Selective Vulnerability of Hippocampus and Disturbances of Memory Storage After Mild Unilateral Ischemia of Gerbil Brain

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SUMMARY The effect of selective injury of hippocampal neurons on the consolidation of memory traces was studied in gerbils (meriones unguiculatus) after production of mild cerebral ischemia. The right carotid artery was permanently ligated, and animals without gross neurological deficits ("symptom-negative" gerbils) were selected. Eight days and eight weeks after vascular ligation, cell counts of hippocampal neurons were carried out and correlated with regional blood flow and the acquisition of operant behaviour. Eight days after carotid artery occlusion, learning behaviour was significantly impaired although the number of hippocampal neurons had not changed and blood flow had even increased above normal. After eight weeks, learning behaviour and blood flow were normal but now a significant loss of pyramidal neurons was present in the CA1 and CA2 sectors of the hippocampus. Our observations demonstrate that it is possible to detect subtle functional disturbances by appropriate behavioural investigation before manifestation of selective injury of the hippocampus. Recovery of integrative function, despite persistent cellular damage, provides further evidence for central nervous plasticity.

THE CENTRAL NERVOUS SYSTEM exhibits different levels of neuronal activity: the relatively simple neuron-circuits of the spinal cord; more complex circuits of medulla, mesencephalon, thalamus; and the highest level of neuronal modules, the cerebral cortex which is the center of integrative functions such as learned behaviour. The evaluation of each of these functional levels requires different methodological approaches. Histological and electrophysiological methods may be adequate to assess basic patterns of neuronal activity but more sensitive behavioural studies are necessary to explore complex integrative processes. Full understanding of normal or abnormal function of the central nervous system, in consequence, is possible only by using a methodological approach which considers the various levels of neuronal integration.

We have combined histological, physiological and behavioural methods to investigate a peculiar phenomenon of cerebral ischemia, i.e. selective vulnerability of pyramidal neurons of the CA1 sector of the hippocampus. In this region, morphological alterations appear after a transient global episode of ischemia, followed by several days of "maturation" during which the cellular lesion becomes manifest. Recently, this model of post-ischemic selective vulnerability has been widely used for studying basic mechanisms of ischemic cell injury. However, it has to be considered that the more common clinical situation is a gradually developing permanent reduction of blood supply. We, therefore, were interested to know if morphological lesions in selectively vulnerable areas of the hippocampus occur not only after transient severe ischemia but also during a mild but permanent reduction of blood supply to the brain.

Such an insult can be produced in a sub-population of gerbils following unilateral carotid artery ligation. In this species, no anatomical connections exist between the carotid and basilary artery system, and the density of ischemia after carotid artery occlusion depends on the collateral blood supply from the opposite hemisphere via the anterior communicating artery. In about 60% of the animals the collateral blood supply is so poor that infarcts with gross neurological deficits develop. These animals are referred to as "sensitive" or "symptom-positive." The other 40% are "symptom-negative" animals which do not exhibit any gross neurological deficits although there are mild disturbances of energy state and of water and electrolyte homeostasis. These animals were selected for investigating the occurrence of hippocampal injury and for studying the question if such injury is associated with disturbances of learning behaviour.

Material and Methods

Experimental Protocol

Adult male Mongolian gerbils (weighing 60–80 g) were subjected to unilateral ligation of the right common carotid artery or sham-operation. Seventy-two randomized and weight-balanced animals, without gross neurological symptoms including their control counterparts, were divided into 10 groups. Each of the following parameters were determined 8 or 56 days after vascular occlusion: In 2 groups, operant conditioning in a Skinner box was performed (n = 10); in 2 further groups, regional cerebral blood flow was measured (n = 6); in another 2 groups, histological examination was carried out using conventional staining with hematoxylin, cresylviolet, Masson's and Bodian's stain (n = 4). The remaining groups served as sham-operated controls.

Surgical Procedures

Animals were anesthetized with 1.2% halothane. The right common carotid artery was exposed and dou-
ble-ligated with silk. After recovery from anesthesia, gerbils without gross neurological symptoms were identified and divided into the above described experimental groups. Sham-operation consisted of anesthesia, exposure and clamping of the carotid artery by silk without ligation.

Operant Conditioning
Conditioning was carried out in a classical Skinner box. The acquisition of operant behaviour was defined as 4 bar presses and appropriate reinforcements within 1 minute, the maximal interval between bar press and reinforcement being 10 seconds. After 24 hours of food deprivation and determination of the operant level, reinforcements were counted until gerbils had achieved the above defined criterion of learning.\textsuperscript{11}

Measurement of Cerebral Blood Flow
Regional cerebral blood flow was measured semi-quantitatively using antipyrine autoradiography according to the method of Sakurada et al.\textsuperscript{12}

Histological Evaluation of the Hippocampus
In 5 µm coronal sections, 1.5 mm posterior to bregma, pyramidal neurons of hippocampal segments were counted in an area covering 0.02 mm\textsuperscript{2}. Homotopic regions of CA1, CA2, CA3, and CA4 segments were evaluated, and changes were expressed as the difference between the right (ischemic) and the left (non-ischemic) hemisphere.

Statistical Evaluation
Means and standard deviations of every experimental and control group were computed, and significant differences between groups were calculated by the Mann-Whitney-Wilcoxon non-parametrical u-test for the independent measurements\textsuperscript{13} of blood flow and operant conditioning, and by Wilcoxon's signed-ranks test for the non-parametrical paired values of hippocampal cell density. Significance was assigned to a one-tailed p-value of less than 0.05 or 0.01, respectively.

Results
Cerebral Blood Flow (fig. 1)
Eight days after carotid artery occlusion, blood flow in the ipsilateral hippocampus (1.5 mm posterior to bregma) was 18% higher than on the contralateral side (p < 0.05). Sham-operated control animals, in contrast, exhibited no side difference. After 8 weeks, no statistical differences were observed in either the experimental or control groups. Hemodynamic changes in other brain regions were absent at any time.

Operant Conditioning (fig. 2)
Eight days after ligation, learning significantly deteriorated. Ischemic animals needed $\bar{x} = 36$ reinforcements to achieve the criterion of learning in comparison to $\bar{x} = 23$ reinforcements in sham-operated gerbils (p < 0.01). Eight weeks after carotid artery occlusion,

**Figure 1.** Blood flow of the right hippocampus 8 days and 56 days after right carotid artery ligation. Dark bars: experimental groups, open bars: sham-operated groups. Values (means ± SD) are expressed as percent of opposite side. *Statistically significant difference, p < 0.05.

**Figure 2.** Operant learning 8 days and 56 days after right carotid artery ligation. Dark bars: experimental groups, open bars: sham-operated groups. Values (means ± SD) are numbers of reinforcements required for acquisition of operant behaviour. **Statistically significant difference from control group, p < 0.01.
operant learning normalized with $\bar{x} = 28$ reinforcements in the experimental and $\bar{x} = 27$ reinforcements in the sham-operated group ($p = 0.427$).

Segmental Cell Density of the Hippocampus (fig. 3)

Eight days after right carotid occlusion, no significant changes in hippocampal cell density were detected. The difference between the ipsilateral and contralateral sides amounted to $\bar{x} = +10$ cells/0.02 mm$^2$ in CA1, $\bar{x} = -5$ cells/0.02 mm$^2$ in CA2, $\bar{x} = +2$ cells/0.02 mm$^2$ in CA3 and $\bar{x} = +4$ cells/0.02 mm$^2$ in CA4. After 56 days, in contrast, a significant loss of pyramidal cells appeared in CA1 and CA2 segments ipsilaterally to carotid occlusion, amounting to $\bar{x} = -22$ and $\bar{x} = -29$ cells/0.02 mm$^2$ respectively ($p < 0.05$). In CA3 and CA4 segments, significant changes were not observed: $\bar{x} = -7$ cells/0.02 mm$^2$ in CA3 and $\bar{x} = +3$ cells/0.02 mm$^2$ in CA4.

Discussion

The results obtained demonstrate that permanent occlusion of the carotid artery in gerbils without any gross neurological deficits results in selective injury of CA1 and CA2 sectors of the hippocampus which resembles closely that observed after a transient period of severe forebrain ischemia. This finding is of considerable interest for the understanding of the mechanisms of selective vulnerability. Ischemia causes a depolarisation of cell membranes, resulting in intracellular flooding of the cytosol with calcium$^{14}$ which may lead, on one hand, to calcium overload of mitochondria$^{15}$ and, on the other, to an activation of calcium-dependent enzymatic processes which in turn will cause delayed inhibition of protein synthesis.$^{16}$ This process is initiated during ischemia and proceeds during the recirculation phase because post-ischemic hyperexcitability results in continuous receptor-dependent influx of calcium.$^{17}$

In the present situation of permanent occlusion of carotid artery in a subgroup of animals without gross neurological deficits, the primary ischemic impact was presumably very mild. In fact, in a previous series of experiments using the same methods, reduction of flow shortly after vessel occlusion was only 20–40% of control, i.e. a flow rate which is distinctly above the threshold of cell membrane depolarisation.$^{18}$ This reduction is reversible as demonstrated by the present measurements of flow after one week, which revealed even an increase as compared to the opposite side. This observation demonstrates that selective vulnerability develops in the absence of persisting flow disturbances; other events such as ischemia-induced hyperexcitability$^{19}$ or inhibition of protein synthesis,$^{16}$ therefore, seem to be of greater importance for the pathological process than the ischemic deterioration of energy metabolism.

The functional disturbance of the hippocampus following mild ischemia is also reflected by the behavioural changes. In agreement with earlier observations made in rats,$^{20}$ hippocampal damage was associated with an impairment of learning behaviour. This is not surprising in view of recent considerations about the biochemistry of memory. According to the hypothesis proposed by Lynch and Baudry,$^{21}$ long-term potentiation, which is thought to be involved in memory formation, is caused by a calcium-induced uncovering of glutamate receptors. Delayed ischemic injury of hippocampal neurons is closely associated with a massive release of glutamate causing an increase of transmembrane calcium fluxes.$^{22}$ It is therefore conceivable that under these conditions the capacity of the hippocampus to exhibit long-term potentiation after physiological stimulation is reduced. In fact, such an inverse relationship has been described after "kindling" of the hippocampus which is also associated with increased calcium fluxes and which causes an increase in glutamate binding sites.$^{23}$ The disturbed memory function, in consequence, may be the first symptom of the same molecular process which eventually leads to the destruction of the hippocampal cell integrity.

In contrast to the previous study in rats, in which ischemia — and hence the hippocampal injury — was bilateral,$^{20}$ behavioural disturbances were reversible in the present investigation. Normal acquisition of operant behaviour after 8 weeks can be explained by reorganization of neuronal connections. The commissural inputs of the opposite hippocampus converge on the ipsilateral dentate gyrus.$^{24}$ The afferent fibers of the ipsilateral dentate gyrus project to CA3 neurons which provide pathways to CA1 and to the neocortical areas.$^{25}$ The ipsilateral reverberating circuit of the limbic system, which is interrupted by the loss of CA1 and CA2 neurons, can be compensated by the contralateral side. It seems that within 8 weeks after carotid artery ligation, new connections have been established be-
between cellular structures involved in the consolidation process of memory traces.

Our observations, in consequence, demonstrate that mild ischemia without gross neurological deficits may cause selective neuronal injury of CA1 and CA2 sectors of the hippocampus which is associated with temporary disturbances of the acquisition of operant behaviour. If such behavioural disturbances are to be shown, the timing of investigation is of crucial importance because the functional deficits may disappear owing to the plasticity of the central nervous system. It is less likely that such a reversal is possible after bilateral hippocampal lesions or in more highly developed species. But even if this should be the case, transient functional deficits following circumscribed lesions of hippocampal subfields cannot be dismissed and should be considered for the interpretation of the pathological process.

References

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