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Research paper

Lentivirally administered glial cell line-derived neurotrophic factor promotes post-ischemic neurological recovery, brain remodeling and contralesional pyramidal tract plasticity by regulating axonal growth inhibitors and guidance proteins

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ABSTRACT

Owing to its potent longterm neuroprotective and neurorestorative properties, glial cell line-derived neurotrophic factor (GDNF) is currently studied in neurodegenerative disease clinical trials. However, little is known about the longterm effect of GDNF on neurological recovery, brain remodeling and neuroplasticity in the post-acute phase of ischemic stroke. In a comprehensive set of experiments, we examined the effects of lentiviral GDNF administration after ischemic stroke. GDNF reduced neurological deficits, neuronal injury, blood-brain barrier permeability in the acute phase in mice. As compared with control, enhanced motor-coordination and spontaneous locomotor activity were noted in GDNF-treated mice, which were associated with increased microvascular remodeling, increased neurogenesis and reduced glial scar formation in the peri-infarct tissue. We observed reduced brain atrophy and increased plasticity of contralesional pyramidal tract axons that crossed the midline in order to innervate denervated neurons in the ipsilesional red and facial nuclei. Contralesional axonal plasticity by GDNF was associated with decreased abundance of the axonal growth inhibitors brevican and versican in contralesional and ipsilesional brain tissue, reduced abundance of the growth repulsive guidance molecule ephrin b1 in contralesional brain tissue, increased abundance of the midline growth repulsive protein Slit1 in contralesional brain tissue and reduced abundance of Slit1's receptor Robo2 in ipsilesional brain tissue. These data indicate that GDNF potently induces longterm neurological recovery, peri-infarct brain remodeling and contralesional neuroplasticity, which are associated with the fine-tuned regulation of axonal growth inhibitors and guidance molecules that facilitate the growth of contralesional corticofugal axons in the direction to the ipsilesional hemisphere.

1. Introduction

With the recent progress in recanalizing therapies, that is, thrombolysis and thrombectomy (Powers et al., 2019), acute stroke treatment has considerably advanced in the last five years. Despite these improvements, the majority of stroke patients still exhibit neurological deficits in the long run. As such, stroke remains the most frequent cause of longterm disability world-wide. The persistent neurological deficits raise the need for treatments that promote brain remodeling and plasticity once ischemic damage has occurred (Hermann and Chopp, 2012). In the ischemic brain, neuronal connectivity is regulated in a highly dynamic way. As such, surviving neurons or axons in the vicinity and at

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distance of the stroke are capable to sprout over short and long distances, contributing to functional neurological recovery (Hara, 2015; Hermann and Chopp, 2012; Herz et al., 2012; Reitmeir et al., 2012). Both the ipsilesional and contralesional motor cortices exhibit post-ischemic neuronal plasticity (Carmichael, 2003; Liu et al., 2009; Reitmeir et al., 2011; Wang et al., 2018). Neuronal plasticity is initiated in the first days post-stroke and continues over several weeks or months (Reitmeir et al., 2012).

Neuronal plasticity considerably depends on the remodeling of peri-infarct brain tissue, which exhibits (a) de novo formation of cerebral microvessels via a process called angiogenesis, (b) proliferation and neuronal differentiation of neural precursor cells (NPC) that migrate along these microvessels and integrate into ischemic brain parenchyma as newly formed neurons and (c) astroglial activation and scar formation, which may either promote tissue neuronal rewiring or prevent rewiring (Dibajnia and Morshead, 2013; Liu and Chopp, 2016; Ruan et al., 2015). Newly formed microvessels, neural precursor cells and astrocytes release considerable amounts of neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) or vascular endothelial growth factor (VEGF) that enhance neuronal plasticity (Kalinowska-Lyszczarz and Losy, 2012; Markus et al., 2002; Reitmeir et al., 2012). Parenchymal cells, specifically astrocytes, are rich sources of axonal growth inhibitors, guidance proteins and midline growth repulsive proteins that modulate axonal growth and path finding (Cramer, 2010; Kilic et al., 2010; Meves and Zheng, 2014).

GDNF was first identified in glioma cells as protein that has neurotrophic activity (Lin et al., 1993). It is a secreted factor, which is mainly produced by neurons under physiological conditions and which acts in a paracrine fashion on neighboring neurons via the so-called rearranged during transfection (RET) receptor (Duarte Azevedo et al., 2020). GDNF is crucial for fetal development and survival (Pascual et al., 2008). Its expression decreases during adolescence, and it is mainly expressed in the cortex, striatum, hippocampus and substantia nigra in the adult brain (Hidalgo-Figueroa et al., 2012). Upon focal cerebral ischemia, GDNF is de novo expressed in the peri-infarct cortex and striatum (Wei et al., 2000). In the subacute stroke phase, GDNF is furthermore produced by astrocytes and microglial cells (Duarte Azevedo et al., 2020; Wei et al., 2000). In addition to its neuroprotective effect after focal cerebral ischemia, traumatic brain injury or optic nerve degeneration (Hermann et al., 2001b; Kilic et al., 2005; Kilic et al., 2003, 2004), neurorestorative actions of GDNF have been shown under conditions of Parkinson's disease, Huntington's disease and spinal cord injury (Blits et al., 2004; Kirik et al., 2000; Ramaswamy et al., 2009). Most recent studies in patients with Parkinson's disease revealed that viral GDNF delivery is well-tolerated and safe (Heiss et al., 2019). Based on the fact that GDNF potently protects dopaminergic neurons in the substantia nigra, clinical trials in human patients using viral GDNF vectors are currently performed (e.g., https://www.clinicaltrials.gov/ct2/show/NCT01621581).

In contrast to neurodegenerative diseases, where solid evidence of functional neurological recovery was provided, the long-term effects of GDNF on post-ischemic neurological recovery, brain remodeling and neuronal plasticity in the post-acute stroke phase have so far not systematically been assessed. To characterize GDNF's therapeutic potential in the post-acute stroke phase, we herein examined the effect of lentiviral GDNF gene delivery on ischemic injury, BBB permeability, angiogenesis, neurogenesis, astroglial scar formation and long-distance plasticity of contralesional pyramidal tract axons that cross the midline to innervate denervated neurons in the ipsilesional hemisphere. By means of Western blots, we performed a comprehensive analysis of expression changes of axonal growth inhibitors (brevican, versican), guidance proteins (ephrin b1, ephrin b2) and midline growth repulsive proteins (Slit1, Robo2), which, as we for the first time show, are dynamically regulated by GDNF and set the stage for contralesional midline-crossing neuroplasticity.

2. Material and methods

2.1. Ethics statement

This study has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the Ethics Committee of Bezmialem Vakif University, Istanbul. Investigators were blinded for experimental groups at all stages of experiments and data analysis.

2.2. Animal sets and groups

Experiments were performed using 88 male C57/Bl6 mice (8–10 weeks, 20–25 g). All animals were kept under regular light conditions with a 12:12 h light-dark cycle. Following an initial experiment, in which GDNF expression was studied by Western blots and quantitative real-time polymerase chain reaction (qrtPCR) 10 days after intrastriatal delivery of a lentiviral vector expressing GDNF and GFP (Lv-GDNF-GFP) transgenes in non-ischemic animals (n = 4 animals), two sets of animals were assigned, which were exposed to intraluminal middle cerebral artery occlusion (MCAO). Set 1 (Fig. 1A) was used for studying effects of GDNF on acute ischemic injury 24 h or 72 h after 90 min or 30 min of MCAO. In these animals, Lv-GFP or Lv-GDNF-GFP vectors were intrastriatally administered, followed by MCAO induction 10 days later (n = 7 animals/group). In set 2 (Fig. 1B), a comprehensive study was performed of long-term effects of GDNF on neurological recovery, brain remodeling and plasticity. Lv-GFP or Lv-GDNF-GFP vectors were again intrastriatally administered, followed by induction of 30 min MCAO after 10 days. Animals were divided into three subsets used for studying (a) neurological recovery and contralesional pyramidal tract plasticity using the anterograde tract tracer biotinylated dextrane amine (BDA) (n = 12 animals/group), (b) immunohistochemistry, which included BrdU incorporation analysis for the evaluation of neurogenesis (n = 7 animals/group), and (c) protein expression analysis by Western blots (n = 7 animals/group). Animals wer sacrified 52 days after MCAO. Non-ischemic animals, ischemic animals belonging to animal set 1 and ischemic animals belonging to subsets (b) and (c) of animal set 2 were killed by decapitation in deep anesthesia. Animals of subset (a) of set 2 were sacrified by transcardial perfusion with 4% paraformadelhyde in 0.1 M phosphate buffered saline (PBS). Brains were frozen with dry ice and cut on a cryostat into 18 μm coronal sections or tissue samples were taken for subsequent molecular biological studies.

2.3. Cloning of Lv-GFP or Lv-GDNF-GFP vectors

A second generation lentivirus packaging system was used as an efficient and safe way for vector production. Total RNA from SH-SY5Y cell cultures was isolated using AllPrep DNA/RNA/Protein Mini Kit (80,004, Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. To obtain complementary DNA (cDNA), DNA was synthesized from the RNA template using Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche, Basel, Switzerland). The coding sequence of human GDNF transcript variant 1 (NCBI Reference Sequence: NM_000514.3) was amplified using appropriate primers (forward: 5′- AGT CAG GTA CCA TGA AGT TAT GGG ATG TCG TGG −3″, reverse: 5′- AGT CAG CGG CCG CGG AGT CAG ATA CAT CCA CAC C -3′) with fast digest restriction enzymes *Kpn*I (FD0524 Thermo Fisher Scientific, Massachusetts, USA) and *Not*I (FD0593, Thermo Fisher Scientific). The PCR product for hGDNF and expression plasmids (pLenti-CMV-GFP-2A-Puro; Applied Biological Materials, Richmond, Canada) were digested by using KpnI and NotI restriction enzymes. Next, they were ligated using T4 DNA ligase (EL0014, Thermo Fisher Scientific). Thereafter, the insert was confirmed by sequencing. pMD2.G and psPAX plasmids, which were kindly provided by Dr. Didier

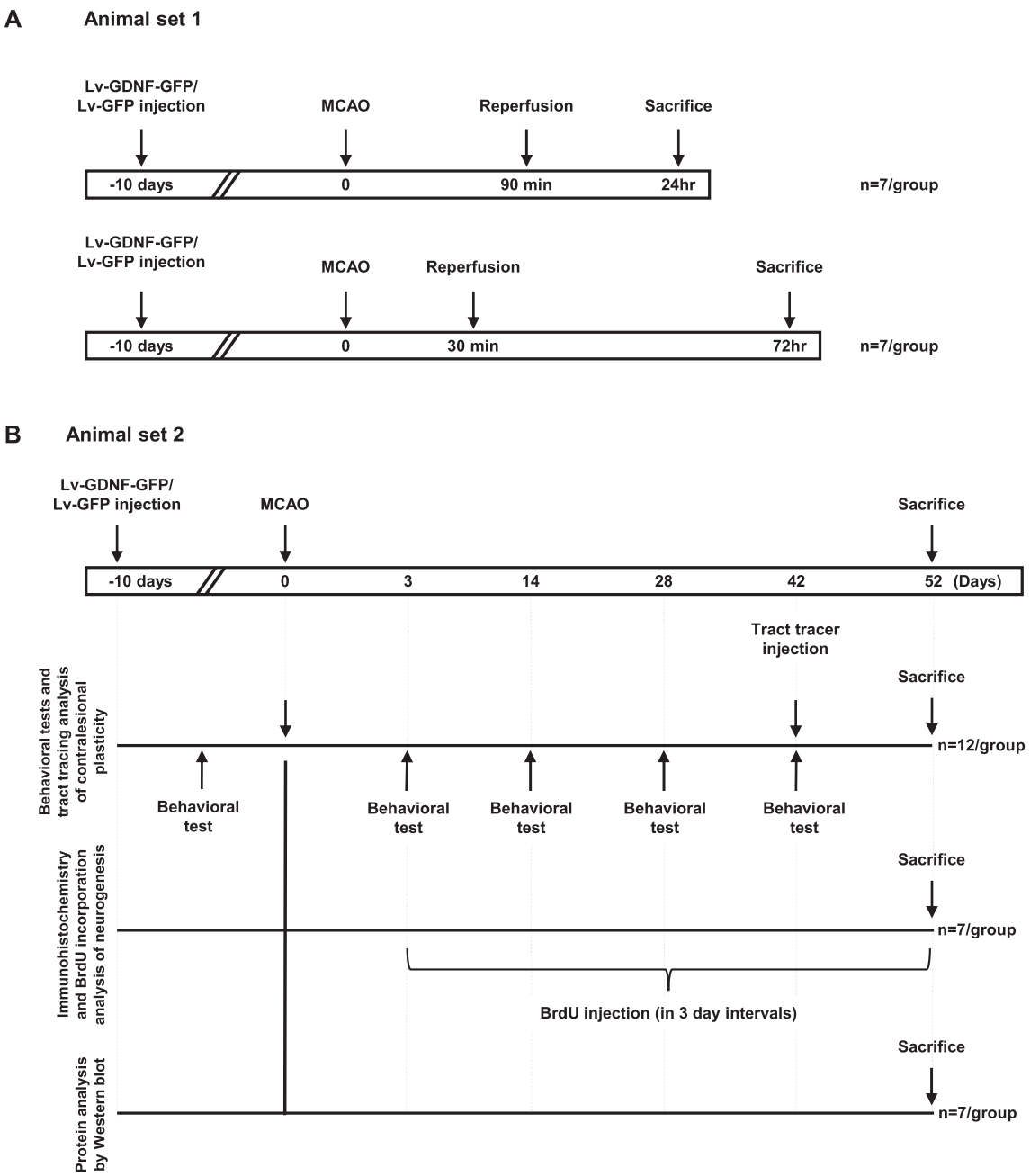

Fig. 1. Animal groups and time lines. In animal set 1, the effects of lentiviral GDNF delivery using the Lv-GDNF-GFP vector on ischemic brain injury were studied in models of 90 min and 30 min middle cerebral artery occlusion (MCAO), which evoke reproducible infarcts of the cortex and striatum (90 min) or disseminate neuronal injury (30 min). In animal set 2, the effects of lentiviral GDNF delivery on (a) neurological recovery and contralesional axonal plasticity (the latter studied by injection of the anterograde tract tracer biotinylated dextrane amine (BDA) into the contralesional motor cortex), (b) brain remodeling and neurogenesis (the latter studied by BrdU incorporation analysis) and (c) the expression of plasticity-associated proteins and proteoglycans were examined in three animal subsets. Animals were sacrified at 24 or 72 h (set 1) or 52 days (set 2) post-MCAO. A Lv-GFP vector was administered as vehicle in the control groups.

Trono (Ecole Polytechnique Federale, Lausanne, Switzerland), were used as complementary vectors for packaging of the lentiviral system (12,259; 12,260, Addgene, UK). HEK293T (6×10^6 cells) were seeded on 10 cm plates (CLS3294, Corning, New York, USA). The next day, transfection was performed using Lipofectamine 3000 (L3000015, Thermo Fisher Scientific) as described by the manufacturer's protocol. Briefly, 7 μg lentiviral vector, 3.5 μg pMD2.G, and 7 μg psPAX were used to prepare DNA-lipid complex. After 10 min incubation at room temperature, the DNA-lipid complex was added to cells drop by drop. Six hours after transfection, the medium was replaced with fresh DMEM (P04–01158, Pan Biotech, Bavaria Germany) and it was incubated at 37 °C in a moist atmosphere containing 5% CO_2. Twenty-four and fifty-two hours after transfection, the entire medium was harvested, centrifuged for 10 min at 2000 rpm and filtered with a low binding filter having 0.45 μm pore size. After ultra-centrifuging at 100,000*g* for 2 h, virus particles were discarded and dissolved in Dulbecco's Phosphate-Buffered Saline (DPBS; P04–3650, Pan Biotech) without calcium and magnesium. Expression plasmid without addition of any insert DNA was used as a control and packaged with the same procedure applied to the GDNF containing one.

2.4. Calculation of virus titer

Ten-fold serially diluted virus particles ranging from 10 to 1 to 10–4

were exposed to HEK293T cells and incubated for 3 days by renewing the medium on each day. After having sufficient GFP signaling, cells were trypsinized, inactivated with culture media, spun and resuspended in cold phosphate buffer saline (PBS), respectively for fluorescence activated cell sorting (FACS) analysis (Becton Dickson Influx cell sorter, USA). In order to determine the titer of the virus particles, wells having cells expressing 1–20% GFP were used. According to the formula "Virus titer (TU/ml) = F x C_n/ V(ml) x Df" multiplicity of infection (MOI) was calculated as 10^8. In this formula F is the frequency of GFP expressing cells, C_n is the total number of cells infected (4×10^5 for this study), V is the volume of the inoculum (1 ml for this study), and Df is the virus dilution factor.

2.5. Administration of Lv-GFP or Lv-GDNF-GFP vectors

Mice were anesthetized with 1% isoflurane (30% O_2, remainder N_2O) and placed in a stereotaxic frame (Stoelting, Illinois, USA). The animals' skull was drilled and Lv-GDNF-GFP or Lv-GFP vectors (10^8 lentivirus particles in 2 μl 0.1 M PBS) were intrastriatally administered into the left hemisphere (ipsilesional) (injection coordinates: AP ± 0.0 mm, ML −2.5 mm and DV +3 mm from bregma) via a microsyringe pump (Micro 4; World Precision Instrument, Florida, USA).

2.6. Induction of focal cerebral ischemia

Male C57bl/6 mice (20–25 g) were anesthetized with 1% isoflurane (30% O_2, remainder N_2O). Rectal temperature was controlled at 36.5–37.0 °C using a feedback-controlled heating system (MAY instruments, Ankara, Turkey). During MCAO and reperfusion, cerebral blood flow (CBF) was monitored by laser Doppler flow (LDF) measurement using a flexible 0.5 mm fiber optic probe (Perimed, Sweden) attached to the intact skull above the MCA territory (AP +2 mm and ML +6 mm from bregma) with tissue adhesive. Focal cerebral ischemia was induced using an intraluminal filament technique (Beker et al., 2015; Beker et al., 2018b). A small midline neck incision was made and the left common and external carotid arteries were isolated and ligated. The internal carotid artery was temporally ligated using microvascular clips (FE691; Aesculap, Frankfurt, Germany). A silicon coated (tip diameter 180–190 μm; Xantropen; Bayer Dental, Japan) 8.0 nylon monofilament (Ethilon; Ethicon, Germany) was inserted through a small incision into the common carotid artery and advanced 9 mm into the internal carotid artery to the middle cerebral artery offspring for MCAO. Thirty or ninety minutes later, reperfusion was initiated by filament removal. After that, LDF recordings were continued for 20 min (for 30 min MCAO) or 30 min (for 90 min MCAO) to control reperfusion. After the surgery, wounds were carefully sutured, anesthesia was discontinued, and mice were placed into their cages.

In mice exposed to 90 min MCAO, neurological deficits were evaluated 24 h after MCAO using the modified Bederson score(Bederson et al., 1986): 0 = normal function; 1 = flexion of torso and of the contralateral forelimb upon lifting of the animal by the tail; 2 = circling to the contralateral side but normal posture at rest; 3 = reclination to the contralateral side at rest; 4 = absence of spontaneous motor activity.

For analysis of contralesional pyramidal tract plasticity, animals belonging to set 2 (a) were re-anesthetized at 42 days post-stroke with 1% isoflurane. Following a small trephination of the skull, the anterograde tract tracer BDA (molecular weight: 10,000 Da; D1956; Thermo Fisher Scientific) was injected into non-ischemic motor cortex (AP: +0.5 mm, ML: +2.5 mm, DV: +1.5 mm), as previously reported (Reitmeir et al., 2011).

For analysis of neurogenesis and gliogenesis, animals belonging to set 2 (b) were intraperitoneally treated with 100 mg/kg 5'bromo-2-deoxyuridine every third day from post-ischemic day 3 until animal sacrifice (BrdU; B5002; Sigma Aldrich, Missouri, USA).

2.7. Functional neurological tests

2.7.1. Grip strength test

The grip strength test apparatus consists of a spring balance interconnected with a Newton meter. A triangular steel wire, which is held by the animals instinctively, is attached to the Newton meter. When pulled by the tail, the animals exert force on the steel wire. In this experiment, grip strength was evaluated at the right forepaw of the animals which was paretic as a consequence of the stroke. During the test, the left non-paretic forepaw was wrapped with adhesive tape. The test was repeated five times on occasion of each test session and mean values for each seesion were calculated (Kilic et al., 2014).

2.7.2. RotaRod test

The RotaRod (model 47,600; UgoBasile, Varese, Italy) is a rotating platform, which, with an accelerating speed from 6 to 40 rpm, allows evaluation of motor coordination skills. At the end of 245 s., the maximum speed was reached and the time at which animals dropped off the drum, was evaluated (maximum testing time: 300 s.) Measurements were performed five times on occasion of each test session, and mean values for all five measurements were calculated. (Kilic et al., 2014)

2.7.3. Open field test

The open field is a round arena (150 cm diameter), which is covered by white plastic floor. A side wall made of white polypropylene at the height of 35 cm high surrounds the platform. It allows to evaluate spontaneous locomotor activity and exploration behavior. The arena is partitioned into three sections, an outer wall zone, an intermediate transition zone and an inner zone. Each mouse was released near the wall of the outer zone and observed for 10 min. Paths traveled by animals were tracked and recorded with an electronic imaging system (Anymaze Version 4.99; Stoelting). In order to determine exploratory behavior and anxiety, total resting and progressing times of the animals and the time spent in each of the three zones were analyzed (Kilic et al., 2014).

2.7.4. Light dark transition test

The light/dark transition test consists of a closed box with a size of 40 cm × 20 cm × 20 cm that is equally divided into two light and dark chambers by a 4 cm × 4 cm door opening at the floor level. The test offers information about exploration behavior and anxiety making use of the animals' instinct of abstaining from illuminated environments when experiencing fear. Each mouse was placed in the distant corner of the light chamber and monitored for 10 min. Paths traveled by animals were tracked and recorded with an electronic imaging system (Anymaze Version 4.99; Stoelting). The time spent in the light or dark chamber was assessed (Kilic et al., 2014).

2.8. Cresyl violet staining

To evaluate infarct volume, brain swelling and brain atrophy, brain sections collected at 2 mm intervals were stained with cresyl violet (C5042; Sigma Aldrich) in animals sacrified 24 h or 52 days post-MCAO. Using the ImageJ software (NIH, USA), infarct area was determined in animals sacrified 24 h post-MCAO by subtracting the non-lesioned area of the injured hemisphere from that of the contralesional hemisphere. Infarct volume was calculated by integrating infarct areas at various brain levels. Brain edema was calculated as the volume difference between the ischemic and the non-ischemic hemisphere and expressed as percentage of volume of the non-ischemic hemisphere. The volumes of both brain hemisphere were determined in animals sacrified 52 days post-MCAO by outlining tissue areas on brain sections, integrating these tissue areas across the forebrain and expressing the volumes determined as percentage of volume of the corresponding contralesional hemisphere.

2.9. IgG extravasation analysis

Brain sections obtained from the bregma level of mice that were exposed to 90 min MCAO were rinsed in PBS for 10 min to remove intravascular IgG and were fixed in 4% PFA (16,005, Merck, Darmstadt, Germany) in 0.1 M PBS. After blocking endogenous peroxidase with methanol/ 0.3% H_2O_2, sections were immersed in 0.1 M PBS containing 5% bovine serum albumin (BSA) and normal swine serum. Sections were incubated in biotinylated goat anti-mouse IgG (sc-2013; Santa Cruz, Texas, USA) for 1 h and stained with diaminobenzidine (DAB) using an avidin peroxidase kit (Vectastain Elite; Vector Labs, California, USA). All sections were processed in parallel. Sections were scanned and IgG extravasation in the ischemic striatum and cortex was densitometrically analyzed (Beker et al., 2015). For correction of background staining, optical densities in corresponding contralesional non-ischemic tissue were subtracted from those in ischemic tissue.

2.10. DNA fragmentation analysis

Brain sections from the bregma level were fixed with 4% PFA and stained by terminal transferase dUTP nick end labeling (TUNEL) using a in situ cell death detection kit (11,684,795,910; Roche, Switzerland) according to the manufacturer's protocol (Beker et al., 2015). Sections were counterstained with 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI). TUNEL positive DNA-fragmented cells were counted in a total of 9 regions of interest (ROI) in the striatum (each of 62,500 μm^2 size) using a LSM 780 confocal microscope (Carl Zeiss, Jena, Germany).

2.11. Immunofluorescence staining

Sections from bregma level were used for conventional immunofluorescence analysis. After immersion fixation in 4% PFA, sections were pretreated with sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) for antigen retrieval, rinsed and immersed for 1 h in 0.1 M PBS containing 0.3% Triton (PBS-T) and 10% NGS. Sections were incubated with anti-BrdU (Ab6326, Abcam, Cambridge, UK; used as cell proliferation marker), anti-NeuN (MAB377c3, Millipore, Massachusetts, USA; used as neuronal marker), anti-GFAP (3656, Cell Signaling, Cell Signaling, USA; used as astrocyte marker), anti-Iba1 (019–1974, Wako, Japon) and/or anti-CD31 (102,416, Biolegend, California, USA; used as endothelial marker) antibodies, which were fluorescently conjugated with Cy3 (anti-NeuN), Alexa Fluor-555 (anti-GFAP) or Alexa Fluor-647 (anti-CD31) or detected by an Alexa Fluor-488 conjugated (ab150157, Abcam; used for detection of BrdU) or Alexa Fluor-555 conjugated (A27039; Thermo Fisher Scientific; used for detection of Iba1) secondary antibody. Nuclei were counterstained with DAPI. Labeled BrdU+ cells, NeuN+ neurons, BrdU+/ NeuN+ newborn neurons, BrdU+/GFAP+ newborn astrocytes, BdU+/Iba1+ microglial cells and CD31+ microvessels were counted in the same 9 ROI of the striatum as above (see section 2.10; each 62,500 μm^2) using the LSM 780 confocal microscope. Glial scar formation was analyzed by measuring the area covered by GFAP+ cells using Zen Blue software (Carl Zeiss) in each brain section. CD31+ micro-vessels were counted in ipsilesional striatum. For the analysis of capillary density, 100 μm-length grids were drawn in a 1 mm^2 area within the striatum and capillaries crossing each line were counted. The total number of capillaries was depicted as CD31+ cell profiles (Kilic et al., 2014).

2.12. Anterograde tract tracing analysis

Brain sections of animals that received BDA injections and that had transcardially been perfused with 4% PFA were blocked with 10% NGS for 1 h, washed and incubated in Alexa Fluor-555 conjugated streptavidin (S21381, Thermo Fisher Scientific) for 2 h. Pyramidal tract fibers were analyzed using the LSM780 confocal microscope at the level of the parvocellular red nucleus (bregma −3.0 to −3.5 mm) for corticorubral projections and at the level of the facial nucleus (bregma −5,8 to −6,3 mm) for corticobulbar projections. For analysis of midline-crossing fibers, a 1.000 μm long straight line was drawn along the midline (Caglayan et al., 2019). The numbers of axons crossing this line were counted for each animal at both levels.

2.13. Western blot analysis

Brain tissue samples were taken from the striatum. Samples of the same groups were pooled and homogenized with radioimmunoprecipitation assay (RIPA) lysis buffer (89,900; Thermo Fisher Scientific) containing protease and phosphatase inhibitor cocktail. Proteins were extracted after 15 min centrifugation at 14,000 rpm. Protein concentrations were measured using the BCA protein assay kit (23,227; Thermo Fisher Scientific). Equal amounts of protein (20 μg) was loaded into 4–20% bis-tris gels, run for 1 h at 150 V and transferred to polyvinylidene fluoride (PVDF) membranes (162–0174; Biorad Life Sciences Research, California, USA). Membranes were blocked with 5% non-fat milk powder dissolved in Tris-buffered saline containing Tween-20 (TBS-T), for 1 h. Membranes were washed with TBS-T and incubated overnight with primary antibodies against GDNF (sc-13,147; Santa Cruz), brevican (610,895; BD Biosciences, USA), versican (ab19345; Abcam), ephrin b1 (sc-515,264; Santa Cruz), ephrin b2 (sc-398,735; Santa Cruz), Slit1 (sc-376,756; Santa Cruz), Robo2 (sc-25,673; Santa Cruz), doublecortin (PA5–28536; Thermo Fisher Scientific) or nuclear factor erythroid 2-related factor-2 (Nrf2; 8882; Cell Signaling) antibody. The next day, membranes were washed with TBS-T and incubated with appropriate horseradish peroxidase (HRP) labeled secondary antibody for 1 h. For normalization of protein loading, membranes were stripped and reprobed with anti-β-actin (4970, Cell Signaling) antibody. Blots were developed via enhanced chemiluminescent (ECL) (K-12043-D10; WesternBright Sirius, USA) labeling that was visualized using a CCD camera (Fusion FX7; Vilber, Germany). Protein levels were densitometrically analyzed using Image J (NIH, USA) software and normalized with optical densities determined on β-actin blots. All proteins were studied in three independent blots.

2.14. Quantitative real-time PCR (qrtPCR)

Brain tissue samples were taken from the striatum. Total RNA was isolated from individual tissue samples using the high pure RNA isolation kit (11,828,665,001; Roche). 100 mg of tissue was homogenized with 500 μl TRIzol reagent (15,596,026; Thermo Fisher Scientific). Chloroform (200 μl) was added onto the homogenate and incubated at room temperature for 1 min. After centrifugation at 14,000 rpm for 15 min, the soluble phase of the supernatant was separated into a clean tube. After addition of 100% ethanol, the mixture was loaded onto DNA elimination colons and centrifuged at 14,000 rpm for 3 min. After washing, the RNA was eluted with RNase free water. After determining the RNA concentration, first strand cDNA was produced (First strand cDNA synthesis kit; 04896866001; Roche). Reactions were performed for 60 min at 50 °C followed by 5 min at 85 °C. Forward and reverse primers against human GDNF and control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were selected from Harvard primer bank data base. For PCR, which were performed using a CFX96 Touch Real-Time PCR system (Bio-Rad), the SensiFAST™ SYBR No-ROX kit (BIO-980005; Bioline, London, UK), which uses Sybr green as non-specific DNA interchelating agent, was used. Gene expression was analyzed by quantifying the threshold cycle (C_T). C_T values for Lv-GDNF-GFP animals were normalized using the endogenous reference GAPDH. Data were analyzed using logarithmic transformation of fold induction ratios according to relative quantification (R_F) formula ($2^{-\Delta\Delta Ct}$) (Beker et al., 2018a).

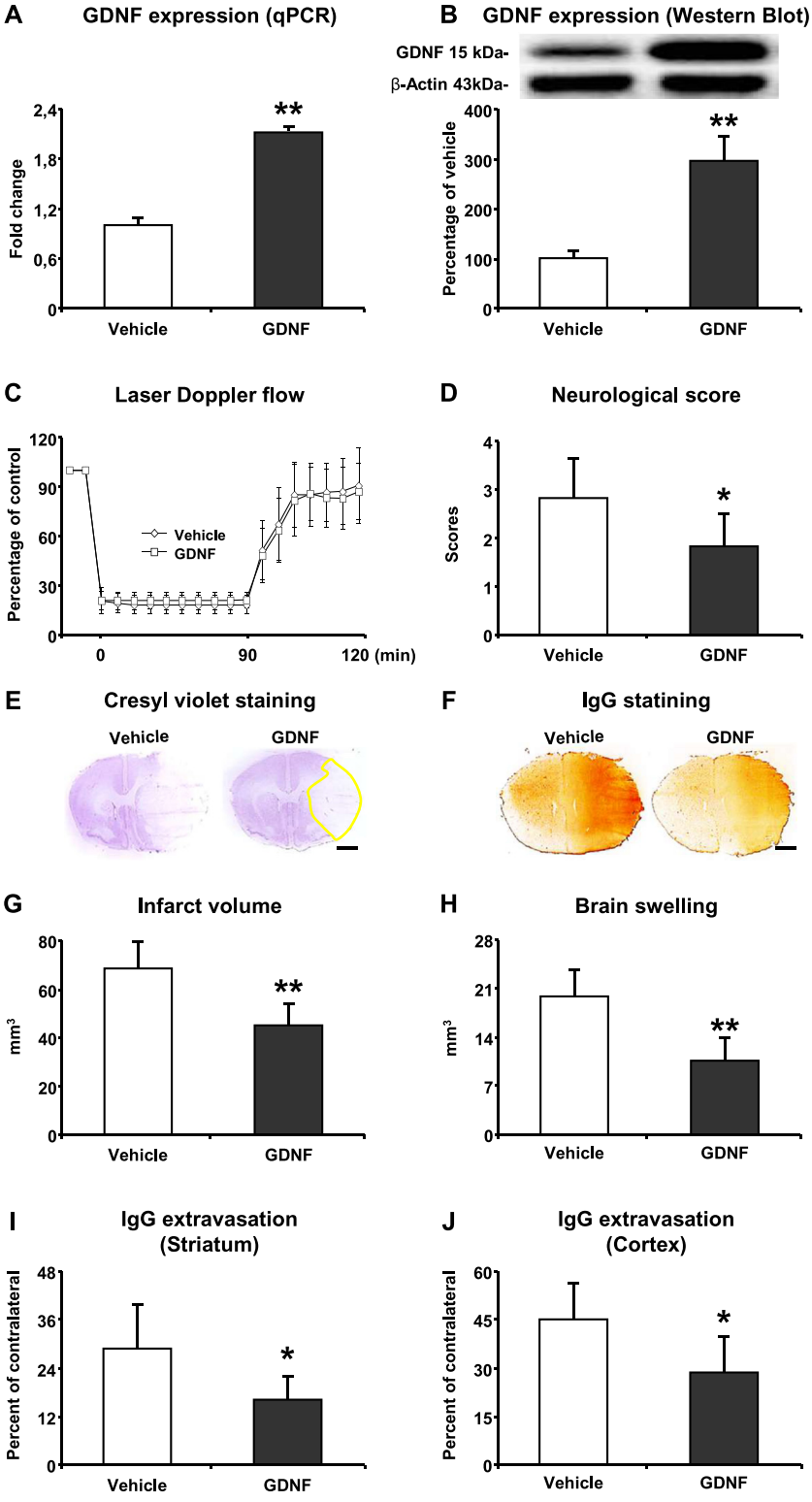

Fig. 2. Lentiviral GDNF reduces neurological deficits, infarct volume, brain edema and blood-brain barrier permeability in the acute stroke phase. GDNF expression (A) on the mRNA level determined by quantitative real-time polymerase chain reaction (qrtPCR) and (B) on the protein level evaluated by Western blot in the striatum of mice receving stereotactic injections of the Lv-GDNF-GFP or Lv-GFP vectors. (C) Cerebral blood flow assessed by laser Doppler flow (LDF) recordings, (D) neurological deficits examined by the Bederson score. (G) Infarct volume and (H) brain swelling measured on cresyl violet-stained brain sections and (I-J) IgG extravasation as marker of blood-brain barrier (BBB) permeability evaluated by immunohistochemistry in the striatum and cortex of mice exposed to 90 min MCAO followed by 24 h reperfusion, in which Lv-GFP (as vehicle) or Lv-GDNF-GFP vectors had intrastriatally been administered. Representative coronal sections of mouse brain stained with cresyl violet or IgG extravasation are shown in (E) and (F). Data are mean ± SD values (n = 7 mice/group). *$p \leq .05$, **$p \leq .01$ compared with vehicle. Scale bar, 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.15. Statistical analysis

Data analysis were done with a standard software package (SPSS for Windows; SPSS Inc., USA). Differences between groups were calculated by independent two-sided t-tests. All values were given as mean ± SD values. p values ≤.05 were considered significant.

3. Results

3.1. Delivery of Lv-GDNF-GFP vector induces GDNF expression on mRNA and protein level

By qrtPCR and Western blot we showed that Lv-GDNF-GFP delivery induced robust GDNF expression at the mRNA and protein level (Fig. 2A, B).

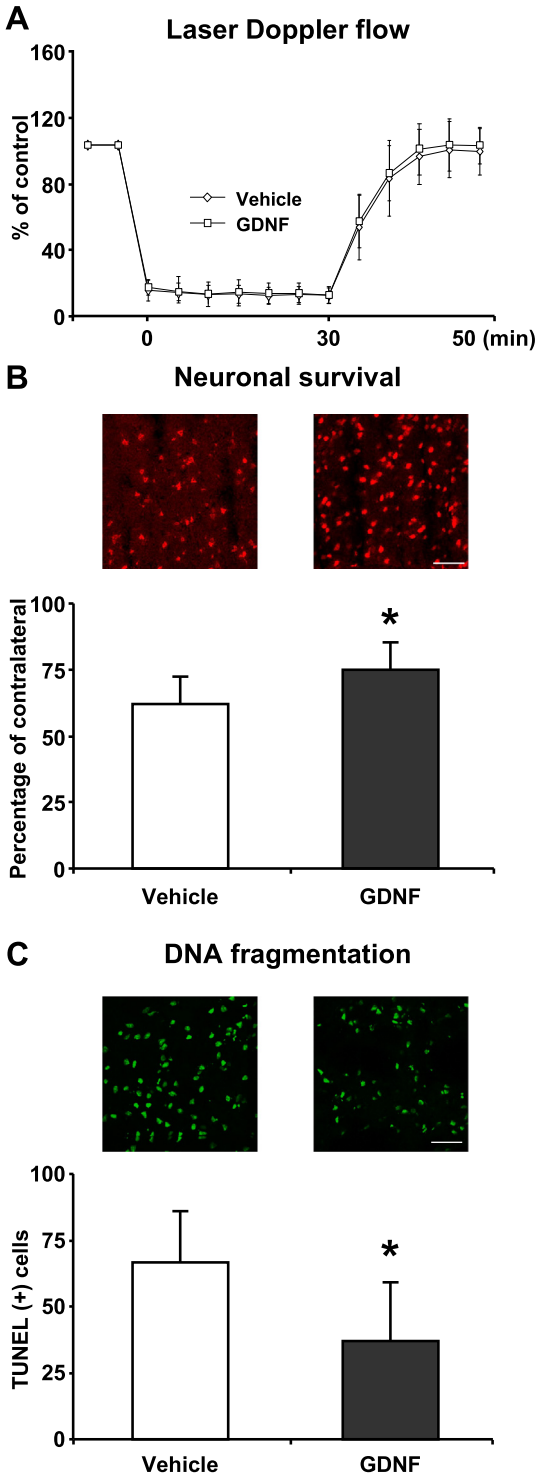

Fig. 3. GDNF reduces disseminate neuronal injury in the striatum in the acute stroke phase. (A) Cerebral blood flow assessed by laser Doppler flow (LDF) recordings, (B) percentage of surviving NeuN+ neurons and (C) density of DNA fragmented TUNEL+ cells in the ischemic striatum of mice exposed to 30 min MCAO followed by 72 h reperfusion, in which Lv-GFP (as vehicle) or Lv-GDNF-GFP vectors had intrastriatally been applied. Representative microphotographs are shown. Data are mean ± SD values (n = 7 mice/group). *p ≤ .05 compared with vehicle. Scale bar, 100 μm.

3.2. Intraluminal MCAO results in highly reproducible ischemia

LDF recordings above the core of the middle cerebral artery territory revealed that LDF levels decreased to ~20% of preischemic control values in animals exposed to 90 min (Fig. 2C) or 30 min (Fig. 3A) MCAO. After reperfusion, LDF rapidly returned to baseline values. Lentiviral GDNF delivery did not influence LDF values neither during nor after MCAO.

3.3. Lentiviral GDNF reduces neurological deficits and ischemic injury in acute stroke phase

In mice submitted to 90 min of MCAO, robust neurological deficits were detected using Bederson's neurological score (Beker et al., 2018b; Bieber et al., 2019) in the acute stroke phase, that is after 24 h (Fig. 2D) which were associated with reproducible infarcts covering the cortex and striatum and ipsilesional brain swelling. The effects of lentiviral delivery of GDNF on infarct volume and brain swelling were measured using cresyl violet staining (Fig. 2E) and effect of GDNF on IgG extravasation were analyzed using IgG staining (Fig. 2F). GDNF delivery reduced the neurological deficits (p = .045; Fig. 2D), decreased infarct volume (p = .003; Fig. 2G) and brain swelling (p = .001; Fig. 2H). IgG extravasation, which was studied as marker of BBB permeability, was reduced by GDNF in the ischemic striatum (Fig. 2I; p = .045) and cortex (Fig. 2J; p = .037).

Mice exposed to 30 min MCAO exhibited disseminate neuronal injury in the ischemic striatum indicated by the decrease in the percentage of surviving NeuN+ neurons (to ~60% of contralesional control values; Fig. 3B) and the appearance of TUNEL+, that is, DNA fragmented cells (Fig. 3C). GDNF increased the percentage of surviving NeuN+ neurons in the ischemic striatum (Fig. 3B; p = .044) and reduced the density of TUNEL+ cells (Fig. 3C, p = .034). Neurological deficits were more subtle in these mice. Behavioral test batteries were therefore used for evaluating these deficits.

3.4. Lentiviral GDNF delivery improves neurological recovery in the post-acute stroke phase

Grip strength and RotaRod tests revealed reproducible motor and coordination deficits in mice exposed to 30 min MCAO, which partly recovered over 42 days (Fig. 4A, B). These motor and coordination deficits went along with reduced spontaneous motor activity in open field tests (Fig. 4C, D) and a preference for the dark zones in light/dark transition tests indicative of anxiety (Fig. 4E, F), which persisted over 42 days. GDNF delivery improved motor and coordination recovery (Fig. 4A, B; p = .026 and p = .045, respectively), attenuated the reduced spontaneous motor activity (Fig. 4C, D; p = .047 each) and reduced anxiety (Fig. 4E, F; p = .045 each).

3.5. Lentiviral GDNF increases brain capillary density, induces neurogenesis, reduces glial scar formation and reduces brian atrophy

In animals exposed to 30 min MCAO, immunohistochemical studies showed that GDNF delivery increased the density of brain capillaries in the ischemic striatum in the post-acute stroke phase, that is, after 52 days (Fig. 5A; p = .010). GFAP immunohistochemistry showed glial scar formation in the dorsolateral ischemic striatum, that was reduced by GDNF delivery (Fig. 5B; p = .010). Whole brain volume at 52 days post-MCAO was significantly increased by GDNF (Fig. 5C; p = .007), indicating that GDNF delivery successfully prevented brain atrophy.

Considering that post-ischemic angiogenesis is closely associated with neurogenesis (Hermann and Zechariah, 2009), we examined the de novo formation of neurons in the ischemic brain tissue by BrdU incorporation analysis. BrdU+ proliferating cells were noted in proximity of the lesion-sided subventricular zone and the adjacent ischemic striatum (Fig. 6A). Double stainings revealed that some of the BrdU+ proliferating cells exhibited evidence of neuronal differentiation indicated by the coexpression of NeuN (Fig. 6B), whereas others exhibited coexpression of the astrocytic marker GFAP (Fig. 6C) or the microglial marker Iba1 (Fig. 6D). GDNF increased the number of BrdU+

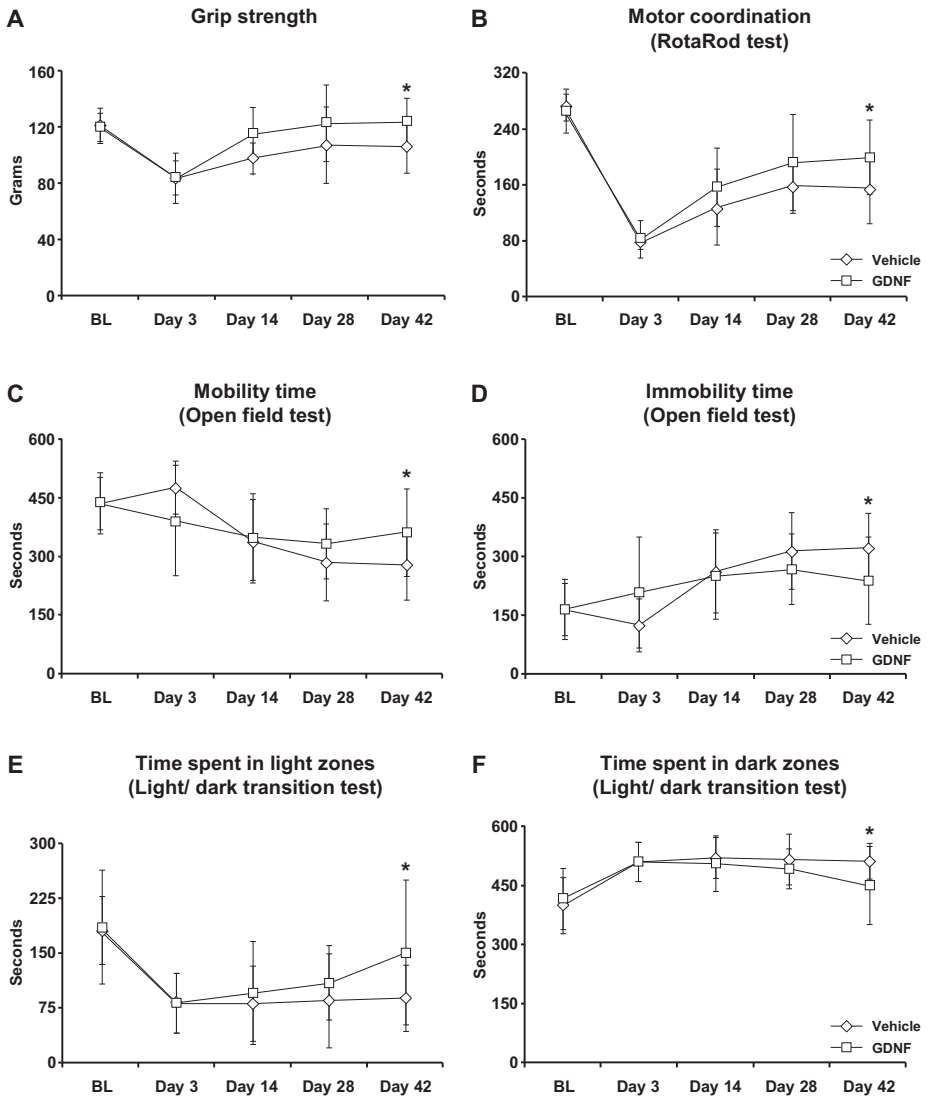

Fig. 4. GDNF promotes neurological recovery in the post-acute stroke phase. (A) Grip strength of the paretic right forelimb, (B) motor coordination performance in the RotaRod test, (C) time of mobility and (D) time of immobility in the open field test, as well as (E) time spent in the light zones and (F) time spent in the dark zones in the light/ dark transition test of mice exposed to 30 min MCAO followed by up to 42 days reperfusion, in which Lv-GFP (as vehicle) or Lv-GDNF-GFP vectors had intrastriatally been delivered. BL, preischemic baseline. Data are mean ± SD values (n = 12 mice/group). *p ≤ .05 compared with vehicle.

proliferating cells (Fig. 6A; $p \leq .001$), increased the number of BrdU+/ NeuN+ new-born neurons (Fig. 6B; $p = .025$) and decreased the number of BrdU+/ GFAP+ cells (Fig. 6C; $p = .012$). However, GDNF treatment did not influence the number of BrdU+/Iba1+ cells when compared with vehicle treated animals (Fig. 6D).

3.6. Lentiviral GDNF induces contralesional pyramidal tract plasticity across the midline

By means of anterograde tract tracing using BDA, we traced terminal axons branching off the pyramidal tract at the level of the parvocellular red nucleus and facial nucleus migrating across the midline in direction to the ipsilesional parvocellular red nucleus and facial nucleus and counted the number of midline-crossing fibers at both rostrocaudal levels. A moderate number of labeled axons was noted in ischemic control mice at the level of the red nucleus (Fig. 7A) and facial nucleus (Fig. 7B). GDNF delivery significantly increased the number of midline-crossing corticorubral and corticobulbar axons at both levels (Fig. 7A, B; $p = .023$ and $p = .045$, respectively).

3.7. Lentiviral GDNF induces finely tuned response of axonal growth inhibitors, guidance molecules and midline growth repulsive proteins

Based on the finding of midline-crossing axonal plasticity that was increased by GDNF delivery, we finally studied the expression of the axonal growth inhibitors brevican and versican (Rhodes and Fawcett, 2004), the guidance molecules ephrin b1 and ephrin b2 (Batista et al., 2014; Giger et al., 2010), the midline growth repulsive extracellular matrix protein Slit1 and its receptor Robo2 (Brose et al., 1999; Erskine et al., 2000), the microtubule stabilizing protein doublecortin (Jean et al., 2012) and the transcription factor Nrf2, which controls the expression of antioxidative and angiogenic genes (Florczyk et al., 2014), in the ipsilesional and contralesional striatum, that is, in vicinity of the pyramidal tracts by Western blots. These blots revealed a decreased abundance of the axonal growth inhibitors brevican and versican in the contralesional and ipsilesional brain tissue, respectively (Fig. 8A, B), reduced abundance of the growth repulsive guidance molecule ephrin b1 in the contralesional brain tissue (Fig. 8C), increased abundance of the guidance molecule ephrin b2 (Fig. 8D), increased abundance of the midline growth repulsive extracellular matrix protein Slit1 in the contralesional brian tissue (Fig. 8E) and increased abundance of Slit1's

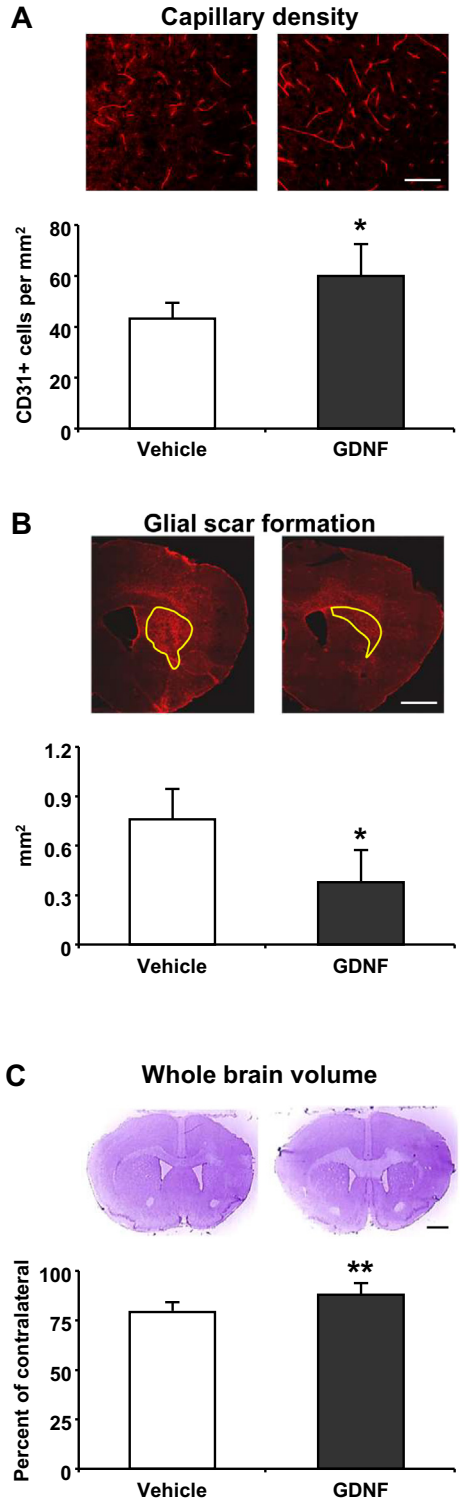

Fig. 5. GDNF increases brain capillary density, reduces glial scar formation and reduces brain atrophy in the post-acute stroke phase. (A) Density of CD31 + brain capillaries in the ischemic striatum, (B) area of the GFAP + astroglial scar in the ischemic striatum and (c) whole brain volume of mice exposed to 30 min MCAO followed by 52 days reperfusion, in which Lv-GFP (as vehicle) or Lv-GDNF-GFP vectors had intrastriatally been administered. Representative microphotographs are presented. Data are mean ± SD values (n = 7 mice/group). *p ≤ .05, **p ≤ .01 compared with vehicle. Scale bar, 50 μm (in (A)) and 1 mm (in (B–C)).

receptor Robo2 in the ipsilesional brian tissue (Fig. 8F) of animals exposed to GDNF delivery. GDNF delivery increased the abundance of the microtubule-stabilizing protein doublecortin and the transcription factor Nrf2 in the ipsilesional brain tissue and decreased Nrf2 abundance in the contralesional brain tissue (Fig. 8G, H). Our study revealed a finely tuned regulation of plasticity-associated proteins which facilitated the growth of contralesional pyramidal tract axons across the midline into the ipsilesional brain hemisphere.

4. Discussion

We herein show that lentivirally administered GDNF induces robust long-term neurological recovery in the postacute stroke phase that is associated with peri-infarct brain remodeling and contralesional neuronal plasticity. Increased angiogenesis, increased neurogenesis and reduced astroglial scar formation were noted in the peri-infarct brain tissue of GDNF treated mice exposed to intraluminal MCAO that were associated with contralesional sprouting of motor cortical axons branching off the pyramidal tract at the levels of the red and facial nucleus, which crossed the midline to innervate the ipsilesional red and facial nuclei. Contralesional corticorubral and corticobulbar plasticity was associated with reduced abundance of the axonal growth inhibitors brevican and versican in the contralesional and ipsilesional brain tissue, respectively, reduced abundance of the growth repulsive guidance molecule ephrin b1 in the contralesional brain tissue, increased abundance of the midline growth repulsive extracellular matrix protein Slit1 in the contralesional brain tissue and increased abundance of Slit1's receptor Robo2 in the ipsilesional brain tissue, which facilitated the growth of contralesional pyramidal tract fibers in direction to the ipsilesional hemisphere, but not of ipsilesional pyramidal tract fibers in direction to the contralesional hemisphere. Our study reveals the finely tuned regulation of neuronal plasticity associated proteins and proteoglycans, which set the stage for neurological recovery.

Previous studies using strategies of topical GDNF delivery (Abe et al., 1997; Kitagawa et al., 1998; Wang et al., 1997), adenovirus or sendai virus mediated GDNF delivery (Abe et al., 1997; Hermann et al., 2001a; Kilic et al., 2003; Kitagawa et al., 1998; Sumbria et al., 2013; Wang et al., 1997; Zhang et al., 2002) or TAT or IgG fusion protein mediated GDNF delivery (Kilic et al., 2005; Kilic et al., 2003; Sumbria et al., 2013) have already shown that GDNF reduces infarct volume, promotes neuronal survival and reduces brain edema in the acute stroke phase after permanent or transient intraluminal MCAO in rats and mice. In these studies, animals were sacrified at 24 to 72 h post-MCAO. GDNF was administered up to 6 h post-MCAO. At the molecular level, reduced NO formation, increased expression of the anti-apoptotic protein Bcl-X_L and reduced activation of caspases-1 and -3 and were found to contribute to GDNF-mediated neuroprotection after MCAO (Hermann et al., 2001a; Kilic et al., 2003; Kitagawa et al., 1998; Shirakura et al., 2004; Wang et al., 1997; Zhang et al., 2002). Using two models of transient focal cerebral ischemia, that is, 90 min MCAO, which is associated with reproducible combined corticostriatal brain infarcts, and 30 MCAO, which associated with disseminate neuronal injury in the striatum, we now confirmed this acute neuroprotective effect for lentiviral GDNF delivery. Besides reduced infarct volume, neuronal injury and brain edema, an attenuation of serum IgG extravasation, indicative of preserved BBB integrity, was noted in the brains of lentiviral GDNF treated mice. These results show that GDNF protects the neurovascular unit from damage.

By means a a comprehensive battery of motor coordination tests, which included the Bederson score, grip strength, RotaRod, open field and light/ dark transition tests, we showed that lentiviral GDNF delivery reduced neurological deficits in the acute stroke phase and increased motor-coordination recovery, spontaneous locomotor activity and exploration behavior in the post-acute stroke phase, that is, up to 42 days post-MCAO. Only few studies so far examined functional neurological recovery in response to GDNF delivery. These studies mostly

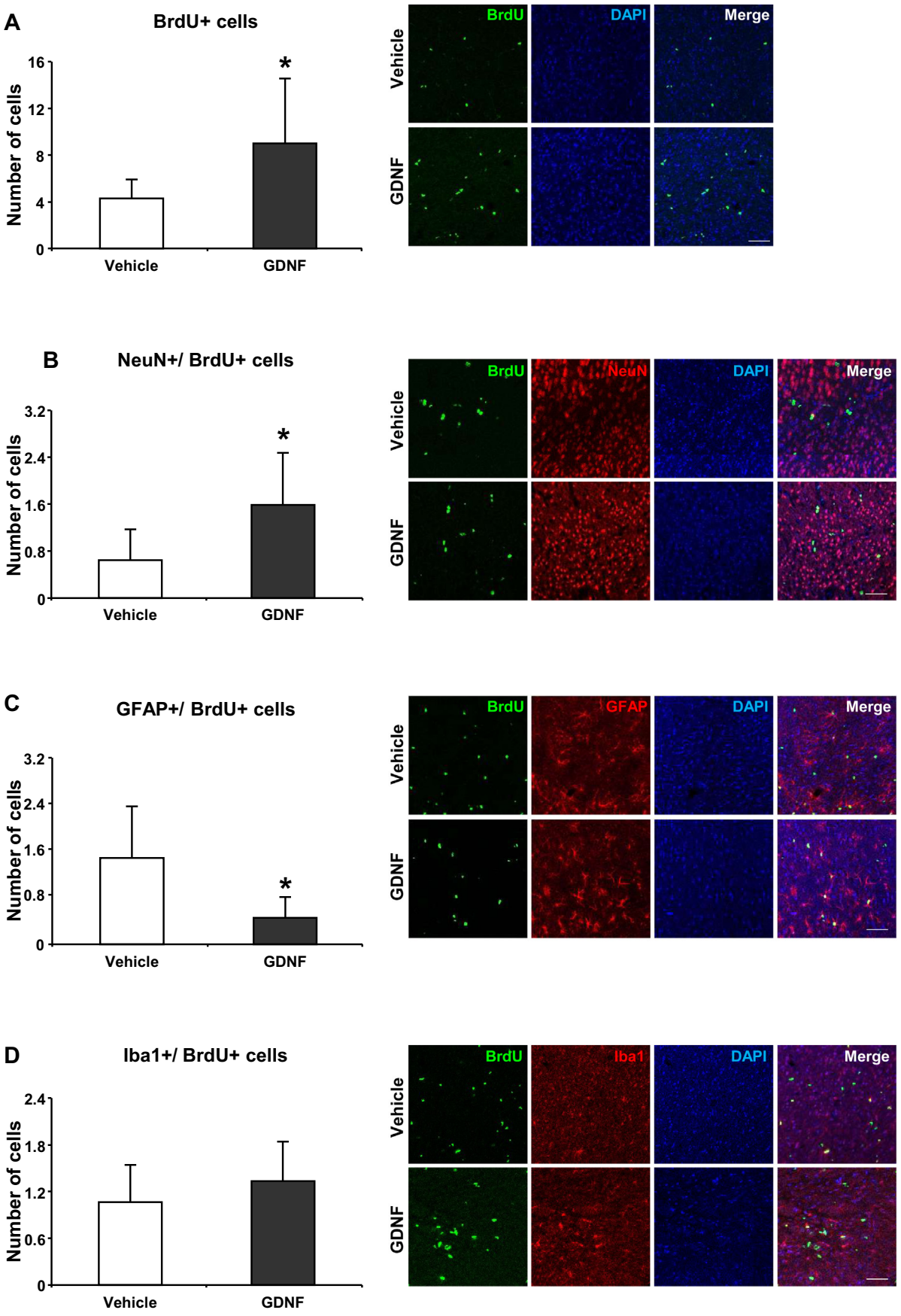

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Fig. 6. GDNF increases post-ischemic cell proliferation and neurogenesis. (A) Cell proliferation, evaluated by BrdU incorporation, (B) newly formed neurons, assessed by the co-expression of NeuN on BrdU incorporating cells, (C) proliferating neural precursor cells (NPCs) co-expressing the astroglial marker glial fibrillary astrocyte protein (GFAP) and (D) proliferating cells co-expressing the microglial marker ionized calcium binding adaptor protein (Iba)-1 in the ischemic striatum of mice exposed to 30 min MCAO followed by 52 days reperfusion, in which Lv-GFP (as vehicle) or Lv-GDNF-GFP vectors had intrastriatally been applied. Representative microphotographs are depicted. Data are mean ± SD values (n = 7 mice/group). *p ≤ .05 compared with vehicle. Scale bar, 50 μm.

used single tests or led to ambiguous conclusions. Using body swing or cylinder tests, a reduction of motor assymmetry was found over up to 28 days in rats exposed to transient peripheral MCAO or transient intraluminal MCAO receiving herpes simplex virus (HSV) and PEP-1 fusion protein mediated GDNF delivery, respectively (Harvey et al., 2003; Liu et al., 2016). In another study, topical cortical application of

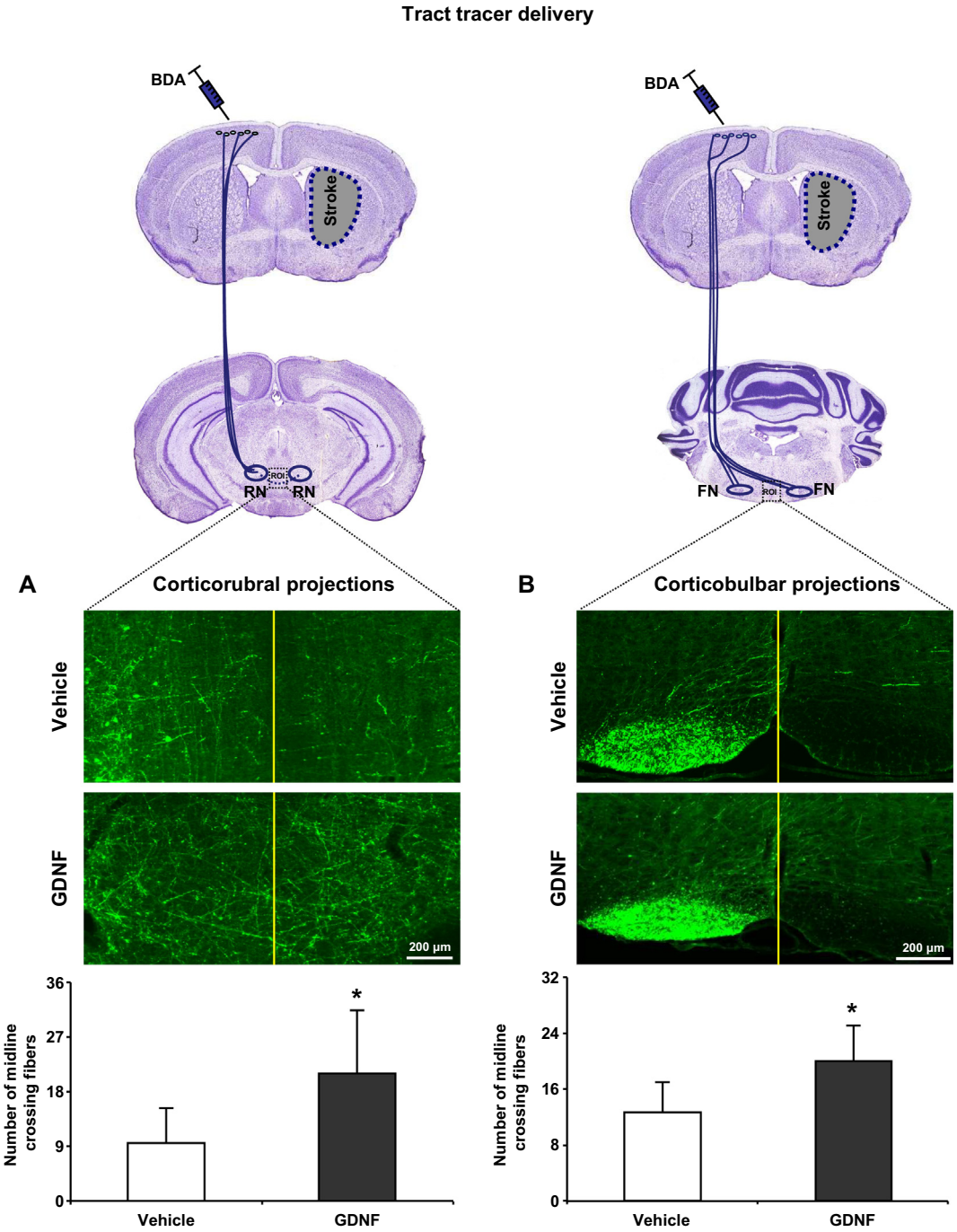

Fig. 7. GDNF induces contralesional corticorubral and corticobulbar sprouting across the midline in direction to the ipsilesional parvocellular red nucleus and facial nucleus. Anterograde tract tracing using biotinylated dextrane amine (BDA) that was injected into the contralesional motor cortex of mice exposed to 30 min MCAO in which Lv-GFP (as vehicle) or Lv-GDNF-GFP vectors had intrastriatally been administered. At the level of (A) the red nucleus (RN) and (B) facial nucleus (FN), midline-crossing fibers branching off the contralesional pyramidal tract in direction to the ipsilesional parvocellular red nucleus and facial nucleus were determined at 52 days post-MCAO. Representative microphotographs are shown. Data are mean ± SD values (n = 12 mice/group). *p ≤ .05 compared with vehicle. Scale bar, 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

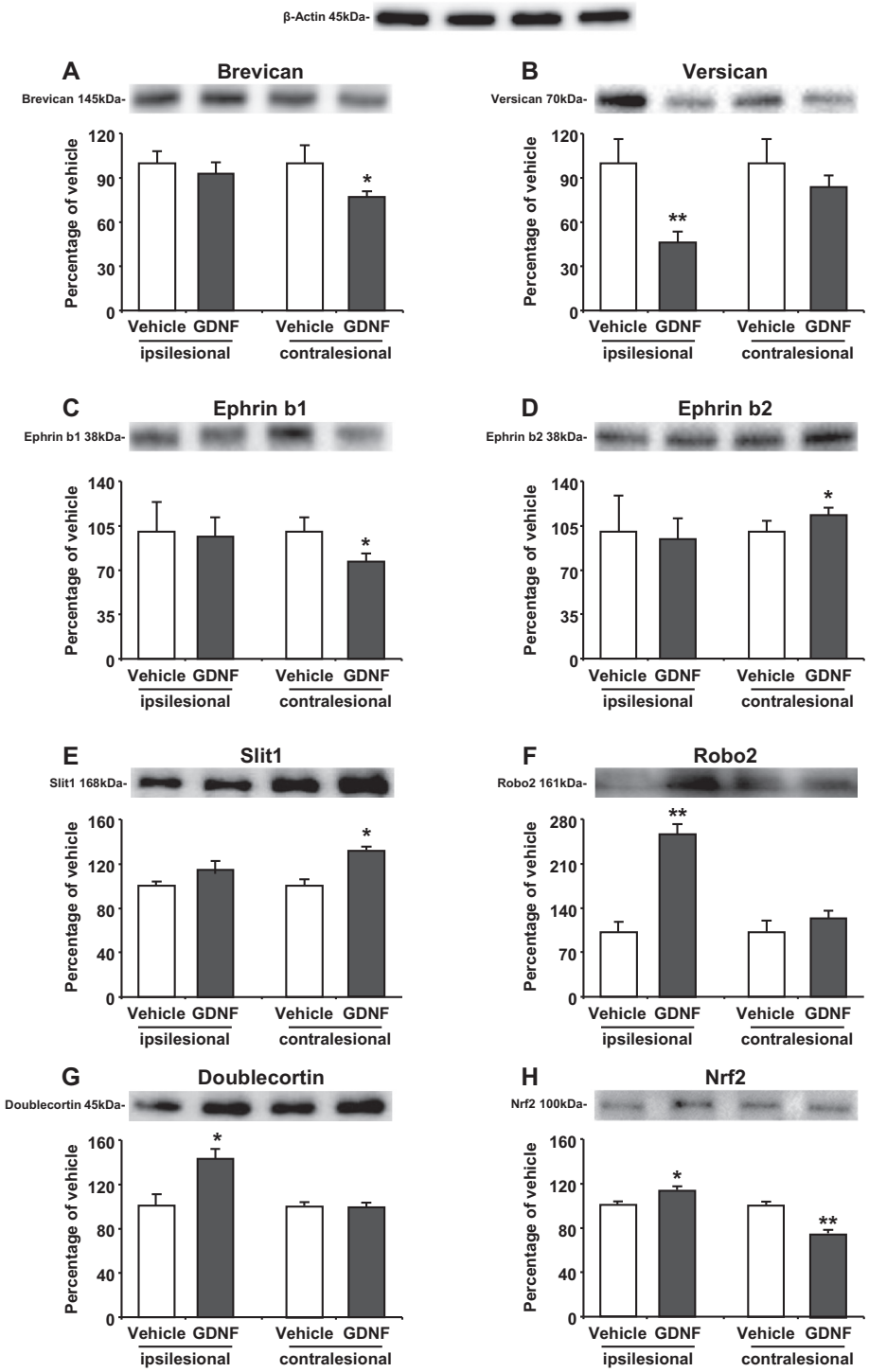

Fig. 8. GDNF induces finely tuned response of axonal growth inhibitors, guidance molecules and midline growth repulsive proteins. Abundance of the axonal growth inhibitors (A) brevican and (B) versican, the guidance molecules (C) ephrin b1 and (D) ephrin b2, (E) the midline growth repulsive protein ligand Slit1, (F) Slit1's receptor Robo2, (G) the microtubule stabilizing protein doublecortin and (H) the transcription factor Nrf2 determined by Western blots in the ipsilesional and contralesional striatum of mice exposed to 30 min MCAO followed by 52 days reperfusion, in which Lv-GFP (as vehicle) or Lv-GDNF-GFP vectors had intrastriatally been applied. Representative microphotographs are presented. Data are mean ± SD values (n = 3 independently processed blots/group). *p ≤ .05, **p ≤ .01 compared with vehicle.

GDNF-fibrin glue, but not of GDNF alone reduced motor-coordination deficits over up to 28 days evaluated by RotaRod and grip strength tests (Cheng et al., 2005). Importantly, the latter study lacked control groups receiving fibrin glue only. For this reason, possible effects of GDNF could not be discriminated from effects of fibrin glue.

Using histochemical studies we found that lentivirally administered GDNF increased angiogenesis as revealed by brain capillary density analysis, increased neurogenesis and reduced gliogenesis without affecting microglial activation as revealed by BrdU incorporation analysis, reduced astroglial scar formation in the peri-infarct brain tissue and increased brain volume. The promotion of post-ischemic angiogenesis by GDNF has to the best of our knowledge not been shown. In mice, successful peri-infarct brain remodeling has been shown to be associated with increased angiogenesis after intraluminal MCAO in response to other growth factors, namely VEGF and erythropoietin (Reitmeir et al., 2011; Reitmeir et al., 2012). That GDNF delivery promotes neurogenesis in the ischemic striatum has already been demonstrated in rats exposed to intraluminal MCAO, in which BrdU was administered 7 days after MCAO followed by animal sacrifice 8 h later (Kobayashi et al., 2006). In this study, survival of new-born neurons which had been labeled with BrdU from day 6 to 13 post-MCAO was furthermore demonstrated over up to 34 days post-MCAO. Unfortunately the latter study did not perform behavioral studies. Thus, the functional consequences of the increased neurogenesis remained

unknown. Reduction of GFAP immunoreactivity by HSV mediated GDNF delivery has previously been reported in the ischemic cortex of rats 28 days after transient peripheral MCAO (Harvey et al., 2003). Reactive astrocytes are rich sources of axonal growth inhibitory proteins and proteoglycans, such as brevican and versican, which prevent post-ischemic neuroplasticity (Jones et al., 2003; Leonardo et al., 2008). The observation of reduced astroglial scar formation in animals receiving lentiviral GDNF delivery prompted us to perform an in depth analysis of lesion-remote axonal plasticity.

By means of the anterograde tract tracer BDA, which we injected into the contralesional motor cortex, we demonstrated that lentivirally administered GDNF promoted the outgrowth of contralesional corticorubral and corticobulbar fibers that branched off the pyramidal tract and crossed the midline in order to innervate denervated neurons in the ipsilesional parvocellular red nucleus and facial nucleus. Promotion of contralesional pyramidal tract plasticity has previously been shown in rat and mouse models of ischemic stroke following delivery of other growth factors (i.e., VEGF and erythropoietin) (Reitmeir et al., 2011; Reitmeir et al., 2012), neural precursor cells (Bacigaluppi et al., 2016), neutralizing antibodies directed against the axonal growth inhibitor NogoA (Lindau et al., 2014; Seymour et al., 2005) or the glutamate inhibitor memantine (Wang et al., 2017). That GDNF promotes contralesional pyramidal tract plasticity is new. At the molecular level, contralesional corticorubral and corticobulbar plasticity was associated with reduced abundance of the axonal growth inhibitors brevican and versican in the contralesional and ipsilesional hemisphere, respectively, reduced abundance of the growth repulsive guidance molecule ephrin b1 in the contralesional hemisphere, increased abundance of the midline growth repulsive protein ligand Slit1 in the contralesional hemisphere, increased abundance of Slit1's receptor Robo2 in the ipsilesional hemisphere, increased abundance of the microtubule-stabilizing protein doublecortin and the anti-oxidative transcription factor Nrf2 in the ipsilesional hemisphere and decreased Nrf2 abundance in the contralesional hemisphere. The growth inhibitory proteins brevican and ephrin b1 have previously been shown to be reduced in the contralesional hemisphere of mice exposed to intraluminal MCAO which received intracerebroventricular VEGF treatment (Reitmeir et al., 2012). Our observation that a growth factor controls the abundance of the midline growth repulsive extracellular matrix protein Slit1, its receptor Robo2, the microtubule-stabilizing protein doublecortin and the anti-oxidative transcription factor Nrf2 is new. The downregulation of axonal growth inhibitors and growth repulsive guidance proteins facilitated the sprouting of contralesional pyramidal tract axons to the ipsilesional hemisphere, whereas the upregulation of the midline growth repulsive extracellular matrix protein Slit1 in the contralesional brain tissue and upregulation of Slit1's receptor Robo2 in the ipsilesional brain tissue specifically may prevent the sprouting of ipsilesional pyramidal tract axons to the contralesional hemisphere. This molecular signature may have provided the mechanistic basis for the directed growth of contralesional pyramidal tract fibers across the midline after stroke.

Author contributions

This work was carried out in collaboration between all authors. MB, ABC and MCB carried out experimental work, analyzed data and helped to write the manuscript. SA and MOA carried out the behavioral tests. RK and AD carried out Western blot and immunofluorescence studies. GTK DMH and EK defined the research theme and revised the manuscript critically.

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Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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