Meta-Analysis of the Efficacy of Granulocyte-Colony Stimulating Factor in Animal Models of Focal Cerebral Ischemia

Jens Minnerup, MD; Jan Heidrich, MD, MSc; Jürgen Wellmann, PhD; Andreas Rogalewski, MD; Armin Schneider, MD; Wolf-Rüdiger Schäbitz, MD

**Background and Purpose**—Recent reports have described the efficacy of the hematopoietic growth factor granulocyte-colony stimulating factor (G-CSF) in animal stroke models. Early clinical multicenter trials evaluating the effect of G-CSF in acute stroke and pilot clinical trials for the subacute phase are ongoing. To guide further development, a meta-analysis was performed to assess the effects of G-CSF on infarct size and sensorimotor deficits.

**Methods**—Using electronic and manual searches of the literature, we identified studies describing the efficacy of G-CSF in animal models of focal cerebral ischemia. Two reviewers independently selected studies and extracted data on study quality, G-CSF doses, time of administration, and outcome measured as infarct volume and/or sensorimotor deficit. Data from all studies were pooled by meta-regression analyses.

**Results**—Thirteen studies including 277 animals for infarct size calculation and 258 animals for assessment of sensorimotor deficit met the criteria for inclusion. Overall efficacy of G-CSF regarding infarct size reduction was 42%. Meta-regression analysis revealed a 0.8% \( (P<0.0001) \) decrease in infarct size per 1-μg/kg increase in G-CSF dose when applied within the first 6 hours and a 2.1% \( (P<0.0001) \) decrease when applied later than 6 hours after induction of ischemia with a significant \( (P=0.0004) \) greater infarct size reduction after delayed treatment. Sensorimotor deficits categorized into 3 subgroups improved between 24% and 40%.

**Conclusions**—Our findings consolidate G-CSF as a drug that both reduces infarct size and enhances functional recovery. These effects are presumably dose dependent. In contrast to most other neuroprotectants, a beneficial outcome may also be achieved when treatment is delayed. (Stroke. 2008;39:1855-1861.)

**Key Words:** stroke ■ hematopoietic cell growth factors ■ meta-analysis ■ animal models

Granulocyte-colony stimulating factor (G-CSF) is a 20-kDa glycoprotein that functions as a growth factor responsible for mobilization and differentiation of hematopoietic stem cells. G-CSF has been in clinical use for >10 years to treat neutropenia in cancer patients and to mobilize stem cells for grafting procedures in patients with hematologic malignancies. Recently, functions of G-CSF in the healthy and diseased brain have been revealed. Under ischemic conditions, G-CSF inhibits programmed neuronal cell death and stimulates neural progenitor cell differentiation. These mechanisms and others, including immunomodulation and blood vessel plasticity, are currently thought to be responsible for infarct size reduction and improved functional outcome in rodent stroke models. Owing to this efficacy in the acute and chronic stroke situation and its multimodal action within the ischemic cascade, G-CSF appears to be an ideal candidate for further clinical drug development.

Unfortunately, the efficacy of candidate neuroprotectants in animal experiments does not reliably predict efficacy in stroke patients. At least 912 drugs have been tested in animal models; 97 of these have been tested in clinical trials; and all of them have failed so far. This discrepancy between efficacy in animal models and that in clinical trials has been discussed exhaustively. Potential reasons include the use of inappropriate animals (animals are young and healthy, whereas patients are typically older and have various comorbidities, such as diabetes or hypertension), the use of inappropriate stroke models (anesthesia with neuroprotective properties, hypothermia in animals versus normothermia in patients), or low study quality due to unblinded assessment of animals and absent randomization of treatment allocation.

To enhance the likelihood of the successful development of new stroke therapies, the Stroke Therapy Academic Industry Roundtable (STAIR) was founded to improve preclinical...
development of candidate drugs. This group made a series of specific recommendations regarding the preclinical evaluation of neuroprotective drugs before proceeding into clinical stroke trials. G-CSF widely fulfills the STAIR criteria as indicated in a recent review. To improve the significance of animal data beyond application of the STAIR criteria, systematic meta-analyses of candidate neuroprotectants in animal experiments may be conducted.17–20 To obtain an overall impression of G-CSF’s efficacy in recently published preclinical studies and for potential guidance of further clinical studies, a meta-analysis was performed. This included a description of the impact of study characteristics on efficacy and the conditions under which maximum efficacy can be achieved. Because of heterogeneity of the interventions, we sought to investigate the combined effect of the aforementioned elements on infarct volume and functional outcome, and for this purpose, we used a meta-regression technique.21

Materials and Methods

Retrieving the Literature

We searched the databases PUBMED (1974 to September 2007), EMBASE, and BIOSIS (2000 to September 2007). This strategy included the words “G-CSF” OR “granulocyte-colony stimulating factor” AND “ischemia” OR “stroke” OR “infarct.” We included only articles in English and German. The bibliographies of relevant articles were further cross-checked to search for articles not referenced in the aforementioned databases. We also manually searched published abstracts of scientific meetings and requested that the senior authors of identified publications provide references of other studies.

Selection of Studies and Data Extraction

All studies were included in the analysis in which outcome was measured as volume of infarction or in which sensorimotor deficits were assessed. Studies of rodents with focal cerebral ischemia that received G-CSF or vehicle starting at or after induction of ischemia were evaluated. We extracted data of mean outcome, standard deviation, and number of animals in treatment and vehicle groups. When only standard errors were presented, they were converted to standard deviations. For comparisons of infarct volumes, the total dose of G-CSF in the first 24 hours was considered, and volumes measured at least 24 hours after induction of ischemia were included.

Because meta-analysis requires a reasonable number of tests, only sensorimotor tests were considered, which represented the most frequently used tests for measuring neurobehavioral outcome in the different studies included in the analysis. We categorized sensorimotor tests into 3 clinically meaningful groups: the Rotarod for running function, neuroscore as gross neurologic deficit score, and limb function (including the foot fault test, adhesive tape removal test, and modified limb placing test). When sensorimotor deficits were assessed at different times, only the last time point was included. For comparison of functional outcome data, the cumulative G-CSF dose was calculated. When values for data were expressed graphically only, we contacted the authors and asked them to provide the required additional data. If requested data were not provided, values were defined by using Corel Draw version 12 (Corel Corporate Communications, Ottawa, Canada).

Quality Assessment

We evaluated the methodological quality of the included studies by applying a modified scale used by Horn et al in a systematic review of nimodipine in experimental focal cerebral ischemia. Eleven aspects of each study were evaluated, as follows: (1) the dose-response relation, (2) randomization of the experiment, (3) optimal time window of treatment, (4) monitoring of physiologic parameters (temperature, glucose level, or blood pressure), (5) blinded outcome assessment, (6) assessment of at least 2 outcomes (infarct size and 1 functional outcome), (7) outcome assessment in the acute phase (1 to 7 days), (8) outcome assessment in the chronic phase (beyond 7 days), (9) appropriate animal model (aged, diabetic, or hypertensive), (10) compliance with animal welfare regulations, and (11) statement of potential conflict of interests. The 11 quality checklist items were categorized into 3 categories (category I, 8 to 11 items; category II, 4 to 7 items; and category III, 0 to 3 items). Two authors (J.M. and W.-R.S.) independently extracted data and assessed study quality. Disagreements were solved after discussion of the study details.

Statistical Analysis

The published results from the studies were displayed graphically and summarized by means of meta-analysis techniques separately for each of the end points, infarct volume and the 3 types of functional tests. In addition, mean infarct volume in treated animals was plotted against infarct volume in the corresponding control group (L’Abbé plot).

For the remaining analyses, ratios of the results in treated and untreated animals were investigated.22 Thus, changes in results due to treatment are expressed as percentages and are therefore comparable across species regarding infarct volume and different measurement scales for the functional tests. Actually, this approach amounts to processing of the logarithms of published mean values. The corresponding standard errors, which are needed to compute weights for the subsequent computations, were approximated by means of the delta method.23 The CIs of ratios for treated and untreated animals, which are displayed in forest plots, have been approximated by Fieller’s method.24

The published results were summarized under the assumption of a random-effects model by standard meta-analytic methods.25 For a first analysis, a single ratio of treated and untreated animals was computed for each trial. If there was >1 treatment group, the corresponding means were averaged by using weights according to the inverse variance method. A test for homogeneity across trials was performed. A weighted mean of the logarithms of these ratios was computed, wherein the weights were determined according to the random-effects approach of DerSimonian and Laird. These results were transformed into weighted geometric means, and CIs and were displayed in forest plots. Finally, percentage changes in the outcomes due to dosage and timing of G-CSF treatment were estimated by applying random-effects meta-regression26 to the logarithms of the results of each treatment and control group. Dosage was entered as fixed, linear term and different slopes were allowed for G-CSF administered <6 hours or 6 hours and more after the onset of ischemia. A random-intercept parameter was allowed for each trial. All analyses were performed with SAS version 9.1 (SAS Inc, Cary, NC). Probability values <0.05 were considered significant.

Results

Study Inclusion and Study Characteristics

From the electronic search, 19 abstracts meeting the eligibility criteria were retrieved. One of the identified studies reported simultaneous treatment with G-CSF and stem cell factor and was therefore excluded. Another study was excluded because the article was written in Chinese, and the authors were unable to provide us with the requested data in English. The study of Yata et al was excluded because it used a neonatal hypoxia/ischemia model. Zhao et al reported a beneficial effect of G-CSF when administered >3 months after the onset of ischemia. This study was excluded because its time of administration differed widely from that of all other included studies, and therefore, meta-regression analysis could not be properly applied. One study was excluded for using genetically modified animals (immunodeficient mice) and for measuring infarct sizes as areas and not volumes. Another study was also excluded for
measuring infarct areas only. Manual searching, searching of bibliographies of relevant articles, and requests to senior authors did not identify any further considerable data. Therefore, the meta-analysis is based on the data of 13 articles, which included 34 comparisons (Table 1). Outcome was assessed in a total of 277 animals for infarct size calculation and of 258 animals for evaluation of functional recovery. Combinations of drug dose and time of administration used in the studies were presented separately for comparisons of infarct volumes and sensorimotor deficits (see supplemental Figure I, available online at http://stroke.ahajournals.org). Obviously, the data points are not evenly distributed across the range of possible combinations of the 2 variables, pointing to considerable heterogeneity in study characteristics.

<table>
<thead>
<tr>
<th>First Author, Year of Publication</th>
<th>Species</th>
<th>Stroke Model</th>
<th>Method of Administration</th>
<th>Outcome Measures, n (Treated/Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schäbitz 2003</td>
<td>Rat</td>
<td>MCAO (1.5 h)</td>
<td>0.5 h after occlusion, 60 μg/kg IV within 24 h</td>
<td>Infarct volume (12/12)</td>
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<tr>
<td>Six 2003</td>
<td>Mouse</td>
<td>MCAO (1 h)</td>
<td>24 h after occlusion, 50 μg/kg SC within 24 h</td>
<td>Infarct volume (9/3)</td>
</tr>
<tr>
<td>Shyu 2004</td>
<td>Rat</td>
<td>MCAO (1.5 h)</td>
<td>24 h after occlusion, 50 μg/kg SC within 24 h</td>
<td>Infarct volume (12/12)</td>
</tr>
<tr>
<td>Gibson 2005</td>
<td>Mouse</td>
<td>MCAO (1 h)</td>
<td>1 h after occlusion, 50 μg/kg within 24 h, total of 50 μg/kg SC</td>
<td>Infarct volume (5/5), Rotarod (12/15), food fault test (12/15)</td>
</tr>
<tr>
<td>Gibson 2005</td>
<td>Mouse</td>
<td>Permanent MCAO</td>
<td>Immediately after occlusion, 50 μg/kg SC within 24 h</td>
<td>Infarct volume (6/6)</td>
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<tr>
<td>Lee 2005</td>
<td>Rat</td>
<td>MCAO (1.5 h)</td>
<td>2, 24, 96, or 168 h after occlusion, 50 μg/kg IP in first 24 h, total of 150 μg/kg IP</td>
<td>Infarct volume (12/12), modified limb placing test (15/15)</td>
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<tr>
<td>Schneider 2005</td>
<td>Rat (a)</td>
<td>MCAO (1.5 h)</td>
<td>2 h after occlusion, 60 μg/kg IV within 24 h</td>
<td>Infarct volume (10/7)</td>
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<td></td>
<td>Mouse (c)</td>
<td>CCA + MCAO (3 h)</td>
<td>1 h after occlusion, 50 μg/kg within 24 h, total of 50 μg/kg IV</td>
<td>Infarct volume (5/5), neuroscore (5/5)</td>
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<td></td>
<td>Rat (b)</td>
<td>Photothrombotic ischemia</td>
<td>1 h after ischemia induction, total of 75 μg/kg IV</td>
<td>Rotarod (7/7), NSS (7/7), adhesive tape removal test (7/7)</td>
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<td>Komine-Kobayashi 2006</td>
<td>Mouse</td>
<td>MCAO (1 h)</td>
<td>30 min after occlusion, 50 μg/kg within first 24 h, total of 50 μg/kg IV</td>
<td>Infarct volume (6/6) and neurologic deficit score (6/6) after 1 (a) and 3 (b) days</td>
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<tr>
<td>Schneider 2006</td>
<td>Rat (a)</td>
<td>MCAO (1.5 h)</td>
<td>4 h after occlusion, 60 μg/kg IV within 24 h</td>
<td>Infarct volume (18/24)</td>
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<td></td>
<td>Rat (b)</td>
<td>Photothrombotic ischemia</td>
<td>24 or 72 h after ischemia induction, total of 100 μg/kg IV</td>
<td>Rotarod (10/10)</td>
</tr>
<tr>
<td>Solaroglu 2006</td>
<td>Rat</td>
<td>MCAO (1.5 h)</td>
<td>1.5 h after occlusion, 50 μg/kg within 24 h, total of 150 μg/kg SC</td>
<td>Infarct volume (6/6), neurologic score (6/6)</td>
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<tr>
<td>Yanqing 2006</td>
<td>Rat</td>
<td>MCAO (1 h)</td>
<td>6 h after occlusion, 10 μg/kg within 24 h, total of 50 μg/kg SC</td>
<td>Infarct volume (6/6) and NSS (6/6) after 7 (a), 14 (b), and 21 (c) days</td>
</tr>
<tr>
<td>Sehara 2007</td>
<td>Rat</td>
<td>MCAO (1.5 h)</td>
<td>1.5 h after occlusion, 50 μg/kg within 24 h, total of 50 μg/kg SC</td>
<td>Infarct volume (5/5), neurologic score (5/5)</td>
</tr>
<tr>
<td>Zhao 2007</td>
<td>Rat (hypertensive)</td>
<td>Permanent MCA ligation</td>
<td>3 h after ligation, 50 μg/kg within 24 h, total of 300 μg/kg SC</td>
<td>Infarct volume (10/10), limb placement test (10/10), foot fault test (10/10)</td>
</tr>
</tbody>
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MCAO indicates middle cerebral artery occlusion; CCA + MCAO, combined common carotid artery/MCAO; and NSS, neurologic severity score.
Study Quality
The median of quality checklist items was 6 (range, 2 to 9). No study investigated the dose-response relation. Only 3 studies were allocated to the highest quality category. Forest plots of studies ordered by quality category did not reveal a relation between study quality and efficacy (Figures 1 and 2). There was significant heterogeneity for 3 of the 4 outcome measures (infarct volume, limb function, and neuroscore).

Overall Efficacy and Impact of Drug Dose and Time of Administration
Animals that received G-CSF had considerably smaller infarct volumes compared with placebo-treated animals. Infarct size was reduced by 42% (95% CI, 34% to 49%; Figure 1). The L’Abbé plot suggests a reduction of infarct volumes proportional to the infarct volumes of placebo-treated animals (Figure 3). Compared with placebo, G-CSF therapy reduced
limb function deficits by 40% (95% CI, 9% to 61%; Figure 2). G-CSF enhanced Rotarod running by 24% (95% CI, 14% to 35%) and improved the neuroscore by 36% (95% CI, 25% to 44%; Figure 2).

Results of a meta-regression analysis demonstrated effects of G-CSF dose and time of treatment initiation on outcome measures (Table 2). An increase in G-CSF dose of 1 μg/kg body weight for doses between 10 and 60 μg/kg body weight reduced infarct volumes by 0.84% (P<0.0001) and 2.06% (P<0.0001) for treatment initiation within the first 6 hours and later than 6 hours after the onset of ischemia, respectively. For an increase of 1 μg/kg for cumulative doses between 50 and 150 μg/kg, time on the Rotarod was extended by 2.1% (P=0.0017) and 2.2% (P<0.0001), respectively. For higher doses, limb function and neuroscore were also significantly improved (P<0.0001). A delay of treatment initiation after the first 6 hours reduced infarct volumes significantly (P=0.0004). The effects of time of treatment initiation on sensorimotor deficits were heterogeneous. As shown in Table 2, there was a significant lower neuroscore and a significant improved limb function when treatment was delayed (P=0.014 and P=0.0076, respectively), whereas time of treatment initiation showed no significant effect on Rotarod performance (P=0.9096).

Discussion

Efficacy of G-CSF in Animal Models

In the present meta-analysis, G-CSF effectively reduced both infarct volumes and sensorimotor deficits in animal models of focal cerebral ischemia. Infarct sizes were reduced by 42%, and sensorimotor deficits, which were categorized into 3 subgroups, were improved between 24% and 40%. Meta-regression identified higher doses of G-CSF to be associated with significantly smaller infarct volumes (infarct size reduction of 0.84% and 2.06% per 1 μg/kg body weight increase in dose for early and late treatment initiation). Also, Rotarod

<table>
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<th>Table 2. Effect of Dose of G-CSF on Infarct Volume and Functional Outcome by Timing of Administration</th>
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<td><strong>Effect Estimate per 1 μg/kg</strong></td>
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<tr>
<td>Infarct volume†</td>
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<tr>
<td>Rotarod test‡§</td>
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<td>Neuroscore‡§</td>
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<td>Limb function‡§</td>
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An effect estimate of 1.0 indicates no difference between treatment and control groups. An effect estimate <1.0 indicates a decline of the outcome parameter per μg/kg body weight G-CSF dose. For infarct volume, neuroscore, and limb function, a value >1.0 indicates improvement, whereas for the Rotarod test, a value >1.0 means a longer stay on the Rotarod and therefore indicates a better outcome.

* P value for difference of effect of dose between early and late administration.
†G-CSF dose is dose within 24 h after induction of cerebral ischemia.
‡G-CSF dose is cumulative dose.
§Model adjusted for species.
test, limb function and neuroscore improved significantly when G-CSF dose was increased. The information of increasing efficacy with higher doses is particularly important because conclusive, experimental, dose-finding data derived from a singular stroke study are currently unavailable. However, these findings have to be interpreted with caution, because only a few different doses were tested in the analyzed studies (supplemental Figure I). Clinical trials published to date have shown that G-CSF is safe in low doses (1 to 10 μg/kg body weight) in stroke patients.12 Currently ongoing data analysis from the multicenter study of G-CSF treatment in acute ischemic stroke (ie, AXIS) will reveal the safety of higher doses (up to 100 μg/kg body weight per day and of cumulative doses up to 180 μg/kg body weight within 3 days) in stroke patients.9

Another important aspect of stroke drugs is the therapeutic time window, which is defined as the period from the onset of ischemia to the maximal delayed time point at which a candidate drug is still effective. The present meta-regression analysis revealed that delayed treatment was as effective as early treatment initiation and may have even led to smaller infarct sizes. This result is particularly surprising, as the time window for most candidate neuroprotectants is narrow.32 The potential of a much longer time window for G-CSF compared with other stroke drugs might be explained by its multimodal actions, consisting of neuroprotective and particularly proreparative properties.4 However, the significance of this analysis is limited due to administration of the drug within the first few hours in most comparisons (see supplemental Figure 1, available online at http://stroke.ahajournals.org). The neuroscore and the limb function also showed a favorable outcome when treatment was delayed, whereas the effects of time of treatment initiation on Rotarod performance were not significant. This might have been caused by the small number of comparisons and the heterogeneity of the tests. For these reasons, findings on functional outcome should be interpreted with caution.

Methodologic Considerations
Our meta-analysis was based on a series of 13 available studies. The median quality of the studies was relatively high (6 of a maximum quality score of 11) compared with other studies of efficacy of neuroprotectants in animal stroke models.17–20 A weakness of meta-analysis of experimental studies in general is potential publication bias due to the fact that negative studies are often not published. To attenuate bias caused by this source, senior authors of identified publications were requested to report negative studies. Even through this way, negative results could not be obtained. However, we cannot rule out the possibility that some relevant data were omitted, but graphical analysis did not suggest the presence of publication bias.

The usual approach of combining results from studies of continuous outcomes that were measured on different scales is to standardize these results by the corresponding standard deviations.25 A drawback of this approach is that the results of the meta-analysis can then only be interpreted in units of standard deviations, which are not easily comprehensible. Furthermore, the standard deviations available from the studies reviewed herein were based on low numbers of animals and might therefore be subject to considerable random error. These drawbacks are avoided when one considers percentage changes in mean values. Moreover, when considering infarct volume, a multiplicative effect of G-CSF is biologically plausible and seems to have been confirmed by the L’Abbé plot (Figure 3).

Implication for Further Studies
The overall relatively good quality of the analyzed studies can be interpreted as a learning effect caused by the STAIR criteria. However, our analysis showed that only 1 study33 investigated G-CSF efficacy in animals with a comorbidity (hypertension), whereas no studies investigated G-CSF in stroke models with other conditions such as diabetes or high age. This lack of information should certainly be addressed in future studies. This meta-regression analysis suggests efficacy even after delayed treatment. However, further studies would be required to assess when the optimum time window closes and to determine the time of administration under which maximum efficacy can be achieved.

Selection of patients for future trials should resemble the situation of animal studies as close as possible to allow a successful transfer of experimental data to the clinical situation. All of the animal models that tested for G-CSF efficacy in this meta-analysis were based on middle cerebral artery occlusion models or photothrombotic ischemia in the anterior circulation. Consequently, only patients with ischemic strokes in the middle cerebral artery territory should be included. Enrolling patients with other stroke subtypes such as lacunes or subcortical white matter infarcts should be avoided. Experimental studies have shown that functional efficacy of candidate neuroprotective drugs can be more sensitively measured with subtle tests of sensorimotor function, such as the Rotarod or adhesive tape removal test instead of gross neurologic scales. In contrast, treatment efficacy in stroke patients is typically measured by relatively imprecise scales such as the modified Rankin Scale. Future studies even in the acute stroke situation could therefore be enhanced by including more sensitive measurement tools such as the Jebsen Taylor test or the Wolf Motor Function Test to detect drug-induced improvements in sensorimotor function.

Conclusions
This meta-analysis further strengthens confidence in the efficacy of G-CSF both for infarct volume reduction and for improvement of functional outcome. Furthermore, this first meta-regression analysis of a neuroprotective drug in animal stroke models reveals that effects were presumably dose related. It was shown that delayed treatment in the analyzed studies was as effective as early treatment initiation, pointing toward the recovery-enhancing effect of the drug, a property that distinguishes G-CSF from almost all other candidate stroke drugs.

Acknowledgment
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Disclosures

W.-R. Schibitz and A. Schneider are inventors on a patent application regarding the neuroprotective effects of G-CSF. All other authors have no conflicts of interest.

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