Postacute Delivery of GABA$_A$ α5 Antagonist Promotes Postischemic Neurological Recovery and Peri-infarct Brain Remodeling

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Background and Purpose—Poststroke, neuronal excitability is tonically reduced in peri-infarct tissue via inhibitory influences of extrasynaptic GABA$_A$ receptors. We hypothesized that GABA$_A$ α5 blockade by the competitive antagonist S44819 enhances postischemic neurological recovery, brain remodeling, and neuroplasticity.

Methods—In an explorative study followed by a confirmation study, male C57Bl6/j mice were exposed to transient intraluminal middle cerebral artery occlusion. Starting 72 hours poststroke, vehicle or S44819 (3 or 10 mg/kg, BID) was delivered orally for 28 days. Neurological recovery, perilesional tissue remodeling, and contralesional pyramidal tract plasticity were evaluated for 42 days, that is, 14 days after completion of S44819 delivery.

Results—S44819, delivered at 10 but not 3 mg/kg, persistently improved motor coordination and spatial memory in both studies. Striatal atrophy was reduced by 10 mg/kg S44819 at 42 days post-treatment onset, and neuronal long-term survival in the peri-infarct striatum was increased. Delayed neuroprotection was associated with reduced peri-infarct astrogliosis, increased peri-infarct brain capillary density, and increased neural precursor cell proliferation and differentiation in proximity to the ipsilesional subventricular zone. Contralesional pyramidal tract plasticity, evaluated by anterograde tract tracing at the level of the red nucleus, was not influenced by S44819. Concentrations of neurotrophic (brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor) and angiogenic (vascular endothelial growth factor and basic fibroblast growth factor) growth factors were elevated by 10 mg/kg S44819 in peri-infarct but not contralesional brain tissue.

Conclusions—Our data demonstrate that S44819 enhances neurological recovery and peri-infarct brain remodeling in the postacute stroke phase.

Visual Overview—An online visual overview is available for this article. (***Stroke***. 2018;49:2495-2503. DOI: 10.1161/STROKEAHA.118.021378.)

Key Words: animals ◼ cell differentiation ◼ mice ◼ neuronal plasticity ◼ neuroprotection ◼ stroke

The peri-infarct cerebral cortex exhibits sustained hypo-excitability poststroke that results from tonic inhibition via extrasynaptic γ-aminobutyric acid (GABA)$_A$ receptors.[1,2] Whereas tonic inhibition may protect ischemic tissue from excitotoxic acute injury, it impairs neuronal remodeling and plasticity during subsequent stroke recovery. In mice exposed to photothrombotic stroke and rats exposed to endothelin microinjection, the deactivation of GABA$_A$ receptors by the inverse GABA$_A$ agonist L655708 improved neurological recovery.[3,4] This effect depended on the presence of the GABA$_A$ α5 subunit in GABA$_A$ receptors. In GABA$_A$ α5$^{-/-}$ mice, L655708 did not restore neurological deficits.[3]

S44819 is a potent and competitive GABA$_A$ receptor antagonist that preferentially binds to α5 subunit-containing GABA$_A$ receptors at their GABA-binding site and which has no affinity to the benzodiazepine site.[5] This GABA$_A$ α5 selectivity is governed by amino acid residues within the α5 subunit F loop—a region associated with GABA binding.[5] In mouse hippocampal CA1 neurons, S44819 enhanced hippocampal long-term potentiation and blocked tonic currents mediated by extrasynaptic α5 subunit-containing GABA$_A$ receptors but had no effect on synaptic GABA$_A$ receptor activity.[5] In rats and mice, S44819 improved memory and reduced anxiety in a variety of cognitive tests, when

administered orally at doses between 0.3 and 10 mg/kg body weight.[6]

Considering its pharmacological profile, S44819 was evaluated in a randomized double-blinded placebo-controlled phase I crossover TMS study (Transcranial Magnetic Stimulation) in healthy young humans, in which single 100-mg doses of S44819 reduced active motor threshold, that is, the intensity needed to produce a motor-evoked potential of 0.5 mV, and the amplitude of the N45 potential, that is, a $GABA_A$-ergic component of the TMS-evoked electroencephalography response.[7] These observations demonstrated that S44819 reaches human cortex to impose an increase in cortical excitability.

Because peri-infarct hypoexcitability impedes stroke recovery,[3,4] we asked whether the pharmacological inhibition of extrasynaptic $GABA_A$ receptors by S44819 might promote postischemic perilesional brain remodeling and contralesional long-distance axonal plasticity, thus enhancing motor coordination recovery poststroke. Thus, we exposed mice to transient intraluminal middle cerebral artery occlusion (MCAO), administering S44819 orally at 2 doses (3 or 10 mg/kg BID, for 28 days) starting at 72 hours poststroke. We evaluated the effects of S44819 on neurological recovery for 42 days posttreatment onset (dpt) using a battery of motor coordination and cognitive tests and studied perilesional brain remodeling at 14, 28, and 42 dpt. Following this exploratory study, we performed a confirmation study, in which mice exposed to transient intraluminal MCAO were identically treated with S44819 (3 or 10 mg/kg BID). In these mice, we again examined S44819 effects on motor coordination and cognitive deficits for 42 days, as well as on poststroke neurogenesis and contralesional pyramidal tract plasticity.

Materials and Methods

Legal Issues, Randomization, and Statistical Planning

Experiments were performed with local government approval (Bezirksregierung Düsseldorf) in accordance to European Union (Directive 2010/63/EU) and ARRIVE guidelines (Animal Research Reporting of In Vivo Experiments). Experiments were strictly randomized and blinded. Statistical planning of each of the 4 sets of studies within the exploratory and confirmatory study assumed an α-error of 5% and a β-error (1 - statistical power) of 20%. Details are presented in Materials and Methods in the online-only Data Supplement. The data that support the findings of this study are available from the corresponding author on reasonable request.

Stroke Model, Drug Dosing, and Animal Groups

Focal cerebral ischemia was induced in male C57BL6/j mice (11–15 weeks, 22–25 g; Charles River Laboratories, Cologne, Germany) anesthetized with 1.0% to 1.5% isoflurane (30% O_2, remainder N_2O) by 40-minute left-sided intraluminal MCAO, as described before.[8,9] Rectal temperature was maintained between 36.5 and 37.0°C. Cerebral blood flow was recorded by measuring laser Doppler flow above the middle cerebral artery territory core. From 72 hours until 31 days poststroke (ie, from 0 until 28 dpt), mice received formulations of vehicle (99.5% aqoat, 0.5% magnesium, suspended in 2% hydroxyethyl cellulose) or S44819 (3 or 10 mg/kg; in 30% aqoat milled extrudate, 69.5% aqoat, 0.5% magnesium, suspended in 2% hydroxyethyl cellulose) that were administered twice daily by oral gavage.

For dose selection, pre-experiments were performed in C57BL6/j mice, in which 3 mg/kg S44819 administered by oral gavage yielded peak plasma, brain, and cerebrospinal fluid concentrations of 105.89±27.26 ng/ml, 41.66±12.60 ng/g, and 39.13±2.57 mg/ml, respectively, after 2 hours.[6] Based on cerebrospinal fluid measurements from phase I studies, this dose was considered equivalent to 100 mg doses orally in human subjects that have previously been reported to increase cortical excitability in the TMS paradigm.[7] To allow for dose-response assessments, an additional dose of 10 mg/kg S44819 was chosen.

In the first study that was scheduled as an exploratory study, 1 set of mice (animal set 1) received detailed assessments of motor coordination and cognitive deficits by rotarod, tightrope, open-field, and Barnes maze tests (Figure IA in the online-only Data Supplement).[8–10] These animals were sacrificed at 42 dpt by transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; n=18 animals per group).

Another set of mice within the exploratory study (animal set 2) was sacrificed at 14, 28, or 42 dpt (each n=6 per group) by transcardiac perfusion with 4% paraformaldehyde in 0.1 M PBS (Figure IB in the online-only Data Supplement). These animals were used for histochemistry.

A third set of mice within the exploratory study (animal set 3) was sacrificed at 14, 28, or 42 dpt (each n=6 per group) by transcardiac perfusion with normal saline (Figure IC in the online-only Data Supplement). Additional animals subjected to 40-minute left-sided MCAO or sham surgery (n=6 per group) neither received vehicle nor S44819. These animals were transcardially perfused with normal saline at 72 hours poststroke or post-sham surgery and used for ELISA.

Following the exploratory study, a confirmatory study (animal set 4) was conducted in mice exposed to 40-minute left-sided MCAO, which were identically treated with vehicle or S44819 (3 or 10 mg/kg) as before (n=18 animals per group; Figure ID in the online-only Data Supplement) and in which rotarod, tightrope, open-field, and Barnes maze tests were again performed. These animals received daily intraperitoneal injections of bromodeoxyuridine (BrdU; 50 mg/kg) on 3 to 8, 8 to 13, or 13 to 18 dpt (6 animals per group each). These animals obtained injections of the anterograde tract-tracer biotinylated dextran amine into the contralesional motor cortex at 42 dpt[8,9] and were sacrificed by transcardiac perfusion with 4% paraformaldehyde in 0.1 M PBS at 49 dpt. These animals were used for studying endogenous neurogenesis and contralesional pyramidal tract plasticity.

Details are presented in Materials and Methods in the online-only Data Supplement. The animal flow is summarized in Table I in the online-only Data Supplement.

Behavioral Tests

Motor coordination deficits was analyzed using the Rotarod and tightrope tests at baseline (ie, before MCAO), before treatment onset (ie, at 3 days after MCAO), and at weekly intervals after treatment onset. Spontaneous motor activity and anxiety were examined by open-field tests at 7 and 28 dpt and spatial memory assessed by Barnes maze tests at 8 to 19 dpt (for details, see Materials and Methods in the online-only Data Supplement).

Analysis of Brain Atrophy

Whole brain, striatal, and hippocampal atrophy were volumetrically evaluated using cresyl violet-stained 20-µm-thick coronal brain sections that had been collected at millimeter intervals throughout the forebrain.[8,9]

Immunohistochemistry for Neuronal, Astrocytic, Microglial, and Endothelial Markers

Neuronal survival, astrocytic reactivity, microglial activation, and brain capillary density were analyzed in 20-µm-thick brain sections using cresyl violet (ie, Nissl) staining and NeuN (neuronal nuclear antigen), GFAP (glial fibrillary acidic protein), Iba1 (ionized calcium-binding adaptor protein), or CD31 (cluster of differentiation 31) immunohistochemistry, as described in Materials and Methods in the online-only Data Supplement. Immunohistochemistries were counterstained with the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI). Cresyl violet+ neurons, which could be identified based on

the appearance of Nissl staining in surviving neurons, NeuN+ neurons, GFAP+ astrocytes, and Iba1+ microglia were blindly quantified under a motorized Zeiss AxioObserver.Z1 inverted epifluorescence microscope equipped with Apotome optical sectioning by measuring cell numbers in 6 defined regions of interest of the striatum ipsilateral to the stroke (size, 500×500 µm; regions of interest centered 1.5 and 2.5 mm lateral to midline/2.5, 3.25, and 4.0 mm below brain surface), as specified in Figure IE in the online-only Data Supplement.[8,9] For cresyl violet and NeuN stainings, neuronal densities determined were multiplied with striatal areas of each section, thus correcting for consequences of brain atrophy. CD31 stainings were evaluated by counting microvessel numbers in the same regions of interest. Optical sectioning was used for correction of cell/capillary overcounts. Mean values were calculated for all regions of interest.

Immunohistochemistry for Endogenous Neurogenesis

Endogenous neural precursor cell (NPC) proliferation and differentiation were examined in 20-µm-thick brain sections obtained from the bregma level by colabeling with anti-BrdU and anti-DCX (doublecortin) or anti-BrdU and anti-NeuN antibodies. Nuclei were counterstained with DAPI. Sections were evaluated using a Carl Zeiss LSM 710 confocal microscope by nonbiased colabeling analysis of maximum-intensity projections obtained from 666×666×10 µm stacks in the ipsilesional subventricular zone, peri-infarct striatum, and peri-infarct cortex using customized automated algorithms (for details, see Materials and Methods in the online-only Data Supplement).

Immunohistochemistry for Biotinylated Dextran Amine

Corticorubral plasticity was evaluated in 2 consecutive 40-µm-thick brain sections obtained from the level of the parvocellular red nucleus that were incubated with avidin-biotin-peroxidase complex and revealed by 3,3′-diaminobenzidine (DAB). Using an Olympus BX42 microscope, the number of tracer-stained fibers crossing a 500-µm-long intersection line on the brain midline were quantified.[8,9,11] These fibers originate from the contralesional pyramidal tract and innervate the ipsilesional red nucleus. The number of fibers counted was divided by the total number of labeled fibers that was evaluated in the pyramidal tract and multiplied with 100, resulting in percent values of fibers crossing the midline (Materials and Methods in the online-only Data Supplement).

ELISA

In homogenates of peri-infarct striatum and cortex and homologous contralesional striatum and cortex, levels of BDNF (brain-derived neurotrophic factor), GDNF (glial cell line-derived neurotrophic factor), VEGF (vascular endothelial growth factor), and basic FGF (fibroblast growth factor) were determined using commercial kits (Materials and Methods in the online-only Data Supplement).

Statistical Analysis

Data were analyzed by 2-way or 2-way repeated measures ANOVA, followed by Bonferroni-corrected 2-tailed *t* tests as post hoc tests. Data were presented as mean±SD (longitudinal comparisons within the same animals) or in box blots as median/mean±interquartile range with minimum and maximum data (all other comparisons between animals or tissue samples). *P* values <0.05 were considered significant.

Results

Postacute Delivery of S44819 Improves Poststroke Motor Coordination Recovery and Spatial Memory

Body weight and laser Doppler flow above the core of the middle cerebral artery territory did not differ between animals treated with vehicle or S44819 (3 or 10 mg/kg), neither in the exploratory study (Figure IIA and IIB in the online-only Data Supplement) nor in the confirmation study (Figure IIIA and IIIB in the online-only Data Supplement) nor when both studies were evaluated in a combined analysis (Figure 1A and 1B). In all groups, laser Doppler flow decreased to ≈15% of baseline during MCAO, followed by a rapid blood flow recovery after reperfusion. Motor coordination performance, assessed by Rotarod and tightrope tests, was markedly impaired 3 days poststroke, before the start of treatment. From 14 dpt, motor coordination deficits were significantly improved by S44819 delivered at a dose of 10 mg/kg but not at 3 mg/kg, both when both studies were evaluated separately (Figures IIC, IID, IIIC, and IIID in the online-only Data Supplement) and in a combined analysis (F [2/105], 19.434; *P*<0.001 for Rotarod/F [2/105], 29.989; *P*<0.001 for tightrope test; Figure 1C and 1D). The improvement of motor coordination skills was maintained until 42 dpt, that is, 14 days after the termination of S44819 delivery.

In line with the motor coordination improvement, spontaneous motor activity, evaluated as distance covered in the open-field test, was not influenced by S44819 (Figure 2A). Anxiety, examined by the time spent in the center of the open-field arena, was moderately but significantly reduced by 10 mg/kg S44819 at 28 dpt (F [2/69], 5.717; *P*=0.005; Figure 2B). The time spent in the border and corner zone was unchanged (Figure 2C and 2D).

In the Barnes maze test, which was conducted between 8 and 19 dpt, spatial memory, evaluated as the number of errors (F [2/105], 3.488; *P*=0.035) and latency (F [2/105], 4.279; *P*=0.016) until finding the target hole, was reduced by 10 mg/kg but not 3 mg/kg S44819 in the combined analysis (Figure IVA and IVB in the online-only Data Supplement). In post hoc tests, target hole latency was significantly reduced by 10 mg/kg S44819 at 19 dpt.

Postacute S44819 Administration Prevents Secondary Striatal Atrophy and Promotes Delayed Neuronal Survival

Cresyl violet stainings revealed progressive shrinkage of the striatum and parietal cortex from 14 to 42 dpt. Notably, the volume of the striatum, which represents the core of the middle cerebral artery territory,[12] was significantly increased by 10 mg/kg but not 3 mg/kg S44819 at 42 dpt (Figure 3A), whereas whole brain volume (Figure VB in the online-only Data Supplement) and hippocampal volume (Figure VC in the online-only Data Supplement) were not significantly altered. Neuronal survival in the striatum was significantly increased by 10 but not 3 mg/kg S44819 at 14 dpt and, more pronounced, 42 dpt, as shown by NeuN (Figure 3B) and cresyl violet (ie, Nissl; Figure VA in the online-only Data Supplement) histochemistry.

S44819 Delivery Reduces Peri-Infarct Astrogliosis and Increases Brain Capillary Density

Peri-infarct astrogliosis, evaluated by GFAP immunohistochemistry in brain sections, was significantly decreased by 10 but not 3 mg/kg S44819 at 14, 28, and 42 dpt (Figure 3C).
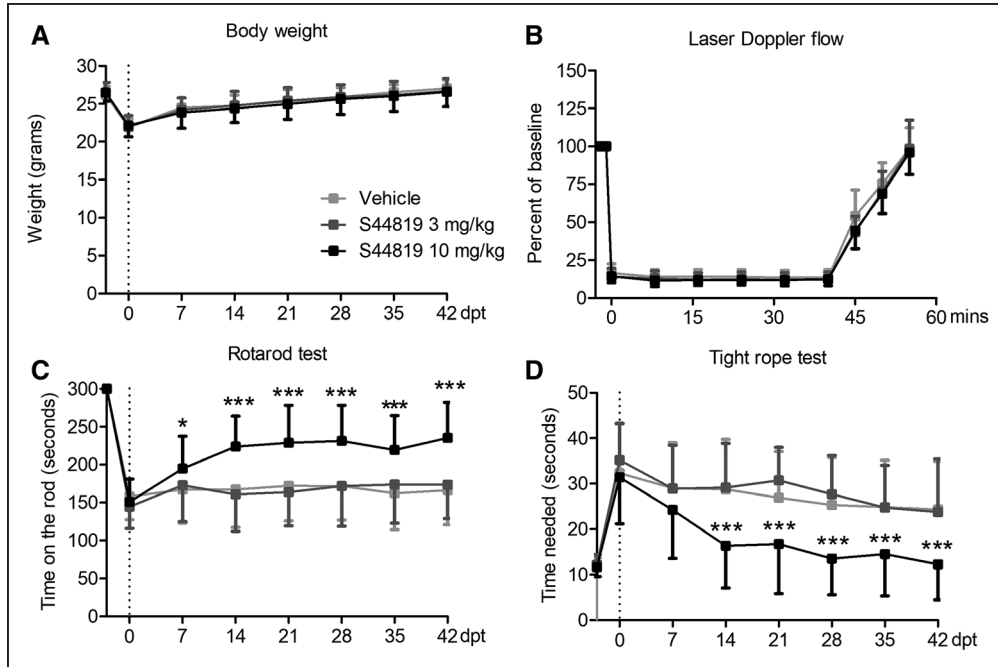
Figure 1. Delayed delivery of S44819 at 10 mg/kg but not 3 mg/kg promotes postischemic recovery of motor coordination deficits. Combined analysis of the exploratory study (animal set 1) and confirmatory study (animal set 4). **A**, Body weight, (**B**) laser Doppler flow (LDF) recordings above the middle cerebral artery territory core, and (**C** and **D**) coordination skills evaluated by Rotarod and tightrope tests in mice exposed to transient middle cerebral artery occlusion (MCAO). Vehicle or S44819 (3 or 10 mg/kg BID) was administered for 28 d starting 72 h after reperfusion. Body weight (**A**) and LDF recordings (**B**) do not differ between groups. Results are means±SD values (n=36 animals per group). *$P<0.05$, ***$P<0.001$ compared with vehicle.

Microglial activation, defined by Iba1 immunoreactivity, was not influenced by S44819 at both doses (Figure VD in the online-only Data Supplement), whereas brain capillary density, examined by CD31 immunohistochemistry, was significantly increased by 10 mg/kg at 42 dpt (Figure 3D).

S44819 Enhances NPC Proliferation and Neuronal Differentiation in the Ipsilesional Subventricular Zone

To test whether the enhanced peri-infarct brain remodeling was associated with increased neurogenesis, we next assessed NPC proliferation and differentiation by BrdU incorporation analysis
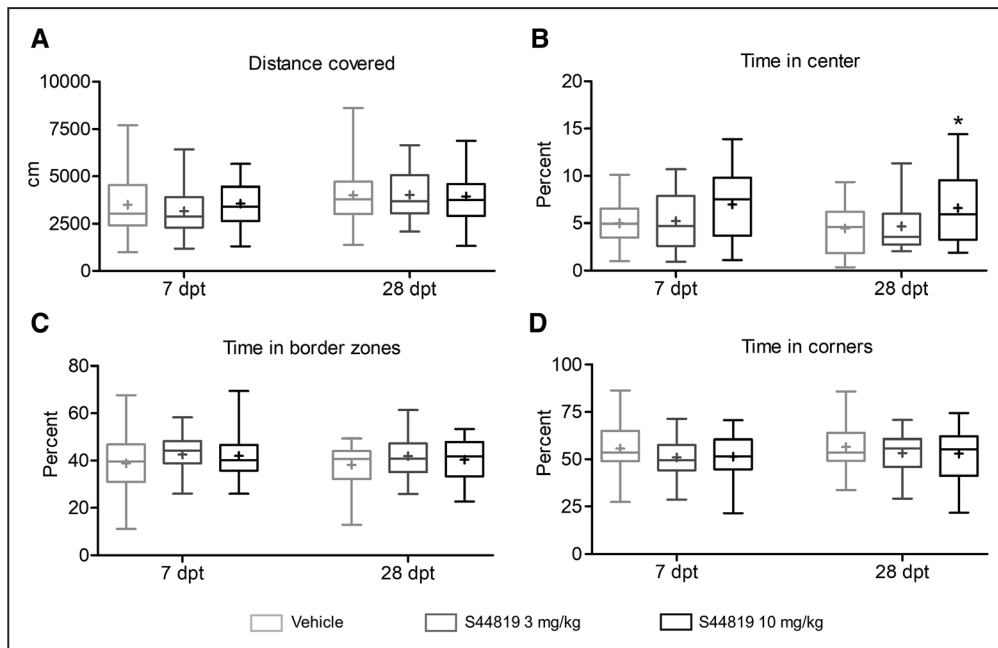
Figure 2. Delayed S44819 delivery at 10 mg/kg but not 3 mg/kg enhances postischemic exploration behavior. Combined analysis of the exploratory study (animal set 1) and confirmatory study (animal set 4). **A**, Distance covered, (**B**) time in center, (**C**) time in border zone, and (**D**) time in corner zone in open-field tests of mice exposed to transient middle cerebral artery occlusion (MCAO). Vehicle or S44819 (3 or 10 mg/kg BID) was administered for 28 d starting 72 h after reperfusion. Results are medians (lines inside boxes)/means (crosses inside boxes)±interquartile range (boxes) and minimum/maximum data (elongation lines; n=36 animals per group). *$P<0.05$ compared with vehicle.
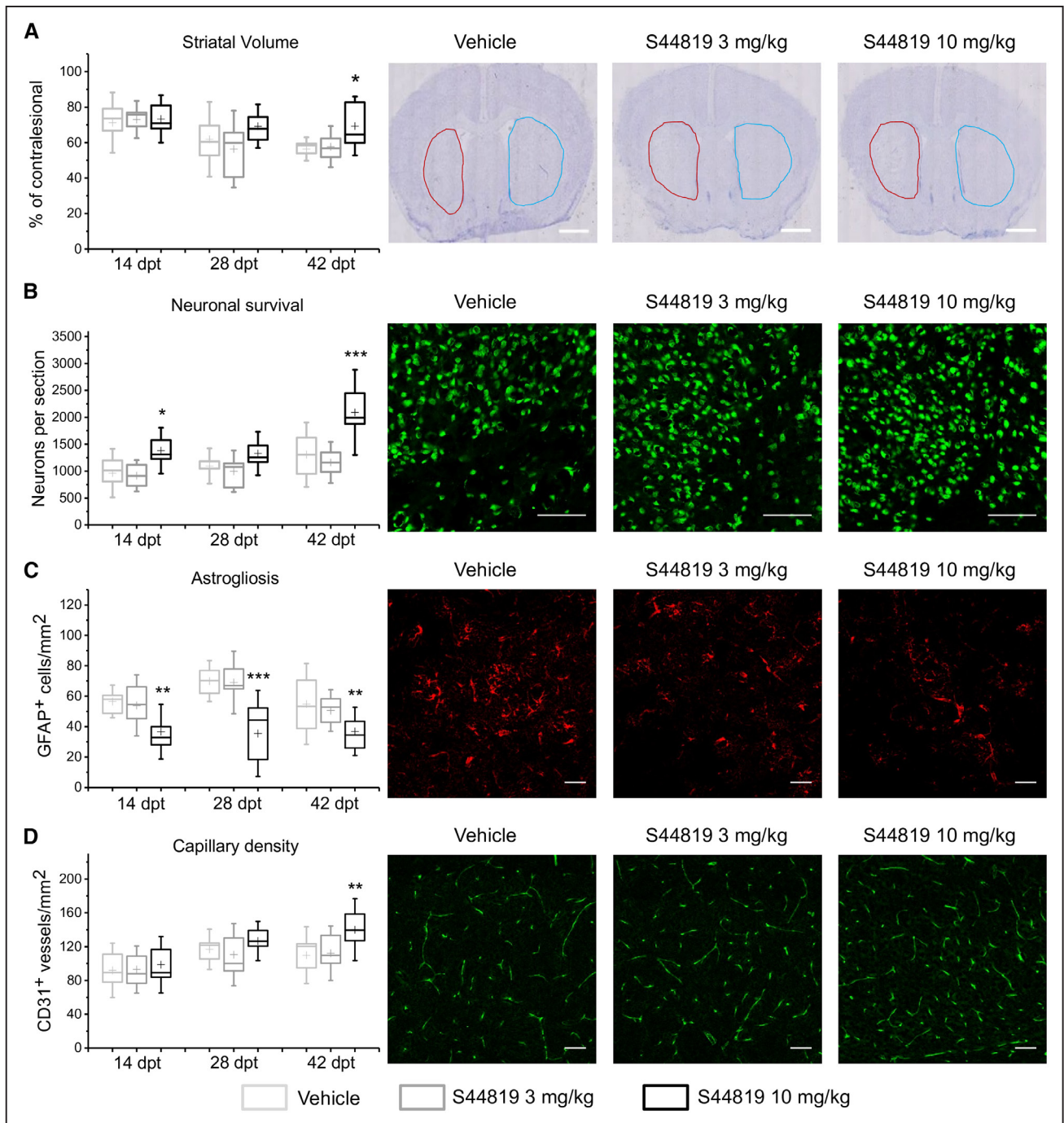
Figure 3. S44819 prevents delayed neurodegeneration and promotes peri-infarct brain remodeling. **A**, Striatal volume, outlined on cresyl violet-stained brain sections; (**B**) neuronal density, evaluated by NeuN (neuronal nuclear antigen) immunohistochemistry; (**C**) astrogliosis, examined by GFAP (glial fibrillary acidic protein) immunohistochemistry; and (**D**) capillary density, analyzed by CD31 (cluster of differentiation 31) immunohistochemistry, in mice exposed to transient middle cerebral artery occlusion (MCAO) that were treated with vehicle or S44819 (3 or 10 mg/kg BID) starting 72 h after reperfusion. Photomicrographs at 42 d post-treatment onset (dpt) are also shown. Cell and capillary densities were assessed in 6 regions of interest within the striatum, as described in Materials and Methods in the online-only Data Supplement and depicted in Figure IE in the online-only Data Supplement. Representative microphotographs taken at 42 dpt are shown. Results are medians (lines inside boxes)/means (crosses inside boxes)±interquartile range (boxes) and minimum/maximum data (elongation lines; n=6 animals per group). Bars=1000 μm (**A**)/50 μm (**B–D**). *P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

in animals, in which BrdU had been administered at 3 defined time intervals, that is, from 3 to 8, 8 to 13, or 13 to 18 dpt. Compared with vehicle-treated animals, enhanced cell proliferation was observed adjacent to the ipsilesional subventricular zone (Figure 4A) but not in the perilesional striatum (Figure VIA in the online-only Data Supplement) or the peri-infarct cortex (not shown) of animals treated with 3 or 10 mg/kg S44819, in which BrdU had been delivered at 13 to 18 dpt. Similarly, NPC differentiation, examined by BrdU colabeling with the immature neuronal marker DCX, but not NPC differentiation, assessed by BrdU colabeling with the mature neuronal marker NeuN, was significantly increased adjacent to the ipsilesional subventricular zone
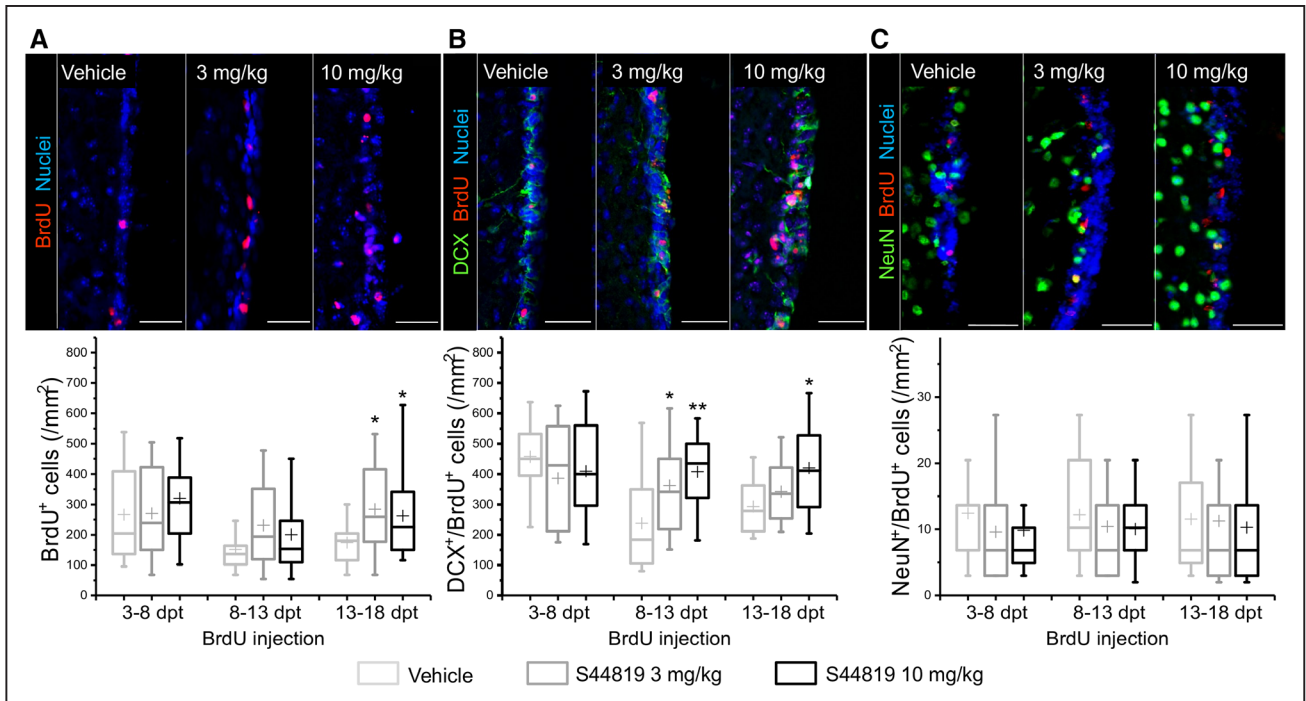
Figure 4. S44819 increases neural precursor cell (NPC) proliferation and differentiation in proximity to the subventricular zone. NPC (**A**) proliferation and (**B** and **C**) differentiation adjacent to the ipsilesional subventricular zone, as revealed by bromodeoxyuridine (BrdU) incorporation and colabeling of BrdU incorporating cells (in red in **A**–**C**) with the immature neuronal marker DCX (doublecortin; in green in **B**) and the mature neuronal marker NeuN (neuronal nuclear antigen; in green in **C**) in mice exposed to transient middle cerebral artery occlusion (MCAO) that were treated with vehicle or S44819 (3 or 10 mg/kg BID) starting 72 h after reperfusion. BrdU was intraperitoneally administered at 3 to 8, 8 to 13, or 13 to 18 d post-treatment onset (dpt), respectively. Photomicrographs for animals receiving BrdU at 13 to 18 dpt are also shown. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; in blue). Results are medians (lines inside boxes)/means (crosses inside boxes)±interquartile range (boxes) and minimum/maximum data (elongation lines; n=6 animals per group). Bars=100 μm. *$P<0.05$, **$P<0.01$ compared with vehicle.

(Figure 4B and 4C) but not in the peri-infarct striatum (Figure VIB and VIC in the online-only Data Supplement) or the peri-infarct cortex (not shown) of animals, in which BrdU had been delivered at 8 to 13 or 13 to 18 dpt. These observations support the existence of a time interval between 8 and 18 dpt (ie, 11–21 days poststroke), in which precursor cell proliferation and differentiation were stimulated adjacent to the ipsilesional subventricular zone. As a matter of fact, this response did not translate into enhanced neurogenesis in previously ischemic brain tissue.

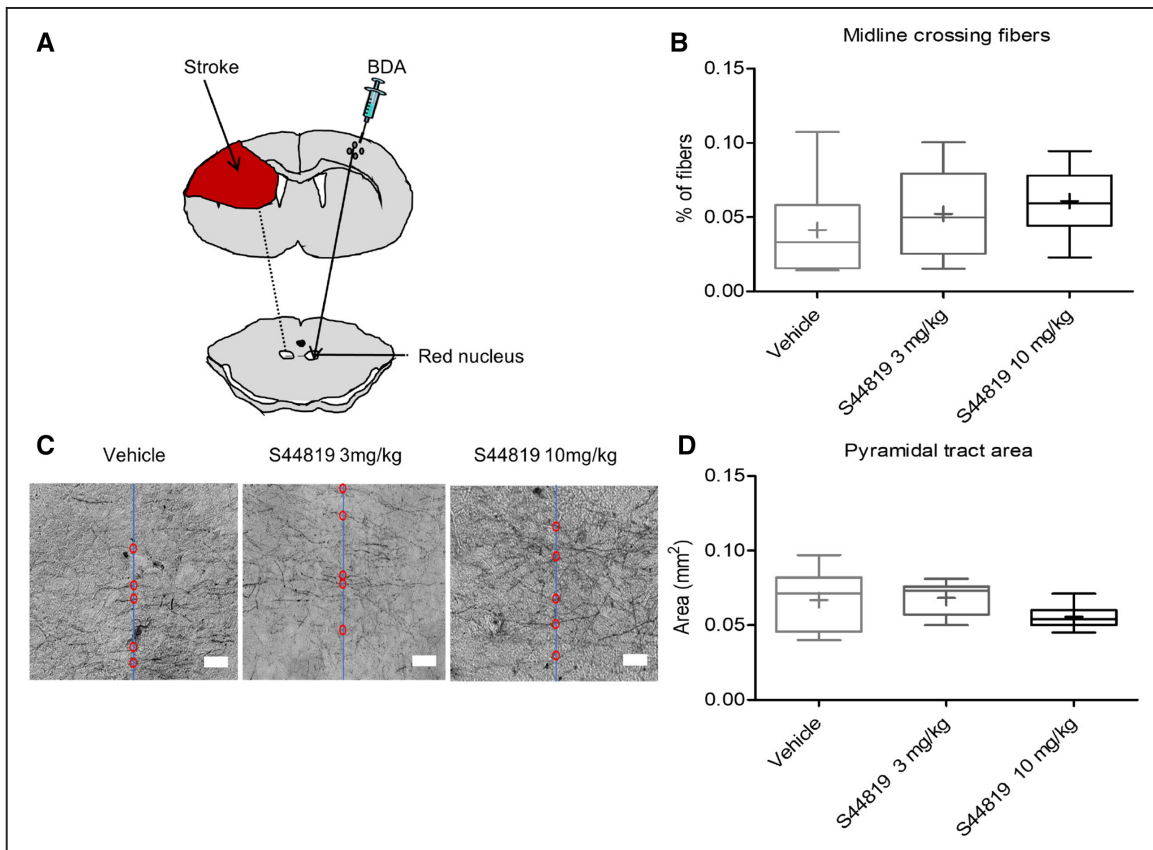### S44819 Does Not Influence Contralesional Pyramidal Tract Plasticity
To evaluate whether neurological recovery induced by S44819 involved contralesional pyramidal tract plasticity, we next evaluated the density of midline-crossing pyramidal tract fibers originating from the contralesional motor cortex in direction to the ipsilesional parvocellular red nucleus after vehicle and S44819 delivery (Figure 5A). This corticorubral projection has previously been shown to exhibit robust sprouting after intraluminal MCAO in response to therapy with different growth factors[8,11] and NPCs.[13] The density of midline-crossing fibers that had anterogradely been labeled with biotinylated dextran amine did not differ between vehicle- and S44819-treated (3 or 10 mg/kg) animals (Figure 5B and 5C), as did pyramidal tract area at the level of the red nucleus (Figure 5D). These findings argued against significant long-distance axonal sprouting that was evoked in the contralesional pyramidal tract system in response to S44819.

S44819 Increases Growth Factor Levels in the Peri-Infarct but Not Contralesional Brain Tissue
To further characterize restorative responses, we finally evaluated concentrations of growth factors in both brain hemispheres before treatment onset and at 14, 28, and 42 dpt by ELISA. Interestingly, concentrations of neurotrophic growth factors (BDNF and GDNF) and preferentially angiogenic growth factors (VEGF and FGF) were significantly elevated in peri-infarct brain tissue by 10, but not 3 mg/kg S44819 at 28 dpt (GDNF and FGF) and 42 dpt (all 4 growth factors; Figure 6A through 6D). Growth factor concentrations in the corresponding contralesional tissue were not influenced by S44819 (Figure VIIA through VIID in the online-only Data Supplement).

Discussion
We herein show that the preferential GABA$_A$ α5 antagonist S44819 induces sustained postischemic neurological recovery, when delivered in the postacute stroke phase starting at 3 days after transient MCAO. To substantiate this finding, we performed 2 behavioral studies, an exploratory study that was endorsed by a subsequent confirmation study, both studies having an adequate statistical power and size to detect recovery-promoting effects. Neurological recovery, which persisted after the completion of S44819 treatment, was associated with peri-infarct brain remodeling, that is, prevention of secondary striatal atrophy, promotion of peri-infarct neuronal survival, inhibition of peri-infarct astrogliosis, promotion of peri-infarct brain capillary density, and promotion of subventricular zone neurogenesis. Contralesional

Figure 5. S44819 does not increase contralesional pyramidal tract plasticity at the level of the red nucleus. **A**, Midline-crossing fibers originating from the contralesional motor cortex in direction to the ipsilesional parvocellular red nucleus were evaluated by injection of the anterograde tract-tracer biotinylated dextran amine (BDA) into the contralesional motor cortex. **B**, Percentage of BDA-labeled midline-crossing fibers at the level of the red nucleus originating from the contralesional motor cortex after intraperitoneal delivery of vehicle or S44819 (3 or 10 mg/kg BID). No significant differences were noticed between groups. Representative microphotographs are depicted in (**C**) (midline shown in blue, midline-crossing fibers labeled as red dots). **D**, Total area of the contralesional pyramidal tract at the level of the red nucleus, which does not change in response to S44819 delivery, indicating the absence of contralesional corticospinal tract degeneration. Data are medians (lines inside boxes)/means (crosses inside boxes)±interquartile range (boxes) and minimum/maximum data (elongation lines). Bars=30 μm (**C**).

pyramidal tract plasticity, evaluated by anterograde tract tracing at the level of the red nucleus, was not influenced by S44819. Concentrations of neurotrophic growth factors (BDNF and GDNF) and angiogenic growth factors (VEGF and FGF) were increased in peri-infarct but not contralesional brain tissue. Our data reveal a profound restorative response in the peri-infarct brain tissue that prevents neurodegeneration and enables neurological recovery (visual overview).

In models of permanent MCAO, that is, photothrombotic stroke in mice and cortical endothelin microinjection in rats, improvement of neurological deficits has repeatedly and consistently been reported after delayed subcutaneous or intraperitoneal delivery of the negative allosteric GABA$_A$ modulator L655708[3,4], which similarly to S44819 reverses tonic GABA-ergic inhibition. The recovery-promoting effect of L655708 was absent in ischemic GABA$_A$ α5$^{-/-}$ mice, indicating that the deactivation of the GABA$_A$ α5 subunit was critical for neurological recovery.[3] S44819 differs from L655708 in that it is the first competitive antagonist of GABA$_A$ receptors that selectively interacts at the GABA-binding site of GABA$_A$ α5 receptors and does not bind to the benzodiazepine site.[5] Our observations for S44819 exceed results from L655708 studies by showing that S44819 reduces secondary brain atrophy, increases long-term

peri-infarct neuronal survival, reduces peri-infarct astrogliosis, increases peri-infarct brain capillary density, and augments NPC proliferation and differentiation in the subventricular zone.

S44819 exhibits high affinity to GABA$_A$ α5 receptors (Ki=6.6×10^{-8} M)[5] and less potent affinity to GABA$_A$ α1, α3, and α4 receptors (Ki=7.1×10^{-7}, 6.6×10^{-6}, and 6.9×10^{-7} M, respectively), while not interacting with GABA$_A$ α2 receptors. In vitro, under conditions mimicking extrasynaptic GABA exposure, S44819 acts as competitive antagonist of recombinant GABA$_A$ α5 and α4 but not GABA$_A$ α1 and α3 receptors in human embryonic kidney (HEK) 293 cells (unpublished data). Ex vivo, S44819 (10^{-5} M) inhibits α5-mediated but not α4-mediated tonic currents in mouse hippocampal and thalamic slices.[5] These data suggest that S44819 is a highly selective GABA$_A$ α5 antagonist. Although effects on GABA$_A$ α4 receptors could not entirely be excluded, effects on other GABA$_A$ receptor subtypes could be excluded. S44819 has previously been administered already to rats twice daily for 28 days at doses 10× above those we used. There was no drug accumulation: S44819 concentrations after morning dosing were very similar on days 1 and 28 (unpublished). S44819 reaches its peak plasma concentrations 1 to 2 hours after oral administration in rats and mice. The compound is rapidly cleared in both species (half-life, ≈5 hours).
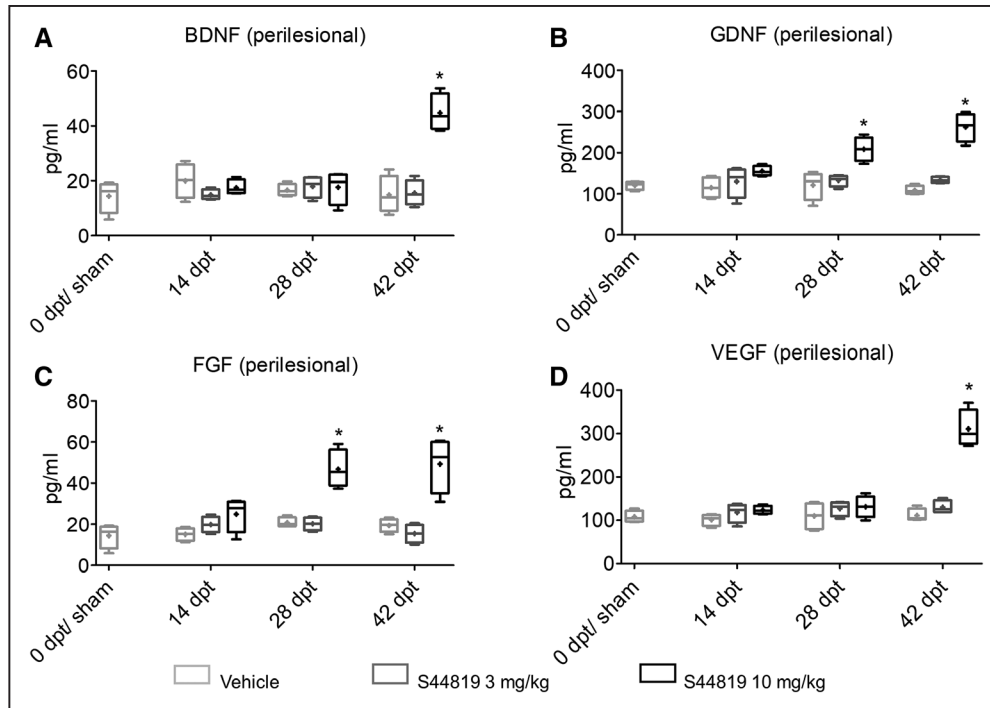
Figure 6. S44819 elevates neurotrophic and angiogenic growth factors in the peri-infarct brain tissue. Concentrations of (**A**) BDNF (brain-derived neurotrophic factor), (**B**) GDNF (glial cell line-derived neurotrophic factor), (**C**) FGF (fibroblast growth factor), and (**D**) VEGF (vascular endothelial growth factor) in peri-infarct striatum and cortex evaluated by ELISA in mice exposed to transient middle cerebral artery occlusion (MCAO) or sham surgery that were treated with vehicle or S44819 (3 or 10 mg/kg BID) starting 72 h after reperfusion. Results are medians (lines inside boxes)/means (crosses inside boxes)±interquartile range (boxes) and minimum/maximum data (elongation lines; n=6 animals per group). *$P<0.05$ compared with ischemic vehicle.

For evaluating effects of S44819, we used a protocol very similar to protocols that we previously used for evaluating effects of growth factors,[8,11] NPCs,[12,13] and other pharmacological compounds[9] in the postacute stroke phase. S44819 delivery was initiated at 3 days after transient intraluminal MCAO, and motor coordination and cognition were studied for 6 weeks, that is, 2 weeks after the completion of treatment, using comprehensive test batteries. Structural brain remodeling was examined using histochemical and BrdU incorporation studies. Interestingly, the time window at which neurological recovery occurred after S44819 administration was faster than after growth factor or NPC delivery, suggesting both a rapid symptomatic and a delayed restorative effect. After growth factor[8,11] or NPC[12,13] delivery, neurological improvements gradually evolved for 4 to 6 weeks posttreatment onset.

S44819 induced a marked inhibition of astrogliosis, which was evident already at 2 weeks post-treatment onset and persisted after the termination of S44819 treatment. Brain astrocytes do express GABA$_A$ receptors,[14] and they may furthermore attenuate tonic inhibition under pathophysiological conditions via GABA uptake.[15] These observations raise the possibility that the effects of S44819 on neurological recovery and secondary neurodegeneration might at least partly be mediated by astrocytes, perhaps by increasing their release of growth factors. Notably, S44819's effects on astrogliosis, brain atrophy, and neuronal survival were noted only at a dose of 10 but not 3 mg/kg S44819, as were effects on neurological recovery. Hence, slightly higher S44819 doses (10 mg/kg) were needed than those predicted in pre-experiments (3 mg/

kg), in which brain and plasma S44819 concentrations had been determined (see Materials and Methods).

Although the proliferation and differentiation of NPCs were elevated adjacent to the subventricular zone by S44819 at 8 to 18 dpt, as revealed by BrdU incorporation analysis and colabeling with the immature neuronal marker DCX, we did not find evidence for NPC proliferation or differentiation in previously ischemic striatum and cortex. GABA is a well-known modulator of subventricular zone NPCs that controls neuronal differentiation both via activity-dependent synaptic and extrasynaptic GABA$_A$ receptors.[16] In an unbiased high-throughput genome-wide study, GABA was shown to control neurogenesis in an activity-dependent way via the transcriptional regulator NFAT (nuclear factor of activated T cells)-c4, which via binding to specific promoter-responsive elements regulated GABA$_A$ α2 and GABA$_A$ α4 subunit expression.[17] In a mouse model of Down syndrome, in which spatial learning and memory are impaired because of excessive tonic inhibition, reversal of tonic inhibition by the GABA$_A$ α5-negative allosteric modulator RO4938581 restored deficits in spatial learning and memory and adult neurogenesis.[18] The role of GABA$_A$ α5 receptors in the stroke brain differs from GABA$_A$ α1 receptors, which, when activated by zolpidem starting 3 days poststroke, enhance neurological recovery by activating phasic GABA$_A$ currents.[19]

In this study, the midline sprouting of biotinylated dextran amine–labeled contralesional pyramidal tract fibers was not increased by S44819, indicating that long-distance axonal plasticity in lesion-remote brain areas was unaffected. With this respect, S44819 differs from growth factors,[8,11] NPCs,[12,13] and the N-methyl-D-aspartate (NMDA) antagonist memantine,[9]

for which our group has previously reported enhanced contralesional pyramidal tract plasticity after transient intraluminal MCAO in mice. That S44819 did not influence neuronal plasticity at distance to the stroke might be attributable to the fact that tonic inhibition is a local phenomenon that is confined to peri-infarct brain tissue.[1,2] In line with this finding, brain tissue levels of the growth factors BDNF, GDNF, VEGF, and FGF were elevated by S44819 in the peri-infarct brain tissue. We did not evaluate receptors or downstream signals of growth factors that establish causal links to neuroplasticity. The GABA$_A$ α5 subunit has previously been involved in neuronal long-term potentiation and synaptic plasticity.[20]

We were surprised to see that GABA$_A$ α5 deactivation increased peri-infarct brain capillary density. This finding may result from elevations of angiogenic growth factors (VEGF and FGF) that promoted microvascular remodeling and growth.

The clear strength of this study is the use of a study protocol that used well-defined batteries of functional neurological tests and sets of histochemical analyses, which our group previously used in the evaluation of other experimental therapies. Each of the 2 behavioral studies was adequately powered to detect improvements of motor coordination deficits with 80% statistical power (1 - β-error) and 5% α-error. Hence, this study fulfilled the principles of a hypothesis-building study that was confirmed by a second independent data set. This study complied with state-of-the-art principles of animal randomization and data blinding. Hence, our study raises hopes that the GABA$_A$ α5 antagonist S44819, which is currently evaluated in a randomized controlled phase IIb stroke trial in 15 countries on 5 continents (RESTORE Brain [Randomized Efficacy and Safety Trial With Oral S44819 After Recent Ischemic Cerebral Event]), might also be effective in human patients.

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