Reduced Mortality and Brain Damage After Locomotor Activity in Gerbil Forebrain Ischemia

W. Stummer, MD; K. Weber, DVM; B. Tranmer, MD; A. Baethmann, MD, PhD; O. Kempski, MD, PhD

Background and Purpose Preischemic spontaneous locomotor activity was distinguished in this laboratory as a factor influencing outcome after 15 and 20 minutes of forebrain ischemia in gerbils. Histological investigations were carried out to analyze potential relations between postischemic survival and a reduction of cerebral damage by spontaneous locomotor activity.

Methods Male Mongolian gerbils were divided into two groups, one with access to running wheels ("runners") and one kept in conventional cages ("nonrunners") for 2 weeks preceding forebrain ischemia of 15 or 20 minutes. A total of 99 gerbils were divided in subgroups and were allowed to recover for 2 weeks for assessment of survival. Other subgroups (n=7 to 9) were killed at day 4 for quantitative histology of selectively vulnerable areas such as hippocampus, cortex, striatum, and thalamus.

Results Two weeks after 15-minute ischemia, 44% of nonrunners had survived compared with 90% of runners (P<.01).

With 20-minute ischemia all runners survived compared with 21% of nonrunners. Quantitative histology (15-minute ischemia) revealed selective nerve cell injury in various cerebral regions in both groups. In runners, however, with the exception of the CA1 sector, damage was attenuated in cortex, striatum, and hippocampus. Furthermore, the extent of thalamic infarction was reduced (P<.05).

Conclusions Locomotor activity before global cerebral ischemia is highly efficient in protecting the brain as demonstrated by enhanced survival and a reduction of tissue damage in Mongolian gerbils. The mechanisms underlying this protection are currently unclear. However, further understanding of this intriguing phenomenon should enhance the understanding of ischemia pathophysiology and lead to the development of new treatment strategies. (Stroke. 1994;25:1862-1869.)

Key Words • cerebral ischemia • neuroprotection • locomotion • gerbils

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The gerbil model of forebrain ischemia has been widely used for the investigation of ischemia pathophysiology as well as for the assessment of pharmacological protection from ischemic damage. In these animals, severe ischemia can be easily induced by transient bilateral occlusion of the common carotid arteries. This experimental advantage notwithstanding, various factors such as body temperature, anesthesia,2 seizure susceptibility,3 and the inspired oxygen concentration4 have been identified as factors influencing outcome. A well-controlled experimental protocol must, therefore, take such aspects into consideration.

Spontaneous locomotor activity of gerbils maintained in treadmill cages for 14 days preceding 15 minutes of forebrain ischemia has been observed as an additional factor markedly enhancing survival.5-7 For further information, the present experiments were designed to determine whether reduced mortality was reflected by similarly attenuated tissue damage in vulnerable regions of the brain. For this purpose, quantitative histology was carried out in Mongolian gerbils subjected to 15 minutes of bilateral occlusion of the common carotid arteries with or without prior voluntary exercise in running wheels. Although numerous studies emphasize cell death in the hippocampal formation, other brain regions such as neocortex, striatum, or thalamus are also susceptible to ischemia8-9 and may be relevant to outcome. Thus, an investigation of these regions was included in the present study. Evaluation was performed by determining the density of surviving neurons in the respective structures, since selective neuronal damage could result in subtle morphological changes not readily detected by gross examination of routine histological preparations.

Materials and Methods

The experiments were conducted according to the ethical standards of the United States Public Health Service. Male Mongolian gerbils of 60 to 90 g body weight were raised from a stock of animals purchased from Hoechst. Gerbils were assigned to three experimental groups. One group was placed in cages with free access to running wheels with a diameter of 34 cm for a period of 14 days before ischemia ("runners"). During the 7 days preceding ischemia, revolutions of the running wheel were monitored by a computer for quantification of locomotor activity. A corresponding group of animals was kept in conventional cages ("nonrunners"). For induction of forebrain ischemia, animals were anesthetized with halothane (1.5%) and maintained at 37°C by a feedback-controlled heating pad. Both common carotid arteries were exposed under the operating microscope and were loosely encircled by 5.0 monofilament thread. Attention was paid to preserve adjacent vagal nerves. Arrest of blood flow was
induced by straining the filaments with a weight of 15 g, resulting in simultaneous occlusion of both arteries. The inspired concentration of halothane was reduced to 0.8\% during ischemia. Carotid occlusion was terminated after 15 or 20 minutes by release of the ligature, followed by closure of the skin and discontinuation of anesthesia. The animals were maintained on the heating pad until they woke and were subsequently returned to conventional cages with free access to food and water. Sham-operated gerbils were subjected to the same protocol except for carotid occlusion. Subgroups of animals were observed for 2 weeks after ischemia to assess survival (15-minute ischemia: nonrunners, n=55; runners, n=11; 20-minute ischemia: nonrunners, n=24; runners, n=9). Other gerbils with and without wheel-running before ischemia were killed at day 4 after 15 minutes of carotid occlusion for assessment of histopathology (nonrunners, n=9; runners, n=8; sham-operated, n=7). These animals were killed in deep ether anesthesia for perfusion fixation of the brain with phosphate-buffered paraformaldehyde (2\%) at pH 7.4 after rinsing with 0.9\% saline. Paraffin-embedded brain tissue blocks were cut serially into coronal slices of 5-\mu m thickness and stained with cresyl violet. Quantitative histology was performed by using standard sections obtained 1.7 mm caudal to the bregma for the study of parietal neocortex, hippocampus, and thalamus or at 0.5 mm rostral to the bregma for the study of striatum and frontoparietal neocortex.

Images of respective structures were obtained by using a light microscope equipped with a x10 lens (Zeiss). The images were projected onto the screen of an Amiga 2000 computer (Commodore) using a color CCD camera (Ikegami) and a genlock interface. Standardized grids were superimposed over the video image using software developed in this laboratory. Dimensions of the grids were calibrated with a microscope ruler (Leitz).

Surviving nerve cells in the hippocampus were quantified in the CA1, CA2, CA3, and CA3/CA4 subsectors. As schematically demonstrated in Fig 1A, the frame for the CA1 sector (0.31x0.12 mm) was adjusted with the lateral end of the upper branch of the dentate gyrus serving as a landmark. The frame for CA2 (0.34x0.13 mm) was placed adjacent to the lateral convexity of the neuronal band. The position of the CA3 frame (0.39x0.15 mm) was centered between the lateral convexity and lateral end of the upper branch of the dentate granule cells. The CA3/CA4 frame (0.31x0.12 mm) comprised nerve cells belonging to both the CA3 sector and the hilus (CA4) and was placed within the branches of the dentate gyrus. The frame for striatum (0.35x0.25 mm) was centered between the medial border of the lateral ventricle and the radiatio corporis callosi (lateral border) in sections taken 0.5 mm rostral to the bregma (Fig 1B). For quantification of surviving neurons in frontoparietal and parietal neocortex, analysis was not based on the neuroanatomic laminae into which the cortex can be subdivided. Because of the ischemic damage present in some specimens, confident identification of these laminae was not always possible. For assessment, therefore, four adjoining frames (0.3x0.2 mm) were superimposed vertically over the width of the neocortical band 3 mm from the sagittal plane (Fig 5, left). This mode of evaluation paid tribute to the laminar arrangement of the cortex but did not depend on a completely intact neuroanatomy. Likewise, because of its complex structure, no attempts were made to quantify neuronal density in the thalamus. Rather, well-defined areas of necrosis (infarcts) of varying size, which were frequently identified within the lateral nuclei of the thalamus, were subjected to planimetric assessment of infarct area.

Counting was conducted by investigators blinded to the respective experimental group. To be classified as viable and thus counted, a neuron was required to meet a number of inclusion criteria. These criteria, as summarized in the Table, are easily recognized at the light-microscopic level.\textsuperscript{10,11} Only cells with a complete presentation of all listed criteria were counted; other cells were rejected. Nerve cell counts obtained from both hemispheres of each animal were averaged for final evaluation.

Additional subgroups of gerbils were used in an initial study to register postischemic physical activity after 15 and 20 minutes of forebrain ischemia (n=14 and n=9, respectively). To do so, gerbils were placed in running wheels 14 days before ischemia to assess their normal daily physical activity. Postischemic running was then expressed as a percentage of the preischemic value.

All data are expressed as mean±SEM with the exception of animal survival, which is demonstrated by Kaplan-Meier probability plots. Differences in survival were tested for significance

Inclusion Criteria for the Identification of Viable Nerve Cells

<table>
<thead>
<tr>
<th>Feature</th>
<th>Criteria</th>
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<tbody>
<tr>
<td>Sharply delineated nucleus</td>
<td>ellipsoid or round shape</td>
</tr>
<tr>
<td>Clearly distinguishable nucleolus</td>
<td>located centrally within the nucleus</td>
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<tr>
<td>Nucleus slightly darker than surrounding neuropil</td>
<td></td>
</tr>
<tr>
<td>Neuronal cytoplasm cleary demarcated from surrounding neuropil</td>
<td></td>
</tr>
<tr>
<td>Less than one third of the neuron surrounded by confluent vacuolization</td>
<td>(&quot;pericellular halo&quot;)</td>
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Results

Locomotor activity was monitored during the final 7 days preceding cerebral ischemia. During this period, activity amounted to 823.5 ± 120.5 revolutions per day, a value corresponding to a running distance of 879.6 ± 128.7 m.

All gerbils regained righting reflexes within 1 hour after ischemia, initially displaying a hunched posture and decreased motor activity. Twenty-four hours later and during the first few days after ischemia, the animals were hyperexcitable on handling. This behavior was more pronounced in the group without preischemic wheel running. However, the level of hyperexcitability was not quantified in the present investigation. During the 2 to 4 days after ischemia, all gerbils lost weight. Those that eventually survived the 2-week observation period resumed uptake of food and water and rapidly compensated for the initial weight loss. On the other hand, moribund animals continued to lose weight, ultimately dying in a cachectic and somnolent state. Most important, access to wheel running during the 14 days preceding cerebral ischemia resulted in significantly enhanced survival (P < .01). With 15-minute ischemia almost all gerbils (91%) with access to running wheels survived the postischemic observation period of 14 days compared with only 44% of the group of animals maintained in conventional cages (Fig 2). After 20 minutes of ischemia all runners survived (not significantly different from 15-minute-ischemia runners) compared with only 21% of nonrunners. Correspondingly, in the subgroup of gerbils killed for histology 4 days after 15-minute ischemia, 2 of 9 nonrunners died beforehand, whereas all animals of the wheel-running group (n=8) survived.

Quantitative histology 4 days after ischemia revealed severe neuronal losses in all sectors of the hippocampus (Fig 3). Losses were most pronounced in the CA1, where no differences between runners and nonrunners were noted. However, in all other sectors of the hippocampus, damage was attenuated by preischemic running. This protection showed regional differences and was most obvious in the CA3 sector, where approximately 50% of neurons were found to survive compared with 10% in the nonrunning group. Two nonrunners displayed complete unilateral obliteration of the hippocampal formation, including the granule cells of the dentate gyrus. Nerve cell damage of such magnitude was not encountered in the gerbils with preischemic wheel running. Fig 4 shows photomicrographs of the hippocampus of animals subjected to forebrain ischemia with and without wheel running. Marked differences in the levels of damage to the cornu ammonis between both groups are obvious.

Histological damage of the neocortex was subtle in most cases. In one gerbil of the nonrunning group, necrotic lesions were present in the cortex of one hemisphere, involving neurons and endothelial and glial cells alike. However, only more severely affected animals displayed a laminar pattern of damage, preferentially of the third cortex layer and in some cases of the second, fifth, and sixth layers. Fig 5, right panel, gives an example of predominantly laminar neocortical damage as observed in a nonrunning gerbil after forebrain ischemia.

As mentioned, quantitative analysis was performed independent of the laminar architecture of the neocor-
Instead, the neocortex was superimposed with four frames of equal size covering its entire width, in which neurons appearing viable were counted. By this method, moderate damage not perceived at first glance was detected in the parietal cortex, as demonstrated in Fig 6A. Here neuronal losses were apparent in the three outermost frames. In runners, however, nerve cell counts in the outermost frame were not decreased, indicating protection in this region. With regard to the laminar arrangement of the neocortex and the pattern of damage visible in severely affected animals, protection by locomotor activity appeared to be predominantly conferred to laminae II and III. Conversely, neuronal losses observed in lamina V were similar in both groups. This pattern of protection was confirmed by data obtained in the frontoparietal section of the neocortex, where neuronal density was reduced in nonrunners in the two outermost frames. No losses were apparent in runners in this area.

Neuronal density was reduced in dorsolateral striatum in both runners and nonrunners, as shown in Fig 7. The number of surviving neurons, however, was significantly higher in the wheel-running group, amounting to 50% of control compared with 10% in gerbils without wheel running. There was no evidence of outright striatal infarction in either group.

Well-demarcated areas of necrosis (infarcts) were found in the lateral thalamic nuclei in 5 of 7 nonrunning gerbils. In 2 animals of this group, infarcts were present in both hemispheres. On the other hand, comparable lesions were observed in only 2 of 8 animals of the wheel-running group and were confined to only one hemisphere. Four days after ischemia (the time of death) these lesions presented as tissue areas in which neurons were completely obliterated, whereas glial and endothelial cell elements were partially preserved (Fig 8A). After 14 days the lesions were characterized by glial proliferation and macrophage infiltration, indica-
ventralis thalami. Larger infarcts found in animals of the nonrunning group also affected the dorsomedial part of the nucleus ventralis thalami and the lateral thalamic nucleus. Planimetric assessment of lesion size in the examined section revealed an average area of $2.24 \pm 1.1 \text{ mm}^2$ in gerbils without wheel running and $0.13 \pm 0.1 \text{ mm}^2$ in runners ($P<.05$). Furthermore, a close relation was found in nonrunning animals between infarct area and the number of surviving neurons in laminae V and VI of frontoparietal cortex ($r=-.98; P<.001$; Figs 5, left panel, and 9).

It appears noteworthy that postischemic wheel-running activity (registered in a separate group of gerbils) was dramatically enhanced during the first week after ischemia. After 15-minute ischemia ($n=14$) maximal hyperactivity reached $420\pm120\%$ of the preischemic value 3 days after ischemia; with 20-minute ischemia ($n=9$) hyperactivity had already reached $460\pm160\%$ by the first postischemic day and remained on an elevated plateau of $400\%$ to $500\%$ for 3 days. Gerbils with pronounced postischemic hyperactivity appeared to have particularly severe hippocampal nerve cell loss. This observation, which remains to be substantiated by a separate, more detailed study, is supported by results of others.$^{14}$

Discussion

The present findings confirm that preischemic locomotor activity effectively enhances survival in gerbils with forebrain ischemia (compare with References 3, 5, and 6). In gerbils both with and without wheel running before ischemia, quantitative histology revealed neuronal damage in those regions known to be particularly susceptible to ischemia, such as the hippocampus, the neocortex, the striatum, and the thalamus.$^{8,9,15-17}$ However, preischemic wheel running attenuated damage in most of these regions. Moreover, wheel running was associated with a reduction of infarct area in the thalamus. On the other hand, no protection was afforded to the CA1 sector of the hippocampus, which was almost completely obliterated in both groups. Consequently, the decrease in mortality observed in runners was not related to the preservation of CA1. More likely, survival was linked to the integrity of neurons in the striatum, the neocortex, or the thalamus. This point is noteworthy because the CA1 sector is a frequent subject in studies of the efficacy of drugs in ischemia (eg, Reference 18).

Histological damage after 20-minute ischemia was only investigated in a pilot study including 9 runners and 5 nonrunners. Neuronal loss was more severe after 20 minutes of ischemia than after 15 minutes, and again nerve cell counts were better in runners than in nonrunners. A somewhat different approach to quantifying histological damage had been chosen in these early experiments; therefore, these data were not included in the present report. They do however confirm the neuronal protection by spontaneous physical activity in the gerbil.

This protection by short-term preischemic wheel running is without precedent and was thoroughly unanticipated. As in all experiments, the validity of the conclusions demands to be questioned by careful scrutiny of the experimental procedures used. In the present case, care had to be taken to rule out more obvious factors.
Quantitative assessment of histology was conducted by blinded investigators after unequivocal positioning of the counting frames with respect to unmistakable anatomic landmarks. In the neocortex, no attempts were made at differentiating neuroanatomic layers, as their reliable identification was not always possible after ischemia. Instead, the cortical band was subdivided for counting into four layers of equal height to avoid misinterpretations in this region. Although the actual counting of neurons for quantitative assessments of cerebral tissue damage may be influenced by error, the determination of postischemic mortality is unambiguous. With respect to this parameter a subjective bias by the investigator can be ruled out.

Finally, to verify the effect of preischemic locomotion on postischemic mortality, additional experiments in which ischemia had been extended to 20 minutes were conducted by a skeptical coauthor (B.T.), confirming the initial results (Fig 2).

Accepting the validity of the present observations, obvious explanations for the protection encountered are not readily at hand. Improved survival in runners may be attributable to physical training, eg, of the cardiopulmonary system, providing for better compensation of systemic blood pressure changes during reestablishment of cerebral perfusion. Global cerebral ischemia is liable to induce systemic hypotension during recirculation, thus preventing adequate restoration of cerebral blood flow. However, continuous measurements of systemic blood pressure before, during, and after forebrain ischemia revealed identical blood pressure levels in both groups of gerbils with and without wheel running (unpublished data, Stummer et al, 1994), making differences in cerebral perfusion pressure unlikely.

Because protection by preischemic wheel running was conferred to regions of the brain that are particularly susceptible to short periods of ischemia, protection may involve the antagonism of mechanisms underlying the phenomenon of selective vulnerability. With regard to presently entertained concepts of this phenomenon, physical exercise may influence the release and the persistence of excitatory amino acids, such as glutamate,

Fig 9. Regression plot of cortical neuron density in laminae V and VI (abscissa) and the planimetrically assessed area of thalamic infarction (ordinate) of animals with and without wheel running before forebrain ischemia. The mean (±SEM) areas of ischemic necrosis in the thalamus of runners (closed bar) and nonrunners (open bar) is given as inset. A highly significant inverse correlation holds in nonrunners between the density of surviving neurons in parietal cortex and thalamic infarct size. The Pearson product-moment correlation yielded $P<.001$ with a polynomial regression: $r=-.984$, $P<.001$. Dotted lines illustrate 95% confidence limits. In runners with mitigation of thalamic damage, no significant relation could be established. Data of one gerbil are outside the 95% confidence limits.

Fig 8. Photomicrographs showing histological brain sections of conventionally maintained gerbils (nonrunners) with 15-minute forebrain ischemia demonstrating thalamic infarction. A, Ischemic necrosis (arrows) at 4 days after ischemia in nucleus ventralis thalami (nvt), which is located cranially and medially to the lemniscus medialis (LM). Note preservation of nucleus reticularis thalami (nrt) (scale bar, 200 μm). B, Ischemic necrosis in nucleus ventralis thalami (nvt) 14 days after ischemia with glial and macrophage infiltration (scale bar, 200 μm). cg1 indicates corpus geniculatum laterale; TO, tractus opticus.

other than wheel running that might determine outcome. Initially, enhancement of survival by preischemic locomotor activity was a coincidental observation during experiments designed to assess locomotor activity after ischemia as a functional measure of outcome. Other factors possibly influencing outcome were identical in both groups of gerbils. All animals were of the same stock, sex, and comparative age and weight and were assigned at random to the different experimental groups. Anesthesia, control of body temperature, surgical preparation, and postischemic handling were identical.

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in the extracellular compartment. Cerebral protection by preischemic locomotor activity might be explained by an interaction of exercise-induced changes of endogenous opioid systems with glutamate transmission. It is known that the N-methyl-D-aspartate (NMDA) receptor displays an affinity to opioids and morphinoids and that binding of these substances results in noncompetitive antagonization of the receptor. It has been repeatedly demonstrated that physical activity and physical stress are associated with an enhanced release of endogenous opioids. If the levels of these compounds are assumed to have been elevated in response to preischemic physical activity, the excitoxic stimulation of NMDA-receptor complexes by glutamate may have been inhibited.

As far as other mediator mechanisms are concerned, there is evidence of a shift in the balance between antithrombotic and prothrombotic eicosanoids by physical training, favoring antithrombotic compounds. It was found, for example, that exercise enhances the release of prostaglandin I2 from endothelial elements together with tissue plasminogen activator and reduces thromboxane release. Consequently, ischemia-induced aggregation of platelets may be suppressed, possibly attenuating postischemic reperfusion disturbances that may evolve in gerbils with cerebral ischemia.

Last but not least, chronic physical exercise requiring the continuing stimulation of cortical and subcortical centers for muscular control might affect the vascular blood supply of these cerebral regions. Isaacs et al. have obtained evidence for increased angiogenesis in the molecular layer of the cerebellum of rats subjected to forced or voluntary exercise by wheel running. The phenomenon was attributed to an increased metabolic demand resulting from enhanced regional synaptic activity. Although the significance of this observation for the presently observed protection is not clear, induction of such a process may have supported an inhibition of nerve cell loss from ischemia.

Taken together, the present findings demonstrate an impressive efficacy of preischemic locomotor activity in reducing both postischemic mortality and nerve cell losses in the brains of Mongolian gerbils. Further information on mechanisms underlying this phenomenon is not yet available. However, it is conceivable that protective mechanisms are operative during the period of early reperfusion. Although the present findings may have only minor consequences for the clinical management of patients, the elucidation of mechanisms involved in this phenomenon may provide a basis for improving the prophylaxis and therapy of ischemic stroke.

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References


Editorial Comment

The article by Stummer et al reported a marked improvement in survival and a corresponding decrease in neuronal damage in gerbils that were allowed to engage in spontaneous locomotor activity before an episode of cerebral ischemia of 15 to 20 minutes in duration. This remarkable finding has no satisfactory explanation at present. It should stimulate research into the mechanisms involved and into its possible implications for human cerebral ischemia. At present, the most likely explanation appears to be an influence of exercise on neurotransmitter release mechanisms in the brain, which in turn may affect neuronal survival. It is hoped that these investigators and perhaps others would pursue this interesting finding and identify the mechanisms involved in the near future.

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