

RESEARCH ARTICLE

Baclofen facilitates sleep, neuroplasticity, and recovery after stroke in rats

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Abstract

Objective: Sleep disruption in the acute phase after stroke has detrimental effects on recovery in both humans and animals. Conversely, the effect of sleep promotion remains unclear. Baclofen (Bac) is a known non-rapid eye movement (NREM) sleep-promoting drug in both humans and animals. The aim of this study was to investigate the effect of Bac on stroke recovery in a rat model of focal cerebral ischemia (isch). **Methods:** Rats, assigned to three experimental groups (Bac/isch, saline/isch, or Bac/sham), were injected twice daily for 10 consecutive days with Bac or saline, starting 24 h after induction of stroke. The sleep–wake cycle was assessed by EEG recordings and functional motor recovery by single pellet reaching test (SPR). In order to identify potential neuroplasticity mechanisms, axonal sprouting and neurogenesis were evaluated. Brain damage was assessed by Nissl staining. **Results:** Repeated Bac treatment after ischemia affected sleep, motor function, and neuroplasticity, but not the size of brain damage. NREM sleep amount was increased significantly during the dark phase in Bac/isch compared to the saline/isch group. SPR performance dropped to 0 immediately after stroke and was recovered slowly thereafter in both ischemic groups. However, Bac-treated ischemic rats performed significantly better than saline-treated animals. Axonal sprouting in the ipsilesional motor cortex and striatum, and neurogenesis in the peri-infarct region were significantly increased in Bac/isch group. **Conclusion:** Delayed repeated Bac treatment after stroke increased NREM sleep and promoted both neuroplasticity and functional outcome. These data support the hypothesis of the role of sleep as a modulator of poststroke recovery.

Introduction

Ischemic stroke is one of the most prevalent neurological conditions and a leading cause of death and long-term disability worldwide.^{1,2} Despite progress made in understanding the mechanisms involved in neuronal damage during ischemia, limited advances have been reached in developing effective treatments for stroke patients.^{3,4} Spontaneous partial recovery after stroke is associated with neuronal plasticity mechanisms. Data from both patients and animal models showed a remodeling of neuronal networks in the hemisphere affected by stroke as well as recruitment of additional circuits from the contralesional hemisphere.^{5–8} Therefore, methods inducing or enhancing neuronal plasticity processes in the

lesioned brain area may represent a novel effective therapeutic strategy for stroke.

There is growing evidence suggesting an important role of sleep in facilitating brain plasticity.^{9,10} Changes in sleep and sleep electroencephalogram (EEG) may not only reflect changes in connectivity within cortical neuronal network but also drive changes in synaptic strength.^{11,12} Moreover, it has been shown that plasticity-related genes and proteins display differential expression in sleep, wakefulness, and following sleep deprivation (SD).¹³ Increased slow-wave activity (SWA) in nonrapid eye movement (NREM) sleep, a marker of sleep intensity, was observed in the brain regions stimulated by intense activities during wakefulness both in humans and animals.^{14,15} Furthermore, specific waking activities can trigger both induction

of plasticity-related genes in the cortex and homeostatic response during the subsequent sleep episode.¹⁶ SD impaired induction and/or maintenance of long-term potentiation (LTP),¹⁷ a basic mechanism thought to underlie neuronal plasticity and memory formation, whereas sleep oscillations induced LTP in the adult¹⁸ and in the developing cat following monocular deprivation.¹⁹ In addition, recent data have shown that NREM sleep has a key role in promoting learning-dependent synapse formation and maintenance on selected dendritic branches.²⁰ Therefore, interaction between EEG markers of sleep and the events mediating plasticity at the molecular, cellular, and network levels provide a link between sleep and brain plasticity.

There is accumulating evidence suggesting an important role for sleep in stroke recovery. Manipulation of sleep after ischemia may in fact affect stroke outcome. Disruption of sleep during acute and subacute phase of stroke aggravated brain damage²¹ and impeded functional recovery in rats.²² On the contrary, administration of γ -hydroxybutyric acid (GHB), considered a sleep-promoting drug, immediately after reperfusion accelerated motor function recovery in mice.²³ Furthermore, a physiological enhancement of sleep (following previous sleep deprivation) occurring immediately after stroke induction, was also associated with a reduction of brain damage.²⁴ Sleep may, therefore, play a dual beneficial role in brain repair, fostering neuroprotection in the acute phase and enhancing neuroplasticity in the delayed phase after stroke.

The aim of the current study was to investigate the effect of sleep enhancement on brain repair and functional recovery after the acute phase of ischemic stroke. We hypothesized that induction of sleep or synchronized neuronal activity would facilitate motor function recovery and brain repair mechanisms in a rat model of focal cerebral ischemia. Considering our strong interest in translational approaches, we decided to use baclofen (Bac), a Gamma-aminobutyric acid (GABA)-B receptor agonist which is known to promote sleep in humans.²⁵ Our group has recently shown that Bac also increases NREM sleep duration in rats.²⁶

Methods

Animals

Adult male Sprague–Dawley rats ($n = 53$; Harlan Laboratories, Horst, Netherlands; Charles–River, Sulzfeld, Germany), 318 ± 17 g at the time of surgery, were maintained on a 12–12 h light–dark cycle at $22 \pm 0.5^\circ\text{C}$ ambient temperature. They were kept individually in Macrolon cages and provided with food and water *ad libitum*, except food restriction during behavioral training. The experiments were carried out with governmental approval according to local guidelines for the care and

use of laboratory animals at the University Hospital Zürich, Switzerland.

Experimental protocol

Two separate experiments were performed. In Experiment 1 (Fig. 1A), rats were implanted with EEG and electromyogram (EMG) electrodes. Animals were then subjected to focal cerebral ischemia (isch) or sham surgery and assigned to one of the three experimental groups: Bac/isch ($n = 6$), saline/isch ($n = 7$), or Bac/sham ($n = 4$). Baclofen (Sigma–Aldrich, Buchs, Switzerland; 10 mg/kg) was diluted in saline (0.9% NaCl) to obtain 3 mg/mL working solution. The drug was administered intraperitoneally (i.p.) 24 h after surgery and then twice daily (1 h after light onset and offset) for 10 consecutive days. Sleep was recorded during baseline preceding surgery and on day 2, 6, and 11 after surgery. All animals were decapitated 1 day after the last injection and their brains were collected for histological analysis.

In Experiment 2 (Fig. 1B), rats were trained in a single pellet reaching task (SPR) for ca. 25 days. During training the preferred paw was identified for every rat. Cerebral ischemia or sham surgery was performed, when animals reached a stable level of performance. Thereafter, rats were subdivided into three groups Bac/isch ($n = 14$), saline/isch ($n = 14$), and Bac/sham ($n = 8$) and subjected to the same pharmacological protocol as in Experiment 1. Motor function was assessed 1 day after surgery before the first drug injection and then weekly starting on the day after the end of the drug administration (days 12, 19, 26, 33, and 40 after surgery).

Proliferation marker, 5-bromo-2'-deoxyuridine (BrdU; Sigma–Aldrich, St. Louis, MO; 50 mg/kg),²⁷ incorporating into DNA during cell division, was administered for 10 days (2 h after light onset, i.p.), and followed Bac or saline injections. BrdU was diluted in saline (concentration 10 mg/mL).

The anterograde tracer, biotinylated dextran amine (BDA, 10%; MW = 10,000 Da; Molecular Probes, Eugene, OR; diluted in 0.01 mol/L phosphate buffer),²⁸ used to evaluate axonal sprouting,^{22, 29} was microinjected at two locations into the motor cortex contralateral to the lesion side (stereotaxic coordinates: antero–posterior (AP) ± 1 mm, medio–lateral (ML) 1 mm, dorso–ventral (DV) 3 mm from the skull). All rats belonging to the Experiment 2 received a total volume of 1 μL of tracer (0.5 μL of each injection, over 10 min) 6 weeks after surgery. Two weeks later rats were sacrificed and brains were collected for further evaluations.

EEG implantation and recording

Rats were implanted epidurally with EEG and EMG electrodes under deep anesthesia (2% isoflurane in 30% O₂

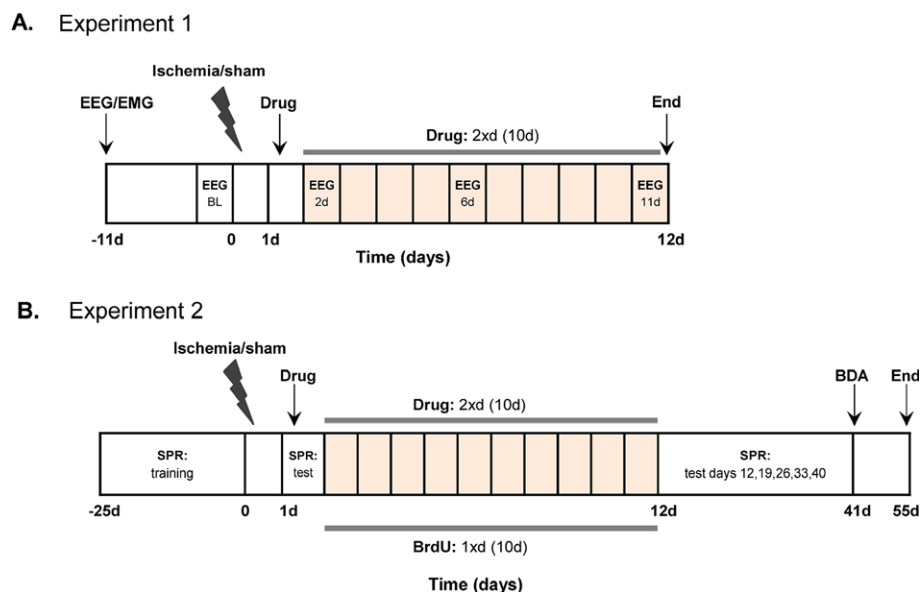


Figure 1. Design of the experiments. (A) In Experiment 1, rats were implanted with EEG/EMG electrodes and 11 days later subjected to ischemia (isch) or sham surgery. Twenty-four hours after surgery animals were treated with the drug (10 mg/kg baclofen, Bac, or saline, Sal) and then twice daily for 10 days. EEG and EMG were recorded during a 24-h baseline (BL) day and on days 2, 6, and 11 following isch/sham surgery. Three treatment groups were designed: Bac/isch ($n = 6$), Sal/isch ($n = 7$), and Bac/sham ($n = 4$). (B) In Experiment 2, rats were trained in the single pellet reaching (SPR) task for 3–4 weeks preceding isch or sham surgery. Twenty-four hours after surgery rats were treated with Bac or Sal and then twice daily for 10 days. All rats received also bromodeoxyuridine (BrdU) injection for ten days. SPR performance was assessed over 3 days preceding surgery (baseline) and on days 1, 12, 19, 26, 33, and 40 following surgery. Forty-one days after surgery all rats received microinjection of biotinylated dextran amine (BDA) and were perfused 2 weeks later. Three treatment groups were designed: Bac/isch ($n = 14$), Sal/isch ($n = 14$) and Bac/sham ($n = 8$).

and 70% N₂O). Four gold-plated mini-screws were positioned in the skull over the motor cortex of the right and left hemispheres (± 2 mm to bregma, 2 mm lateral to midline). Electrodes were connected to stainless steel wires and fixed to the skull with dental cement. Two gold wires were inserted bilaterally in the neck muscles for EMG recording. At least 8–10 days were allowed for recovery. Before baseline recordings, rats were habituated to the sleep recording apparatus for 4–5 days.

EEG and EMG were sampled at 200 Hz. Signals were amplified, filtered, and analog-to-digital converted. Hardware EMBLA and software Somnologica-3 (Medcare Flaga, Reykjavik, Iceland) were used. Activity in the 50 Hz band was discarded from the analysis because of power line artifacts. The EEG was subjected to a discrete Fourier transformation yielding power spectra (range: 1–25 Hz; frequency resolution: 0.25-Hz bins; time resolution: consecutive 4-sec epochs; window function: hamming).

Three vigilance states – NREM sleep, REM sleep, and wakefulness – were visually scored according to standard criteria at 4-sec epochs.³⁰ In addition to these three conventional vigilance states we introduced a new state, distinct from physiological sleep or wakefulness, which was

observed after Bac administration. This state was characterized by atypical behavior and abnormal hypersynchronous EEG pattern (“drug-induced” state), as described previously.²⁶ The state lasted 195.5 ± 7.6 and 197 ± 8.3 min in the Bac/isch group (light and dark phase, respectively), and 103.5 ± 32 and 189.1 ± 11.5 min in the Bac/sham group. Epochs were assigned to a specific vigilance state when more than half of the epoch fulfilled the criteria for that state. Epochs containing EEG artifacts were identified and excluded from subsequent spectral analysis in both derivations (25% of recording time, most of them [17%] occurred during wakefulness).

Induction of focal cerebral ischemia

Stroke was induced by the three-vessel occlusion method (3Vo) with permanent occlusion of the distal middle cerebral artery (MCA) and the ipsilateral common carotid artery (CCA), superimposed by temporal occlusion of the contralateral CCA under general anesthesia with 2% isoflurane.^{22, 31} A small piece of the skull overlying the MCA was removed and the dura was retracted. The MCA and its three main branches were occluded by bipolar electro-

coagulation. The CCA ipsilateral to the occluded MCA was ligated permanently with a 4-0 silk suture, whereas the contralateral CCA was temporarily occluded for 60 min with an aneurysm clip. Rectal temperature was maintained between $36.5 \pm 0.5^\circ\text{C}$ by a warm lamp during the surgery. Sham-operated rats were subjected to the same procedure except for occlusion of the MCA and CCA. Both ischemia and sham surgeries were performed on the hemisphere contralateral to the preferred forelimb assessed by SPR task.

SPR task

SPR task was used to assess fine motor skills.³² Rats had to use their preferred forelimb to retrieve a food pellet located in a well outside the test chamber.²² Briefly, animals were placed in a clear Plexiglas box ($41 \times 27 \times 37$ cm) with a vertical slit (1×15 cm) placed in the middle of the front wall, 1 cm above the floor. A 2-cm wide shelf with small wells was mounted in the front of the slit, but outside the box wall. Animals were trained to reach a food pellet (45 mg dustless precision pellet; Bio-Serv, Frenchtown, NJ) placed in the well on the shelf. Rats received daily training sessions consisting of 50 pellets for 3–4 weeks. A pellet was placed in the well on the side contralateral to the preferred paw. A single reaching attempt was permitted. Reaching attempts were classified as successful or failed. In the successful attempt the rat was expected to make a single reach, grasp the pellet from the well, bring it to the mouth and eat it. During the test sessions before and after surgery rats were given 50 pellets and the session ended when rats made 50 attempts or when 15 min elapsed. Success rate was computed as the percentage of successfully obtained pellets out of 50 possible attempts. The baseline (BL) performance was computed as the average of the 3 days immediately preceding surgery. Improvement in poststroke motor performance was computed as a difference in success rate between days 40 and 1 after ischemia.

To increase motivation all animals underwent a food restriction schedule with 20 g of chow per day during the training weeks and at the days preceding the test sessions. During this time rats were maintained at 95% of their normal body weight.

Tissue collection

Two weeks after BDA administration rats were deeply anesthetized and perfused transcardially with ice-cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were removed, postfixed in PFA for 2 h, and cryoprotected in 15% and 30% sucrose in the

ascending manner. The tissue was stored at -80°C for further evaluations.

Analysis of lesion volume and corpus callosum thickness

To determine lesion size and corpus callosum thickness, 40- μm thick coronal sections were cut with a cryostat at six predefined levels: 2.7 (L1), 1.7 (L2), 0.7 (L3), -0.3 (L4), -1.3 (L5), and -2.3 (L6) mm to bregma.³³ Brain sections were then stained with cresyl violet and digitized. Measurements were done with ImageJ (NIH, Bethesda, MD). Brain damage was computed on one section for each level as a difference between intact hemisphere and the nonlesioned area of the ischemic hemisphere. Lesion volume was estimated by multiplying obtained brain damage values by the size of each level (section thickness plus distance between levels). The corpus callosum thickness was measured using coronal sections from L6 level.

Immunohistochemistry

For detection of BDA, free-floating brain sections were incubated overnight with avidin–biotin–peroxidase complex (Elite ABC kit; Vector Laboratories, Burlingame, CA) and revealed with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich, Buchs, Switzerland).²² The sections were digitized and the area of BDA-labeled axons was quantified by determining the number of pixels above the intensity gray-scale threshold using ImageJ (NIH) as described previously.²⁹ Briefly, the mean surface area of BDA-labeled axons was measured in the primary motor cortex and striatum at the levels L2–L4 of coronal sections. Ratios between ipsilesional and contralesional cortical surface areas (corresponding to the corticocortical projections) and between ipsilesional striatal and contralesional cortical areas (corresponding to the corticostriatal projections) were used as an index of axonal sprouting. For each rat 2 adjacent sections were averaged on each level ($n = 9$, $n = 10$, $n = 7$ for corticocortical and $n = 7$, $n = 7$, $n = 6$ for corticostriatal in the saline/isch, Bac/isch and Bac/sham group, respectively). Ratios near zero indicated a low BDA penetration from the contra- to the ipsilesional side and, therefore, limited axonal sprouting.

Double immunofluorescence staining with the antibody against BrdU together with antibodies against specific cell-type marker was used to detect cell proliferation and to assess the type of proliferating cells. Free-floating sections were incubated in 2 mol/L HCl for 2 h at room temperature (RT) to denature DNA and then washed four times in PBS pH = 7.4. After preincubation in blocking solution (0.01 mol/L PBS containing 2% appropriate normal sera and 0.3% Triton-X), sections were

incubated overnight at +4°C with the rat anti-BrdU antibody (1:200; Abcam, Cambridge, UK) and one of the following antibodies for a specific cell-type marker: mouse anti-NeuN (1:200; Millipore, Billerica, MA; marker for neurons), rabbit anti-gial fibrillary acidic protein (GFAP, 1:200; Dako, Carpinteria, CA; marker for astrocytes) or rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1, 1:600; Wako Chemicals, Osaka, Japan; marker for microglia). Sections were then incubated for 1.5 h at RT with the fluorophore-conjugated secondary antibodies Alexa Fluor-488 (green; for detection of BrdU) or Cy3 (red; for detection of other markers) (1:200; Jackson ImmunoResearch, West Grove, PA) against the appropriate host species of the primary antibodies. Finally, sections were rinsed three times with PBS, mounted on gelatin-subbed slides (Southern Biotechnology Association Inc, Birmingham, AL, USA) and coverslipped.

Cell counting and microscopy analysis

The number of BrdU-positive (+), NeuN+/BrdU+, GFAP+/BrdU+, and Iba1+/BrdU+ cells were quantified in the peri-infarct region of ischemic animals and in the corresponding cortical region of sham animals using the optical fractionator probe (Stereo Investigator version 8.2; MicroBrightField Inc., Williston, VT) at 40× magnification on a fluorescence microscope equipped with a motorized x-y stage (Zeiss Axio Imager Z1, Jena, Germany; 20×/0.5 EC Plan-Neofluar objective). The peri-infarct area was outlined on a 10× magnification using the tracing function of Stereo Investigator. Several parameters were then determined on optical fractionator: counting frame (200 × 200 μm; x-y plane), optical dissector height (27–36 μm; z plane), distance between sampling regions (600 μm in x and y direction) and the grid size. A computer-driven motor stage allowed to analyze sections at each of the counting frame location under a 40× magnification. This procedure provided unbiased stereological quantification, because once the region of interest was outlined, sampling sites were evenly and randomly distributed throughout the marked region. Quantification was performed at levels L2 and L3 and averaged on two sections per animal ($n = 7$ per ischemic groups, $n = 6$ per sham group). The data are presented as the average of cell number per mm². All histological and immunohistochemical analyses were determined in a blinded way.

Statistical analysis

Effects of Bac treatment and time on motor performance in the SPR task were evaluated by a repeated measures ANOVA (SAS software, SAS Institute, Cary, NC). Effects of treatment on sleep, neurogenesis, axonal sprouting,

corpus callosum thickness, and brain damage were evaluated by one-way ANOVA. Post hoc paired and unpaired *t*-tests, Wilcoxon and Kruskal–Wallis or Tukey–Kramer test for multiple comparisons were performed if the results of the ANOVA reached statistical significance ($P < 0.05$). All provided values are means ± SEM. Pearson correlation coefficients were calculated between SPR performance and BDA or BrdU parameters; $P < 0.05$ was considered of statistical significance.

Results

Effects of Bac on vigilance states and EEG power spectrum

Bac increased the amount of NREM sleep during the dark period on days 2, 6, and 11 after stroke compared to saline administration ($P < 0.05$, unpaired *t*-test; Fig. 2A). We found no changes in the amount of REM sleep after Bac treatment. Amount of NREM sleep was similar between treatment conditions on the baseline day (364.5 ± 32.7 vs. 361.6 ± 36.6 min during the light phase and 218.8 ± 59.7 vs. 207.3 ± 55.4 min during the dark phase in saline/isch vs. Bac/isch group, respectively).

EEG power spectra were affected mostly in the hemisphere ipsilateral to the lesion (Supplementary material S1). Stroke led to a significant reduction of EEG power density in NREM sleep throughout most of the frequency range (Fig. 2B). Thus, in the saline/isch group it was reduced below BL values in the frequencies >3.75 Hz during both light and dark phases on days 2 and 6 after stroke and during light phase on day 11 (Fig. 2B left). Bac treatment resulted in a partial recovery of power. Hence, a significant reduction of power density in the frequencies >4.75 Hz was observed only during the light phase on day 2 after stroke (Fig. 2B right). EEG power was below BL values in the frequencies between 5.75 and 7.75 Hz on days 6 and 11 (only light phase) and above 17 Hz on day 6 (Fig. 2B right).

Effects of Bac on functional recovery

Effects of Bac administration on the recovery of grasping ability were assessed by SPR task. All rats showed similar performance in the task prior to ischemia. The success rate of pellet retrieval was $51.14 \pm 2.75\%$, $51.36 \pm 2.81\%$, and $45.50 \pm 2.74\%$ in Bac/isch, saline/isch, and Bac/sham groups, respectively. Rat performance dropped to zero (rats were not able to perform the task) immediately after stroke in both ischemic groups, but remained stable in the sham-operated animals (Fig. 3A). Slow spontaneous recovery was observed in the saline/isch group in the course of the following 6 weeks. However, saline/isch rats never reached the performance level of sham-operated animals ($P < 0.005$,

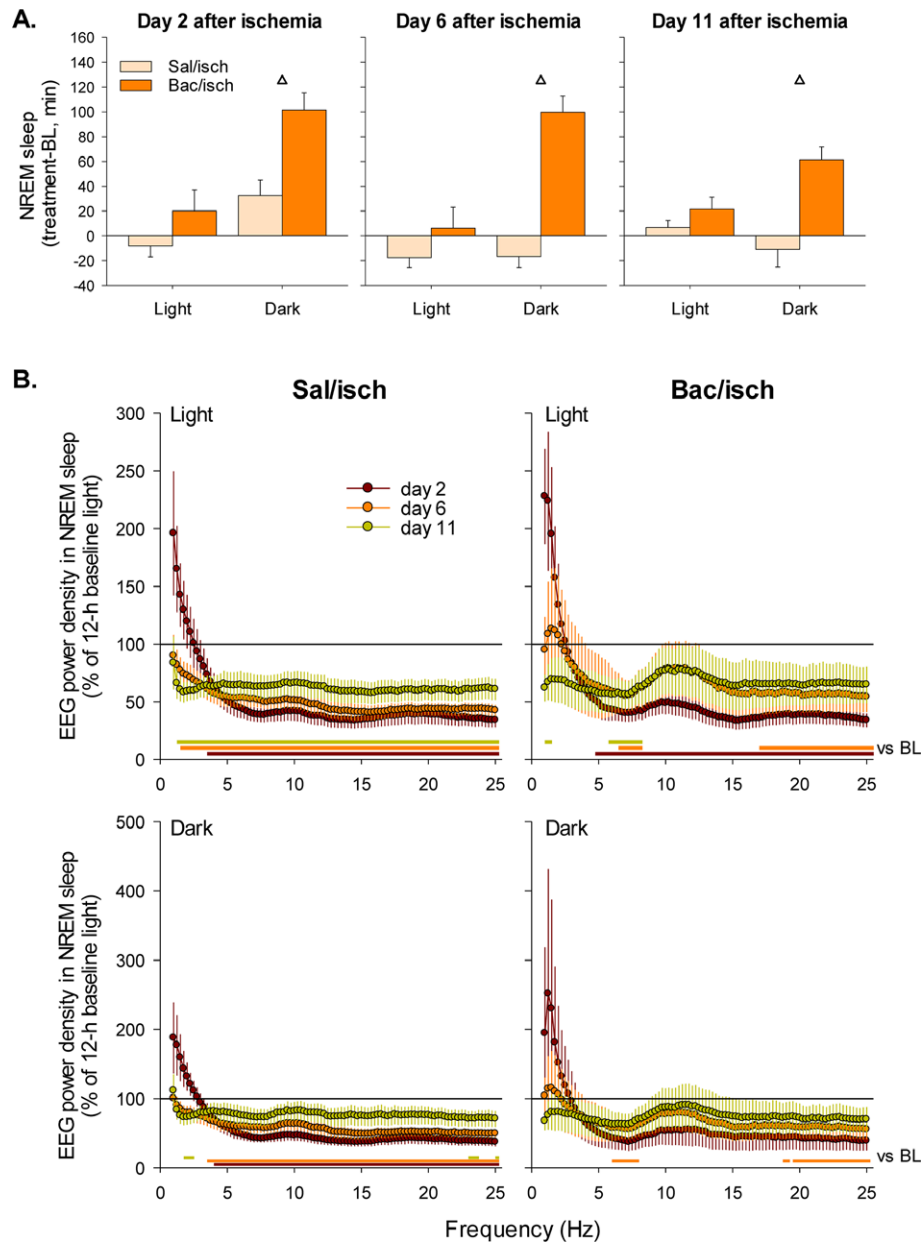


Figure 2. Effect of baclofen (Bac) or saline (Sal) treatment on NREM sleep amount (minutes) (A) and EEG power density in NREM sleep (B) during the light and dark phase of days 2, 6, and 11 after stroke. (A) Amount of NREM sleep (mean \pm SEM) was computed over the 11-h period following drug injection as a difference between treatment and corresponding baseline (BL) level. $\Delta P < 0.05$, unpaired *t*-test. (B) Power in each frequency bin after Bac or Sal treatment was normalized to the corresponding mean 12-h light BL value of the same bin. The curves connect mean values \pm SEM during the light and dark phase. Lines indicate differences between the treatment day and BL: $P < 0.05$, paired *t*-test.

Kruskal–Wallis after rANOVA interaction “group” \times “day” $F_{12,198} = 7.58$, $P < 0.0001$; Fig. 3A). In contrast, pellet retrieval of the Bac/isch group did not differ significantly from the Bac/sham group starting from day 33 post-stroke. Moreover, ischemic rats treated with Bac had generally a higher success rate than saline-treated animals ($P < 0.01$, Tukey–Kramer “group” after rANOVA factor “group” $F_{2,33} = 12.51$, $P < 0.0001$; Fig. 3A).

It has been shown previously that the side of the brain lesion may affect the dynamics of functional recovery.³⁴ Therefore, we investigated the effect of the lesion side on the success rate of pellet retrieval. Bac facilitated motor function recovery only in rats with right hemispheric stroke ($P < 0.0001$, Tukey–Kramer “group” after rANOVA factor “group” $F_{2,17} = 9.68$, $P = 0.0016$; Fig. 3B). Improvement in performance was detected already at day 19 after surgery.

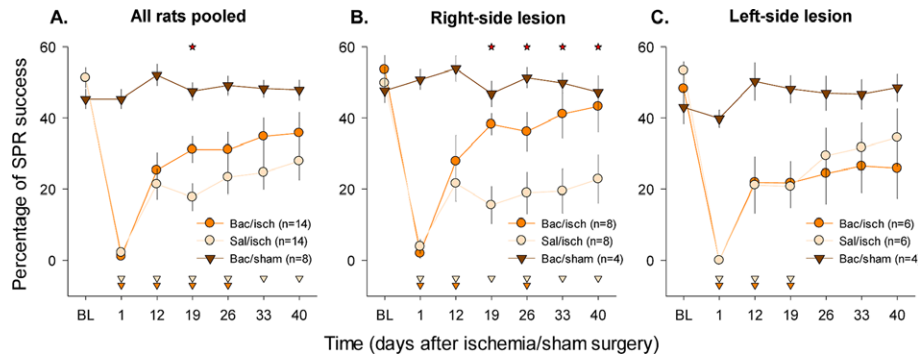


Figure 3. Effects of baclofen (Bac) or saline (Sal) treatment on motor function recovery in the single pellet reaching (SPR) task following brain ischemia (isch) or sham surgery: all rats pooled (A), rats with lesion in the right (B) or left hemisphere (C). The SPR success (number of successful attempts) was expressed as percentage of the total reaching attempts made (mean \pm SEM). Triangles indicate difference between Bac/sham and Bac/isch (orange) or Sal/isch (light orange) groups; Star between Bac/isch and Sal/isch group: $P < 0.05$, unpaired t -test following significant ANOVA.

No such facilitation was observed in rats with left hemispheric stroke (Tukey–Kramer “group” n.s.; Fig. 3C). Moreover, both ischemic groups with left hemispheric stroke showed no significant difference in sham-operated animals starting from day 26 after surgery (Fig. 3C).

Effects of Bac on axonal sprouting

Functional recovery can be related to neuroanatomical changes of tracts of fibers originating in the unlesioned cortex.⁵ Therefore, we evaluated axonal sprouting from neurons in the contralesional motor cortex toward the ipsilesional hemisphere (cortex and striatum; Fig. 4). Ratios reflecting corticocortical and corticostriatal projections were close to zero in the Bac/sham rats, indicating that treatment did not affect axonal sprouting in the control situation (Fig. 4B). In the saline/isch group only the corticostriatal ratio showed a significant increase compared to sham values ($P < 0.005$, Tukey–Kramer after ANOVA factor “group” $F_{2,19} = 57.10$, $P < 0.0001$; Fig. 4B). In contrast, Bac treatment in ischemic rats resulted in a twofold increase in both ratios compared to either saline/isch or Bac/sham group ($P < 0.0001$, Tukey–Kramer after ANOVA factor “group” $F_{2,25} = 54.46$ and $F_{2,19} = 57.10$, $P < 0.0001$ for corticocortical and corticostriatal ratios, respectively; Fig. 4B), indicating pronounced axonal sprouting from contra- to ipsilesional hemisphere. Moreover, ratios reflecting corticocortical and corticostriatal projections correlated positively with the improvement in motor performance ($r = 0.53$, $P < 0.01$, $n = 26$ and $r = 0.47$, $P < 0.05$, $n = 20$, respectively; Supplementary material S2).

Effects of Bac on neurogenesis

After stroke the peri-infarct area shows increased neuroplasticity, which allows sensorimotor functions remap-

ping.^{35,36} In order to evaluate the effect of Bac treatment on neurogenesis, we assessed the number of BrdU+ cells in the peri-infarct region 8 weeks after ischemia. Consistent with previous reports,^{22,37} we found a prominent BrdU labeling in both ischemic groups in contrast to sham animals, which showed a low level of cell proliferation. Moreover, there was a 60% increase in the number of BrdU+ cells in the Bac/isch (527 ± 32 cells/mm²) compared to the saline/isch group (317 ± 24 cells/mm²; $P < 0.0001$, Tukey–Kramer after ANOVA factor “group” $F_{2,26} = 59.22$, $P < 0.0001$).

To characterize the fate of BrdU+ cells, we examined double immunostaining with different cell-specific markers (NeuN for neurons, GFAP for astrocytes and Iba1 for microglia) (Fig. 6). Quantification of double labeled cells revealed that Bac administration after ischemia induced a significant increase in all targeted cell types compared to both saline/isch and Bac/sham groups. The number of NeuN+/BrdU+, GFAP+/BrdU+ and Iba1+/BrdU+ cells was significantly above saline/isch values 55 days after stroke ($P < 0.0001$, Tukey–Kramer after ANOVA factor “group” $F_{2,19} = 70.53$, $F_{2,19} = 74.89$, and $F_{2,19} = 18.11$, $P < 0.0001$, respectively; Fig. 5A). In addition, the number of proliferating neurons, astrocytes, and microglia correlated positively with the improvement in SPR task ($r = 0.71$, $P < 0.0005$; $r = 0.74$, $P < 0.0005$; $r = 0.57$, $P < 0.01$, $n = 20$, respectively; Supplementary material S3).

In all groups the majority of BrdU+ cells colocalized with the neuronal marker NeuN (Fig. 5B). The percentage of NeuN+/BrdU+ cells in both ischemic groups was significantly higher compared to sham-operated animals ($P < 0.001$, Tukey–Kramer after ANOVA factor “group” $F_{2,19} = 12.08$, $P < 0.05$). No difference was found between the two ischemic groups. Interestingly, the proportion of GFAP+/BrdU+ cells was significantly higher in Bac/isch

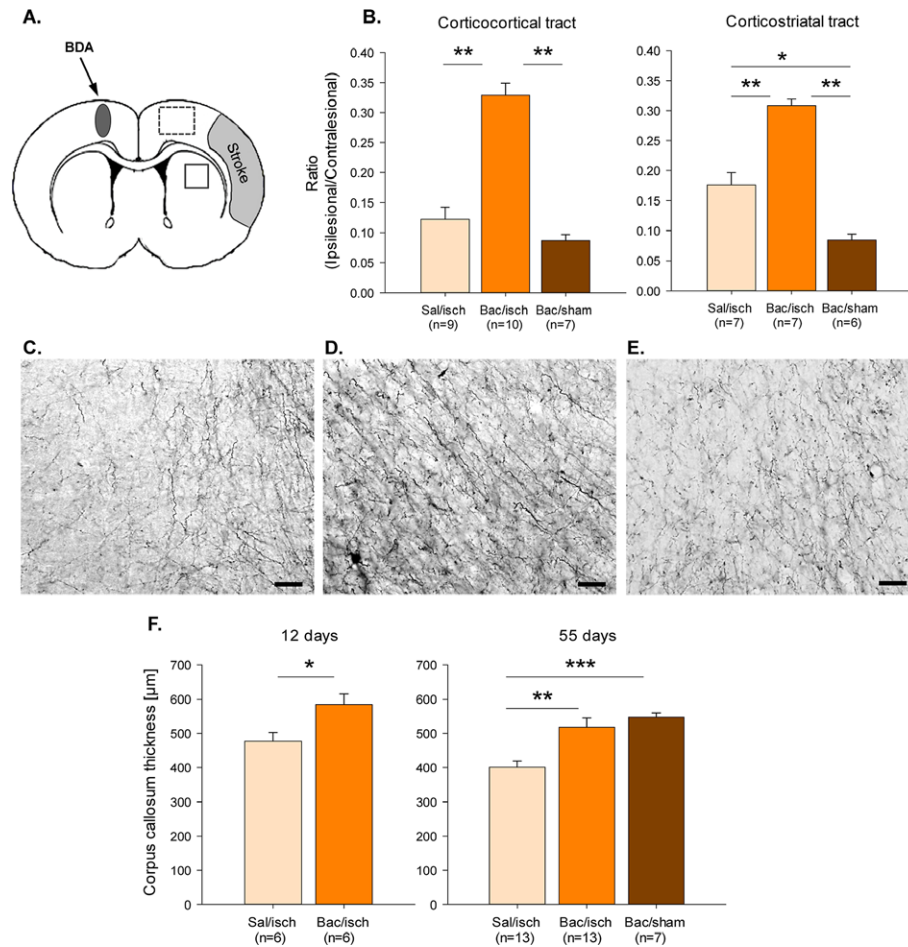


Figure 4. Effect of baclofen (Bac) or saline (Sal) treatment on axonal sprouting (mean \pm SEM) into the lesion-sided motor cortex and striatum following ischemia (isch) or sham surgery. (A) Schematic coronal brain section illustrating site of the lesion and placement of biotinylated dextran amine (BDA) tracer injection. (B) Quantification of corticocortical (left panel, dash-boxed area in A) and corticostriatal (right panel, black-boxed area in A) projections stained with BDA. Differences between groups: $*P < 0.005$, $**P < 0.0001$, Tukey–Kramer following significant ANOVA. (C–E) High-power magnification photographs from representative Sal/isch (C), Bac/isch (D), and Bac/sham (E) rats showing BDA traced fibers in the ipsilesional motor cortex (dash-boxed area in A). Scale bars, 50 μm . (F) Effect of Bac or Sal treatment on stroke-induced corpus callosum atrophy (mean \pm SEM) 12 and 55 days after isch or sham surgery. $*P < 0.05$, unpaired *t*-test; $**P < 0.005$, $***P < 0.001$, Tukey–Kramer following significant ANOVA.

compared to both saline/isch and Bac/sham groups ($P < 0.0001$, Tukey–Kramer after ANOVA factor “group” $F_{2,19} = 13.53$, $P < 0.05$; Fig. 5B). Additionally, the proportion of NeuN+/BrdU+ and GFAP+/BrdU+ cells correlated positively with the recovery of postischemic SPR success ($r = 0.69$, $P < 0.001$; $r = 0.65$, $P < 0.005$; $n = 20$, respectively; Supplementary material S4). The percentage of BrdU+ cells coexpressing Iba1 did not differ between groups.

Effects of Bac on lesion volume and corpus callosum thickness

Bac treatment had no effect on the extension of the sensorimotor cortex damage. Lesion size differed between

Bac- and saline-treated animals neither 12 (55.5 ± 10.1 vs. $57.5 \pm 8.8 \text{ mm}^3$, respectively; n.s.) nor 55 days after ischemia (61.8 ± 11.1 vs. $71 \pm 9.6 \text{ mm}^3$, respectively; n.s.). The mean volume of the right hemisphere lesion (66.8 ± 14.9 and $76.4 \pm 10.9 \text{ mm}^3$; Bac and saline group, respectively) did not differ significantly from the left hemisphere lesion (55 ± 17.9 and $62.4 \pm 18.8 \text{ mm}^3$).

It has been shown that ischemia results in corpus callosum atrophy, which is considered to be an indirect marker of neuronal loss.³⁸ To further evaluate the effects of Bac treatment on the ischemic brain, we determined thickness of corpus callosum. Thickness of corpus callosum was reduced in the saline/isch group when compared to the Bac/sham group ($P < 0.001$, Tukey–Kramer after ANOVA factor “group” $F_{2,32} = 11.18$, $P = 0.0002$, $n = 33$;

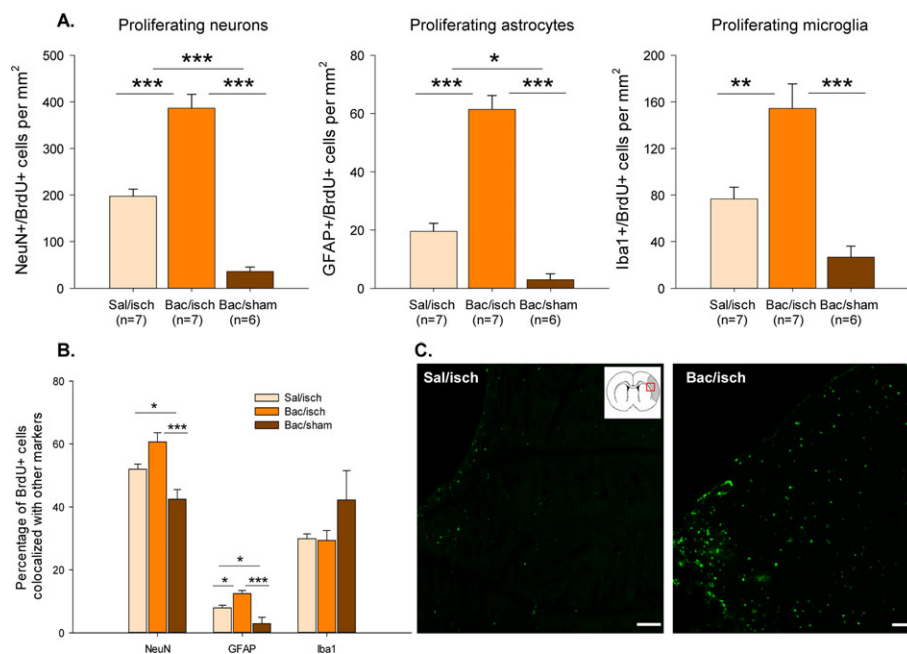


Figure 5. Effects of baclofen (Bac) or saline (Sal) treatment on cell proliferation in the peri-infarct region 55 days after ischemia (isch) or sham surgery. (A) Number (mean \pm SEM) of proliferating neurons (NeuN+/BrdU+ cells), astrocytes (GFAP+/BrdU+ cells) and microglia (Iba1+/BrdU+ cells) per mm². (B) Proportion of BrdU+ cells expressing NeuN, GFAP or Iba1 in the three experimental groups. Differences between groups: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$, Tukey–Kramer after significant ANOVA. (C) Representative confocal images of BrdU-positive cells in the peri-infarct region (red-boxed area on the brain scheme) of Sal/isch and Bac/isch groups. Scale bars, 100 μ m.

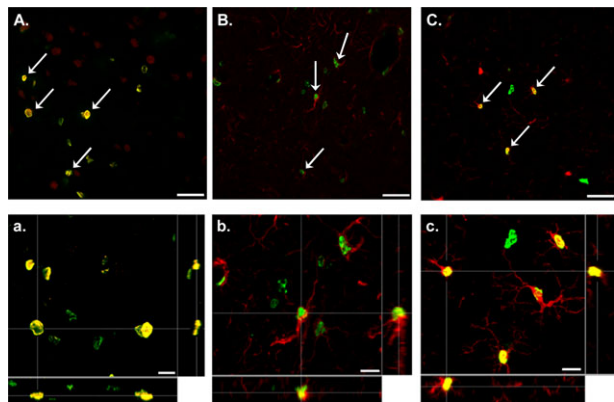


Figure 6. Phenotype of BrdU-positive cells (representative confocal images). Coronal sections were double labeled for BrdU (green) and NeuN (A), GFAP (B) or Iba1 (C) (red). Scale bars, 30 μ m. (A–C) Orthogonal reconstructions confirming double labeling in x – z (bottom) and y – z (right) planes. Scale bars, 10 μ m.

Fig. 4F). Bac treatment of ischemic rats decreased the atrophy of the corpus callosum significantly compared to saline treatment both at 12 ($P < 0.05$, unpaired t -test, $n = 12$; Fig. 4F) and 55 days after ischemia ($P < 0.005$, Tukey–Kramer after ANOVA factor “group” $F_{2,32} = 11.18$, $P = 0.0002$, $n = 33$; Fig. 4F).

Discussion

In the present study we show for the first time, to the best of our best knowledge, that pharmacological sleep promotion with baclofen (Bac) (1) has beneficial impact on long-term functional recovery after stroke in rats and (2) this improvement in behavioral performance is associated with an enhancement in endogenous brain restorative processes, such as axonal sprouting and neurogenesis.

Sleep has been suggested to be important during the recovery process after stroke. We have previously shown that sleep disruption after cerebral ischemia impairs functional and structural outcomes,²² and that SD before stroke, leading to sleep rebound in the acute phase of ischemia, is instead neuroprotective.²⁴

In this study the main goal was to test the effects of pharmacological sleep promotion with Bac starting 24 h after stroke onset, in order to avoid the reported neuroprotective effects of this compound.^{39,40}

In accordance with the previous studies in rodents,^{41,42} ischemic lesion resulted in a broad inhibition of the faster part of the EEG spectrum in the lesioned hemisphere. Bac administration following ischemia resulted in a significant increase in NREM sleep amount, particularly during the dark period. Bac given at the beginning of the light phase was less effective probably because of a ceiling effect. Fur-

thermore, Bac led to a recovery of the power in the higher frequencies (above 8 Hz). Noteworthy, recent observations in rats, linked motor function recovery with EEG frequencies >7 Hz.^{41,43} SWA during NREM sleep has been associated with neuroplasticity processes^{9,17–19} and therefore could be proposed to play a role in the promotion of endogenous restorative mechanisms and functional improvement during stroke recovery. Low-frequency synchronous neuronal activity has been suggested to have an important role in the anatomical reorganization and axonal sprouting after brain lesion.²⁹ Therefore, the changes in EEG activity observed immediately after Bac injections could play a role in improving the functional outcome.

In the present study cerebral ischemia was induced in the somatosensory cortex, thus leaving the motor cortical areas anatomically intact. Nevertheless, we observed a remarkable drop in the SPR performance after stroke. This effect was expected since motor cortex function was severely disrupted by extensive remodeling processes, during the recovery period following ischemia even when the area itself is spared by the lesion.⁴⁴ Spontaneous gradual improvement of motor function was present in the stroke animals injected with saline. However, Bac treatment accelerated performance improvement after stroke.

After Bac treatment, we found increased BDA labeling in both cortex and striatum, indicating an enhancement in the number of axons and/or increased axonal transport. Axonal sprouting, or the ability of brain to form new connections in areas denervated by the lesion, is a well-known phenomenon.^{5,29} Ischemic damage of the sensorimotor cortex induced sprouting of axons into the perilesion cortex from the homotopic cortex in the contralateral hemisphere²⁹ and into the striatum below the lesion.⁴⁵ Axonal sprouting after stroke is regulated by various neuroplasticity-related genes, including growth-promoting and growth-inhibiting molecules.⁴⁶ Many of these genes and proteins associated with neuroplasticity have been shown to be modulated by sleep.^{13,47} Previous studies showed that sleep deprivation after stroke induced an increase in the expression of neurocan (the main growth-inhibiting molecule to axonal sprouting),²¹ while GHB decreased it.²³ Therefore, changes in sleep may affect molecules crucial for post-stroke axonal sprouting. Furthermore, Carmichael et al. demonstrated the strong correlation between axonal sprouting and the periodic synchronized neuronal activity.²⁹ Bac, besides its effects on sleep, also induced a transient electrophysiological hypersynchronous pattern during the subanesthetic state, which could play a role in axonal growth and functional outcome. In this frame of reference it is possible to hypothesize that the positive effects of Bac on axonal sprouting and neurogenesis after cerebral ischemia are related to sleep-induced mechanisms.

Noteworthy, the corpus callosum atrophy was significantly reduced in Bac/isch group, despite the absence of the beneficial effect of Bac on the extent of brain damage. Because corpus callosum is the conduit for the interhemispheric communication, its thickness is a sensitive indicator of ischemic neuronal loss and a relevant marker influencing recovery of function after stroke. Our finding that Bac administration significantly reduced stroke-induced corpus callosum atrophy supplements the data showing a Bac-related boost in axonal sprouting.

Repeated administration of Bac also boosted neurogenesis in the ischemic brain. We found an increase in the number of proliferating cells. Several reports indicated that ischemic injury induces increased cell proliferation,^{37,48} associated with migration of newborn cells to the lesion sites.^{49–51} However, only a small fraction of newborn cells display a long-term survival. Therefore, enhancement of endogenous neurogenesis, primarily by improving survival of newborn cells, would be a plausible strategy for restorative therapies. In our study, extensive BrdU staining was noted in the peri-infarct region in both ischemic groups. Peri-infarct area is critical for rehabilitation, it shows intensified neuroplasticity, allowing remapping of sensorimotor function.^{35,36} Bac-treated rats had almost twofold increase in BrdU+ cells 55 days after stroke onset. Hence Bac might not only increase cell proliferation but also prolong survival of newborn cells and, therefore, enhance endogenous neurogenesis.

Although the majority of newly formed cells found in the peri-infarct region expressed the neuronal marker NeuN, we also observed an increase in the number of proliferating cells expressing glial markers after Bac treatment. Interestingly, the percentage of cells that differentiated into astrocytes was significantly higher in Bac/isch group, indicating that Bac might also affect differentiation of newborn cells. Glial cell activation has been demonstrated to accompany cerebral ischemia. However, there is a disagreement whether such gliosis is neuroprotective or harmful.⁵² Recent experimental evidence indicates that astrocytes and microglia play a dual role in tissue repair and reorganization.^{53–55} The results of the present study are in accordance with the emerging view that glial cells are active participants in the maintenance of a functional central nervous system and play an important role in the recovery from the brain lesion.^{56–58}

We observed that the increase in axonal sprouting and neurogenesis in Bac treated animals was positively correlated with the improvement of functional recovery. Several studies have previously suggested an association between neuronal plasticity and neurological recovery. In experimental stroke, enhancement of axonal sprouting improves functional outcome after brain damage.^{59–61} In addition, disruption of neurogenesis impedes functional

recovery after stroke, whereas treatment strategies aimed at augmenting neurogenesis are associated with functional improvement.^{62,63} We suggest that Bac treatment, most likely by promoting NREM sleep, enhanced endogenous mechanisms underlying neuronal plasticity and, therefore, improved functional recovery.

One intriguing finding of this study was that facilitation of functional recovery by Bac depended on the location of the lesion. Right hemisphere lesioned rats treated with Bac recovered faster, although the stroke extension/volume was similar on both sides. Brain asymmetry in motor and other functions was found in humans and rodents.^{64,65} There are several mechanisms that could account for the effects observed in the present study. The course of spontaneous recovery has been reported to be worse after lesions in the right, compared to the lesions in the left hemisphere.³⁴ Hence, the difference between Bac and saline-treated animals after the right-side injury could result from the poorer spontaneous recovery, allowing the drug to reveal its efficacy. Another possible explanation could be that this lateralized recovery promoted by Bac was the consequence of neuroanatomical or chemical asymmetries in the brain. It was shown that only right cortical or subcortical lesions led to the lateralized behavioral response and this effect was related to biochemical changes (particularly in dopaminergic (DA) and noradrenergic (NA) transmission) generated by the lesion.^{66–68} Therefore, if DA and NA activity level is changed only after right-side lesion, and Bac is known to affect both DA in ventral tegmental area (VTA)⁶⁹ and NA in locus coeruleus (LC),⁷⁰ it might be that the pronounced effect of Bac in right-lesioned rats is caused by normalization of the balance disturbed by stroke. The biochemical and anatomical origin of lateralized behavioral responses remains however poorly understood.

In summary, we have shown that an increase in NREM sleep induced by the delayed administration of baclofen promotes neuroplasticity and functional outcome in a rat model of stroke. Further studies are needed to understand the mechanisms responsible for these sleep-associated favorable effects.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of baclofen (Bac) or saline (Sal) treatment on EEG power density in NREM sleep in the contralesional hemisphere during the light and dark phase of days 2, 6, and 11 after stroke. Power in each frequency bin after Bac or Sal treatment was normalized to the corresponding mean 12-h light baseline (BL) value of the same bin. The curves connect mean values \pm SEM during the light and dark phase. Lines indicate differences between the treatment day and BL: $P < 0.05$, paired t -test.

Figure S2. Correlation between improvement in the single pellet reaching (SPR) performance and corticocortical (A) or corticostriatal (B) projections. Values are expressed as mean \pm SEM.

Figure S3. Correlation between improvement in the single pellet reaching (SPR) performance and the number of proliferating neurons (NeuN+/BrdU+ cells) (A), astrocytes (GFAP+/BrdU+ cells) (B) and microglia (Iba1+/BrdU+ cells) (C) per mm^2 . Values are expressed as mean \pm SEM.

Figure S4. Correlation between improvement in the single pellet reaching (SPR) performance (mean \pm SEM) and the percentage of NeuN+/BrdU+ (A) and GFAP+/BrdU+ (B) cells.