$) or vehicle control was delivered ($n = 1$ vehicle group). Two weeks after hydrogel or BDNF administration, animals were euthanized and tissue harvested for BDNF ELISA. Samples were taken from frontal, temporal, and parietal peri-infarct areas within 1 cm of the infarct; from the ipsilateral frontal and temporal lobes >2cm from the infarct edge; from the contralateral frontal and temporal regions and the hydrogel mass. MRI was performed prior to euthanasia.[30]

Statistics

Studies were conducted in compliance with the ARRIVE guidelines. Sample size for behavioral studies was calculated based on mean and standard deviations from closely related experiments as greater than or equal to 10.[3–5,7,8] Sample size for tissue outcome measures was calculated based on mean and standard deviations from related stroke studies as equal to or greater than 4.[3–5,7] Animals were randomly allocated to treatment groups (www.randomizer.org). All studies were analyzed blinded to experimental condition. Data were tested with multiple comparisons ANOVA and Tukey's or Dunnett's post-hoc tests.

Results

Hydrogel-delivered BDNF is slowly released in peri-infarct tissue

To slowly release BDNF from the infarct cavity, a thiolated HA hydrogel with thiolated denatured collagen was admixed with two different doses of BDNF. This hydrogel slowly polymerizes through the covalent crosslinking action of polyethylene glycol diacrylate to eventually have similar viscoelastic properties as brain,[31] allowing the solution to be injected into the brain as a liquid and, in a predictable temporal window, gel *in situ*.[27] We injected the hydrogel-BDNF combination at a dose of maximum aqueous solubility of BDNF in saline prior to hydrogel admixture (hydrogel + medBDNF). To test maximized BDNF delivery, we also directly dissolved BDNF within the aqueous hydrogel rather than in saline prior to hydrogel mixing (hydrogel + highBDNF). These hydrogel-BDNF combinations were compared with soluble BDNF (BDNF alone) injected into a model of deep subcortical stroke in the striatum (Figure 1a and b). The injections were delivered 1 week after stroke and then measured up to 3 months after stroke; these are time points during the normal periods of tissue repair and recovery in peri-infarct tissue.[2–5,7,8,29] There were no adverse events from stroke or hydrogel injection. Hydrogel-delivered BDNF produces a sustained and significant release for 3 weeks in striatal stroke (Figure 1c). Injection of BDNF alone produced elevated levels for 1 week compared with the control non-stroke brain (Figure 1c). A higher dose of BDNF in the hydrogel (hydrogel/highBDNF) did not produce BDNF release. Examination of this high-dose hydrogel revealed insoluble BDNF deposits in the gel. To determine the release characteristics in cortical stroke, the sustained effective dose of BDNF in the striatal stroke (hydrogel + medBDNF) and BDNF alone was injected into the infarct core in forelimb motor cortex 1 week after stroke. As with the deep striatal stroke injection, this cortical stroke injection produced sustained BDNF release over 3 weeks compared with the control cortex and with peri-infarct cortex prior to hydrogel delivery (Figure 1d). Injection of BDNF alone produced elevated levels for 3 weeks, but with a sharp decline between weeks 1 and 3 (Figure 1d) and at a significantly lower level than hydrogel + medBDNF. In both stroke models, the endogenous pattern of BDNF release after stroke is seen in the experimental groups in which BDNF is not delivered. In stroke alone, BDNF is significantly elevated only at 1 week and only in cortical stroke. Thus, these two stroke data sets include cortical and subcortical stroke and indicate that the release characteristics of the hydrogel do not vary by brain region, and produce sustained BDNF release to peri-infarct tissue above that seen in the condition of normal stroke.

A goal in the development of a biological therapy is to track the delivered therapeutic. A defining characteristic of HA hydrogels is their water absorption,[32] suggesting the possibility of tracking these hydrogels in the brain via MRI. Mice with cortical stroke were imaged with MRI over the course of the hydrogel treatment time course, before the hydrogel was injected (7 d after stroke) and then for 10, 14, 22, 28, and 67 d after stroke. The infarct cavity appears as a dark tissue cavity prior to hydrogel transplant. The transplanted hydrogel is clearly demarcated as a bright signal in T2-weighted MRI (Figure 1e and f) and can be followed out to at least 60 d after transplantation (67 d after stroke) as a clearly distinguishable bright signal in the otherwise dark infarct cavity (Supplementary Figure 1a). Quantifying this hydrogel signal over time indicates that the hydrogel is a stable volume over time, from 3 d after injection to 60 d (Figure 2g, Supplementary Figure 1B), ranging from 28% to 46% of the infarct core (Supplementary Figure 1b). Injection

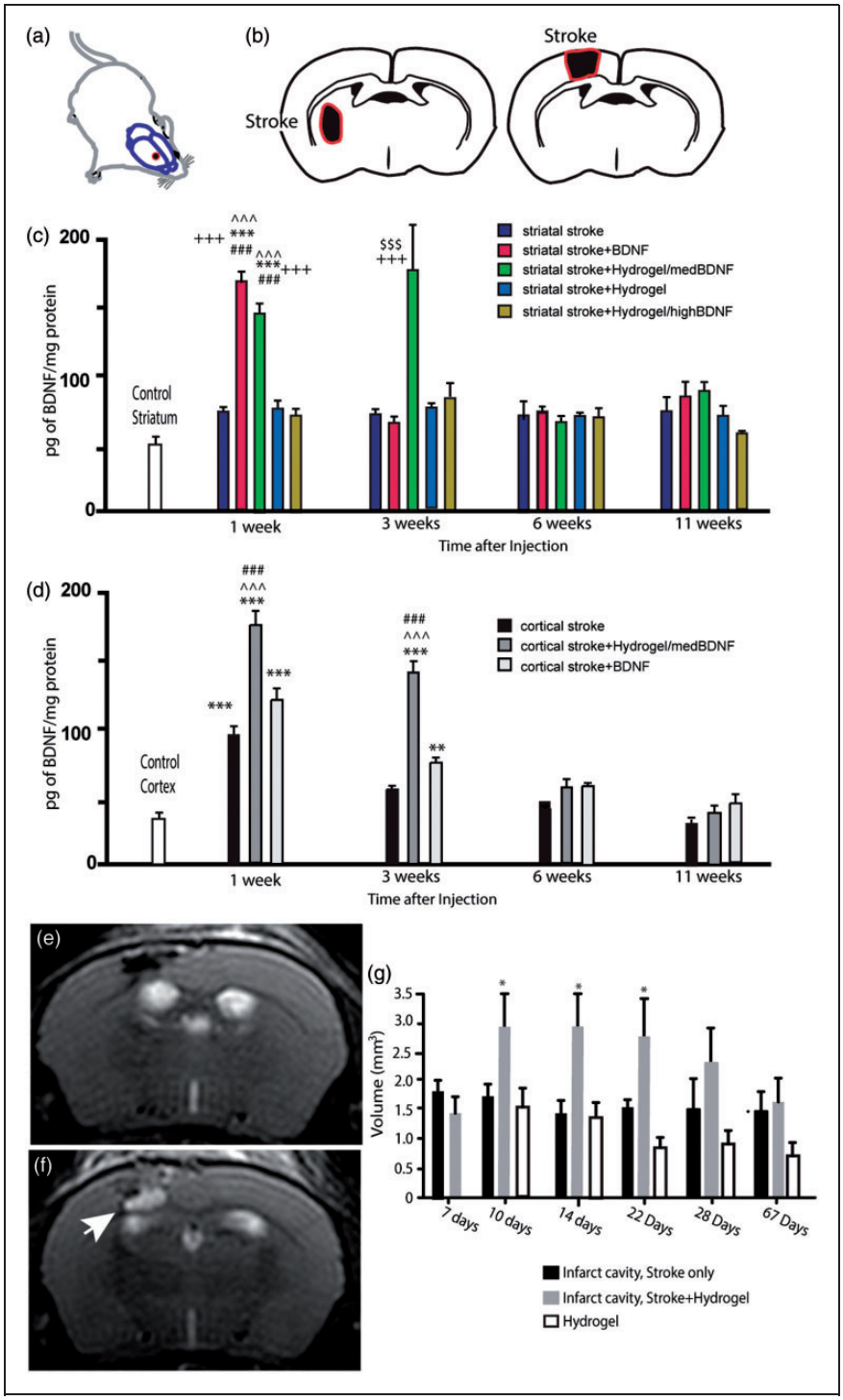

Figure 1. Hydrogel release of BDNF and visualization in MRI. (a) Top panel is cortical stroke in forelimb motor cortex; bottom panel shows location of subcortical stroke in striatum. (b) Timeline for studies. (c) ELISA for BDNF in mouse brain striatum, after striatal stroke and after striatal stroke with hydrogel, hydrogel-BDNF at two doses, and high dose BDNF alone (soluble). ***$p < 0.001$ versus stroke+hydrogel at 1 week, ###$p < 0.001$ versus striatal stroke at 1 week; ^^^$p < 0.001$ versus stroke+highBDNF at 1 week;-+++$p < 0.001$ versus control striatum; $$$p < 0.001$ versus all conditions at 3 weeks. (d) ELISA for BDNF in mouse cortex, after cortical stroke and after cortical stroke with hydrogel/medBDNF and hydrogel+BDNF (high dose, soluble). ^^^$p < 0.005$ versus cortical stroke cortex; ^^$p < 0.05$ versus control cortex, ###$p < 0.005$ versus cortical stroke+BDNF, ***$p < 0.005$ versus control cortex, **$p < 0.01$ versus control cortex. (e and f) T2-weighted MRI images of mouse with motor cortex stroke 15 d after hydrogel-BDNF injection in control stroke (e) and in stroke+hydrogel (f). Arrow points to bright signal of hydrogel. (g) Size of stroke, hydrogel, and total size of stroke+hydrogel as measured in MRI images. *$p < 0.01$, stroke+hydrogel versus stroke. Stroke only and stroke-hydrogel are separate cohorts of mice ($n = 4$). The hydrogel values are taken from the stroke+hydrogel mice.

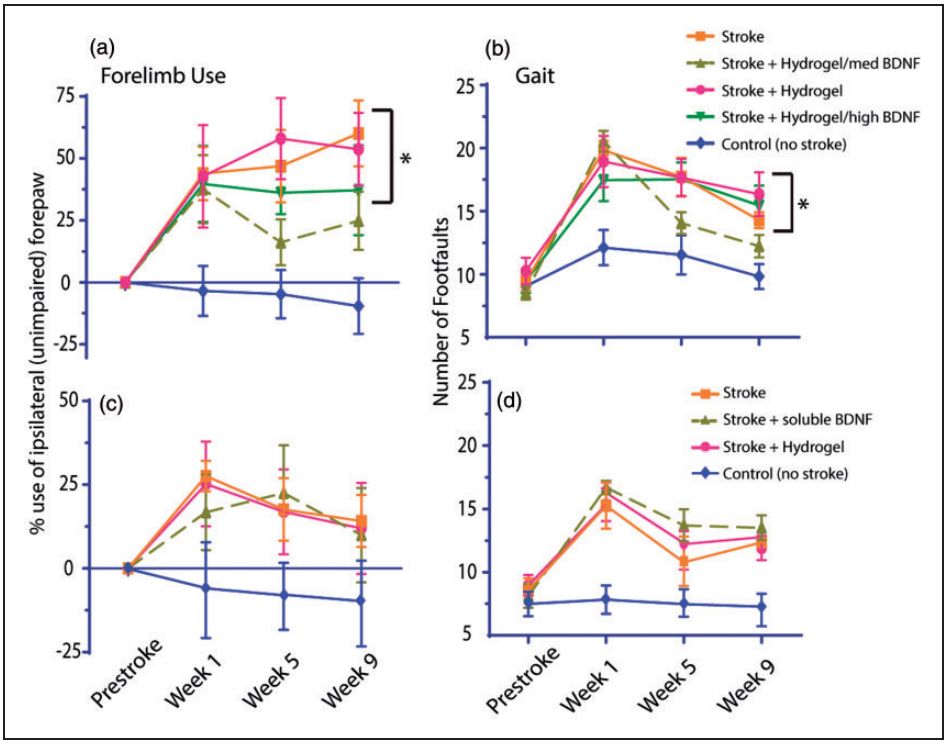

Figure 2. Behavioral effect of hydrogel-delivered BDNF. (a) Effect of hydrogel-BDNF on forelimb use in exploratory rearing after stroke (cylinder task). All stroke conditions are statistically different from control, non-stroke mice (*$p < 0.05$) except for stroke+hydrogel+medBDNF at weeks 5 and 9 after stroke. (b) Effect of hydrogel-BDNF on forelimb use in gait. Stroke causes an approximate doubling of the number of footfaults in walking on a grid at one week (before hydrogel injection), with no difference across stroke treatment groups. Beginning on weeks 5 and 9 only stroke+hydrogel+medBDNF is not different from control performance (*$p < 0.05$). (c) Effect of BDNF alone (no hydrogel) on exploratory rearing. There is no significant difference between stroke and stroke+BDNF or stroke+hydrogel groups. (d) Effect of BDNF alone on gridwalking. There is no significant difference between stroke and stroke+BDNF or stroke+hydrogel.

of the hydrogel into the infarct core results in overall larger measurement of the infarct from 3 d to 15 d after hydrogel injection, seen in MRI as a larger overall infarct cavity (Figure 1g). This data indicate that there is a transient early period, 2 weeks after injection, in which the hydrogel expands the infarct cavity. The hydrogel volume is stable in the infarct cavity for months, and the infarct cavity size does not differ between normal stroke and hydrogel-injected stroke in the long term.

Hydrogel-delivered BDNF promotes motor recovery

To assess if sustained local delivery of BDNF from an HA hydrogel in the infarct core promotes motor recovery, mice were given a stroke in the motor cortex and 1 week later were treated with either hydrogel-delivered BDNF, hydrogel alone, or BDNF alone. There is no difference in forelimb motor impairments in all groups prior to introduction of treatment. However, beginning at 5 weeks after stroke, hydrogel + medBDNF produces a statistically significant improvement in impaired forelimb use in rearing (Cylinder task) and in gait (grid walking task), which continued to 9 weeks after injection (Figure 2a and 2b). This improvement in motor function was not seen with delivery of BDNF alone, hydrogel alone, or a high dose of BDNF in hydrogel (Figure 2). There is no difference in stroke size across these experimental groups (Supplementary Figure 2a, a ratio of ipsilateral hemisphere to stroke/contralateral hemisphere: stroke alone 0.77 ± 0.1, stroke + hydrogel + medBDNF 0.78 ± 0.07, stroke + hydrogel 0.76 ± 0.3, stroke + highBDNF 0.77 ± 0.11, and control (no stroke) 0.97 ± 0.05). These data indicate that hydrogel release of BDNF from the infarct core in motor cortex stroke is correlated to improved behavioral recovery during and after the release, resulting in a persisting effect of the growth factor in peri-infarct tissue.

Hydrogel-delivered BDNF promotes axonal sprouting in motor system

Stroke induces the formation of new connections (axonal sprouting) in cortical and subcortical areas,[2] which has been causally associated with motor recovery

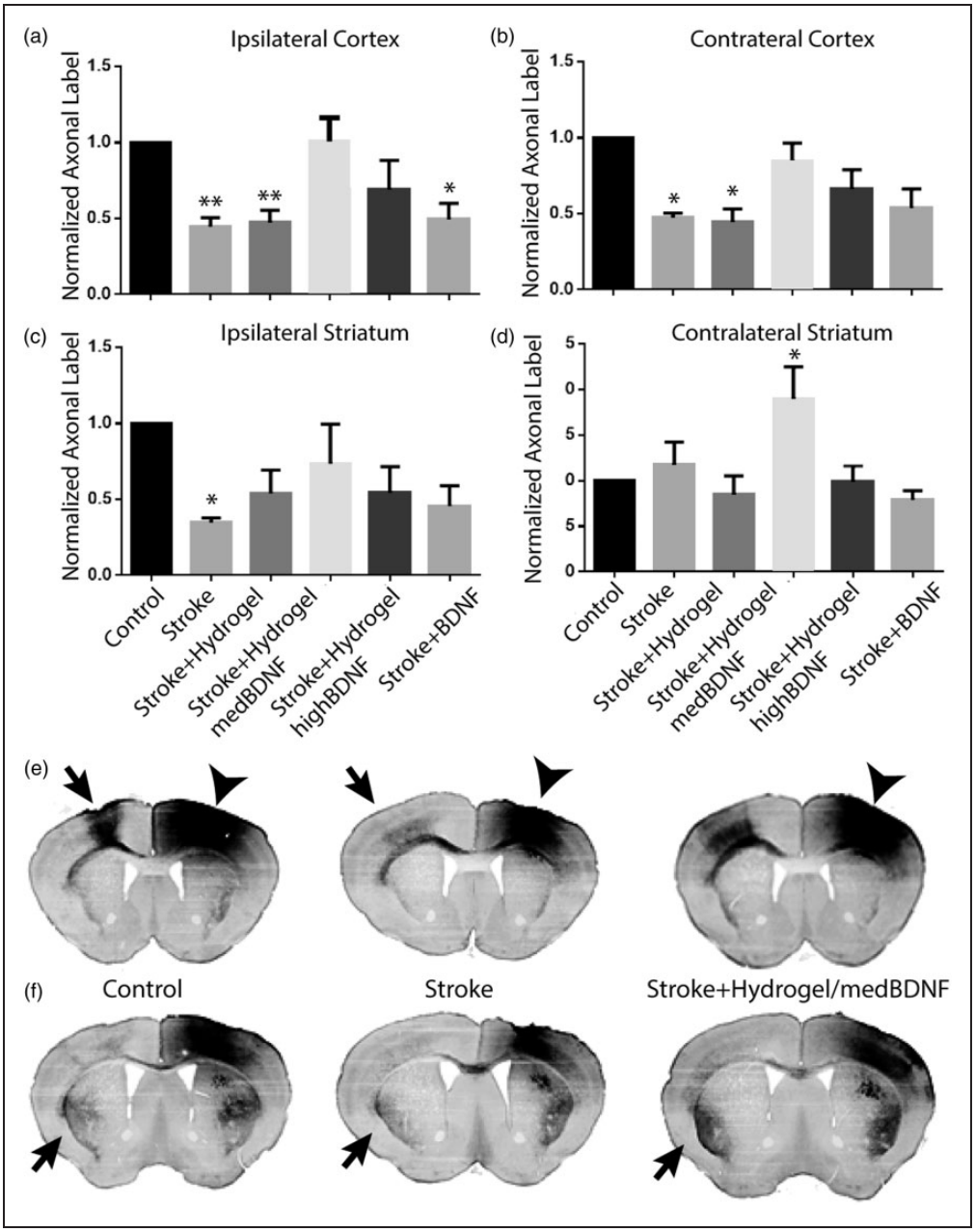

Figure 3. Motor cortex axonal connections in stroke and stroke+BDNF. (a–d) Quantification of axonal label from BDA injection into the forelimb motor cortex ipsilateral to the stroke at 9 weeks after stroke. All values are normalized to the axonal label that is present in the control, naïve mouse brain. In (a–d), $*p < 0.05$ and $**p < 0.01$ versus naïve. (e and f) Two representative coronal sections through the frontal cortex in each condition. These sections are anterior to the stroke site. Arrowheads denote location of BDA injection. Arrows show highlight contralateral cortex (e) or contralateral striatal (f) projections in which the pattern of axonal labeling is similar in stroke+hydrogel+medBDNF to that of control (top row) or in which there is an increase in axonal label in contralateral striatum in stroke+hydrogel+medBDNF compared to stroke and to control (bottom row).

in peri-infarct cortex.[4,5] To determine the effect of hydrogel-delivered BDNF on axonal sprouting in peri-infarct cortex, we densitometrically measured motor system axonal connections[28] after BDNF delivery in prominent axonal systems of the motor cortex: ipsilateral connections to adjacent cortical areas, to contralateral motor cortex, and to ipsilateral and contralateral striatum. The axonal tracer BDA was microinjected into forelimb motor cortex that is preserved, anterior to the stroke site, 4 weeks after BDNF delivery (5 weeks after stroke), a time period in which axonal sprouting is seen in this stroke model.[3–5] This approach will measure the pattern of axonal connections of the cortex anterior to the stroke, and the analysis focuses

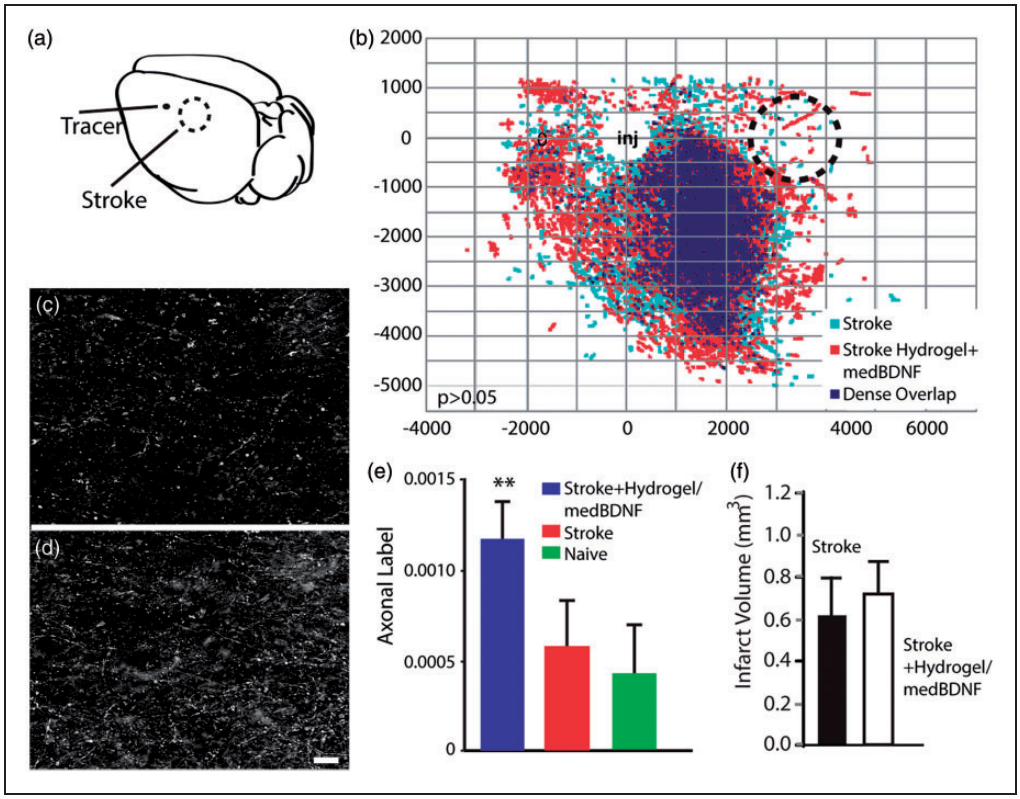

Figure 4. Cortical motor maps in stroke and stroke+hydrogel+medBDNF. (a) Schematic view of mouse brain with location of BDA tracer injection (black) and stroke in motor cortex (dotted circle). (b) Flattened map of tangential sections of the entire ipsilateral cortex to the stroke. Red is the location of projections from motor cortex in stroke+hydrogel+medBDNF animals ($n = 4$). Light blue represents motor cortex projections in stroke-only ($n = 4$) and dark blue is the region of dense overlap of the two projections. "Inj" is the region of the tracer injection and dense neuronal labeling. The dotted circle is the schematic location of the stroke. There is no significant difference between the spatial mapping of motor cortex projection maps in stroke-only and stroke+hydrogel+med/BDNF. (Hotelling's t2 test; $p > 0.05$). (c and d) Photomicrographs of axonal label in motor cortex in stroke-only (c) and stroke+hydrogel/BDNF (d). Bar=20 μm. (e) Quantification of the total axonal label in the ipsilateral hemisphere across conditions. $^{**}p < 0.01$ ($n = 4$). (f) Infarct size in studies with quantitative mapping of connections in (a)–(d).

on connections of this rostral motor region with local cortical areas and with contralateral cortex and striatum. Axonal connections were compared across hydrogel + medBDNF, hydrogel + highBDNF, direct delivery of soluble BDNF, and to hydrogel injection alone using linear fluorescent measurements which significantly correlate with axonal counts.[28] Hydrogel + medBDNF induces significant axonal sprouting from peri-infarct motor cortex to contralateral striatum, as seen by increased axonal labeling within this projection compared with the control brain (Figure 3d and f). Stroke causes a loss of motor system connections within ipsilateral cortex to the infarct and in the projections of motor cortex to contralateral cortex and ipsilateral striatum (Figure 3a and c). Hydrogel + medBDNF and hydrogel + highBDNF prevent this loss, as there is no significant difference in ipsilateral cortical and striatal connections and contralateral cortical connections in the motor system with both medium and high BDNF concentrations delivered with the HA hydrogel after stroke (Figure 4a). There is no difference in infarct size or size of injection site (Supplementary Figure 2c and 2d: stroke only 0.62 ± 0.22, stroke + hydrogel 0.73 ± 0.2, stroke + hydrogel + medBDNF 0.64 ± 0.17, stroke + hydrogel + highBDNF 0.67 ± 0.16, and stroke + soluble BDNF 0.75 ± 0.15; injection size in mm^3: control $0.133 + /-0.07$; stroke only $0.152 + /-0.08$, stroke + hydrogel 0.181 ± 0.05, stroke + hydrogel + medBDNF 0.152 ± 0.7, stroke + hydrogel + highBDNF 0.142 ± 0.04, and stroke + soluble BDNF 0.17 ± 0.04).

Outside of the formation of new distant connections, stroke induces a re-mapping of cortical connections within local cortical areas.[3–5,29] We used quantitative mapping of forelimb motor cortex connections to determine the motor system maps within peri-infarct cortex in control, stroke, and stroke with hydrogel + medBDNF. The locations of all labeled connections from peri-infarct motor cortex were digitized in each mouse, collapsed across groups for a common motor cortex map by condition and statistically compared

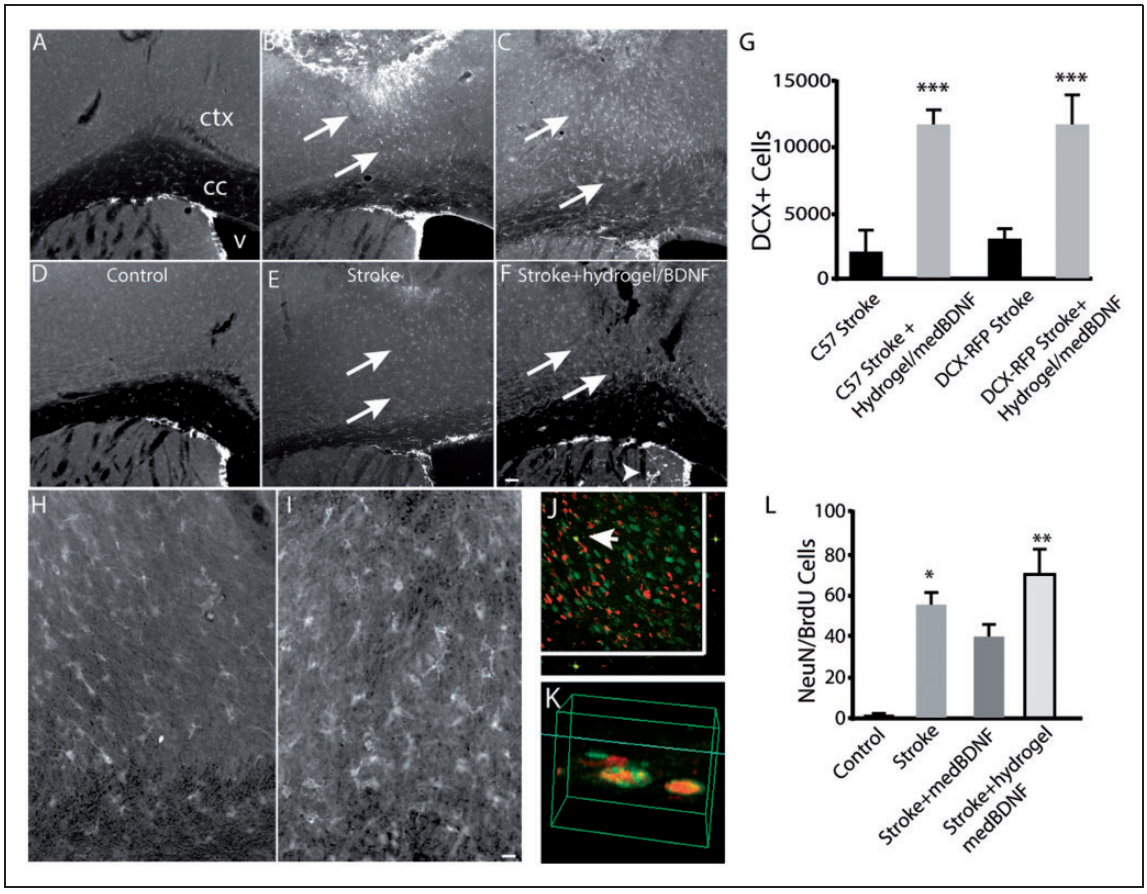

Figure 5. Post-stroke neurogenesis in stroke and stroke+hydrogel+medBDNF. The initial response of migration of immature neurons from the SVZ to peri-infarct tissue was measured (a–f) as well as the long term survival of these cells (j–l). (a–c) Images from C57Bl/6 mice stained for doublecortin (white) 2 weeks after stroke/one week after hydrogel injection. (d)–(f) doublecortin positive cells in DCX-RFP mouse under same time conditions. Arrows in (b and e) show area of DCX + cells in peri-infarct cortex. Arrowhead in (f) shows DCX + cells in striatum in DCX-RFP+hydrogel/BDNF. In (a), *v*, ventricle; cc, corpus callosum; ctx, cortex. (g) Guantification of doublecortin positive cells in both C57Bl6 and DCX-RFP lines. ***$p < 0.001$ versus C57 stroke, DCX-RFP. (h and i) Photomicrographs with higher magnification of area of DCX + cells in peri-infarct cortex in stroke alone (h) and stroke+hydrogel/BDNF (i). These images are taken from region adjacent to arrows in (b and e). (j) Representative confocal image stack showing a double positive NeuN/BrdU cell (yellow, arrow) in maximum projection of a Z stack in peri-infarct cortex, panels on right and bottom show *y*/*z* and *x*/*z* stacked images. In (j) and (k), green is NeuN and red is BrdU. (k) Three-dimensional reconstruction of double positive cell for NeuN and BrdU from confocal image stack. Note that BrdU and NeuN are tightly co-localized. (l) The number of NeuN/BrdU double positive cells per mouse in peri-infarct cortex at 9 weeks after stroke. Stroke * $p < 0.05$ versus control, **$p < 0.01$ versus control. Bar in $F = 100\,\mu m$. Bar in $I = 20\,\mu m$.

across condition ($n = 4$ per group). There is no difference in the pattern of cortical maps of motor system connections across condition (Figure 4b). This indicates that hydrogel-delivered BDNF does not alter the cortical areas that are interconnected within the forelimb motor cortex network in the hemisphere ipsilateral to the stroke. We next analyzed the density of labeled motor system connections within the ipsilateral cortex. The density of axonal connections in the ipsilateral cortical hemisphere in stroke treated with hydrogel + medBDNF exceeds that seen in stroke and in naïve motor cortex (Figure 5e). There is no difference in infarct size or size of injection site across groups (Supplementary Figure 2d: infarct size ipsilateral hemisphere/contralateral hemisphere stroke 0.63 ± 0.17; stroke + hydrogel + medBDNF 0.76 ± 0.15; infarct size in mm^3 control 0.19 ± 0.06; stroke 0.148 ± 0.04, stroke + hydrogel medBDNF 0.17 ± 0.08). This finding indicates that hydrogel delivery of BDNF promotes local axonal sprouting within the pre-existing areas of motor system connections after stroke rather than the formation of new patterns of connections in adjacent brain areas. This finding using quantitative mapping of cortical connections in the ipsilateral hemisphere to the

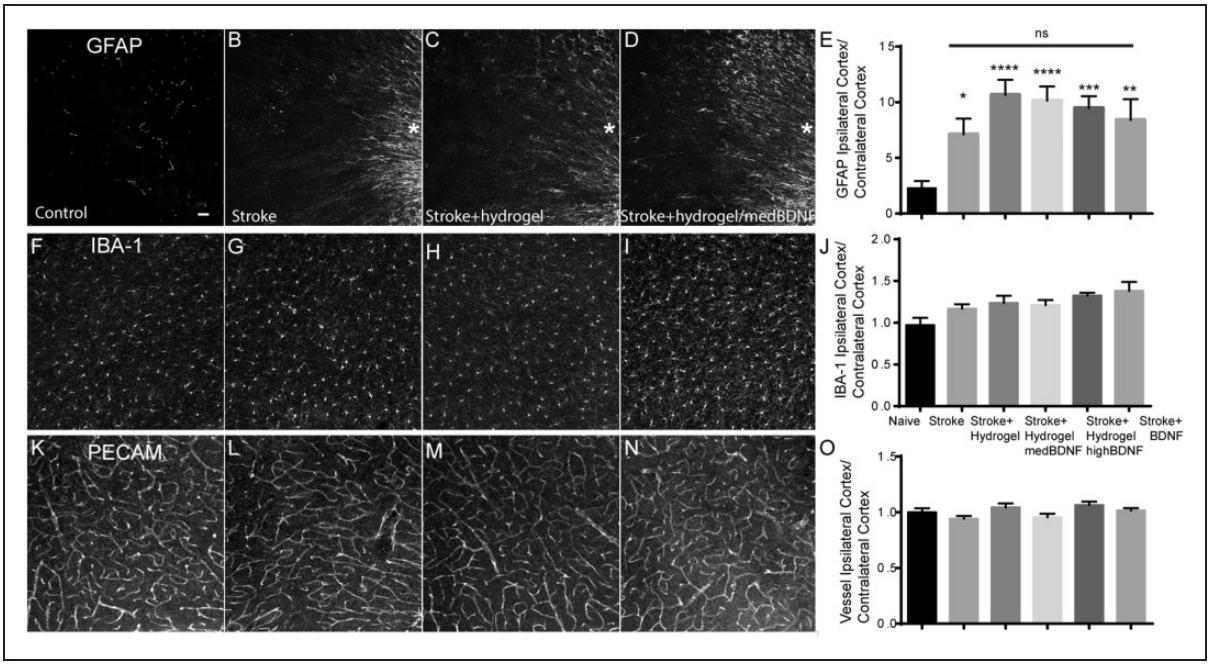

Figure 6. Effect of hydrogel/BDNF on reactive tissue changes after stroke. Brains were analyzed 5 weeks after hydrogel injection/ 6 weeks after stroke. First column is from control (no stroke), second column is from stroke only, third column is from stroke+hydrogel, and fourth column is from stroke+hydrogel+medBDNF. (a)–(d) Immunohistochemical stain for GFAP to demarcate reactive astrocytes. *Edge of infarct. Scale bar in A = 40 μm and applies to all photomicrographs. (e) Quantification of GFAP immunoreactivity across conditions. *$p < 0.05$ versus naïve, **$p < 0.01$ versus naïve, ***$p < 0.005$ versus naïve, ****$p < 0.001$ versus naive. (f)–(i) Immunohistochemical stain for the microglial/macrophage marker IBA-1 in peri-infarct cortex. (j) Quantification of IBA-1 immunoreactivity across conditions. There are no statistically significant differences across conditions. (k)–(n) Immunohistochemical stain for PECAM to indicate endothelial cells in peri-infarct cortex. (o) Quantification of PECAM immunoreactivity across conditions. There are no statistically significant differences across conditions.

stroke is consistent with the findings in the densitometric measurements of axonal connections throughout the forebrain (Figure 3), and supports the conclusion that sustained delivery of BDNF is not causing axonal sprouting into new brain areas after stroke but increasing the density of connections within the normal striatal and cortical projection zones of motor cortex near the stroke site.

Hydrogel-delivered BDNF induces neurogenesis after stroke

Stroke induces a response from the subventricular zone (SVZ) in which newly born immature neurons (neuroblasts) migrate from the SVZ to areas of damage.[7] Ablation of newly born neurons after stroke worsens outcome.[33] BDNF has a prominent role in normal neurogenesis in the adult brain and during brain development.[34] To test the effect of hydrogel-delivered BDNF on post-stroke neurogenesis, we measured the initial migration of neuroblasts after stroke and the subsequent long-term survival of these newly born neurons. Stroke was induced and 1 week later hydrogel + medBDNF or hydrogel alone were injected into the infarct core. Two weeks later animals were euthanized and brains stained for doublecortin, a marker of immature neurons.[7,35] To quantify immature neurons independently of single antibody stains a genetic marker of doublecortin cells was used. In these studies, the neurogenesis experiments were repeated in a doublecortin reporter line, in which red fluorescent protein is driven by the doublecortin promoter.[35]

Stroke itself induces a substantial migration of immature neurons into peri-infarct cortex (Figure 5). However, this is potentiated in hydrogel + medBDNF, with tens of thousands more cells tracking from the SVZ into this the region around the cortical stroke both in C57Bl6 mice (Figure 5a–c and f) and in the DCX-RFP reporter mouse line with hydrogel/ BDNF treatment (Figure 5d–f). These data indicate that in two separate experiments and in mice in two different lines, hydrogel + BDNF promotes post-stroke neurogenesis in peri-infarct motor cortex. The effect of BDNF to show increased neuroblasts in peri-infarct could either be through inhibition of neuroblast apoptosis or enhancement of migration and survival or both.

To determine if hydrogel-delivered BDNF promotes the long-term survival of newly born neurons in

peri-infarct cortex, the thymidine analog BrdU was administered for the first week after hydrogel injection (2 weeks after stroke). Cells labeled by both the BrdU and the mature neuronal marker NeuN (Fox3) were stereologically quantified using the *Z* plane reconstruction of confocal image stacks (Figure 6j and k) at 9 weeks after stroke. Hydrogel + medBDNF promoted long-term survival of newly born neurons after stroke, with greater numbers of these neurons compared with control (Figure 6). There is no difference infarct size across experimental groups in neurogenesis studies (Supplementary Figure 2b: ipsilateral to contralateral hemisphere ratio, C57Bl6: stroke 0.68 ± 0.08, stroke + hydrogel + medBDNF 0.71 ± 0.07, stroke long-term survival 0.65 ± 0.1, stroke + medBDNF 0.7 ± 0.11; DCX-RFP stroke 0.62 ± 0.23, stroke + hydrogel medBDNF 0.64 ± 0.26). It should be noted that the BrdU administration protocol may underestimate the number of newly born, mature neurons in these stroke conditions, as it is given only for a week after hydrogel injection, and may not label all proliferating neuroblasts.

Hydrogel-delivered BDNF does not alter reactive tissue changes after stroke

BDNF can promote other aspects of reactive tissue change after stroke and in normal brain, including angiogenesis,[36] microglial activation,[37] and astrocytosis.[34] This HA hydrogel alone does not alter angiogenesis, reactive astrocytosis or microglial inflammation above that seen in normal stroke.[5] To determine the effect of the hydrogel plus BDNF, we measured its effects on adjacent tissue to the stroke cavity at the time that it is having its functional effect in promoting behavioral recovery, 5 weeks after stroke. Hydrogel alone, hydrogel-delivered BDNF, or BDNF alone had no effect on blood vessel density in peri-infarct cortex or microglial responses (Figure 6). There were no significant differences in reactive astrocytosis across all stroke conditions where an injection was made into the infarct core (Figure 6e), possibly reflecting an effect of this manipulation of the stroke site.

Hydrogel-delivered BDNF release in non-human primates

A question in the evaluation of pre-clinical studies of a neural repair therapy is the biodistribution of the therapeutic in the much larger, gyrencephalic human brain compared with the lissencephalic rodent brain. To understand the brain biodistribution of BDNF delivered from the hydrogel in a clinically relevant model with infarct cavity volumes more comparable with those in human, we measured the tissue levels of BDNF 2 weeks after injection into the infarct core in non-human primates in hydrogel + medBDNF, hydrogel alone, or soluble BDNF alone. In hydrogel + medBDNF, BDNF levels were measured at sites greater than 2 cm distant from the infarct in ipsilateral cortex (Figure 7). BDNF levels are elevated after hydrogel-BDNF in peri-infarct tissue in frontal, parietal, and temporal cortex (Figure 7). Due to inter-animal variability, this reached significance only in one site: central peri-infarct tissue ($p < 0.05$). There was no increase in tissue BDNF with soluble BDNF injection into the stroke cavity (Figure 7) or in contralateral cortex in any condition (data not shown). These data indicate that hydrogel delivery of BDNF in chronic stroke in a large gyrencephalic brain can produce elevated BDNF levels in peri-infarct tissue at distances that exhibit plastic changes in motor or sensory maps that are associated with recovery in humans.[38–40] This BDNF delivery to peri-infarct brain requires the release from the hydrogel matrix, as injection of BDNF alone does not produce an increase in peri-infarct BDNF levels. In the non-human primate, the hydrogel is visible on T1-weighted MRI sequences as a dark mass in the infarct core as compared with stroke without hydrogel, making it possible to track the hydrogel over time (Figure 7g).

Discussion

Sustained BDNF release from an HA hydrogel inside the infarct cavity at a time point of subacute stroke promotes motor recovery in the mouse. This effect is seen only with the prolonged release from the hydrogel, as immediate injection of BDNF does not alter motor recovery. Motor recovery induced by hydrogel-delivered BDNF is associated with axonal sprouting in peri-infarct cortex and to contralateral striatum. This process of post-stroke axonal sprouting does not alter the location of motor cortex connections or their distribution, but increases the density of connections in the areas in which they are already present. Hydrogel-delivered BDNF also increases post-stroke neurogenesis in peri-infarct cortex. With HA hydrogel delivery, BDNF diffuses over time into peri-infarct cortex in the non-human primate brain after stroke, to distances that are relevant for neural repair and recovery in humans. The HA hydrogel can be visualized on MRI and followed over time in both mice and primates. This approach provides a clinically relevant tissue engineering therapeutic for neural repair, as a biopolymer is microinjected as a liquid, polymerizes in the tissue cavity of the stroke core, and then promotes neural repair and recovery over the ensuing weeks in adjacent peri-infarct brain tissue.

There are many potential biomaterial approaches to CNS regeneration and repair. Copolymers of polylactic

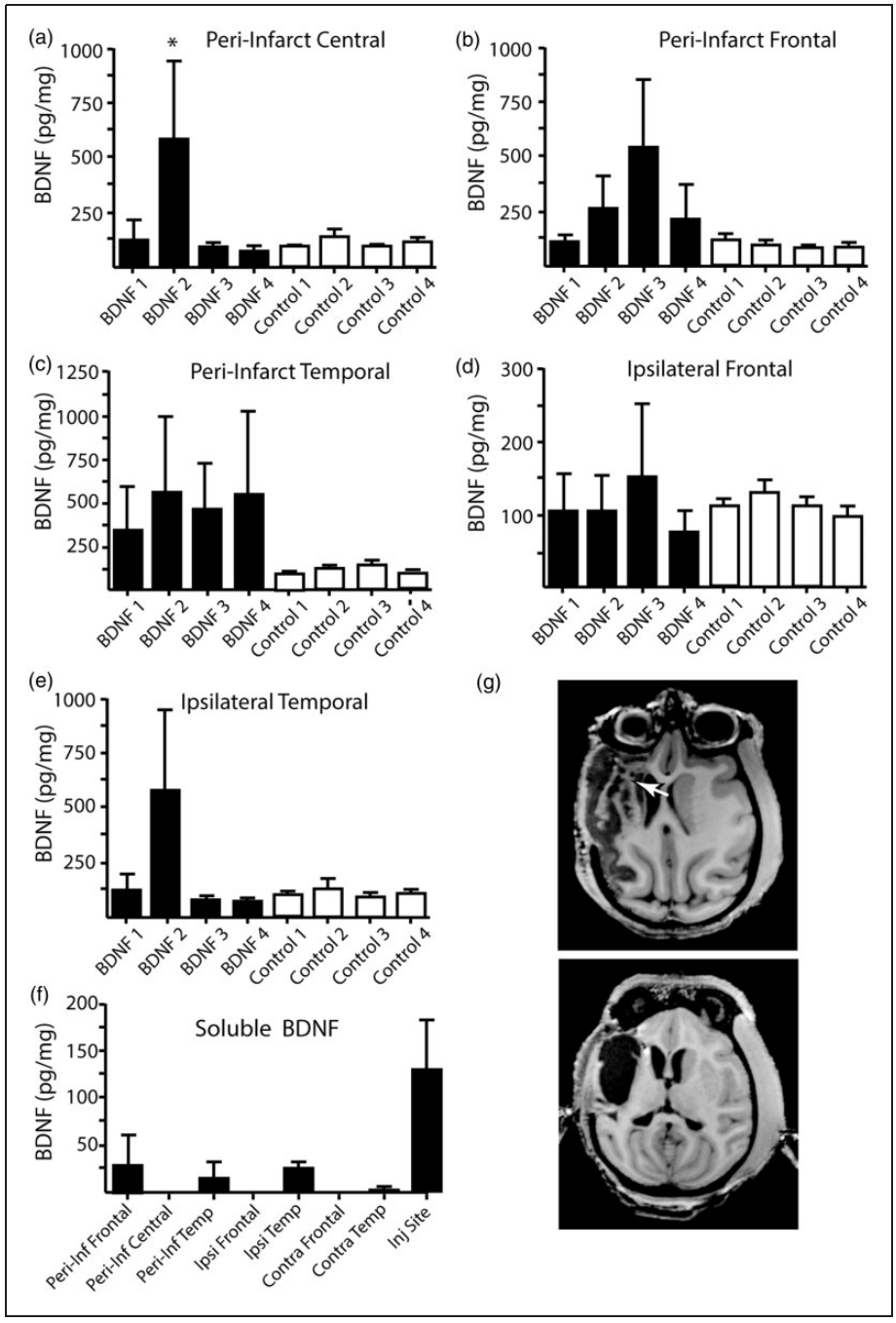

Figure 7. Release of BDNF from hydrogel in non-human primate stroke. Hydrogel+medBDNF or hydrogel alone was injected into the infarct core of a stroke in the non-human primate 3 months after the infarct and BDNF levels measured 2 weeks later in regions close to the infarct (a–c) or more distant to the infarct in the ipsilesional hemisphere (d and e) $^{*}p = 0.05$. (f) Soluble BDNF was injected into the infarct core in the non-human primate 3 months after stroke and BDNF levels measured 2 weeks later. (g) MRI of non-human primate *in vivo* with stroke alone (left panel) or stroke+hydrogel+medBDNF (right panel). The hydrogel appears as dark mass in infarct.

acid and polyglycolic acid, diblock copeptide polymers, alginates, chitin derivatives, and biocomposites have all been studied for tissue scaffolds or molecular release depots in nervous system repair.[41,42] The goal in the present approach was to create a growth factor depot from a self-assembling scaffold of polymers normally present in the CNS that recapitulates the viscoelastic properties of brain.[31] We sought a minimally invasive approach to fill the infarct cavity, which requires micro-injection of a liquid, necessitating only a small injection

needle, and then polymerization *in situ* for sustained growth factor release. Other CNS approaches to sustained drug delivery require invasive placement of bioengineered scaffolds or catheters, which will be associated with the damage from the delivery approach.[42–44] A translational goal in tissue bioengineering is to track the therapeutic to determine its correct targeting and persistence. MRI with routine imaging sequences was effective in tracking the present hydrogel following injection in both mouse and non-human primate.

Hyaluronic acid is one polymer used in preparing CNS-biocompatible scaffolds that are consistent with these translational goals. HA is a non-sulfated, linear glycosaminoglycan consisting of repeating units of (b, 1–4) glucuronic acid-(b, 1–3)-*N*-acetyl glucosamine and is rich in the brain extracellular matrix.[32] HA hydrogels can be transplanted into the brain without provoking injury of adjacent tissue and can diminish inflammatory attack on stem/progenitor cells that are embedded in the hydrogel.[5,27] We and others have shown the ability for HA-based hydrogels to modulate the delivery of several candidate neural repair molecules to the brain including IGF-1,[4] erythropoietin,[45] epidermal growth factor,[46] and blockers of myelin proteins, such as Nogo.[47]

Several possible growth factors could influence post-stroke neural repair. IGF-1, EGF, and HB-EGF have been associated with alterations in the neural stem cell compartment after stroke.[48–50] However, when released from a HA hydrogel in the infarct cavity, we have shown that IGF-1 functions as a neuronal survival factor rather than promoting aspects of tissue repair, such as axonal sprouting.[4] EGF stimulation not only promotes neurogenesis after stroke but also dramatically enhances astrocyte responses.[48] Several converging lines of evidence indicate a central role for BDNF in tissue repair and recovery after stroke. Systemic or intraventricular BDNF delivery enhances recovery after stroke.[9,10] The Val/Met mutation in the *BDNF* gene, which produces a genetic reduction in functional BDNF, retards recovery in transgenic mice[36] and in some studies in humans.[51] Rehabilitative therapy enhances recovery after stroke in a process that is BDNF dependent.[52] Specific blockade of BDNF in peri-infarct cortex blocks motor recovery.[8] A recent report found that hydrogel-release of BDNF modestly promotes motor recovery in a mouse model of stroke.[25] This study extends these findings and this last study on several levels. We tested higher BDNF doses, identifying tissue release for longer intervals after stroke. These higher doses generated a substantial motor recovery. The present study characterized biological effects of hydrogel-BDNF on cellular mechanisms of post-stroke repair in distinct mouse models, tested the delivery of BDNF in the non-human primate, and identified MRI characteristics of the hydrogel for tracking *in vivo* over time. These data suggest that locally boosting BDNF from the infarct cavity into the adjacent tissue may enhance recovery in the subacute to chronic stages of stroke.

BDNF has long been known to be upregulated after neuronal injury and to play a role in axonal sprouting.[53] Stroke induces brain BDNF levels only in a transient manner early after the insult.[54,55] Persistent stimulation of neurotrophins is necessary for a tissue effect.[56] In the present studies, endogenous BDNF was elevated only in the cortical stroke at 1 week, and not at in the striatal stroke at this time point. The hydrogel + BDNF approach provides persistent BDNF release for up to 3 weeks after delivery. The improvement in motor function with hydrogel-delivered BDNF was associated with increased axonal density in the motor cortical projections to the contralateral hemisphere and within the cortex ipsilateral to the stroke. In both cases, this axonal sprouting was seen as an increase in axonal density within the overall cortical projection – new termination areas were not present. These results indicate that BDNF action from the stroke cavity onto adjacent motor neurons causes axonal branching and increased innervation within the existing motor system connections rather than the formation of novel projection patterns. On the contrary, blocking glial growth inhibitors, such as Ephrin A5 or Nogo, does result in new projection patterns in cortex and in the spinal cord after stroke.[2,5,6] Thus, BDNF-stimulated axonal sprouting after stroke is still shaped by the local tissue microenvironment and contained by this environment within the axonal termination fields that are normally present.

BDNF delivery from the HA hydrogel significantly effects post-stroke motor system connections and neurogenesis. Axonal sprouting within the motor or somatosensory systems has been repeatedly demonstrated in many rodent and in primate stroke models.[2–5,57] In mechanistic studies in which a specific axonal projection is blocked after stroke, motor-to-premotor axonal sprouting in the stroke model used in this study was shown to have a causal role in recovery.[5] In larger strokes in the rodent, axonal sprouting from the contralateral cortex to the stroke site has a causal role in recovery.[6] The contribution of neurogenesis to stroke recovery is less clear.[58] Acute ablation of newly born neurons after stroke worsens outcome.[33] However, this approach is likely to impact acute stroke progression and an approach to ablate newly generated neurons in a more chronic time point after stroke is necessary to establish a causal role for this process in recovery. Mechanistic studies to determine whether hydrogel-BDNF improves stroke recovery through axonal sprouting, neurogenesis or some other cellular process will require methodological development in the future.

The biodistribution of BDNF from the hydrogel in both rodent and primate brain corresponds to reported areas of neuroplasticity after stroke. In rat and mouse, stroke triggers a molecular growth program and changes in axonal connections in the region 1–2 mm adjacent to the infarct cavity,[3–5,29] which are causally related to motor recovery.[4,5] In monkeys, somatosensory remapping after stroke and axonal sprouting occur within several millimeters of the infarct.[59,60] In humans, cortical stroke modulates the somatosensory or motor representation in adjacent peri-infarct cortex, shifting the location particularly of hand maps within millimeters to tens of millimeters from the infarct[38,39,61] or altering overall cortical structure and somatosensory responses in this peri-infarct region.[40] In the macaque monkey with a middle cerebral artery territory infarct, hydrogel/BDNF release attains these distances for at least 2 weeks after delivery. Neuroplasticity and functional remapping after stroke is clearly complex, differs by infarct location, and involves alterations in distributed brain networks.[38,62] The present study shows that local modulation of one element in a motor network by using a hydrogel depot to release BDNF can improve recovery and change the connectivity and tissue repair processes of the motor system after stroke.

These studies have limitations. They have established the behavioral effect, MRI appearance, tissue biodistribution, and neural repair effects of hydrogel/BDNF in distinct stroke models and in rodents and primates. While showing that hydrogel/BDNF induces post-stroke axonal sprouting and neurogenesis, these studies did not establish a causal mechanism for the behavioral recovery effect. Such an effort might include selectively ablating neuroblasts after stroke in a suitable genetic mouse line, such as with inducible expression of the diphtheria receptor in doublecortin positive cells. Also, specific blockade of axonal sprouting after delivery of hydrogel/BDNF might allow testing of the role of this process in recovery. These are challenging methodologies that are currently under study.

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Declaration of conflicting interests

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Author contributions

STC and DJC conceived the project. STC, DJC, TIZ, and IL.-L designed the experiments. HHC, CN, ASC, MM, IL-L, DJC, and STC performed the experiments and analyzed data. STC and DJC wrote the manuscript.

Supplementary material

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data

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