Effects of Neural Progenitor Cells on Sensorimotor Recovery and Endogenous Repair Mechanisms After Photothrombotic Stroke

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Background and Purpose—Intravenous neural progenitor cell (NPC) treatment was shown to improve functional recovery after experimental stroke. The underlying mechanisms, however, are not completely understood so far. Here, we investigated the effects of systemic NPC transplantation on endogenous neurogenesis and dendritic plasticity of host neurons.

Methods—Twenty-four hours after photothrombotic ischemia, adult rats received either 5 million NPC or placebo intravenously. Behavioral tests were performed weekly up to 4 weeks after ischemia. Endogenous neurogenesis, dendritic length, and dendritic branching of cortical pyramid cells and microglial activation were quantified.

Results—NPC treatment led to a significantly improved sensorimotor function measured by the adhesive removal test. The dendritic length and the amount of branch points were significantly increased after NPC transplantation, whereas endogenous neurogenesis was decreased compared to placebo therapy. Decreased endogenous neurogenesis was associated with an increased number of activated microglial cells.

Conclusions—Our findings suggest that an increased dendritic plasticity might be the structural basis of NPC-induced functional recovery. The decreased endogenous neurogenesis after NPC treatment seems to be mediated by microglial activation. (Stroke. 2011;42:1757-1763.)

Key Words: neural progenitor cells ■ recovery ■ stroke

Neural stem cell (NPC) transplantation is a promising approach to increase recovery from behavioral deficits after stroke. In contrast to the initial assumption that cell replacement is the main mechanism of NPC-induced functional recovery, recent experimental stroke studies do not support this hypothesis. Only a small proportion of the intravenously transplanted cells reach the brain and express neuronal markers, and there is only little evidence that those cells integrate into neural networks. The brain repair potential of NPC seems to be related to their ability to promote brain remodeling and survival of at-risk cells. As recently shown, NPC display anti-inflammatory, antiapoptotic, and glial scar-inhibitory effects after brain injury and thereby improve functional recovery. The impact of NPC on other components relevant for poststroke recovery, such as host neurons, endogenous neural precursor cells, and neuronal networks, is less well-characterized. Certain brain injuries such as stroke induce the generation of new neurons from progenitor cells. Endogenous neurogenesis occurs in distinct areas of the brain, namely the subventricular zone (SVZ) and the hippocampus, and it is assumed to be part of a physiological repair mechanism that contributes to restore neurological function. Dendritic plasticity was also identified to be an important underlying mechanism of functional recovery improvement after focal cerebral ischemia. In the present study, we investigated the effects of systemically transplanted NPC on functional recovery, endogenous neurogenesis, and dendritic remodeling in a rat model of focal cortical ischemia.

Materials and Methods

Cell Culture
To initiate derivation, OG2/Rosa26 mouse embryonic stem cells were detached from the plastic substratum and cultured as monolay-
ers in absence of mouse embryonic fibroblast medium (stage 1) in embryonic stem cell culture medium supplemented with 10 mg/mL leukemia inhibitory factor and 10 ng/mL bone morphogenetic protein 4 on gelatin-coated 6-cm dishes (2.5×10^4 cells/dish) for 24 hours. Medium was then replaced with modified N2B27 medium (neural induction medium; 1:1 mixture DMEM/F-12 medium supplemented with N2 and Neurobasal medium supplemented with B27) and renewed each day for 7 to 10 days (stage 2). In neural induction culture, cells had formed neural colonies that were mechanically picked and dissociated into single cells by trypsinization. One million cells from these trypsinized colonies were resuspended into a 6-cm dish in neural expansion medium comprising DMEM/F12 medium supplemented with modified N2, 2 mM/L L-glutamine, and 10 ng/mL of each epidermal growth factor and fibroblast growth factor-2. Within 3 to 5 days in neural expansion culture, cells had formed floating aggregates (stage 3). After harvesting, each aggregate was replated into each well of a gelatin-coated 48-well plate in neural expansion medium (stage 4). Aggregates immediately attached onto the plate and bipolar NPC grew (stage 5). Outgrowing cells were then dissociated and expanded on gelatin-coated dishes using neural expansion medium.9 Cells were LacZ-labeled before transplantation.

**Photothrombotic Ischemia Model**

Experimental protocols were approved by the local ethics committee. Experiments were performed on adult male Wistar rats (270–310 grams). Animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight; Ketanest) and xylazine hydrochloride (8 mg/kg body weight). The left femoral vein was cannulated with a PE-50 tube for Bengal Rose infusion. The rectal temperature was maintained at 37°C by a thermostatically controlled heating pad (Föhr Medical Instruments). Photothrombotic ischemia was induced in the right frontal cortex.10 For illumination, a laser spot of 8 mm diameter (G Laser Technologies) was placed stereotactically onto the skull 0.5 mm anterior to the bregma and 3.5 mm lateral from the midline. The skull was illuminated for 20 minutes. During the first 2 minutes of illumination, the dye Bengal Rose (0.133 ML/kg body weight, 10 mg/mL saline) was injected intravenously.

**Cell Transplantation**

Animals were randomly assigned to receive NPC (n=15) or placebo (n=17) treatment. Twenty-four hours after the induction of ischemia, rats were administered 5 million NPC in 1 mL phosphate-buffered saline or phosphate-buffered saline alone over the course of 10 minutes intravenously. Because the optimal NPC dose for stroke therapy is unknown, the number of cells in our experiments was based on previous studies that investigated the efficacy of NPC therapy after stroke.1,2 For immunosuppression, verum and control animals received daily injections of cyclosporine A (10 mg/kg body weight, intraperitoneal) throughout the experiment, commencing 18 hours before transplantation. Starting 1 day after photothrombotic ischemia, dividing cells were labeled with daily bromodeoxyuridine (BrdU) injections (50 mg/kg, intraperitoneal) for 5 days.

**Behavioral Testing**

In all animals, sensorimotor tests were performed during the light cycle 1 day before ischemia (baseline) after a training period of 3 days and at 1, 7, 14, 21, and 28 days after ischemia by an investigator blinded to the experimental groups. For the adhesive removal test, 2 pieces of adhesive-backed paper dots (113.1 mm^2) were used as bilateral tactile stimuli occupying the palmar surface of each forepaw.10,11 The time to remove each paper dot from the forelimbs was documented in 3 trials per day for each forepaw. An asymmetry score was calculated as follows: (time to remove ipsilateral dot – time to remove contralateral dot) / (time to remove ipsilateral dot + time to remove contralateral dot). For the cylinder test, the rats were placed in a transparent cylinder (16-cm diameter, 21-cm height) and videotaped from underneath for 2 minutes.11 Spontaneous wall and ground touches of both forelimbs were counted and an asymmetry score was calculated as described.

**Immunohistochemistry**

Twenty-nine days after ischemia, animals (placebo group, n=11; NPC group, n=9) were reanesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The brains were fixed overnight in 4% paraformaldehyde at 4°C. The tissue was then cryoprotected via 3-day immersion in 30% sucrose solution and stored at −80°C until analysis. Immunohistochemistry was performed on sagittal free-floating 40-μm sections with the following antibodies: mouse anti-β-galactosidase (1:200; Biozol), rabbit anti-DCX (1:500; Abcam), rabbit Calbindin (1:1000; Abcam), rabbit anti-GFAP (1:500; Dako), rat anti-BrdU (1:500; Abcam), mouse anti-NeuN (1:200; Millipore), goat anti-Iba1 (1:200; Abcam), and rabbit anti-CD3 (1:200; Abcam). All BrdU-positive cells in the dentate gyrus and in the SVZ were counted on 7 sections (every 12th section, 440-μm intervals) per hemisphere. All analyzed sections were taken from the same area of the brain (between 4.2 mm and 1.5 mm lateral to the bregma). To determine the percentage of neurons among the newly generated cells, 50 randomly selected BrdU-positive cells within the dentate gyrus and 50 randomly selected BrdU-positive cells within the SVZ were analyzed for BrdU/NeuN co-labeling. For histological quantification of inflammation, all Iba1-positive cells showing morphological features of activated microglia and all CD3-positive cells in the dentate gyrus and in the SVZ were counted.12

**Golgi-Cox Staining and Morphological Analysis of Dendrites**

The brains of 6 NPC and of 6 placebo animals were used in this part of the experiment. Twenty-nine days after ischemia, rats were deeply anesthetized and transcardially perfused with 0.9% saline. Brains were placed in Golgi-Cox solution and then stored in the dark at room temperature for 14 days.13 For another 2 days, the brains were stored in a 20% sucrose solution in the dark at 4°C. Afterward, they were cut on a vibratome in 200-μm-thick coronal slices. Sections were mounted on TESPA-coated (Merck) slides and treated with H2O for 1 minute, with ammonium hydroxide for 30 minutes in the dark, with H2O for 1 minute, with Agfax for film for 30 minutes, with H2O for 1 minute, and then dehydrated in ascending alcohol concentrations. Golgi-impregnated pyramid cells (layer 5) of the cortex 0.6 mm anterior to the bregma, laterally to the ischemic lesion, which was clearly distinguishably from surrounding healthy tissue, and pyramid cells of the corresponding cortex of the contralateral hemisphere were examined (for illustration, see the Supplemental data and Supplemental Figure, http://stroke.ahajournals.org).1,2,14 Three neurons from each area were selected and traced using the Neurolucida Image Analysis System (Microbrightfield). Only fully and equally stained neurons directly adjacent to the infarct were chosen, with soma and dendrites clearly distinguishable from somas and dendrites of other neurons. The total dendritic length and the amount of dendritic branch points (nodes) were calculated and used for statistical comparison. All histological analyses were performed by an investigator blinded to the experimental groups.

**Effects of Cyclosporine on Functional Recovery and Morphological Outcomes**

Because recently published studies showed that cyclosporine may affect neurogenesis and dendritic plasticity, we performed additional experiments to investigate the effects of cyclosporine A on functional recovery and morphological markers of regeneration (Supplemental Methods and Results, http://stroke.ahajournals.org).1,5,16

**Statistical Analysis**

The values presented in this study are means ± SD. Two-way repeated-measures ANOVA was used to analyze sensorimotor deficits. Student t test was used to compare data between 2 groups. All analyses were performed with the statistical software SPSS (version 15.00). P<0.05 was considered statistically significant.
Results

Functional Outcome

Rectal temperature, body weight, and mortality were not different between the NPC and the control group. NPC-treated rats had a more favorable recovery measured by the adhesive removal test over time compared with controls. An ANOVA with repeated measures revealed significant effects of group (F₁,₃₀, 27.965; \( P < 0.001 \)), trial (F₁,₃₀, 34.110; \( P < 0.001 \)), and group by trial interaction (F₁,₃₀, 24.650; \( P = 0.001 \); Figure 1A). Post hoc tests revealed significant treatment effects (\( P < 0.001 \), t test) after 3 and 4 weeks. The cylinder test showed no significant differences between the 2 groups (ANOVA group, \( P = 0.387 \); trial F₁,₃₀, 11.129 and \( P = 0.164 \); group by trial interaction, \( P = 0.528 \); Figure 1B).

Analysis of Transplanted NPC, Endogenous Neurogenesis, and Inflammation

Transplanted NPC were predominantly located on the ischemic side of the brain and were sparsely detected at the contralateral side. On the ischemic side, NPC were located within the boundary zone of the ischemic lesion (Figure 2). Overall, as expected after systemic transplantation, only a few of the transplanted cells were detected within the brain and quantification of this observation was not possible because of the low counts. The largest subpopulation of the transplanted cells expressed DCX, which is a marker for neuronal precursors (Figure 2). Beta-galactosidase/GFAP and \( \beta \)-galactosidase/Calbindin double-positive cells were not detected.

We investigated whether NPC transplantation would alter endogenous neurogenesis in the dentate gyrus and in the SVZ after photothrombotic ischemia. NPC treatment led to a significant decrease in the amount of BrdU-positive cells in the dentate gyrus (\( P < 0.05 \); Figure 3A). There was no statistical significant difference between NPC and placebo-treated rats regarding BrdU-positive cells in the SVZ (\( P = 0.07 \); ipsilateral to the lesion). Counting the BrdU/NeuN double-positive cells, we found that NPC transplantation decreased the number of newly generated neurons in the dentate gyrus on the side of the lesion (\( P < 0.05 \); Figures 3B, 4). The number of BrdU/NeuN double-positive cells in the SVZ did not significantly differ between the groups (\( P = 0.90 \)). In the contralateral hemisphere, the number of newly generated neurons after NPC transplantation was smaller but not statistically significant (dentate gyrus, \( P = 0.20 \); SVZ, \( P = 0.47 \); Figure 3B, 3D) compared to placebo treatment.

Because inflammation is known to decrease endogenous neurogenesis,\(^{17,18}\) we analyzed the number of Iba1-positive cells with retracted processes and round cell bodies, representing activated microglia, and the number of CD3-positive cells for detection of T-lymphocytes. After NPC transplantation, the number of Iba1-positive activated microglial cells was significantly increased in the dentate gyrus (\( P < 0.05 \); Figures 3B, 4). The number of BrdU/NeuN double-positive cells in the SVZ did not significantly differ between the groups (\( P = 0.90 \)). In the contralateral hemisphere, the number of newly generated neurons after NPC transplantation was smaller but not statistically significant (dentate gyrus, \( P = 0.20 \); SVZ, \( P = 0.47 \); Figure 3B, 3D) compared to placebo treatment.

Figure 1. Behavioral testing. A, Adhesive removal test. Neural progenitor cell-treated rats had a significant improved recovery compared to placebo rats (\( P < 0.001 \); analysis of variance, t test). B, Cylinder test. No significant differences between the 2 groups were detected.

Figure 2. Phenotypic characterization of neural progenitor cell. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). A–C, The majority of \( \beta \)-Gal-positive cells (green) expressed the neuronal precursor marker doublecortin (DCX) (red). Beta-galactosidase/DCX double-positive cells showed wispy cell processes (scale bar = 20 \( \mu \)m).

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Activated microglial cells in the SVZ (P<0.08) ipsilateral to ischemic lesion (Figure 5A, B). In the contralateral hemisphere, there were no significant differences regarding the number of Iba-1 microglia cells in the dentate gyrus (P=0.12) and in the SVZ (P=0.24; Figure 5A, B). T-lymphocytes were not detected in the SVZ or in the dentate gyrus.

Morphological Analysis of Host Pyramid Cells
To examine the effect of NPC transplantation on dendritic remodeling, we calculated the total dendritic length and the amount of dendritic branch points (nodes) from traced pyramidal cells (layer 5) of the cortex laterally to the photothrombotic lesion and of the corresponding contralateral cortex. In NPC-treated animals, the dendritic length was significantly increased compared to placebo-treated animals (mean dendritic length NPC, 624.06±62.85; mean placebo, 375.70±80.83; P<0.05; Figure 6A). The amount of branch points was also significantly increased after NPC transplantation (mean branch points NPC, 9.2±0.7; mean placebo, 5.1±1.1; P<0.05; Figure 6b). In the contralateral hemisphere, there were no significant differences between NPC-treated and placebo-treated rats regarding the number of newborn cells.
hemisphere, there were no statistical differences in dendritic length ($P=0.10$) and the number of branch points ($P=0.10$; Figure 6A, B).

**Discussion**

In the present study, NPC-treated rats showed a significantly improved functional recovery compared to placebo rats measured by the adhesive removal test. The gradual sensorimotor deficit improvement over weeks and the pronounced improvement 3 and 4 weeks after treatment suggest a true recovery-enhancing effect of NPC therapy after stroke. Our findings thereby support results of previous studies that demonstrated neurobehavioral deficit improvement by intravenous NPC treatment that was initiated beyond established time windows of neuroprotection.$^{1,2}$ The cylinder test showed no significant differences between NPC-treated and placebo-treated rats. A potential reason that might hinder the detection of potential differences between the 2 groups includes the surprisingly good functional recovery improvement of the placebo group. However, we cannot rule out that effects of NPC therapy on functional recovery improvement in our study were not as robust as in previously published studies. $^{1,2}$ Even marginal differences in cell preparation procedures may account for a lower efficacy.$^{19}$ Previous studies that showed an improved functional recovery after NPC treatment in stroke models used cells of different sources and growth factors used for cell differentiation differ considerably from that used in our study.$^{1,2}$ Because studies that systematically evaluate different cell sources and culture media in experimental stroke do not exist, the impact of cell preparation on functional recovery improvement is unknown so far. However, in concordance with previous studies we showed that NPCs reach the brain only in small amounts.$^{1,2}$ The majority of transplanted cells within the brain expressed DCX, a marker for immature neurons, whereas Calbindin/X-gal double-positive cells, indicating mature neurons, were not detected. Our data therefore confirm that differentiation of transplanted cells and integration in existing neuronal networks is highly unlikely to be the key mechanism of intravenous NPC treatment-induced functional recovery after stroke.$^{1,2}$ Recently identified so-called bystander effects are much more likely to be the mode of action underlying functional recovery of NPC transplantation in stroke.$^{1}$ Besides immunomodulating effects of NPC, angiogenic and antiapoptotic actions after intracerebral transplantation of NPC over-expressing vascular endothelial growth factor or the antiapoptotic factor Akt 1 were reported.$^{3,4}$

**Figure 5. Microglia response.** A–D, Neural progenitor cell treatment increased the number of activated microglial cells in the dentate gyrus (DG) ipsilateral to the infarct ($P=0.05$). There were no statistical significant differences between the 2 groups regarding the number of activated microglia in the subventricular zone (SVZ) of both hemispheres and in the dentate gyrus contralateral to the ischemic lesion. C, D, Examples of Ionized calcium-binding adaptor molecule 1 (Iba)-1–positive cells (red) indicating activated (round) and quiescent (ramified) microglial cells. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue; scale bars=160 μm and 20 μm).
When analyzing effects on host neurons, we found a significantly increased dendritic length and dendritic arborization of dendrites of cortical pyramid cells ipsilateral to the infarct in NPC-treated rats. Dendritic plasticity after stroke was shown to be associated with functional recovery by numerous experimental studies.\(^7,8\) The growth of dendrites and axons is regulated by multiple mechanisms comprising a complex composition of growth-promoting and growth-inhibitory molecules.\(^20\) In fact, NPC were shown to promote neuritic growth by the expression and secretion of the nerve growth factor and the brain-derived neurotrophic factor in an animal model of spinal cord injury.\(^21\) After experimental stroke, bone marrow stromal cells were found to increase neuritic growth and thereby improve functional outcome.\(^22\) Overall, we conclude that an increased dendritic plasticity might be a mechanism of NPC-induced functional recovery after stroke and that NPC growth factor release might be the mediator of this effect.

Here, we investigated for the first time to our knowledge whether exogenous NPC transplantation affects endogenous neural precursor cell proliferation. We found a decreased number of endogenous neural precursor cells in the dentate gyrus of the infarcted hemisphere after NPC treatment compared to placebo treatment. Generally, cerebral injury such as stroke increases endogenous neurogenesis.\(^5,6\) Several recovery-enhancing stroke therapies, including growth factors\(^22\) and bone marrow stromal cells, were shown to increase the number of newly generated neurons after cerebral ischemia and therefore were assumed to improve functional outcome by a stimulation of the endogenous neurogenesis.\(^24\) Regarding the impact of brain-derived neurotrophic factor on neural progenitor cells, existing results are conflicting because suppression of stroke-induced neurogenesis as well as an increased neurogenesis after focal cerebral ischemia were reported.\(^10,25\) However, our finding of decreased neurogenesis and improved functional recovery is not necessarily conflicting, because it is unknown whether neurogenesis is causally related to or merely associated with functional improvement.

Brain inflammation and, in particular, microglia activation were clearly identified to decrease endogenous neurogenesis.\(^17,18\) We therefore investigated the impact of NPC transplantation on the local inflammation in the SVZ and the dentate gyrus. After NPC treatment, the number of activated microglia cells was significantly increased in the dentate gyrus ipsilateral to the infarct. We supposed that NPC transplantation causes a microglial activation, which in turn decreases endogenous neurogenesis. Our finding is in line with that of previous studies that also found an activation of microglial cells in NPC-treated rats after neonatal ischemic brain injury.\(^26\) Beyond its detrimental effect on endogenous neurogenesis, activated microglia was assumed to be beneficial for recovery after stroke by eliminating excess excitotoxins and by producing neuroprotective molecules.\(^27\) Whether the activation of microglia is of relevance regarding function recovery after NPC treatment is, however, speculative. Moreover, heterogeneous results exist whether transplanted NPC decrease or increase microglial cell activation.\(^1,26\)

Recent research suggests that cyclosporine A affects neurogenesis and dendritic plasticity.\(^15,16,28\) To address this issue, we performed experiments that compared animals treated with cyclosporine A with a daily dose of 10 mg/kg body weight and placebo. In these experiments, we found no significant differences between the 2 groups regarding functional recovery improvement, neurogenesis in the dentate gyrus, microglial activation, and dendritic regeneration. Reasons for the unchanged neurogenesis and dendritogenesis after cyclosporine treatment in our experiments compared to previously published studies might include the use of higher cyclosporine doses in
these studies and that experiments were performed with healthy animals. However, although both groups of the cell therapy experiments received immunosuppression, we cannot completely rule out that cyclosporine A might confound our results because we performed no experiment that contains all 3 groups, NPC-treated animals and placebo-treated animals with and without immunosuppression.

Conclusions

In conclusion, we demonstrated that intravenous NPC treatment improved functional recovery after photothrombotic stroke. An increased dendritic plasticity might be the structural basis of the improved functional outcome. In addition, NPC decrease the endogenous neurogenesis after stroke, potentially by activating microglia cells.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Supplemental Methods

The Induction of photothrombotic ischemia, BrdU injections, behavioural testing and transcardial perfusion of the animals were performed as described in the main text of the manuscript. Animals were randomly assigned to receive daily injections of cyclosporine A i.p. (10mg/kg body weight, n=7) or placebo i.p. (n=7) throughout the experiment.

For the detection of neurogenesis and microglial activation immunohistochemistry was performed as described in the main text of the manuscript. Since immunohistochemistry and Golgi-Cox stainings can not be performed within the same sections we performed βIII-tubulin immunohistochemistry as follows to visualize dendrite regeneration. First of all the endogenous peroxidase was blocked by a 0,3% H₂O₂ solution in Methanol/PBS. After washes in PBS and PBST the sections were preincubated with 5% normal horse serum (NHS; vector, Burlingame, CA, USA) in PBST for 30 minutes. Then the slices were incubated with a mouse monoclonal anti-βIII-tubulin antibody in 5% NHS/PBST solution over night at 4°C (1:1600, clone TU-20, Millipore, Billerica, MA, USA). The biotinylated universal secondary antibody (Vectastain kit, Vector, Burlingame, CA, USA) was diluted 1:85 in NHS/PBS and incubated for 30 minutes at room temperature. Immunoreactivity was visualized by the avidin-biotin complex method. The sections were developed in diaminobenzidine (Thermo-Fisher, Fremont, CA, USA). Finally they were mounted on glass slides, dried at room temperature and coverslipped with cytoseal XYL (Microm, Walldorf, Germany). Sagittal sections were scanned in equal light conditions with a digital camera (Roper Scientific, Ottobrunn/Munich, Germany) and digitized with the MCID image analysis system (Imaging Research Inc, St. Catharines, Ontario, Canada). Technical settings were not changed during image acquisition. For semi-quantitative analysis of neuroplasticity, optical density (OD) of βIII-tubulin immunoreactivity was determined in the immediate vicinity of the lesion and adjacent cortex both cranial and caudal to the infarct according to a modified protocol of Shyu and colleagues. In addition, the corresponding contralateral
cortex was also analyzed accordingly. Optical density of the stratum oriens was used as reference value for background staining.

All experiments were performed in a blinded fashion.

**Supplemental Results**

Rectal temperature, body weight and mortality were not different between the cyclosporine A and the placebo group. The adhesive removal test and the cylinder test showed no significant differences between the two groups (ANOVA P = 0.073 and P = 0.593, asymmetry scores (SD) for post-stroke days 1, 7, 14, 21 and 28 in the cyclosporine A and in the control group were -0.78 (0.15), -0.51 (0.22), -0.29 (0.37), -0.21 (0.13), 0.20 (0.20) and -0.71 (0.19), -0.61 (0.16), -0.49 (0.20), -0.45 (0.25), -0.40 (0.15) for the adhesive removal test and -0.17 (0.11), -0.24 (0.19), -0.09 (0.13), -0.14 (0.25), -0.11 (0.28) and -0.22 (0.09), -0.18 (0.08), -0.23 (0.06), -0.18 (0.08), -0.11 (0.06) for the cylinder test, respectively). There were no significant differences regarding BrdU-positive cells in the dentate gyrus (ipsilateral P = 0.85, contralateral P = 0.25), regarding BrdU/NeuN-double-positive cells in the dentate gyrus (ipsilateral P = 0.99, contralateral P = 0.09) and regarding the number of activated microglial cells in the dentate gyrus (ipsilateral P = 0.61, contralateral P = 0.21). There were no differences in the intensity of tubulin immunostaining between cyclosporine and placebo animals neither in the ipsilateral cortex (caudal to the infarct P = 0.85, cranial to the infarct P = 0.51) nor in the corresponding cortex of the contralateral hemisphere (caudal to the infarct P = 0.90, cranial to the infarct P = 0.71).
Online figure. Illustration of the area chosen for the dendrite analysis of Golgi-Cox stained neurons. A sagittal and a coronal view to the brain are shown. The darker orange area indicates the infarct. The red arrows indicate the areas chosen for neuron analysis.
Supplemental References
