G-CSF is a potent hematopoietic factor that enhances survival and drives differentiation of myeloid lineage cells, resulting in the generation of neutrophilic granulocytes. Here, we show that G-CSF passes the intact blood-brain barrier and reduces infarct volume in 2 different rat models of acute stroke. G-CSF displays strong antiapoptotic activity in mature neurons and activates multiple cell survival pathways. Both G-CSF and its receptor are widely expressed by neurons in the CNS, and their expression is induced by ischemia, which suggests an autocrine protective signaling mechanism. Surprisingly, the G-CSF receptor was also expressed by adult neural stem cells, and G-CSF induced neuronal differentiation in vitro. G-CSF markedly improved long-term behavioral outcome after cortical ischemia, while stimulating neural progenitor response in vivo, providing a link to functional recovery. Thus, G-CSF is an endogenous ligand in the CNS that has a dual activity beneficial both in counteracting acute neuronal degeneration and contributing to long-term plasticity after cerebral ischemia. We therefore propose G-CSF as a potential new drug for stroke and neurodegenerative diseases.
The hematopoietic factor G-CSF is a neuronal ligand that counters programmed cell death and drives neurogenesis

G-CSF is a potent hematopoietic factor that enhances survival and drives differentiation of myeloid lineage cells, resulting in the generation of neurophilic granulocytes. Here, we show that G-CSF passes the intact blood-brain barrier and reduces infarct volume in two different rat models of acute stroke. G-CSF displays strong anti-apoptotic activity in mature neurons and activates multiple cell survival pathways. Both G-CSF and its receptor are widely expressed by neurons in the CNS, and their expression is induced by ischemia, which suggests an autocrine protective signaling mechanism. Surprisingly, the G-CSF receptor was also expressed by adult neural stem cells, and G-CSF induced neuronal differentiation in vitro. G-CSF markedly improved long-term behavioral outcome after cortical ischemia, while stimulating neural progenitor response in vivo, providing a link to functional recovery. Thus, G-CSF is an endogenous ligand in the CNS that has a dual activity beneficial both in counteracting acute neuronal degeneration and contributing to long-term plasticity after cerebral ischemia. We therefore propose G-CSF as a potential new drug for stroke and neurodegenerative diseases.

Introduction
Stroke remains one of the most urgent medical problems of our times, growing in importance due to the demographic changes in industrialized societies. Treatment with tissue-plasminogen activator is limited by side effects and by the fact that it must be initiated within a short window of time, so that only a small percentage of all stroke patients undergo thrombolysis (1). Numerous neuroprotective strategies aiming at important mechanisms such as glutamate toxicity or free radical formation have failed due to lack of efficacy or intolerable side effects. It is therefore believed that a successful treatment strategy should be well tolerated, not interfere with essential brain physiology, and approach several pathophysiological mechanisms in parallel. In addition to effects on acute infarct evolution, novel strategies should also impact long-term functional outcome. Recovery of specific functions and improvement of activities of daily living are caused by intrinsic changes in existing neurons or networks or by the generation of new neurons from progenitor cells. Regarding the latter, enhancement of neurogenesis in the post-ischemic brain now appears to be an attractive strategy. Neural progenitor cells residing in the adult brain can indeed initiate a compensatory response to ischemic events that results in the production of new neurons (2, 3).

G-CSF is a 19.6-kDa glycoprotein commonly used to treat neutropenia (4, 5). Known sources of G-CSF in the body include monocytes, mesothelial cells, fibroblasts, and endothelial cells, and receptors for G-CSF are present on precursors and mature neurophilic granulocytes, monocytes, platelets, and endothelial cells. At the myeloid progenitor cell level, G-CSF stimulates the growth of neutrophil granulocyte precursors (6). G-CSF crucially regulates survival of mature, i.e., postmitotic, neutrophils (7) by inhibition of apoptosis (8).

We have recently uncovered the neuroprotective potential of G-CSF in an acute stroke model (9). Here, we explore the mechanisms responsible for that property and report on a dual functionality of G-CSF in the brain that parallels its activity in the hematopoietic system: inhibition of programmed cell death and stimulation of neuronal progenitor differentiation.

Results
G-CSF has robust neuroprotective activity in 2 different rodent stroke models and crosses the blood-brain barrier. We recently reported infarct-reducing activity of G-CSF in the acute stroke model middle cerebral artery occlusion (MCAO) in rats when treatment was initiated 30 minutes after onset of ischemia (9). When we initiated treat-

Nonstandard abbreviations used: BBB, blood-brain barrier; bFGF, basic FGF; CCA, common carotid artery; DCX, doublecortin; DIV, days in vitro; GFAP, glial fibrillary acidic protein; MCA, middle cerebral artery; MCAO, MCA occlusion; NSE, neuron-specific enolase; NSS, neurological severity score; PARP, poly-ADP-ribosyl polymerase; PLP, proteolipid protein; SVZ, subventricular zone; TTC, 2,3,5-triphenyltetrazolium chloride.


A prerequisite for a direct action of G-CSF on the brain would be penetration of the blood-brain barrier (BBB). We determined the amount of iodinated G-CSF ([131I]G-CSF) in brain and serum at 1, 4, and 24 hours after i.v. injection in noninjured rats and calculated the brain/serum ratios of [131I]G-CSF and [131I]albumin as an index of BBB permeability. At every observation point, G-CSF showed a higher brain/serum ratio, which indicated passage of G-CSF through the intact BBB (Figure 1D).

G-CSF receptor and ligand are neuronally expressed and induced by cerebral ischemia. The G-CSF receptor showed a broad, predominantly neuronal expression throughout the rat brain, with particularly high immunopositivity in large principal neurons. Among brain regions, there was a high expression in the cortex (most pronounced in layers II and V) (Figure 2A), the hippocampus, the subventricular zone (SVZ), the cerebellum (particularly in Purkinje cells) (Figure 2B) as well as deep cerebellar (Figure 2C) and brainstem nuclei plus the mitral cells in the olfactory bulb. Importantly, a corresponding neuronal staining pattern was also confirmed for human (Figure 2D: frontal cortex, layer V) and mouse (data not shown) brain tissue. Also, specificity of the G-CSF receptor signal was demonstrated in neural and extraneural tissues by preincubation with the target peptide (Supplemental Figure 2). In a search for sources for the ligand in the CNS, we found expression of G-CSF in all brain regions where its receptor was expressed. For example, we observed strong expression in the hippocampus CA3 field (Figure 2E), the hilus and subgranular zone of the dentate gyrus (Figure 2E, arrows), neurons in the entorhinal cortex (Figure 2F), neurons in the olfactory bulb (Figure 2G), several cerebellar and brainstem nuclei (Figure 2H), and cells in the SVZ (Figure 2I). Positive control stainings using the identical staining protocol and antibody employed in Figure 2, F–I, yielded the expected published staining patterns for G-CSF in extraneural tissues (Supplemental Figure 3).

In addition, neuronal expression was also detected using a second, unrelated antibody against G-CSF (Supplemental Figure 4).

We then performed a series of experiments to confirm the true neuronal expression of this secreted protein. As 1 report mentioned described G-CSF expression in stimulated astrocytes in vitro (11), we performed double-immunohistochemistry with the astrocytic marker glial fibrillary acidic protein (GFAP). There was no appreciable astrocytic expression of G-CSF detectable in vivo in all brain regions examined, both in noninjured and ischemic brains. Figure 3 shows examples of the dentate gyrus hilus (Figure 3A) and cortex (Figure 3B). In contrast, co-staining with the neuronal marker NeuN demonstrated perfect colocalization with G-CSF (Figure 3C). Moreover, the pattern of G-CSF mRNA detection in neurons of brain areas by in situ hybridization corresponded to the staining pattern obtained by immunohistochemistry, which confirmed neuronal synthesis of G-CSF (Figure 3, D–I). Note the distinct staining in selected cells in the subgranular zone of the dentate gyrus (Figure 3H). Finally, we performed laser-capture microdissection of neurons and astrocytes from the mouse frontal cortex and assayed these samples for G-CSF mRNA expression by PCR. Indeed, using this highly sensitive approach, we could readily detect G-CSF expression in the neuronal sample but not in astrocytes, even after 50 cycles of PCR amplification (Figure 3J). Thus, G-CSF is a neuronally expressed protein in the CNS.

Astonishingly, G-CSF localized to neurons expressing its receptor in all areas examined (Figure 4), which suggests an autocrine activation mechanism of the receptor. We asked whether this ligand/receptor system reacts to cerebral ischemia. Indeed, after
In addition, we could also detect ligand induction in a global model (Figure 5A) that was accompanied by induction of G-CSF mRNA on the ipsilateral hemisphere, and it was no longer detectable at 20 hours of reperfusion. This upregulation of the ligand was accompanied by a more modest induction of the receptor. At 6 hours following ischemia, this induction became more specific to the ischemic hemisphere, and it was more distinct than the contralateral hemisphere. A more distinct induction of the ligand was seen in periinfarct cortex samples from a rat cortical photothrombotic model (Figure 5C). This mRNA upregulation by cerebral ischemia was confirmed on the protein level by immunohistochemistry at 6 hours following ischemia (Figure 5, D–O). Induction of receptor and ligand was most clearly seen in the periinfarct area, e.g., in the MCAO model (Figure 5, D [receptor] and G [ligand]), or in cortical photothrombotic ischemia, where the infarct borders are easily recognizable (Figure 5, J [receptor] and M [ligand]). In conclusion, G-CSF and its receptor are coexpressed in neurons in the rodent CNS and are upregulated by ischemic stimuli.

We then asked whether these data from the rodent are likely relevant to the human system. Indeed, when comparing the cortical periinfarct area from a human stroke case 3 days after stroke onset (Figure 6A) with the corresponding contralateral cortex (Figure 6B) or a neuropathologically normal matched control brain (Figure 6C), we found clear induction of G-CSF receptor expression in neurons (see insets), which suggests comparable activity of the G-CSF system in the human brain.

G-CSF activates antiapoptotic pathways in cultured neurons. As G-CSF blocks apoptosis in cells of the myeloid lineage, we hypothesized that G-CSF might also interfere with programmed cell death in neurons. Cortical neurons from rat cortex at E18 invariably expressed the G-CSF receptor (Figure 7A). G-CSF protected cortical neurons against programmed cell death caused by the apoptosis inducer camptothecin (Figure 7B). This activity appeared to be mediated via the neuronal G-CSF receptor, as an antibody against the receptor was able to abolish protection (Figure 7C). Activity of G-CSF against neuronal cell death resulting from other apoptosis-inducing agents could also be observed. An apoptotic stimulus for neurons with high relevance to stroke pathophysiology is NO. G-CSF reduced NO-induced poly-ADP ribose polymerase (PARP) and caspase-3 cleavage in primary neurons (Figure 7D). This antiapoptotic activity was not specific to cells of rodent origin but could also be seen in NO-challenged human SHSY-5Y neuroblastoma cells (Figure 7E), which also expressed the G-CSF receptor (data not shown). We therefore analyzed the activation of antiapoptotic pathways after G-CSF stimulation in primary cortical neurons.

One important known transduction factor of G-CSF in the hematopoietic system is STAT3. Although activation of STAT3 by phosphorylation was detected in neurons after 5 minutes of G-CSF exposure (Figure 8, A and B), this induction was rather moderate and very transient. STAT3 is phosphorylated by the JAK2 kinase, which is recruited to the intracellular domains of the G-CSF receptor upon ligand binding. STAT3 activation appeared to be specifically mediated via the known pathway involving the G-CSF receptor present on neurons (Figure 8A, bottom lane), as AG490, a specific JAK2 inhibitor, strongly reduced STAT3 phosphorylation 5 minutes after addition of G-CSF (Figure 8A, right). Typical for the kinetics of G-CSF–activated STAT3 in the hematopoietic lineage (12), STAT phosphorylation decreased rapidly over a time course of 60 minutes (Figure 8B). G-CSF also led to a long-lasting (at least 8 hours), but overall moderate increase in protein expression.
levels of the STAT3 target Bcl-XL, a potent antiapoptotic factor in neurons (13) (Figure 8C).

Next, we determined activation levels of the ERK family of kinases. ERK1/2, which has been linked to both pro- and antiapoptotic events in neurons (e.g., refs. 14, 15) was only weakly and transiently activated by G-CSF (Figure 8D). In contrast, the newly described ERK5 kinase demonstrated a strong and lasting activation pattern (Figure 8D, bottom 2 rows). Interestingly, a recent report connects ERK5 activation to survival signals elicited by trk receptors (16).

One of the most potent antiapoptotic transduction pathways in all cell types including neurons known to date is the PI3K/Akt pathway (17). Akt is activated via PI3K and 3'-phosphoinositide–dependent protein kinase (PDK), and the amount of active Akt can be determined based on Ser437 phosphorylation. In untreated neurons, there was only a faint band visible corresponding to phosphorylated Akt (Figure 8E, bottom 2 rows). However, 5 minutes after G-CSF exposure, levels of phosphorylated Akt dramatically increased, and they remained elevated for at least 1 hour. The kinetics of Akt activation following G-CSF exposure corresponded well with the phosphorylation of PDK1, the protein kinase immediately upstream of Akt in the PI3K/Akt pathway (Figure 8E, top 2 rows). The phosphorylation of Akt 5 minutes after addition of G-CSF was completely blocked by the PI3K inhibitor LY294002 (Figure 8E, right). Thus, Akt is a prominent signal induced by G-CSF in neurons and appears to be activated via the known PI3K/PDK pathway originating at the G-CSF receptor. Inhibition of PI3K by LY294002 was able to partially block G-CSF–mediated protection against apoptosis in neurons (Figure 8F) or in human neuroblastoma cells (Figure 8G), which suggests that Akt activation indeed is a crucial factor in G-CSF’s antiapoptotic activity.

In conclusion, these results indicate that G-CSF counteracts pro‐programmed cell death in neuronal cells, an activity that is at least partially mediated by the PI3K/Akt pathway.

**G-CSF drives neuronal differentiation in vitro**. We noted that G-CSF receptor and ligand were expressed in the dentate gyrus by neurons of the subgranular zone and the hilus region (for examples, see Figure 2E, arrows and Figure 3, H and I). Expression was also noted in cells of the SVZ (Figure 2F). As these regions are known
to harbor neuronal progenitor cells and G-CSF has a potent role in progenitor cell differentiation in the hematopoietic system, we asked whether G-CSF might have a functional role in differentiation of adult neural stem cells. Indeed, adult neural stem cells isolated from the rat SVZ or hippocampal region that grow as neurospheres in culture expressed the G-CSF receptor at the mRNA level (Figure 9A). By immunocytochemistry we could also detect colocalization with the stem cell marker nestin (Figure 9B). We therefore examined the effects of G-CSF treatment on adult neural stem cells.

G-CSF dose-dependently induced activity of the promoter of the mature neuronal marker β-III-tubulin (Figure 9C) with a maximal induction greater than that reached by the most standard neuronal induction with the stem cell marker nestin (Figure 9B). We therefore determined whether these basic properties of G-CSF had consequences for differentiation of adult neural stem cells. Indeed, adult neural stem cells isolated from the rat SVZ or hippocampal region that grow as neurospheres in culture expressed the mature neuronal marker nestin (Figure 9B). We therefore asked whether G-CSF might have a functional role in differentiation of adult neural stem cells.

G-CSF improves functional outcome after cerebral ischemia. To determine whether these basic properties of G-CSF had consequences for long-term postischemic behavioral changes and for neurogenesis in vivo, we designed an experiment wherein G-CSF was given for 5 consecutive days at a dose of 15 µg/kg body weight following photothrombotic induction of ischemia in the sensorimotor cortex. This model has the advantage of producing defined neurological deficits without affecting survival. The dosage chosen is similar to the clinically used regimen in neutropenic patients (10 µg/kg/d for up to 14 days).

Sensorimotor deficits were obvious in vehicle-treated ischemic animals compared with sham-operated rats in the rotarod and adhesive tape removal test, and in the neurological severity score (NSS), which included the results of the beam balance test, for up to 6 weeks after the insult (Figure 10, A–D, compare red [ischemia + vehicle] and green [sham]). G-CSF–treated ischemic rats performed significantly better in all test paradigms than vehicle-treated animals when group means per time point were compared...
G-CSF stimulation of neural progenitor cells in vitro. We analyzed progenitor cells of the lateral ventricle wall by immunofluorescence against doublecortin (DCX), a microtubule-associated protein that is specifically expressed in neural progenitor cells and immature neurons (19, 20). The development into new neurons was detected by colabeling of DCX with the mature neuronal marker NeuN (21).

Migration of neuronal progenitor cells from the lateral ventricle wall to the lesioned neocortex has previously been reported (22, 23). When we analyzed the distribution of DCX-expressing cells in the ventricle wall as well as overlying corpus callosum and cortex, we found a visible recruitment of progenitor cells into the ischemic area of the neocortex. This response was visibly enhanced by peripheral infusion of G-CSF (Figure 11, A–I). Area and intensity of DCX immunoreactivity were significantly increased in ischemic G-CSF–treated (ischemia + G-CSF) animals compared with vehicle-treated (ischemia + vehicle) animals (area by 300% and intensity by 225%; P < 0.05). As an indicator of ongoing neuronal differentiation of the DCX-positive cells, coexpression with NeuN was frequently detected in cells surrounding the lesion site (see Supplemental Figure 6 for Z-stack analysis). However, using BrdU injections during the first 5 days after ischemia to label newly generated cells, we found that BrdU was not incorporated into NeuN-expressing cells in the cortical areas surrounding the lesion site.

The striatum has previously been described to have some degree of progenitor activation and neurogenesis after ischemia due to its proximity to the subventricular pool of neural stem and progeni-
We analyzed DCX-positive cells in the striatum at 6 weeks after cortical phot thrombosis and peripheral G-CSF infusion. Although the data suggest a trend toward more DCX-positive cells in the striatum of ischemia + G-CSF animals, no significant differences between ischemia + vehicle and ischemia + G-CSF animals were detected (data not shown).

The hippocampus has substantial renewal capacity for granule cells throughout the life of the rat. Since several studies have shown that hippocampal neurogenesis is upregulated after global and focal ischemia (2, 24–27), we investigated whether G-CSF treatment would alter the response of dentate gyrus progenitor cells to cortical injury. Using BrdU injections on days 1–5 after cortical phot thrombosis, we determined the number of cells in the ipsilateral side of the lesion, corresponding to long-term behavioral improvement.

Using BrdU injections on days 1–5 after cortical phot thrombosis, we investigated whether G-CSF treatment would alter the number of cells that are immunoreactive for BrdU and NeuN (Figure 12, A–E).

When looking at the total number of BrdU-positive cells in the hippocampus, we noticed an expected strong increase in vehicle-treated animals subjected to cortical phot thrombosis on the side of the infarct (ipsilateral + vehicle) but also contralaterally (contralateral + vehicle) (Figure 12F, compare sham-operated, vehicle-treated [sham + vehicle] with ipsilateral + vehicle and contralateral + vehicle). Although there was a slight increase in BrdU-positive cells in the ipsilateral dentate gyrus upon G-CSF treatment (Figure 12F, ipsilateral + vehicle and ipsilateral + G-CSF), this was not statistically significant. However, G-CSF induced a significant rise in newly generated cells in the dentate gyrus in sham-operated animals (Figure 12F, sham + vehicle vs. sham + G-CSF).

Counting NeuN/BrdU-double-positive cells, we found that G-CSF indeed increased the number of newly generated granule cells after ischemia on the side of the lesion (Figure 12G, ipsilateral + vehicle vs. ipsilateral + G-CSF; P < 0.01). In the contralateral (unlesioned) dentate gyrus, the increase in newly generated granule cells after G-CSF treatment was smaller and was not statistically significant (Figure 12G, contralateral + vehicle vs. contralateral + G-CSF). However, G-CSF significantly increased neurogenesis in sham-operated, nonischemic animals (Figure 12G, sham + vehicle vs. sham + G-CSF; P < 0.05). Thus, peripheral administration of G-CSF increases hippocampal neurogenesis not only in ischemic animals, but also in the intact, nonischemic rat.

**Discussion**

Here we have demonstrated that the hematopoietic factor G-CSF is an endogenous, neuronally expressed ligand that is upregulated upon ischemia and provides protection against programmed cell death in neurons, which is reflected by robust neuroprotective activity in acute stroke models in vivo. In addition, G-CSF displays a strong neurogenic potential in vitro and in vivo, corresponding to long-term behavioral improvements after ischemia.

We found that G-CSF is expressed by neurons in many areas of the CNS, which implies important new functions of this protein in the CNS. This discovery is particularly surprising as G-CSF is a long-known protein that was cloned many years ago as a growth factor in the hematopoietic system (28). To our knowledge, the only reports that suggest expression of G-CSF in neural cell types deal with stimulus-induced expression in astrocyte cultures (11, 29). We have therefore carefully examined the cellular origin of G-CSF and could not detect any astrocytic G-CSF expression in vivo, even in the acute cerebral ischemia paradigms studied. It is, however,
certainly possible that G-CSF expression in astrocytes might be evoked in vivo by other stimuli or occur under different ischemic conditions or at different postischemic time points.

Neuronally expressed G-CSF was induced more than 100-fold at the level of transcription by focal cerebral ischemia. To the best of our knowledge, this is the strongest regulation of any gene by ischemic events in the brain that has been reported so far, implicating an important adaptive response in neurons. In the MCAO model, we found transcriptional induction of this protein early after ischemia in both hemispheres, a phenomenon frequently encountered in this model (30). However, induction became more specific to the ipsilateral hemisphere at 6 hours and was transient, with elevated mRNA levels no longer detectable at 20 hours following ischemia. Importantly, induction was seen not only in focal but also in global ischemic models.

G-CSF’s actions in the brain appear to be specifically mediated through the G-CSF receptor, which has an astonishingly broad, predominantly neuronal expression pattern in the CNS. Indeed, G-CSF localized to neurons expressing its receptor. Moreover, the G-CSF receptor itself was induced at 6 hours after ischemia. Immunohistochemistry demonstrated a strong induction in the perinfarct zone of both receptor and ligand at this time point. The perinfarct zone is known to harbor neurons at risk of dying, which suggests that G-CSF and its receptor likely function as an autocrine adaptive system in neurons. An autocrine signaling mechanism has indeed been discussed for a number of neuroprotective growth factors in the brain, such as brain-derived neurotrophic factor (BDNF; 31), erythropoietin (EPO; 32), VEGF (33), and neurotrophin-3 (NT-3; 34).

Systemically given G-CSF was able to pass the intact BBB, a property that is shared with other hematopoietic factors such as EPO (35) and GM-CSF (36), and was neuroprotective in 2 different models of focal cerebral ischemia. In vitro, G-CSF displayed strong antiapoptotic activity in neuronal cells. G-CSF evoked very modest and transient activation of STAT3 and ERK1/2 and a strong lasting activation of ERK5, which has recently been implicated in promoting neuronal survival (16). ERK5 was also shown to be activated by G-CSF in non-neural cell types (37). However, the most dramatic effect of G-CSF was seen on the PI3K/Akt pathway, and inhibition of PI3K indeed interfered with the antiapoptotic activity of G-CSF. Since the original description of antia apoptotic activities of Akt in neurons (17), a number of reports have confirmed the powerful central regulatory role of this kinase for neuronal survival (38). In hematopoietic cells, G-CSF activates intracellular
signaling pathways including STAT3 (39) and Akt (40), which are both linked to suppression of apoptosis and proliferation. Therefore, G-CSF signaling and its role in suppressing apoptosis seem to be preserved both in cells of the hematopoietic lineage and in neurons. The involvement of the antiapoptotic PI3K/Akt pathway in the infarct volume–reducing effect of G-CSF in neurons is most likely one crucial mechanism for the robust acute infarct volume–reducing effect of G-CSF.

In the hematopoietic system, G-CSF’s functions are dual, as they involve inhibition of apoptosis but also differentiation of hematopoietic stem cells. This function seems to be preserved in the CNS, as we found expression of receptor and ligand on adult neural stem cells and induction of a neuronal phenotype in these cells by addition of G-CSF in vitro. G-CSF induced functional recovery, which correlated with increased neuronal progenitor activation in the periphery of the ischemic lesion (cortex and corpus callosum) and with enhanced neurogenesis in the dentate gyrus.

Cortical lesions have previously been reported to enhance neuronal progenitor proliferation in the lateral ventricle wall. Similarly, we have observed that the amount of DCX-positive cells outside the ventricle wall and rostral migratory stream are visibly enhanced in lesioned animals as compared with sham-lesioned controls. We observed an additional increase in the amount of DCX-positive cells under G-CSF treatment that were in close proximity to the lesion site. Although a large number of DCX-positive cells was detected in the immediate periphery of the lesion, the colabeling of BrdU with mature neuronal marker did not indicate substantial maturation of progenitor cells into neurons. Neurogenesis in the lesioned cortex has been reported only by Magavi and colleagues (41), who used a very selective apoptotic elimination of individual cortical neurons, but not by Arvidsson and colleagues (42), who used an MCAO model. Neurogenesis in the striatum was slightly increased after G-CSF treatment, but again the number of newly generated cells was rather small. This corresponds to the findings of Arvidsson et al. that demonstrated that less than 0.2% of the damaged striatal neuronal population was replaced by newly generated cells after MCAO (42).

The most striking effect of peripherally administered G-CSF on the brain was seen in the dentate gyrus, where G-CSF increased the number of newly generated neurons under ischemic conditions but also in nonischemic, sham-operated animals. It is therefore intriguing to speculate that G-CSF may enhance structural repair and function even in healthy subjects or at long intervals after stroke.

Generation of new differentiated cells from stem cells involves an intricate interplay among proliferation, differentiation, and selective survival. In our in vitro experiments, G-CSF induced neuronal differentiation of adult stem cells without apparently elevating the number of immature stem cells, at least at the time points examined (see Figure 9D). In vivo, the significant increase in hippocampal neurogenesis (BrdU+/NeuN+ cells, Figure 12F) was based on the fact that a much higher fraction of BrdU+ cells turned into granule cells in ischemic, G-CSF–treated animals compared with ischemic, untreated animals, although the total number of BrdU+ cells was unaltered (Figure 12G). This indicates a predominant role of G-CSF in survival and differentiation of progenitor cells in the postischemic brain.

Although more work needs to be done to better understand the balance of effects evoked by G-CSF during neurogenesis in the adult, it is likely that one basic property of G-CSF in neurons, antiapoptosis, also plays a part in the observed neurogenesis. In the hematopoietic system, counteraction of the built-in apoptosis program in progenitor cells by G-CSF is intricately linked to self-renewal and generation of mature cell types of the blood (43). In the neurogenic regions of the adult brain, the majority of proliferative cells are eliminated by apoptosis before reaching a mature phenotype (44–46). Moreover, the involvement of apoptotic signaling in adult hippocampal neurogenesis has been highlighted by a recent study on Bax-deficient mice (47). A combined mechanism of proliferative and survival-promoting effects on adult brain progenitor cells in vivo has indeed been proposed for the action of several other brain- and blood-derived growth factors, such as BDNF and VEGF (48–51).

The cortical phototethrombosis model used in this study has the most prominent impact on sensorimotor behavior, which was also measured in the test battery performed, whereas hippocampal formation is most frequently linked to learning and memory processes.

Figure 10
G-CSF treatment improves long-term functional outcome after cortical ischemia. (A–D) G-CSF significantly improved motor recovery as measured by rotarod performance (A) and NSS, which included the results of the beam balance test (B), compared with those in nontreated, ischemic control animals. Sensory-motor function as measured by adhesive tape removal was significantly better in G-CSF–treated, ischemic animals compared with ischemic controls when the contralateral forepaw was tested (C) and borderline significant in the ipsilateral forepaw (D). Bar graphs represent an analysis of area under the curve (AUC) for each rat over time in an experimental group. *P < 0.05; **P < 0.01; ***P < 0.001.
However, there is also a wealth of data supporting a possible role of the hippocampus in functional recovery from motor deficits. The hippocampus produces a slow-wave activity known as rhythmical synchronous activity ($\tau$ rhythm), which is thought to be involved in sensorimotor integration and movement initiation (52–54). Type 1 $\tau$ activity gives a direct indication of the level of activation of the motor systems involved in voluntary motor activity, whereas type 2 $\tau$ activity indicates the processing of sensory information (52). In the context of our experiments, sensorimotor integration is most crucial to the adhesive-removal paradigm, where the treatment effect of G-CSF was most prominent (see Figure 10C). Cortical lesions have in fact been shown to disrupt the hippocampal $\tau$ activity patterns (55). The connection between the motor system and hippocampal neurogenesis is further supported by the finding that voluntary running is a strong activator of the latter in mice (56–58). We therefore hypothesize that the observed G-CSF–induced increase in hippocampal neurogenesis directly impacts recovery from cortical lesion–induced sensorimotor deficits.

G-CSF signaling appears to be a novel protective system in the brain that is involved in countering acute neurodegeneration and regulating the formation of new neurons. G-CSF’s principal cellular functions in the CNS appear to be remarkably similar its functions in the hematopoietic system. Thus, G-CSF’s basic functions have apparently been conserved and utilized in 2 different body compartments. While the direct actions of G-CSF uncovered here appear sufficient to fully explain the observed in vivo effects of this protein, it is certainly possible that additional mechanisms such as the mobilization of bone marrow stem cells have a role in G-CSF–mediated neuroprotection, although proof for this hypothesis is lacking at present (59, 60). We have concentrated here on examining the effects of G-CSF in vivo by using the most clinically relevant application scheme of peripheral administration. For the further dissection of mechanisms of action, and to study the role of brain-endogenous G-CSF, studies with transgenically modified mice, such as neuron-specific knock-outs for the G-CSF receptor, are warranted.

In therapeutic terms, G-CSF fulfills the criteria of a novel type of stroke drug discussed in the introduction. Its multimodality, together with the ability to penetrate the BBB and its documented history as a well-tolerated drug, make G-CSF an ideal drug candidate for treatment of stroke. Our data suggest a comparable functionality of the neural G-CSF system in the human. First, the G-CSF receptor is neuronally expressed in the human brain in a pattern of distribution similar to that in rodents. Second, a human neuroblastoma line expresses the receptor and is protected against programmed cell death. Finally, the G-CSF receptor is induced in the ipsilateral infarcted cortex shortly after stroke. We have therefore started a phase IIa trial to establish the safety of i.v. administered G-CSF in acute stroke patients.

The broad expression of the G-CSF receptor in many brain areas, the passage of G-CSF through the intact BBB, the effect on neurogenesis in the nonischemic animal, and the favorable tolerance profile suggest that G-CSF might be beneficial for a number of other neurodegenerative and psychiatric disorders in which neuronal cell death and/or disturbances in neurogenesis are involved.

**Methods**

*Ischemic models*

*Intraluminal occlusion model.* Animals received inhalation anesthesia with 70% N₂O, 30% O₂, and 1% halothane. The femoral artery was cannulated...
for recording of continuous arterial blood pressure and blood sampling for gas analyses. The right femoral vein was used for drug delivery. During the experiment, core body temperature was monitored and maintained at 37°C by a thermostatically controlled heating pad (FMI GmbH). MCAO was induced with a silicon-coated (Provil Novo; Heraeus Kulzer) 4-0 nylon filament (ETHICON) that was introduced into the common carotid artery (CCA) and advanced into the internal carotid artery as described previously (9). Successful MCAO was verified by laser Doppler flowmetry (Perimed 4000) with a probe positioned 4 mm posterior to the bregma and 4 mm lateral from the midline. After 90 minutes MCAO, the filament was withdrawn to allow for reperfusion. Two hours after onset of occlusion, 60 µg/kg G-CSF (NEUPHEN; Amgen Inc.) was infused i.v. over 20 minutes. Infarct volumes were determined by 2,3,5-triphenyl tetrazolium chloride (TTC) staining as described previously (9). Two-millimeter sections were cut using a color scanner and infarct areas determined using ImageJ version 1.32j (http://rsb.info.nih.gov/ij). Edema correction was performed as described previously (9). For the MCAO model, animals with no or minimal infarcts (<60 mm³) were excluded from the analysis before unblinding.

**Combined CCA/distal middle cerebral artery occlusion model.** Transient left combined CCA/middle cerebral artery (CCA/MCA) occlusion model was achieved as described previously (10). Briefly, animals fasted overnight were anesthetized with chloral hydrate (0.45 g/kg i.p.). The right femoral vein and artery were cannulated for recording of arterial blood pressure and blood sampling. The right femoral artery and vein were cannulated for continuous arterial blood pressure and blood gas analyses. The right femoral vein was cannulated by a PE-50 tube for treatment gases. The right femoral vein was used for drug delivery. During the experiment, rectal temperature was monitored and maintained at 37°C by a thermostatically controlled heating pad (FMI GmbH). Photothermotic ischemia was induced by filling the right femoral artery with 4 mg/kg body weight ketamine hydrochloride and 4 mg/kg body weight xylazine hydrochloride if necessary. A PE-50 polyethylene tube was inserted into the right femoral artery for continuous monitoring of mean arterial blood pressure and blood gases. The right femoral vein was cannulated by a PE-50 tube for treatment infusion. During the experiment, rectal temperature was monitored and maintained at 37°C by a thermostatically controlled heating pad (FMI GmbH). Photothermotic ischemia was induced in the rat parietal cortex according to the method of Watson et al. (61). Animals were placed in a stereotaxic frame, and the scalp was incised for exposure of the skull surface. For illumination, a fiber-optic bundle with a 1.5-mm aperture was placed stereotaxically onto the skull 4 mm posterior to the bregma and 4 mm lateral from the midline. The skull was illuminated with a cold, white light beam (150 W) for 20 minutes. During the first 2 minutes of illumination, the dye rose bengal (0.133 ml/kg body weight, 10 mg/ml saline) was injected i.v. Sham-operated animals underwent the same experimental procedures as described above without infusion of rose bengal and illumination. After surgery, the catheters were removed, and the animals were allowed to recover from the anesthesia and given food and water ad libitum. For treatment, mals that displayed a cerebral perfusion of 10–15% of the initial value on the LDF scale (expressing relative values of cerebral perfusion) were included in the study. G-CSF (50 µg/kg) was infused i.v. over 20 minutes starting 60 minutes after induction of ischemia. After 180 minutes of combined CCA/distal MCA occlusion model, reperfusion was established through reversal of the occlusion procedure. After 72 hours of reperfusion, animals were reanesthetized and transcardially perfused with 50 ml of saline. Perfused isolated brains were transferred into ice-cold PBS for sectioning. Infarct volumes were determined by TTC staining (see above).

**Photothermotic ischemia model.** Male Wistar rats weighing 280 to 320 g were anesthetized with an intramuscular injection of 100 mg/kg body weight ketamine hydrochloride (Ketamin 2; Medistar Arzneimittelvertrieb GmbH) and 8 mg/kg body weight xylazine hydrochloride (Rompun; Bayer). Anesthesia was maintained with administration of 50 mg/kg body weight ketamine hydrochloride and 4 mg/kg body weight xylazine hydrochloride if necessary. A PE-50 polyethylene tube was inserted into the right femoral artery for continuous monitoring of mean arterial blood pressure and blood gases. The right femoral vein was cannulated by a PE-50 tube for treatment infusion. During the experiment, rectal temperature was monitored and maintained at 37°C by a thermostatically controlled heating pad (FMI GmbH). Photothermotic ischemia was induced in the rat parietal cortex according to the method of Watson et al. (61). Animals were placed in a stereotaxic frame, and the scalp was incised for exposure of the skull surface. For illumination, a fiber-optic bundle with a 1.5-mm aperture was placed stereotaxically onto the skull 4 mm posterior to the bregma and 4 mm lateral from the midline. The skull was illuminated with a cold, white light beam (150 W) for 20 minutes. During the first 2 minutes of illumination, the dye rose bengal (0.133 ml/kg body weight, 10 mg/ml saline) was injected i.v. Sham-operated animals underwent the same experimental procedures as described above without infusion of rose bengal and illumination. After surgery, the catheters were removed, and the animals were allowed to recover from the anesthesia and given food and water ad libitum. For treatment, mals that displayed a cerebral perfusion of 10–15% of the initial value on the LDF scale (expressing relative values of cerebral perfusion) were included in the study. G-CSF (50 µg/kg) was infused i.v. over 20 minutes starting 60 minutes after induction of ischemia. After 180 minutes of combined CCA/distal MCA occlusion model, reperfusion was established through reversal of the occlusion procedure. After 72 hours of reperfusion, animals were reanesthetized and transcardially perfused with 50 ml of saline. Perfused isolated brains were transferred into ice-cold PBS for sectioning. Infarct volumes were determined by TTC staining (see above).

**G-CSF increases neurogenesis in the dentate gyrus.** (A) Example of BrdU/NeuN-double-positive cells within the basal layer of the dentate gyrus (scale bar: 40 µm). The arrow in A indicates the enlarged double-stained cell in B (scale bar: 10 µm). (C) DCX in red. (D) BrdU in green. (E) NeuN in blue. (F) G-CSF increased the number of newly generated neurons (BrdU+/NeuN*) on the side of the ischemic lesion (red bars, ipsilateral + vehicle vs. ipsilateral + G-CSF; **P < 0.01). Contralateral to the lesion, there was a trend toward an increase in newly generated neurons compared with vehicle-treated ischemic animals that was not statistically significant (blue bars, contralateral + vehicle vs. contralateral + G-CSF). However, G-CSF increased neurogenesis in sham-operated, nonischemic animals (green bars, sham + vehicle vs. sham + G-CSF; *P < 0.05). (G) The total number of BrdU* cells in the dentate gyrus was not significantly further increased by G-CSF treatment in the ischemic animals (red and blue bars), which implies a true induction of neuronal differentiation by G-CSF in the postischemic brain. In contrast, sham-lesioned animals showed an elevation of the total number of BrdU* cells after G-CSF treatment (green bars; *P < 0.05).
ischemic or sham-operated animals were given 15 μg G-CSF/kg body weight i.v. or vehicle 1 hour after the procedure. Daily repeated i.v. bolus infusions via the tail vein (G-CSF or vehicle) followed on days 2–5. Four hours after each tail vein injection of G-CSF or vehicle on days 1–5, dividing cells were labeled with BrdU by i.p. injections (50 mg/kg/d). We perfused animals 37 days after the last injection in order to histologically assess the amount of progenitor cells and newly generated neurons.

All animal experiments were performed in accordance with national and international regulations and were approved by the Regierungssprisdium Karlsruhe (Karlsruhe, Baden-Württemberg, Germany) and the Animal Welfare Committee of the University of Texas – Houston (Houston, Texas, USA). All experiments were done in a fully randomized and blinded fashion.

**Behavioral measurements**

*Combined CCA/distal MCA occlusion model.* All sensorimotor tests were performed during the light cycle between morning and early afternoon. Animals were tested at 72 hours just before they were killed for infarct volume determination. Testing was performed by an investigator blinded to the experimental groups. A neurological deficit score (0 to 18) was calculated by combining the scores of the following 4 tests: forelimb placing (both forepaw), foot-fault, and cylinder tests. Tests were done as an age- and sex-matched control were obtained from routine autopsy cases. Permission for storage and use of human autopsy tissue was obtained from the Ethikkommission der Medizinischen Fakultät der Universität Münster in Münster, Germany. Clinical records, autopsy reports, as well as radiological findings were reviewed in order to determine onset of neurological symptoms and to exclude malignancy, hematological disorders, sepsis and concomitant neurological disease. The 81-year-old female stroke patient had died 3 days upon documented onset of neurological symptoms due to progressive brain edema. The control patient (female, 82 years old) had died upon acute gastrointestinal bleeding. The intervals between death and autopsy were 67 hours and 56 hours, respectively. After antigen retrieval (boiling in 10 mM citrate buffer, pH 6), sections from the ipsilateral and contralateral cortex (stroke patient) or frontal cortex (control) were stained with specific antibodies against the G-CSF receptor (1:100; Santa Cruz Biotechnology Inc.), appropriate secondary antibodies, and the ABC technique using an automated staining system (TechMate; DakoCytomation).

**Histology**

During deep anesthesia, animals were transcardially perfused with 4% paraformaldehyde, and brains were removed and either prepared as free-floating cryosections (30 μm) or embedded in paraffin. For G-CSF or G-CSF receptor immunohistochemistry, sections of paraffin-embedded tissues (2 μm) were deparaffinized and microwaved (in citrate buffer at 500 W for 10 minutes). Afterwards, sections were incubated at room temperature with the respective antisera (1:500; Santa Cruz Biotechnology Inc.) for 1 hour in a humid chamber. Staining was visualized using the avidin–biotin complex (ABC) technique with DAB as chromogen (DakoCytomation). For double immunofluorescence, sections were incubated with the G-CSF receptor antisemur (1:100), and following incubation with an anti-rabbit FITC-conjugated secondary antibody (1:200; Dianova), either the G-CSF (1:100), GFAP, or NeuN antisera (1:100; Chemicon International) were applied. For detection, the sections were incubated with an appropriate TRITC-conjugated secondary antibody (1:200; Dianova), and nuclear staining was performed using DAPI. For negative controls, the primary antiserum was omitted. All double-fluorescence experiments were controlled by parallel single stainings and checked for any fluorescence cross-talk between detection channels. Double-fluorescence stainings were also performed with switched chromophores for the secondary antibody.

**Human autopsy samples**

Formaldehyde-fixed, paraffin-embedded brain tissues samples from a patient suffering from acute ischemic stroke within the anterior circulation as well as an age- and sex-matched control were obtained from routine autopsy cases. Bilateral brain sections of the left and right hemispheres, the control patient (female, 82 years old) had died upon acute gastrointestinal bleeding. The intervals between death and autopsy were 67 hours and 56 hours, respectively. After antigen retrieval (boiling in 10 mM citrate buffer, pH 6), sections from the ipsilateral and contralateral cortex (stroke patient) or frontal cortex (control) were stained with specific antibodies against the G-CSF receptor (1:100; Santa Cruz Biotechnology Inc.), appropriate secondary antibodies, and the ABC technique using an automated staining system (TechMate; DakoCytomation).

**Immunocytochemistry**

Neural stem cells were dissociated and plated on poly-L-ornithin/laminin–coated 96-well plates at a density of 30,000 cells/well. After 2 days, stem cells were washed with PBS (Invitrogen Corp.) (37°C) and fixed with 4% paraformaldehyde for 10 minutes on ice. Then cells were washed with PBS (4°C) and stored at 4°C. Cells were incubated for 10 minutes in 50 mM glycine in PBS and then washed with PBS. After permeabilization on ice using 0.2% Triton X-100 (Sigma-Aldrich) in PBS, cells were incubated with blocking solution (1% BSA in PBS) at room temperature. The G-CSF receptor antiserum (1:100; Santa Cruz Biotechnology Inc.) and the nestin antisemur (1:100; BD Transduction Laboratories) were incubated overnight at 4°C. Cells were then washed with 0.1% BSA in PBS and incubated for 1 hour with the secondary antibodies (anti-rabbit FITC and anti-mouse TRITC, 1:200; Dianova) at room temperature. Cells were then washed briefly in 0.1% BSA in PBS and stained with Hoechst 33342 (Invitrogen Corp.) (1:10,000 in PBS).

**In situ hybridization**

Riboprobes were generated from PCR-generated templates of cloned human G-CSF cDNA using T7 (antisense probe) or T3 (sense probe) polynucleotide (Roche Diagnostics Corp.). Transcripts were labeled with rUTP-biotin. Specificity and concentration of transcripts were verified by denaturing PAGE. Two-micrometer paraffin sections were deparaffinized, prehybridized (50% formamide; 1% sarcosyl; 0.02% SDS, 5x SSC, 2x washing reagent from the DIG Wash and Block Buffer Set [Roche Diagnostics Corp.]), partially digested with proteinase K, washed with 0.1 M glycine/ PBS, and incubated with 500 ng/ml probe overnight at 60°C. Sections were washed, RNase A treated (buffer: 5 mM Tris-HCl, pH 8.0; 250 mM NaCl, 0.5 mM EDTA), and incubated for 2 hours with Streptavidin-AP (Roche Diagnostics Corp.), and stainings were developed using nitro blue tetra-
For receptor blocking experiments, primary cortical neurons from the rat were pooled in 75 µL RNA lysis buffer (Qiagen) and stored at room temperature until further processing. RNA was isolated using the RNeasy Micro Kit (Qiagen). RNA quality and quantity were checked by analyzing 1 µL of RNA on the Agilent 2100 Bioanalyzer using the RNA 6000 LabChip Kit (Agilent Technologies). T7-RNA polymerase-mediated linear amplification was performed according to optimized protocols for low-input RNA amounts. Briefly, after first- and second-strand cDNA synthesis, RNA was transcribed with the T7 MEGAscript Kit (Ambion Inc.) at 37°C for 16 hours. Amplified antisense RNA was purified with the RNeasy Mini Kit (Qiagen) and precipitated. First-strand cDNA was synthesized using random primers, which was followed by second-strand synthesis. PCR was performed for cyclophilin B (mm-cycB1s, TTGTCTGAGC-CATGGTCAAC; mm-cycB1as, ATTTCAGTCTTGCAATGCGATCAG, product length 371 bp), GASP (mGFAPas, CCCCCATCGCTGATCTTAC; mGFAPas, TGCTTCCCTACCTGCCCACCAAT, product length 280 bp), and G-CSF (GCSF-790s, GGAGCTCTAAGCTTCTAGATC; GCSF-1154as, AGCAGCGGCAGACACAGCGGGCTCTTCCTCTACCAA; G-CSF-345s, CATTTGCCATGGACAAGATG). Primers were used: rat G-CSFR-frag-32s, CCATTGTCCATCTTGGGGATC; rat G-CSFR-frag-265as, CCTGGAAGCTGTTGTTCCATG; G-CSF-862as, AGCAGCGGCAGACACAGCGGGCTCTTCCTCTACCAA; G-CSF-1154as, AGCAGCGGCAGACACAGCGGGCTCTTCCTCTACCAA; G-CSF-345s, CATTTGCCATGGACAAGATG). Products were visualized by agarose gel electrophoresis until further processing. RNA was isolated using the RNeasy Micro Kit (Qiagen) and precipitated. First-strand cDNA was synthesized following conditions: GFAP and cyclophilin were amplified over 30 cycles at 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. Melting curves were determined using the following parameters: 95°C to 50°C; ramping to 99°C at 0.2°C/second. The following primer pairs were used: rat G-CSFR-sense-32s, CCTTGTCCATCTTGGGGATC; rat G-CSFR-frag-265as, CCTTGTCCATCTTGGGGATC; G-CSF-345s, CATTTGCCATGGACAAGATG). Specificity of product was ensured by melting point analysis and agarose gel electrophoresis. CDNA content of samples was normalized to the expression level of cyclophilin (primers: G-Cys, ACCCCCCGCGGCTTCTTCGAC; acyc300, CATTGTCCATGGACAAGATG). Relative regulation levels were derived after normalization to cyclophilin.

Detection of G-CSF mRNA in microdissected cells

Horizontal cryostat brain sections (8 µm) were prepared from mouse brain and were mounted on frame slides (POL-membrane, 0.9 µm; Leica Microsystems). Sections were stained with Alexa 488-conjugated NeuN or GFAP antibodies (Chemicon International) using a rapid immunohistochemistry protocol. Identified single neurons or astrocytes were isolated from the frontal cortex using laser microdissection (Leica). One hundred cells were microdissected in 75 µL RNA lysis buffer (Qiagen) and then treated with 20 μM camptothecin to induce apoptosis. G-CSF was added to a final concentration of 50 ng/ml. Incubation was continued for 5 hours, and caspase-3/7 activity was determined by the Caspase-Glo Assay (Promega). For inhibition of PI3K, the inhibitor LY294002 (Merck-Calbiochem) was added at 50 μM final concentration 30 minutes prior to cell death stimuli and G-CSF. Caspase-3/7 activity was determined after 5 hours by the Caspase-Glo Assay (Promega), and luminescence was measured with a plate reader (Mithras; Berthold Technologies GmbH). Eight to 16 independent data points were generated for each treatment.

Western blots analyses

For time series pathway analyses, rat primary cortical neurons (21 days in vitro [DIV]) were treated with G-CSF (NEUPOGEN; Amgen Inc.) and harvested at 5, 15, 30, and 60 minutes. Experiments were repeated at least twice with independent preparations of neurons. For determination of PARP cleavage, neurons (21 DIV) were treated with 150 μM NOR3 with or without G-CSF (50 ng/ml). Primary neurons were scraped off the plate and washed twice in ice-cold PBS containing 2.5 mg/ml pepstatin (Sigma-Aldrich) and apotinin (1:1,000, Sigma-Aldrich). Pellets were resuspended in 1 volume 2% SDS (40 μL), and 5 μL Benzonase solution (40 μL 100 mM MgCl2 and 9 μL Benzonase; Roche Diagnostics Corp.) was added. After solubilization, 1 volume PBS was added and the protein concentration determined (BCA Protein Assay, Pierce). After denaturing at 95°C for 5 minutes, 100 μg were run on 8% SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Protran BA79; Schleicher & Schuell) using a semi-dry blotting chamber (Whatman Biometa). Blots were blocked with 5% milk powder in PBS/0.02% Tween-20, washed 3 times with PBS/0.02% Tween-20, and incubated for 1 hour at room temperature with the primary antibody (anti–cleaved PARP antibody, 1:1,000 [Cell Signaling Technology]; anti–Bcl-2 antibody, 1:500 [BD Transduction Laboratories]; anti–STAT3 and anti–phosphorylated STAT3 antibodies, 1:500 [Cell Signaling Technology]; all other antibodies were from Cell Signaling Technology). After washing, the blots were incubated with the secondary antibody (anti-rabbit anti serum HRP-coupled or anti-mouse anti serum HRP-coupled, 1:4,000; Dianova) for 1 hour at room temperature. Signals were detected using the SuperSignal chemiluminescence system (Pierce) and exposed to Hyperfilm-ECL (Amersham Biosciences). Intensities of bands for phosphorylated Stat3 and Stat3 were quantified on scanned autoradiographs using Windows ImageJ version 1.29.

Quantitative PCR analysis

RNA of brains was isolated using the acidic phenol extraction protocol followed by Qiagen RNeasy Mini Kit purification according to the manufacturer’s recommendations. cDNA was synthesized from 5 μg total RNA using oligo-dT primers and Superscript II Reverse Transcriptase (Invitrogen Corp.). Quantitative PCR analysis was performed using the LightCycler system (Roche Diagnostics Corp.) with SYBR green staining of DNA double strands. Cycling conditions were as follows: 5 minutes at 95°C, 5 seconds at 95°C, 10 seconds at 66°C, 30 seconds at 72°C, 10 seconds at 84°C for 50 cycles. Melting curves were determined using the following parameters: 95°C to 50°C; ramping to 99°C at 0.2°C/second. The following primer pairs were used: rat G-CSFR-sense-32s, CCTTGTCCATCTTGGGGATC; rat G-CSFR-frag-265as, CCTTGTCCATCTTGGGGATC; G-CSF-345s, CATTTGCCATGGACAAGATG). Primers were used: rat G-CSFR-sense-32s, CCTTGTCCATCTTGGGGATC; rat G-CSFR-frag-265as, CCTTGTCCATCTTGGGGATC; G-CSF-345s, CATTTGCCATGGACAAGATG). Specificity of product was ensured by melting point analysis and agarose gel electrophoresis. CDNA content of samples was normalized to the expression level of cyclophilin (primers: G-Cys, ACCCCCCGCGGCTTCTTCGAC; acyc300, CATTGTCCATGGACAAGATG). Relative regulation levels were derived after normalization to cyclophilin.

Primary neuronal cultures

Ten to 12 cortices or hippocampi were dissected from Wistar rat embryos on E18. The tissue was dissociated using 10 mg/ml trypsin, 5 mg/ml EDTA/DNase (Roche Diagnostics Corp.) in HBSS (BioWhittaker Molecular Applications). The digestion was stopped using 4 parts neurobasal medium containing 1X B-27 supplement (Invitrogen Corp.), 0.5 mM l-glutamine, and 25 µM glutamate. After centrifugation, the cell pellet was dissolved in 5 mL medium and plated at a density of 250,000 cells per well of a 24-well plate on glass coverslips coated with poly-L-lysine (for immunocytochemistry or Western blot analyses) or into 96-well plates at 5 x 10^4 cells/well (for cell death assays).

Caspase activity assays

For caspase-3/7 assays, we used the human neuroblastoma cell line SHSY-5Y or rat primary cortical neurons. Cells were seeded into 96-well plates (5 x 10^4 cells/well) for 2 (SHSY-5Y) or 14–21 days (neurons). To elicit programmed cell death, we treated cells with either the NO donor NOR3 ([1(e)-4-ethyl-2(E)-hydroxyimino]-5-nitro-3-hexenamide) (Sigma-Aldrich) at 150 µM, 1 mM staurosporine, or 20 µM camptothecin (both Merck-Calbiochem) in 5 hours with or without recombinant human (NEUPOGEN; Amgen Inc.) or murine (R&D Systems) G-CSF at 50 ng/ml. For receptor blocking experiments, primary cortical neurons from the rat were preincubated for 1 hour with 1 µg/ml anti–G-CSF receptor antibody (SC 9173, Santa Cruz Biotechnology Inc.) and then treated with 20 µM
Neurogenesis detection in vivo

Progenitor activity and neurogenesis were visualized by immunofluorescence as previously described (44, 67), but a brief description is given below. The following antibodies were used: rat anti-BrdU (1:500; Accurate Chemical & Scientific Corp.), mouse anti-NeuN (1:500, Chemicon International), goat anti-DCX C-18 (1:500, Santa Cruz Biotechnology Inc.), anti-rat FITC, anti-goat rhodamine X, anti-mouse Cy5 (Jackson Immunoresearch Laboratories Inc.). Free-floating sections were treated with 0.6% H2O2 in Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 minutes. BrdU-labeled nuclei were detected by immunofluorescence after DNA denaturation: 2 hours incubation in 50% formamidex2x SSC (2x SSC: 0.3 M NaCl, 0.03 M sodium citrate) at 65°C, 5 minutes rinse in 2x SSC, 30 minutes incubation in 2 M HCl at 37°C, and 10 minute rinse in 0.1 M boric acid, pH 8.5. Thereafter, incubation in TBS/0.1% Triton X-100/3% normal donkey serum (TBS-TS) for 30 minutes was followed by incubation with primary antibodies for 48 hours at 4°C. After washing in TBS-TS, sections were incubated with secondary antibodies for 2 hours, extensively washed in TBS, and mounted on glass slides. Fluorescence was detected using a confocal scanning laser microscope (Leica).

Histological quantification

A systematic, random counting procedure was used as previously described (44, 67). Series of every 10th section (400-μm interval) were analyzed. For the dentate gyrus, all BrdU-positive cells in the granule cell layer were counted on approximately 6 sections per animal. For colabeling with neuronal marker NeuN to estimate the percentage of neurons among the newly generated cells, 100 randomly selected BrdU-positive cells per animal were analyzed under the confocal microscope. Multiplying the total number of BrdU-positive cells by the percentage of NeuN/BrdU-double-positive cells yielded the number of new neurons in the dentate gyrus. For the lateral ventricle wall, striatum, and cortex, we systematically analyzed 2 sections per animal by counting cells in 25 adjacent frames (250 x 250 μm each) arranged in an array of 5 x 5 frames starting adjacent to the lesion site. BrdU-positive, DCX-positive, and NeuN/DCX–double positive cells were counted, and the density for each cell type was calculated.

Cultivation of adult neural stem cells

Neural stem cells were obtained from the hippocampus or SVZ of 4- to 6-week-old male Wistar rats as described in ref. 68. Briefly, animals were sacrificed, and brains were dissected and washed in ice-cold Dulbecco’s PBS (DPBS) containing 4.5 g/l glucose (DPBS/Glc). The hippocampus and SVZ from 6 animals were dissected, washed in 10 ml DPBS/Glc, and centrifuged for 5 minutes at 1,600 × g at 4°C. Tissue was minced using scissors. Tissue pieces were washed again and centrifuged for 5 minutes at 800 g and the pellet resuspended in 0.01 % (wt/vol) papain, 0.1 % (wt/vol) Dispase II (Roche Diagnostics) (neutral protease), 0.01 % (wt/vol) DNase I, and 12.4 mM manganese sulfate in HBSS. Tissue was incubated for 40 minutes at room temperature.

Subsequently, the suspension was centrifuged at 4°C for 5 minutes at 800 g and the pellet washed 3 times in 10 ml DMEM/Ham’s F-12 medium containing 2 mM t-glutamine, 100 U/ml penicillin/streptomycin. Cells were then resuspended in 1 ml neurobasal medium containing B27 (Invitrogen Corp.), 2 mM t-glutamine, 100 U/ml penicillin/streptomycin, 20 ng/ml EGF, 20 ng/ml BFGF, and 2 μg/ml heparin. Cells were seeded into 6-well plates at a concentration of 25,000–100,000 cells/ml and incubated at 37°C in 5% CO2. Two-thirds of the medium volume was changed weekly (68).

Assessment of differentiation markers in vitro

Quantitative PCR. Cultured neurospheres (39 DIV) derived from the SVZ were stimulated once with the following G-CSF concentrations: 10 ng/ml, 100 ng/ml, and 500 ng/ml. Four days after addition of recombinant human G-CSF (NEUPOGEN; Amgen Inc.), cells were harvested for the RNA preparation. Untreated cells served as control. RNA of the G-CSF–treated and untreated neurosphere of the SVZ was isolated using the QIAGEN RNeasy Mini Kit according to the manufacturer’s recommendations. cDNA was synthesized from 2 μg total RNA using oligo-dT primers, Superscript II Reverse Transcriptase (Invitrogen Corp.). Quantitative PCR was performed using the LightCycler system (Roche Diagnostics Corp.) with SYBR green staining of DNA double strands. Cycling conditions were as follows: nestin and NSE, 3 minutes at 95°C, 5 seconds at 95°C, 10 seconds at 58°C, 30 seconds at 72°C, 10 seconds at 81°C for 50 cycles; β-III-tubulin, 3 minutes at 95°C, 5 seconds at 95°C, 10 seconds at 65°C, 30 seconds at 72°C, 10 seconds at 87°C for 50 cycles; β-III-tubulin, 3 minutes at 95°C, 5 seconds at 95°C, 10 seconds at 87°C for 50 cycles; β-III-tubulin, 3 minutes at 95°C, 10 seconds at 81°C for 50 cycles. Melting curves were determined using the following parameters: 95°C cooling to 50°C; ramping to 99°C at 0.2°C/second. The following primer pairs were used: rat nestin-plus, AGGAAGAAGCTGCAGACAG; rat nestin-minus, TTCACCTGTGGGCTCTAT; rat NSE-plus, GGCAAGGTGCCACTATG; rat NSE-minus, AGGGTGACGAGGAGGACTGA; rat β-III-tub-716s, CCACCTACGGGGACCTCAAC; rat β-III-tub-1022as, GACATGCCTTCTCACGC; rat GFAP-927as, AAAGGACGAAGGCTGAAGT; rat GFAP-3123s, CTTTCTTATGATGATGAG; rat GFAP-1245as, GTACATGATATCGAAGATG; Relative regulation levels were derived after normalization to cyclophilin and comparison with the untreated cells.

Luciferase assay. To amplify the β-III-tubulin gene promoter (fragment –450 to +54) (69), rat genomic DNA was used as a template for PCR. The amplified fragment was inserted into the MluI/Xhol site of the pG3L-Basic firefly luciferase reporter vector (Promega) to generate the pG3L-β-III-tubulin experimental vector. For DNA transfection, adult neural stem cells were dissociated and plated on poly-t-ornithin/laminin–coated 96-well plates at a density of 35,000 cells/well. After 24 hours cultivation, cells were washed once with 1× DPBS (Invitrogen Corp.). Cotransfection with the pG3L-β-III-tubulin vector (150 ng/well) and a Renilla luciferase construct (100 ng/well) was carried out with the Lipofectamine method (Invitrogen Corp.). The pG3L-Basic firefly luciferase reporter vector served as negative control. The DNA-Lipofectamine 2000 complexes were added to each well after removal of the DPBS, without addition of neurobasal medium. Following the incubation of transfected cells for 24 hours, cells were stimulated with various concentrations of G-CSF in neurobasal medium (5 ng/ml, 10 ng/ml, 100 ng/ml) for 48 hours. As a positive control for in vitro differentiation, stem cells were treated by withdrawal of mitogens and addition of 5% FCS. Using the Dual-Luciferase Reporter Assay System (Promega), we obtained the ratio of luminescence signals from firefly and Renilla luciferase (Mirhas LB 940; Berthold Technologies GmbH).

FACS analysis. For differentiation experiments, adult neural stem cells were plated in 15 cm2 culture flasks at a density of 4 million cells and were treated once with 100 ng/ml G-CSF. A single-cell suspension was made by triturating the neurospheres in 1-ml plastic pipettes and then collected by centrifugation. After resuspension in 1× PBS, the cells were fixed with 1% paraformaldehyde. The cells were incubated for 15 minutes on ice, washed once with 1× PBS, and then permeabilized by resuspension in 0.2% Tween-
20. After an incubation on ice for 15 minutes, FCS was added in a 1:50 dilution for blocking. The cells were incubated for 2 hours on ice with a MAP2 antibody (1:50, Sigma-Aldrich) and washed 3 times with 0.1% Tween-20. Following incubation for 30 minutes on ice with a donkey anti-mouse FITC-conjugated secondary antibody (DianoVa), the cells were washed again three times with 0.1% Tween-20 and finally resuspended in 1x PBS for FACS analysis. Flow cytometry of cells was performed on a FACSCalibur (BD).

Data and statistical analysis

The values are presented as mean ± SEM. The 2-tailed Student’s t-test was used to determine significant difference between infarct volumes. Behavioral measurements were analyzed using the Mann-Whitney rank sum test or the 2-tailed Duncan test were used to determine the statistical significance of differences in physiological parameters and neurogenesis. Statistical analysis was performed with NCSS 2004 software (NCSS).

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