Successful Regeneration After Experimental Stroke by Granulocyte-Colony Stimulating Factor Is Not Further Enhanced by Constraint-Induced Movement Therapy Either in Concurrent or in Sequential Combination Therapy

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Background and Purpose—Both application of granulocyte-colony stimulating factor (G-CSF) and constraint-induced movement therapy (CIMT) have been shown to improve outcome after experimental stroke. The aim of the present study was to determine whether concurrent or sequential combination of both therapies will further enhance therapeutic benefit and whether specific modifications in the abundance of various neurotransmitter receptors do occur.

Methods—Adult male Wistar rats were subjected to photothrombotic ischemia and assigned to the following treatment groups (n=20 each): (1) ischemic control (saline); (2) CIMT (CIMT between poststroke Days 2 and 11; (3) G-CSF (10 μg/kg G-CSF daily between poststroke Days 2 and 11; (4) combined concurrent group (CIMT plus 10 μg/kg G-CSF daily between poststroke Days 2 and 11; and (5) combined sequential group (CIMT between poststroke Days 2 and 11 and 10 μg/kg G-CSF daily between poststroke Days 12 and 21, respectively). Rats were functionally tested before and up to 4 weeks after ischemia. Quantitative receptor autoradiography was performed for N-methyl-D-aspartate, AMPA, and GABA<sub>A</sub> receptors.

Results—Significant improvement of functional outcome was seen in all groups treated with G-CSF alone and in either combination with CIMT, whereas CIMT alone failed to enhance recovery. Infarct sizes and remaining cortical tissue did not differ in the various treatment groups. Failure of significant benefit in the CIMT group was associated with a shift toward inhibition in perilesional and remote cortical regions.

Conclusions—Our findings disclose G-CSF as the major player for enhanced recovery after experimental stroke, preventing a shift toward inhibition as seen in the CIMT group. (Stroke. 2012;43:00-00.)

Key Words: CIMT ■ G-CSF ■ FAU ■ receptor autoradiography ■ regeneration

Although most patients with stroke show some degree of spontaneous neurological recovery, there is urgent need to identify therapeutic strategies to strengthen poststroke repair mechanisms. There is evidence from studies, both in humans and rodents, that divergent therapeutic approaches such as constraint-induced movement therapy on the one hand and pharmacological treatment with growth factors on the other hand can significantly improve neurological outcome after cerebral ischemia. Whether the combination of such different therapeutic approaches will have a synergistic and more beneficial effect has hardly ever been investigated systematically. Determination of the optimal timeframe for therapeutic intervention after stroke is another major problem, potentiated when combining 2 different therapies.

Therefore, one major goal of the present study was to clarify whether combined treatment with constraint-induced movement therapy (CIMT) and granulocyte-colony stimulating factor (G-CSF) will enhance the therapeutic benefit and, if so, whether concurrent or sequential therapy will result in the best behavioral outcome. CIMT has been shown to increase postischemic plastic processes and to enhance neureogenesis, and we know from recent studies in our laboratories that G-CSF promotes differentiation of neuronal precursor cells and increases their survival, even when treatment...
starts with some delay. Therefore, we designed our study to start with CIMT followed by G-CSF treatment. Because there is evidence of an altered posts ischemic balance between excitatory and inhibitory neurotransmitter receptors as one underlying molecular mechanism, we analyzed postischemic ligand binding densities of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and γ-aminobutyric acid Type A (GABA<sub>A</sub>) receptors in perilesional and remote cortical areas.

**Methods**

**Experimental Groups**

All animal procedures were carried out according to the guidelines of the German animal protection law and approved by government authorities. Experiments were performed on adult male Wistar rats (Charles River, Sulzfeld, Germany; 280–320 g body weight), which had free access to food and water throughout the experiments. Animals were randomly assigned to the following experimental groups (n = 20 each; Figure 1): (1) control group (ischemia, treatment with 0.5 mL saline 0.9% starting 48 hours after ischemia until Day 11 after ischemia); (2) CIMT group (ischemia, CIMT starting 48 hours after ischemia until Day 11 after ischemia); (3) G-CSF group (ischemia, treatment with 10 μg/kg G-CSF as daily subcutaneous injection starting 48 hours after ischemia until Day 11 after ischemia); (4) combined concurrent treatment group (ischemia, treatment with 10 μg/kg G-CSF as daily subcutaneous injection and CIMT starting 48 hours after ischemia until Day 11 after ischemia); and (5) combined sequential treatment group (ischemia, treatment with 10 μg/kg G-CSF as daily subcutaneous injection starting 48 hours after ischemia until Day 11 after ischemia); and (5) combined sequential treatment group (ischemia, CIMT starting 48 hours after ischemia until Day 11 after ischemia, subsequent treatment with 10 μg/kg G-CSF as daily subcutaneous injection starting on Day 12 until Day 21 after ischemia). Randomization was carried out by the computer software Research Randomizer (Urbaniaik GC, Plous S. Research Randomizer, Version 3.0; 2011. www.randomizer.org; accessed April 22, 2011). After being operated on, all the animals were recoded by an assistant to ensure necessary blinding and a different technical assistant ensured the administration of G-CSF and saline. A total of 107 animals have been used in this study in which 7 animals died during surgeries.

**Phototrombogenic Ischemia**

Animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight; Ketanest) and xylazine hydrochloride (8 mg/kg body weight; Ceva GmbH), and anesthesia was maintained if necessary. Although ketamine has been shown to exhibit neuroprotective activity, it was impossible to use the preferred inhalation anesthesia (ie, nitrous oxide, oxygen, halothane, and isoflurane) because phototrombogenic stroke was induced by applying laser light to the skull. Furthermore, because animals of all experimental groups were anesthetized with ketamine, it was to be expected that possible neuroprotective effects would be present in all the animals. The left femoral vein was cannulated with a PE-50 tube for Rose Bengal infusion. During the experiment, rectal temperature was maintained at 37°C by a thermostat-controlled heating pad (Föhr Medical Instruments). Animals were placed in a stereotactic frame, and the scalp was incised for exposure of the skull surface. For illumination, a laser spot of 8 mm in diameter (G Laser Technologies) was stereotactically placed onto the skull of the right hemisphere 0.5 mm anterior to the bregma and 4 mm lateral from the midline and then the skull was laser-illuminated for 20 minutes. During the first 4 minutes of illumination, the dye Rose Bengal (0.3 mL/kg body weight, 10 mg/mL saline) was injected intravenously. After surgery, the catheter was removed and the animals were allowed to recover from anesthesia.

**Constraint-Induced Movement Therapy**

Forty-eight hours after induction of ischemia, CIMT-treated animals were fitted with 1-sleeve plaster casts. Animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight; Ketanest) and xylazine hydrochloride (8 mg/kg body weight; Ceva GmbH). The upper torso and the ipsilateral right forelimb were wrapped in soft felt and then the forelimb was positioned in a naturally retracted position against the animal’s sternum. A single plaster of Paris strip was wrapped around the immobilized limb and upper torso.

**Functional Testing**

In all animals, behavioral tests were performed before ischemia (baseline) as well as on Days 1, 12, 21, and 28 after ischemia (Figure 1) by an investigator (K.D.) blinded to the experimental groups. Motor deficits were examined by means of the cylinder test. For this purpose, the rats were placed into a transparent cylinder and videotaped from below for 3 minutes. Spontaneous wall and ground touches of the forepaws were counted. An asymmetry score calculated for each animal was expressed by the following ratio: wall and ground touches of the ipsilateral forepaw—wall and ground touches of the contralateral forepaw/wall and ground touches of the ipsilateral forepaw + wall and ground touches of the contralateral forepaw.

Somatosensory deficits were measured using the adhesive-tape removal test. Two small pieces of adhesive-backed paper dots of equal size used as bilateral tactile stimuli occupying the distal–radial region were placed at the wrist of each forelimb. The time to remove each stimulus was documented. An asymmetry score calculated for each animal was expressed by the following ratio: time to remove the ipsilateral dot—time to remove the contralateral dot/time to remove the ipsilateral dot+time to remove the contralateral dot. For more details, see online-only Data Supplement (http://stroke.ahajournals.org).

**Tissue Processing**

The brains of 10 rats from each treatment group were used for both volumetry and receptor autoradiography. Rats were euthanized 28 days after ischemia. Brains were rapidly removed, frozen in isopentane at –30°C for 10 minutes, and stored at –80°C until analysis. For assessment of the infarct area and receptor autoradiography analysis, serial cryostat sections at –20°C and of 12-μm thickness were cut at the level of the striatum and the hippocampus, respectively, and mounted on TESPA-coated slides.

![Figure 1. Experimental design and treatment groups. G-CSF indicates granulocyte-colony stimulating factor; CIMT, constraint-induced movement therapy; G-CSF + CIMT, combined concurrent treatment.](http://stroke.ahajournals.org/)

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Brain Tissue Calculations

Because photothrombosis only produces well-defined cortical infarcts, lesion volumes were estimated by measurement of the maximum diameter and measurement of the maximum infarct area on the slides, as previously described. Because infarct size and tissue loss do not always match, an additional analysis of the remaining cortical tissue was performed at the level of the largest infarct extension.

Receptor Autoradiography

Quantitative in vitro receptor autoradiography studies were performed using [3H]MK-801, [3H]AMPA, [3H]muscimol, as ligands for NMDA, AMPA, and GABAA receptors, respectively. Unspecific binding was determined by coincubation of alternating sections with labeled ligands and an excess of appropriate unlabeled competitor. For further details, see online-only Data Supplement. Quantitative analysis of radioactivity was performed in the infarct border zone and the cortical perilesional Par1 region as well as in the remote cortical areas Fr1, Par1, and Par2, both ipsilateral and contralateral to the lesion (Figure 2). The respective brain areas were identified according to the atlas by Paxinos. Regions of interest were marked on the monitor if possible and the gray values automatically assessed by the imaging software. In all cases, gray value of the corpus callosum was used as reference value for nonspecific binding and subtracted from the total binding.

Statistical Analysis

Values are presented as mean±SEM. Statistical analyses were calculated using the Statistical Package of Social Sciences (Version 15.0; SPSS Inc, Chicago, IL). The normality distribution of the data were verified by Shapiro-Wilk test (P>0.05). Nonnormal distribution in some cases was due to single extreme values as assessed by graphical examination of the histograms. Sensorimotor measurements were analyzed with 2-way repeated-measures analysis of variance followed by the Fisher protected least significance difference test. An α error rate of 0.05 was taken as the criterion for significance. For analysis of ligand binding data, 1-way analysis of variance was used for comparison between the different therapy groups by calculating comparative values for each region and hemisphere. Significant group effects between different groups were confirmed by Fisher least significant difference post hoc test. For all tests, P<0.05 was considered statistically significant. Due to the exploratory design of the receptor autoradiography, no adjustments for multiple comparisons were made there for the calculated analysis of variance. However, the post hoc tests following significant analysis of variance were corrected for multiple comparisons. Analysis was performed using the general statistics module of Analyze-it for Microsoft Excel (Analyze-it Software Ltd, Leeds, UK). Values were normalized to the control groups and presented as mean±SEM. The investigator carrying out the analysis was blinded to group identity.

Results

Functional Testing

Because the photothrombotic stroke model causes distinct deficits in somatosensory and motor functions, we tested for both qualities. Motor recovery was assessed by the cylinder test and somatosensory recovery by means of the adhesive-tape removal test (Figure 3).

The animals treated with G-CSF alone as well as those treated with the 2 combinations of G-CSF and CIMT (concurrent and sequential, respectively) had a more favorable motor recovery compared with the control group (Figure 3A). This effect reached significance (P<0.05, Fisher least significant difference post hoc test after significant 2-way repeated-measures analysis of variance) on Day 21 after ischemia for the 2 combined treatment groups and on Day 28 after ischemia for the G-CSF-only group. These effects persisted until the end of the experiment (Day 28) for all aforementioned treatment groups. CIMT treatment alone, however, did not significantly improve motor recovery compared with the control group.

In analogy to the improved motor function, the animals treated with G-CSF alone or with either of the G-CSF-CIMT combinations exhibited enhanced somatosensory recovery compared with the control group (Figure 3B). This effect reached significance (P<0.05, Fisher least significant difference post hoc test after significant 2-way repeated-measures analysis of variance) on Day 12 after ischemia for the combined concurrent treatment group and on Day 21 after ischemia for the combined sequential treatment group as well as for the G-CSF-only group. These effects persisted until the end of the experiment (Day 28). Exclusive CIMT treatment, in contrast, did not improve somatosensory recovery compared with the control group.

Brain Tissue Calculation

Infarct volumes were 3.63±1.24 mm³ (control), 4.57±1.09 mm³ (CIMT), 4.76±1.89 mm³ (G-CSF), 3.98±1.14 mm³ (combined concurrent), and 4.46±1.25 mm³ (combined sequential), respectively. Among the individual groups, the differences were not significant (means±SEM; P>0.05, analysis of variance). Analysis of the remaining cortical tissue did not
Receptor Autoradiography

\[^{[H]}MK-801\] Ligand Binding

The various treatments after photothrombotic ischemia were associated with complex alterations of \[^{[H]}MK-801\] ligand binding densities. In the ischemic border zone, binding values were mostly increased, whereas in the perilesional Par1 region, binding densities were predominantly lowered compared with ischemic controls. However, no treatment-specific pattern could be observed. In the corresponding contralateral Par1 region \[^{[H]}MK-801\], labeling was significantly enhanced in Layers II to V in the combined sequential group. In the remote cortical areas Par1, Par2, and Fr1, no significant differences among the various treatment paradigms were detectable apart from a few single outliers, both ipsi- and contralateral (for details see Supplemental Figure I).

\[^{[H]}AMPA\] Ligand Binding

AMPA binding values revealed a more treatment-specific pattern. In the border zone and the perilesional Par1 region, the deeper layers of all treatment groups except for the combined sequential treatment group showed significantly increased AMPA binding densities. A similar pattern, but in this case throughout all the layers, was seen contralaterally. In all remote areas, the lowest binding densities were mostly revealed in the combined sequential group. In the contralateral remote Par2 region, G-CSF treatment showed significantly enhanced labeling compared with controls (for details, see Supplemental Figure II).

\[^{[H]}Muscimol\] Ligand Binding

Binding to inhibitory GABA\(_{\text{A}}\) receptors revealed the most specific treatment patterns. Particularly CIMT was associated with the highest binding values in perilesional and remote areas, reaching significance in the border zone compared with controls and in many layers of the ipsi- and contralateral remote areas compared with the combined sequential therapy paradigm (Figure 5). In the border zone, \[^{[H]}Muscimol\] binding was also significantly enhanced in the deeper layers of the combined concurrent group.

Ratio Between Ligand Binding to Excitatory and Inhibitory Receptors

In view of the complex alterations of posts ischemic ligand binding in the various treatment groups and to get an impression of the net shift in the balance of excitation and inhibition, the ratio between \[^{[H]}AMPA\] plus \[^{[H]}MK-801\] versus \[^{[H]}Muscimol\] was calculated. Although in the border zone and perilesional Par1 region, the ratio was largely within the same range for all treatment groups, the ratio was lowest in the remote Par1, Par2, and Fr1 regions in CIMT-treated rats (Figure 6).

Discussion

Our present study was designed to determine whether the combination of 2 divergent therapies, that is, CIMT and treatment with G-CSF, would result in improved neurological outcome after ischemic stroke compared with the respective uncombined therapies. We were further interested in clarifying whether the effects of the combined therapy depend on a strict temporal sequence of application. The major finding revealed by our tests was the fact that G-CSF alone, but not CIMT alone, significantly improved functional outcome both for motor and somatosensory recovery. G-CSF treatment in combination with CIMT, however, did not result in any enhanced neurological benefit either in concurrent or in sequential application compared with the G-CSF-only therapy. Importantly, these effects cannot be attributed to any significant differences in infarct size or reductions of the remaining cortical tissue between the various treatment groups (Figure 4). As one potential underlying molecular mechanism, we identified an increased and widespread shift
in the balance of excitatory and inhibitory neurotransmitter receptors toward inhibition in the CIMT-only group. Although CIMT, like forced arm use, has been shown to improve sensorimotor function after stroke in humans, the results from experimental studies are rather controversial. In general, the larger the lesion, the higher the risk to exaggerate the damage by forced therapy. In ischemia models with only small lesions such as photothermotic stroke, early CIMT treatment seems to be safe but may be without any significant benefit. In a previous study, we could demonstrate that treatment with another growth factor, the brain-derived neurotrophic factor, outmatched physical therapy with CIMT. Therefore, we hypothesized that the combination of CIMT with a growth factor could overcome this problem and finally result in an additive beneficial effect. The regenerative potential of G-CSF, apart from its neuroprotective power in the acute phase of stroke, has been shown in various studies. In the present study, we can show that postischemic G-CSF treatment, in fact, causes an increased functional outcome concerning motor and sensorimotor qualities. However, its combination with CIMT, by concurrent application as well as by sequential treatment, failed to further enhance the beneficial effect. As already shown in our recent study, CIMT alone in contrast to G-CSF was not able to significantly improve behavioral outcome compared with controls, although results of CIMT treatment did not significantly differ from the various G-CSF groups. Although for other growth factors, like epidermal growth factor and erythropoietin, sequential application of both factors has proved to be a prerequisite for improvement of the neurological outcome after experimental stroke, our data clearly indicate that the major player for successful postischemic regeneration is G-CSF but not CIMT.

Several mechanisms underlying the preregenerative postischemic effect of G-CSF are under discussion, including enhancement of neurogenesis and mobilization of bone marrow stem cells. In our present study, we checked for a specific modulation of postischemic excitatory and inhibitory neurotransmitters as one potential component of the plastic changes induced by G-CSF. In the acute postischemic phase, G-CSF prevented loss of inhibitory GABA<sub>A</sub> receptors protecting neurons at risk from excitotoxic damage. In the chronic phase, the postischemic modifications of receptor-binding densities in the various ipsi- and contralateral perilesional and remote cortical regions yielded very complex and heterogeneous regulation patterns. Therefore, to get an impression of the net shift in the balance between excitatory glutamate and the inhibitory GABA<sub>A</sub> receptor, we calculated the ratio between binding of <sup>3</sup>H]MK-801 plus <sup>3</sup>H]AMPA versus <sup>3</sup>H]muscimol. This approximation resulted in a surprisingly specific pattern of binding densities, which correlated with failure in significant functional recovery, that is, CIMT treatment tended to be associated with a shift toward inhibition in most regions analyzed. This finding is well in line with results from our recent study comparing postischemic receptor regulation after CIMT and brain-derived neurotrophic factor treatment. Similarly, the best functional outcome was seen after brain-derived neurotrophic factor treatment and correlated with a relative shift toward excitation. These findings further corroborate the hypothesis that, during the chronic phase after stroke, postischemic hyperexcitability may enhance functional outcome in the long run. Although excitotoxic overactivation of glutamate receptors is assumed to play a key role in triggering neuronal death in the early postischemic phase, which can be prevented by NMDA receptor antagonists, it has now become clear that this period is surprisingly short. Using a mouse model of head injury, Biegon and colleagues could convincingly demonstrate that hyperactivation of NMDA receptors occurred only during the first hour after the excitotoxic stimulus but then was followed by a profound and long-lasting functional loss. Consequently, stimulation of NMDA receptors 24 and 48 hours after injury significantly improved functional outcome. However, an absolute downregulation of NMDA receptor binding values after transient focal ischemia has recently been described and is thought to be the correlate of postischemic cognitive deficits, which cannot be explained by the infarct alone.22

![Figure 4](http://stroke.ahajournals.org/)

**Figure 4.** Remaining cortical tissue. No significant differences were detectable between the various experimental groups. Absolute values are presented at the left and differences in percent at the right side. CIMT+G-CSF indicates concurrent treatment; CIMT/G-CSF, sequential treatment; means ± SEM; *P* > 0.05, ANOVA. CIMT indicates constraint-induced movement therapy G-CSF, granulocyte-colony stimulating factor; ANOVA, analysis of variance.
Figure 5. [3H]Muscimol ligand binding in perilesional, contralateral, and more remote cortical areas. Although complex alterations are present, CIMT treatment is mostly associated with the highest binding values (C, F, G, C1, C2; significant difference compared with control, CIMT, G-CSF, combined concurrent, or combined sequential group, respectively; means±SEM; \(P<0.05\); ANOVA all pairwise, LSD; compare with Figure 6). CIMT indicates constraint-induced movement therapy; G-CSF, granulocyte-colony stimulating factor; ANOVA, analysis of variance; LSD, least significant difference.
Ratio between ligand binding to excitatory and inhibitory receptors. To get an impression of the net shift in the balance of excitation and inhibition, the ratio between \([3H]MK-801\) plus \([3H]AMPA\) vs \([3H]muscimol\) was calculated. Although in the border zone and the perilesional Par1 region, the ratio was largely within the same range for all treatment groups, the ratio was the lowest in the remote Par1, Par2, and Fr1 regions of CIMT-treated rats (means). AMPA indicates \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CIMT, contraint-induced movement therapy.

Figure 6. Ratio between ligand binding to excitatory and inhibitory receptors. To get an impression of the net shift in the balance of excitation and inhibition, the ratio between \([3H]MK-801\) plus \([3H]AMPA\) vs \([3H]muscimol\) was calculated. Although in the border zone and the perilesional Par1 region, the ratio was largely within the same range for all treatment groups, the ratio was the lowest in the remote Par1, Par2, and Fr1 regions of CIMT-treated rats (means). AMPA indicates \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CIMT, contraint-induced movement therapy.
Conclusions

Treatment with G-CSF significantly improved functional outcome after photothrombotic stroke. Its combination with CIMT, either by concurrent application or by sequential treatment, failed to further enhance the beneficial effect, thus suggesting that G-CSF is the major player for successful regeneration. The failure of CIMT treatment was associated with a relative shift from excitation toward inhibition, which may represent one important component inhibiting therapeutic success.

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Disclosures

C.S. and W.R.S. are inventors on the patent application “Hematopoietic factors for treatment of neurological condition,” including stroke and other diseases. Recently a part of the application (ALS) was granted. C.S. and W.R.S. transferred their rights to Sygnis and received a minor financial compensation upfront. In case of efficacy, compensation in the form of honoraria as Principal Investigator was granted. C.S. and W.R.S. transferred their rights to Sygnis and W.R.S. received compensation in the form of honoraria as Principal Investigator of the AX200 for the Treatment of Ischemic Stroke (AXIS) I study.

References

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SUPPLEMENTAL MATERIAL

Successful regeneration after experimental stroke by G-CSF is not further enhanced by CIMT neither in concurrent nor in sequential combination therapy

Supplemental Methods

Functional Testing

Motor deficits were examined by means of the cylinder test. For this purpose, the rats were placed into a transparent cylinder (16 cm in diameter, 21 cm high) and videotaped from below for 3 minutes. Spontaneous wall and ground touches of the forepaws were counted. Scoring by an experimenter blinded to the condition of the animal was carried out using a digital video player with slow-motion and clear stop-frame capacity. An asymmetry score calculated for each animal was expressed by the following ratio: wall and ground touches of the ipsilateral forepaw minus wall and ground touches of the contralateral forepaw / wall and ground touches of the ipsilateral forepaw plus wall and ground touches of the contralateral forepaw. Somatosensory deficits were measured using the adhesive-tape removal test. All rats were familiarized with the testing environment. In the initial test, 2 small pieces of adhesive-backed paper dots of equal size (113.1 mm²) used as bilateral tactile stimuli occupying the distal–radial region were placed at the wrist of each forelimb and, then, the rats were returned to their cage. The time to remove each stimulus from the forelimbs was documented by the mean of 3 trials per day for each forepaw. Individual trials were separated by a time shift of 60 seconds. Before surgery, the animals were trained for 3 days. Once the rats were able to remove the dots within 10 seconds, they were subjected to ischemia. An asymmetry score calculated for each animal was expressed by the following ratio: time to remove ipsilateral dot minus time to remove contralateral dot / time to remove ipsilateral dot plus time to remove contralateral dot.

Receptor Autoradiography

Quantitative in-vitro receptor autoradiography studies were performed using [3H]MK-801, [3H]AMPA, [3H]muscimol, as ligands for NMDA, AMPA and GABA_A receptors, respectively. Ligands were purchased from Perkin Elmer, Inc. (Boston, MA, USA) and American Radiolabeled Chemicals. (St. Louis, MO, USA). In brief, to remove endogenous ligands, sections were incubated with the respective buffer prior to ligand incubation with [3H]MK-801, [3H]AMPA and [3H]muscimol. In order to demonstrate the maximal binding of [3H]MK-801 to NMDA receptors, the binding assay was performed in a magnesium- and zinc-free solution (50 mM Tris-HCl buffer, pH 7.2) and in the presence of 30µM glycine and 50µM spermidine with 5nM [3H]MK-801 (specific activity 27.5 Ci/mmol) at 22°C for 60 min. Washing in cold buffer (2 x 5 min) and in H2O (2 sec) terminated the incubation. AMPA receptors were labeled with 10 nM [3H]AMPA (specific activity 42.1 Ci/mmol) in 50mM Tris-acetate buffer (pH 7.2, containing 100 mM KSCN) for 45 min at 4°C. Incubation was terminated by rinsing (3 x 4 sec) in cold buffer and by post-fixation with rinses (2 x 2 sec) in acetone/glutaraldehyde (100 : 2.5) solution. GABA_A receptors were incubated with 3nM [3H]muscimol (specific activity 25.5 Ci/mmol) in 50mM Tris-citrate buffer (pH 7.0) for 40 min at 4° C. Incubation was terminated by rinsing (3 x 4 sec) in cold buffer. After the final rinsing procedure, the [3H]AMPA incubated slides had to be placed under hot air for 1-2 sec while all other slides were carefully dried in a stream of cool air. Air-dried, tritium-labeled sections were co-exposed with [3H]plastic standards (Autoradiographic [3H] Microscales®; Amersham Biosciences, Freiburg, Germany) to a tritium-sensitive phosphor screen for 2 weeks. Autoradiographies were analyzed with the Cylone® Plus Storage Phosphor System (Perkin Elmer Inc., Shelton, CT, USA) and digitized with the MCID image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada). Gray value images of the co-exposed plastic standards were used for calibration.

Supplemental Figures S1 and S2
[\textsuperscript{3}H]MK-801 ligand binding

ipsilateral           contralateral

border zone

Par1 (perilesional)

Par1 (remote)

Par2

Fr1

CIMT | G-CSF | CIMT+G-CSF (concurrent) | G-CSF+CIMT (sequential)
Suppl. Fig. 1: [³H]MK-801 ligand binding. The various treatments after photothrombotic ischemia were associated with complex alterations of NMDA receptor binding densities. In the ischemic border zone and in the perilesional Par1 no treatment specific pattern could be observed. In the corresponding contralateral Par1 region [³H]MK-801 labeling was significantly enhanced in layers II to V in the combined sequential group. In remote cortical areas Par1, Par2 and Fr1 again no significant differences between the various treatment paradigms were detectable apart from a few single outliers both ipsi- and contralateral (C, F, G, C1, C2: significant difference compared with control, CIMT, G-CSF, combined concurrent, or combined sequential group, respectively; means±SEM; p<0.05; ANOVA all pairwise, LSD; c.f. Fig. 6).
Su ppl. Fig. 2: $[^3H]$AMPA ligand binding. AMPA binding values showed a more treatment specific pattern. In the border zone and the perilesional Par1 the deeper layers of all treatment groups but the combined sequential arm showed significantly increased AMPA binding densities. A similar pattern but throughout all layers was seen contralaterally. In all remote areas the combined sequential group revealed mostly the lowest binding densities. In the contralateral remote Par2 region G-CSF treatment showed significantly enhanced labeling compared to controls (C, F, G, C1, C2: significant difference compared with control, CIMT, G-CSF, combined concurrent, or combined sequential group, respectively; means±SEM; p<0.05; ANOVA all pairwise, LSD; c.f. Fig. 6).