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The Density and Distribution of Ischemic Brain Injury in the Rat Following 2–10 min of Forebrain Ischemia*

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Summary. The density and distribution of brain damage after 2–10 min of cerebral ischemia was studied in the rat. Ischemia was produced by a combination of carotid clamping and hypotension, followed by 1 week recovery. The brains were perfusion-fixed with formaldehyde, embedded in paraffin, subserially sectioned, and stained with acid fuchsin/cresyl violet. The number of necrotic neurons in the cerebral cortex, hippocampus, and caudate nucleus was assessed by direct visual counting.

Somewhat unexpectedly, mild brain damage was observed in some animals already after 2 min, and more consistently after 4 min of ischemia. This damage affected CA4 and CA1 pyramids in the hippocampus, and neurons in the subiculum. Necrosis of neocortical cells began to appear after 4 min and CA3 hippocampal damage after 6 min of ischemia, while neurons in the caudoputamen were affected first after 8–10 min.

Selective neuronal necrosis of the cerebral cortex worsened into infarction after higher doses of insult. Damage was worst over the superolateral convexity of the hemisphere, in the middle laminae of the cerebral cortex. The caudate nucleus showed geographically demarcated zones of selective neuronal necrosis, damage to neurons in the dorsolateral portion showing an all-or-none pattern. Other structures involved included the amygdaloid, the thalamic reticular nucleus, the septal nuclei, the pars reticularis of the substantia nigra, and the cerebellar vermis.

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Introduction

With the advent of small animal models allowing long term recovery following brain ischemia it has become increasingly clear that the final brain damage incurred may require many hours or even days to "mature", and that the evolution of irreversible injury can be preceded by a free interval of many hours, during which morphologic changes are still subtle or absent (Ito et al. 1975; Kirino 1982; Pulsinelli et al. 1982a; Suzuki et al. 1983a, b; Kirino and Sano 1984a, b). Thus, unless a sufficiently long recovery period is allowed, the density of cell damage may be grossly underestimated. Furthermore, since the rate of evolution of irreversible neuronal injury varies between brain structures, prematue sampling of tissue can give biased information on the susceptibility of various neuronal populations to ischemic injury.

Results obtained in the gerbil have given the surprising information that only 5 min of bilateral carotid artery occlusion cause extensive neuronal death in the CA1 sector of the hippocampus (Kirino 1982; Suzuki et al. 1983a, b). This type of neuronal damage was of the delayed type, was preceded by a free interval of at least 1 day, and occurred in parallel with a faster degeneration of a minority of CA4 pyramids, but spared CA3.

Recent data on rats have confirmed the presence of "maturation" of neuronal damage and have extended the analysis to forebrain structures other than the hippocampus (Pulsinelli et al. 1982a). With this model (Pulsinelli and Brierley 1979), assumed to produce

virtually complete ischemia in forebrain structures (Pulsinelli et al. 1982b), some brain damage was observed in most, but not all, animals after 10 min of vascular occlusion. Within the forebrain, the rank order of vulnerability was: hippocampal h1 (or CA1), h3–5 and paramedian zone (or CA4 and subiculum) pyramids, neocortical layer 3, layers 5 and 6, or both, and small to medium sized striatal neurons (Pulsinelli et al. 1982a). Results obtained with a model of *global* ischemia, i.e., one affecting infratentorial structures as well, suggest that CA1 pyramids and Purkinje cells in the cerebellum are equally sensitive to ischemic insults (Diemer and Siemkowicz 1981).

The question arises whether gerbils are more vulnerable to ischemia than the rat. It has been suggested that this is so, and that gerbils are susceptible because they are prone to develop seizures (Brown et al. 1979). One further possibility is that postischemic hypotension extends the period of ischemia into the recirculation period (Ito et al. 1980). However, some evidence exists to the contrary. Thus, recording of unit activity from the CA1 sector failed to disclose seizure activity, and evidence of inadequate reflow was lacking (Suzuki et al. 1983a, b). Furthermore, recent data on local flow rates during four-vessel occlusion in the rat demonstrate that CBF in forebrain structures are higher than presumed and variable (Blomqvist et al. 1984). It seems possible, therefore, that rats and gerbils are equally susceptible to ischemia provided that dense ischemia is induced.

The objective of this study was to assess the minimal periods of ischemia which gives rise to brain damage in the rat, and to examine the localization of such damage. To that end, we used a model of forebrain ischemia which consistently gives flow rates of less than 5% of control in neocortical, limbic, and striatal regions (Smith et al. 1984). In all experiments the animals were allowed a recovery period of 7 days prior to transcardiac perfusion fixation and subserial sectioning, allowing evaluation of cell damage by direct visual counting of dead neurons.

Material and Methods

Thirty-eight male Wistar rats of an SPF strain (Møllegaards Avlslaboratorium, Copenhagen, Denmark), weighing 320–390 g, were used for the experiments. The animals were divided into six groups: controls, and those subjected to 2, 4, 6, 8, and 10 min of ischemia ($n = 6$ in each group except 10 min ischemia, where $n = 8$), all with a survival time of 7 days. The animals were starved during the night preceding the operation but were allowed tap water ad libitum. Five additional animals were used to assess local CBF (l-CBF) in the recirculation period.

Anesthesia was induced with 3.5% halothane, and the rat was intubated and connected to a Starling type respirator delivering 0.7% halothane and 30% O_2 in N_2O. Muscle paralysis was maintained with 1 mg i.v. boluses of suxamethonium chloride (Celocurin, Vitrum AB, Stockholm, Sweden), repeated every 15–20 min.

Tail artery and venous catheters were inserted, and the common carotid arteries were isolated via a neck incision. In 14 animals, two each in the 2, 4, 6, and 8 min groups and all the controls, a silicone catheter was advanced into the inferior caval vein via the right jugular vein to minimize the exsanguination time at the onset of ischemia (see below). For EEG recording a pair of needle electrodes were inserted in the muscles lateral to the skull bone.

The halothane was then discontinued, and the rat allowed a steady state period of 30 min, body temperature being maintained at 37° C,. arterial PO_2 at 90–100 mm Hg and PCO_2 at 35–40 mm Hg. Prior to the sampling of blood for the first blood gas measurement, 50 IU of Heparin (Vitrum AB, Stockholm, Sweden) was given i.v.

Following discontinuation of suxamethonium chloride administration, ischemia was induced by infusion of trimethaphan camphor sulfonate (Arfonad, Roche, Basel, Switzerland, 5 mg · ml^{-1}) to a blood pressure level of about 100 mm Hg, carotid clamping and arterial or central venous exsanguination to a blood pressure of 50 mm Hg, at which point a timer was started (see Smith et al. 1984). At this point, the animals designated as controls were reinfused with the shed blood, and the carotid clasps were released. In the experimental animals, the cessation of EEG activity was confirmed. Following the desired period of ischemia, blood pressure was restored by rapid reinfusion of shed blood, and the carotid clasps were removed. A volume of 0.5 ml of 0.6 M sodium bicarbonate was injected i.v. to counteract systemic acidosis. When the blood pressure had reached preischemic levels the caval catheter was removed and the neck incision was closed by satures.

Five animals were used to assess local CBF in the immediate recirculation period. In two of them, an infusion with ^{14}C-iodoantipyrine (25 μCi) was started 45 s following the removal of the carotid clasps and continued for 30 s. In these, blood pressure was close to 100 mm Hg. In the other animals, isotope infusion was begun after 60 s and continued for 30 s. These animals had blood pressure of 130–140 mm Hg. In all animals, arterial blood was sampled at 3-s intervals, and they were instantaneously decapitated at the end of the 30-s infusion period. Local CBF was estaminated with quantitative autoradiography as described in detail in an earlier communication (Kågström et al. 1983a).

In animals used for histopathologic analyses, blood pressure was continuously recorded during the early recovery period, and arterial blood samples were taken for control of PO_2, PCO_2, and pH. The temperature was kept near 37° C. After a recovery period of 30–60 min, the animals had regained consciousness, resumed spontaneous respiration, and could be disconnected from the respirator. They were subsequently extubated, and the tail catheters were removd. The animals were housed in cages with access to tap water and pellet food. During the week of survival the rats were weighed and examined daily to assess the nutritional state and neurological status.

On day 7 after the experiment, the animals were reanesthetized with halothane, connected to the respirator, and perfusion-fixed via the ascending aorta with 4% formaldehyde buffered to pH 7.35, preceded by a 30 s rinse with saline (see Auer et al. 1984a). Both solutions were prewarmed to 37° C and infused at a pressure of 135 mm Hg. The brains were allowed to stabilize in situ until removal the next day and subsequent storage in cold fixative.

The brains were cut coronally into 2.8 mm thick slices and dehydrated in graded strengths of ethanol over 2 days. Following clearing in xylol and embedding in paraffin they were subserially sectioned at 8 μm on a Reichert sledge microtome and stained

with cresyl violet and acid fuchsin. Sectioning intervals were adapted to obtain specific standard levels of caudate nucleus, the cerebral cortex and hippocampus for quantification of brain damage (see below).

The caudate nuclei were quantified at the level of the septal nuclei at their widest point, the cerebral cortex at the level of the subfornical organ, and the hippocampus at seven levels throughout its septotemporal extent. Quantification of damaged neurons was performed by direct visual counting of pink, acidophilic neurons at a magnification of 400×, using a two channel laboratory cell counter (Clay Adams, Parsippany, NJ, USA).

Plasma glucose levels were measured with a Beckman Glucose Analyzer 2 (Beckman Instruments, Inc., Fullerton, CA, USA).

Results

All animals except five, which were used to assess postischemic CBF, were allowed to survive for 7 days, and were then reanesthetized for perfusion fixation of the brain. Before giving an account of the histopathology, we describe changes in physiologic variables and neurologic status.

Physiologic Variables

Figure 1 gives a representative example of changes in arterial blood pressure during and following the ischemic period; it also illustrates how the period of ischemia was defined. During the period marked by *arrowheads*, an infusion of Arfonad was given. When the pressure was reduced to about 100 mg Hg, the carotid clasps were applied (*arrow*) and arterial bleeding started (*black bar*). The carotid clamping gave rise to a transient hypertensive response. The ischemic period was assumed to begin when blood pressure reached 50 mm Hg, and was terminated after 2, 4, 6, 8, or 10 min by removal of the carotid clasps and reinfusion of the shed blood. In spite of the fact that no pressor agent was given, blood pressure increased to >100 mm Hg within 1 min and then gradually rose to preischemic values.

It cannot be excluded that ischemia of sufficient magnitude to affect cerebral energy state occurred before the pressure reached 50 mm Hg. For each experiment we therefore calculated the time elapsed between the application and the removal of the carotid clasps. Table 1 gives the mean ischemic times, so defined, with the standard errors of the mean (SEM). We observe that mean clamping times exceeded the nominal ischemic times by about 0.5–1 min. Comparable times were obtained by calculating the periods during which blood pressure was reduced below 100 mm Hg (data not shown). Thus, a conservative

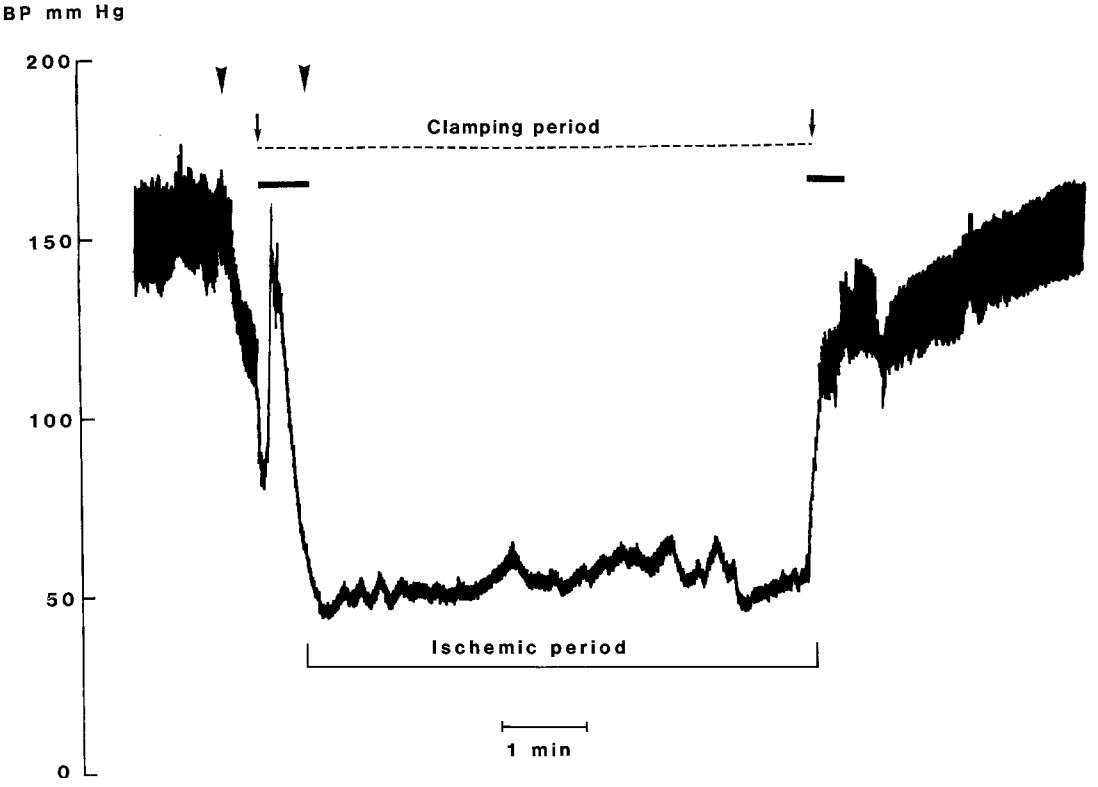

Fig. 1. Blood pressure changes in the ischemic period and in the immediate recirculation period. *Arrowheads* mark the start and termination of trimethaphan infusion. The start and end of the clamping period are marked by *arrows*, and *horizontal black bars* denote the periods of exsanguination and reinfusion of blood, respectively

Table 1. Period of carotid artery occlusion in groups with 2–10 min of ischemia

All values are given as mean ± SEM

Controls $n=6$	2 min $n=6$	4 min $n=6$	6 min $n=6$	8 min $n=6$	10 min $n=8$
0.50 ± 0.06	2.65 ± 0.10	4.78 ± 0.24	6.87 ± 0.30	9.06 ± 0.32	11.00 ± 0.21

Table 2. Physiologic parameters in control animals and in animals subjected to 2, 4, 6, 8, and 10 min of ischemia

	Temp °C		BP mm Hg		$PaCO_2$		PaO_2		pH	
	Pre-Isch.	15′ Recirc.	Pre-Isch.	15′ Recirc.	Pre-Isch.	15′ Recirc.	Pre-Isch.	15′ Recirc.	Pre-Isch.	15′ Recirc.
Control $n=6$	37.2 ± 0.1	37.0 ± 0.1	144 ± 4	144 ± 4	35.3 ± 0.9	37.0 ± 0.8	97 ± 3	102 ± 2	7.37 ± 0.01	7.37 ± 0.01
2′ isch. $n=6$	37.0 ± 0.2	37.0 ± 0.1	138 ± 7	131 ± 7	34.8 ± 0.9	34.8 ± 0.8	102 ± 4	108 ± 6	7.39 ± 0.02	7.39 ± 0.02
4′ isch. $n=6$	37.0 ± 0.3	37.1 ± 0.1	136 ± 3	134 ± 3	36.6 ± 0.8	36.4 ± 0.9	103 ± 4	103 ± 3	7.38 ± 0.01	7.40 ± 0.02
6′ isch. $n=6$	37.0 ± 0.3	37.1 ± 0.1	136 ± 3	142 ± 3	37.5 ± 0.9	36.3 ± 0.7	100 ± 4	108 ± 4	7.37 ± 0.01	7.40 ± 0.02
8′ isch. $n=6$	37.0 ± 0.2	37.0 ± 0.2	137 ± 6	137 ± 5	37.4 ± 1.2	35.3 ± 0.6	101 ± 2	105 ± 4	7.37 ± 0.01	7.40 ± 0.02
10′ isch. $n=8$	37.1 ± 0.1	37.6 ± 0.2	136 ± 4	136 ± 5	38.4 ± 0.6	38.6 ± 1.2	103 ± 3	113 ± 4	7.40 ± 0.01	7.42 ± 0.01

All values are mean ± SEM

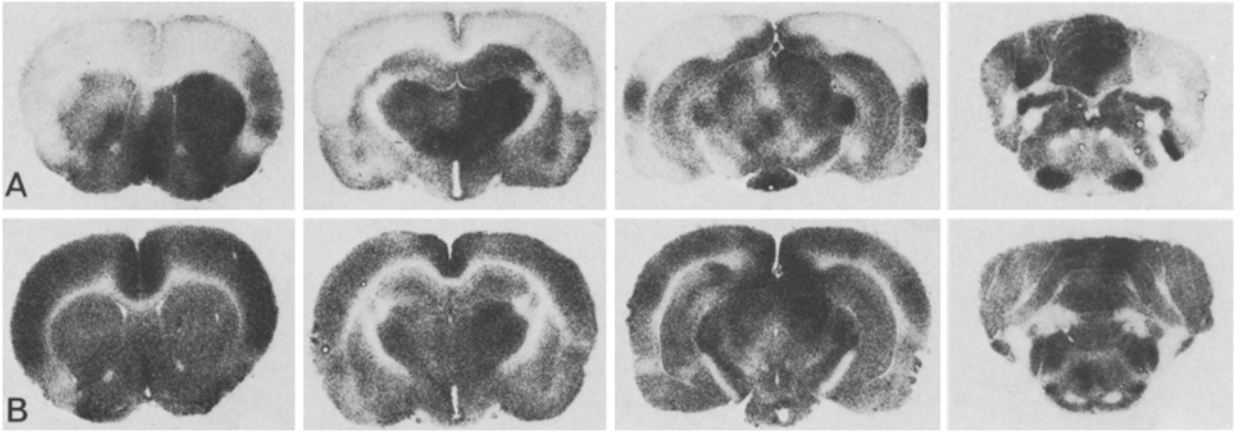

Fig. 2A, B. Autoradiographic pictures of local blood flow of two brains in the early reperfusion period after 10 min of ischemia. **A** is from an animal with a blood pressure of 100 mm Hg and a recirculation time of 1 min. **B** is from an animal with blood pressure 135 mm Hg and with recirculation for 1 min 15 s. Note perfusion defects in neocortex of rat **A**, and their absence in rat **B**

estimate is that the ischemic periods could have been approximately 0.5 min longer than those given (see Discussion).

Table 2 gives the mean values for physiologic variables just before and 15 min after the termination of the ischemia. All groups remained normothermic, had blood pressures of 130–140 mm Hg, $PaCO_2$ values around 35 mm Hg, PaO_2 values around 100 mm Hg, and pH values close to 7.40. Preischemic plasma glucose concentrations were normal or only slightly increased. Spontaneous breathing was resumed in about 40 min. Postischemic hypotension, hypercapnia, or hypoxia did not develop. However, to secure adequate oxygenation of arterial blood after extubation, oxygen was delivered through a tube positioned 1–2 mm from the animal's nose until it regained motility.

Since very short ischemic periods gave rise to brain damage (see below) the question arose as to whether removal of the carotid clasps and reinfusion of shed blood caused prompt recirculation. To study this, quantitative autoradiography was performed as described above (Fig. 2). Both animals infused with ^{14}C-iodoantipyrine with in a period of 45–75 s (MABP about 100 mm Hg) had deficient reflow of neocortical areas but hyperemic flow rates in all other structures, including the hippocampus and caudoputamen. In the animals infused with tracer with in a period of 1–1.5 min (blood pressure 130–140 mm Hg), only one animal had some neocortical

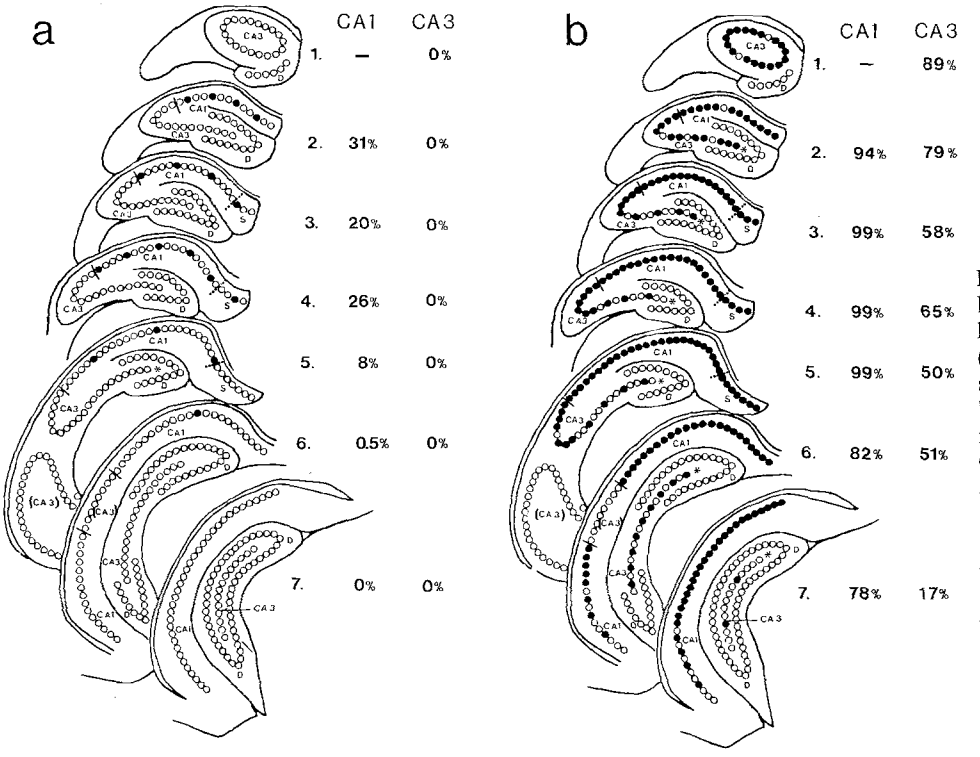

Fig. 3. Schematic figures showing hippocampal damage at seven levels after 4 min (**a**) and 10 min (**b**) of ischemia, followed by seven days of restitution. The border between CA1 and CA3 is marked by a line. The parts of CA3 within parentheses at levels 5 and 6 were not counted. A *dotted line* shows the border between CAD1 and subiculum (*S*). *D* dentate crest. An *asterisk* in the dentate hilus denotes CA4 damage. *Filled circles* neuronal necrosis. Damage in percent of total cells in each level in CA1 and CA3 is given on the right

areas with reduced CBF; in the other two, the forebrains were markedly hyperemic (CBF values 200% – 300% of control). Notably, all animals had CBF values in the vermis of the cerebellum which were higher than those of the cerebellar cortex. We conclude the adequate recirculation occurs within the 1st min within all structures, and that neocortical flow is resumed within the first 1.5 min.

Neurologic Behavior

All animals studied showed a similar pattern in regaining consciousness. The first sign to recover was reaction to painful stimuli, followed by recovery of spontaneous respiratory movements after approximately 30 min recovery. Good respiratory efforts were demonstrated after a mean time of 40 min recovery, allowing the animal to be discontinued from the respirator. Within 1 h after the ischemic insult, the righting reflexes were regained, and the animal was extubated. During the following days, all animals at first lost weight, but they started to eat and drink within the first few days after the insult (with the exception of one 10 min animal which lost weight during the entire recovery period).

Control and experimental animals in the 2 and 4 min groups were indistinguishable from normal rats during the week of survival. Rats in the 6 min group were mildly hyperexcitable on handling, and this was more accentuated in the 8 and 10 min groups. All of the 10 min animals and some of the 8 min animals showed a distinct postural pattern characterized by a "hunchback" kyphosis of the spine, and walking on extended limbs (Smith et al. 1984).

Histopathology

As stated above, the brains were subserially sectioned at 8 μm for histopathologic assessment. Figure 3 shows the levels at which damage of the hippocampus was evaluated, and gives the subdivisions of hippocampal pyramidal cells into the subiculum, CA1, CA3, and CA4 sectors.

Control Animals. Acidophilic neurons were absent in all areas examined. However, all animals showed occasional pyknotic neurons in CA4. The nature of these neurons will be subject to further study. Presently, only acidophilic neurons will be considered.

Ischemia 2 min. Four of six animals showed acidophilic neurons in CA4, and three of six showed such neurons in CA1 and in the subiculum. In the worst affected animal (clamping time 2 min 30 s, showing unilateral damage) 9%, 5%, 8%, and 8% of the CA1 cells in levels 2 – 5, respectively, were dead. The corresponding figures for the subiculum of levels 3 – 5, were 10, 16, and 1.5%. In all animals, the CA3 sector and the dentate gyrus were separed.

Ischemia 4 min. The above pattern of hippocampal damage was found in this group as well. In addition,

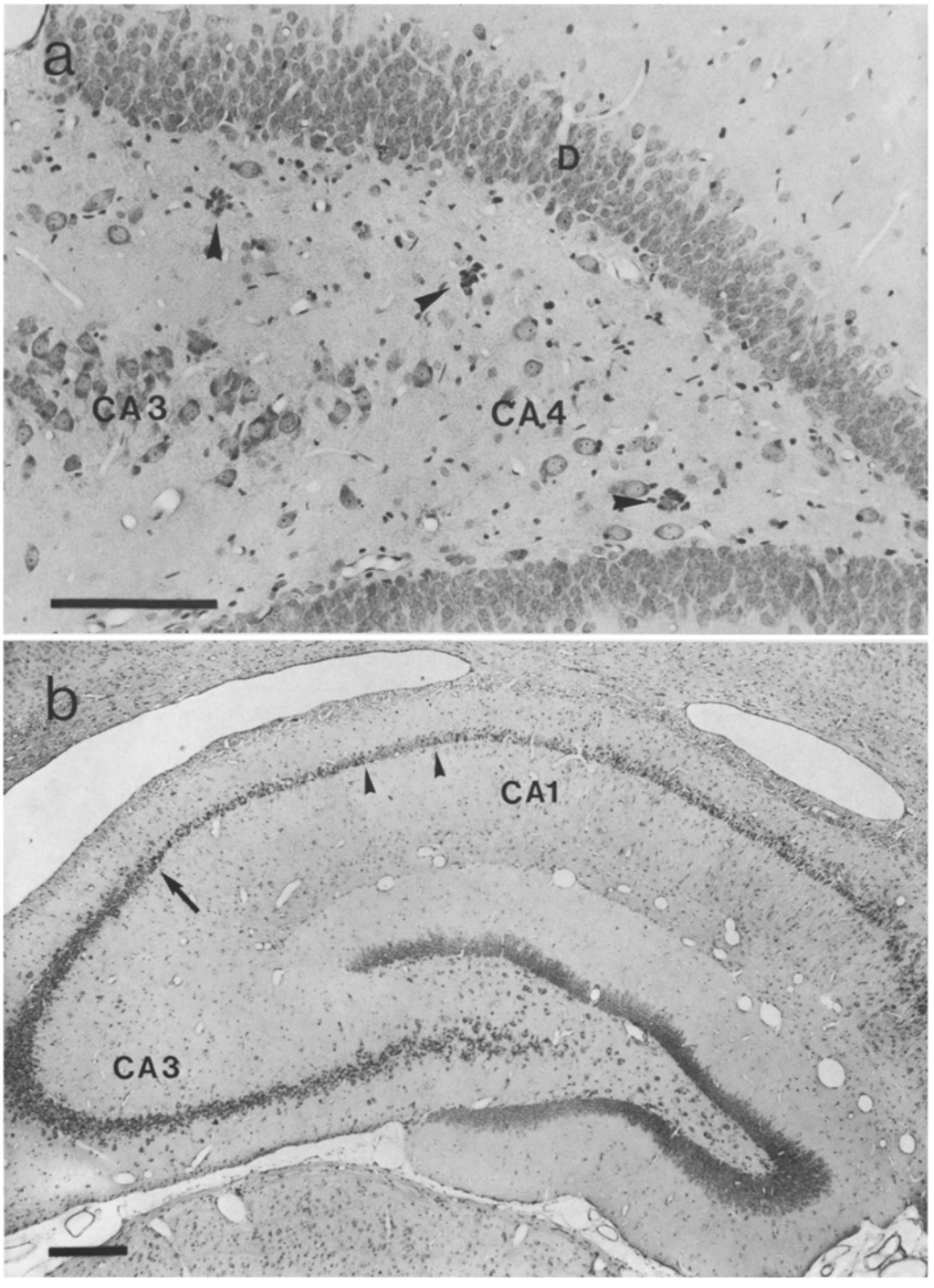

Fig. 4. Hilar region of hippocampus (**a**), 1 week after 4 min of ischemia, including the dentate gyrus (*D*), CA4, and CA3 zones. There are three neuronophagic figures in the hilus proper (*arrowheads*). All of the CA4 cells were never recruited even after relatively severe damage. A lesser degree of this form of damage was seen in the hilus already after 2 min of ischemia. An overview of the hippocampus (**b**), shows selective neuronal necrosis of the CA1 pyramidal neurons, extending to the border (*arrow*) with CA3. Stretches of preserved neurons, measuring about 200 μm, (between *arrowheads*) are sometimes seen. The adjacent stratum radiatum is hypercellular due to dendritic phagocytosis, except in unaffected portions of the gyrus. Note hydrocephalus. Ten minutes of ischemia, 1 week survival. Acid fuchsin/cresyl violet. Bars = 200 μm

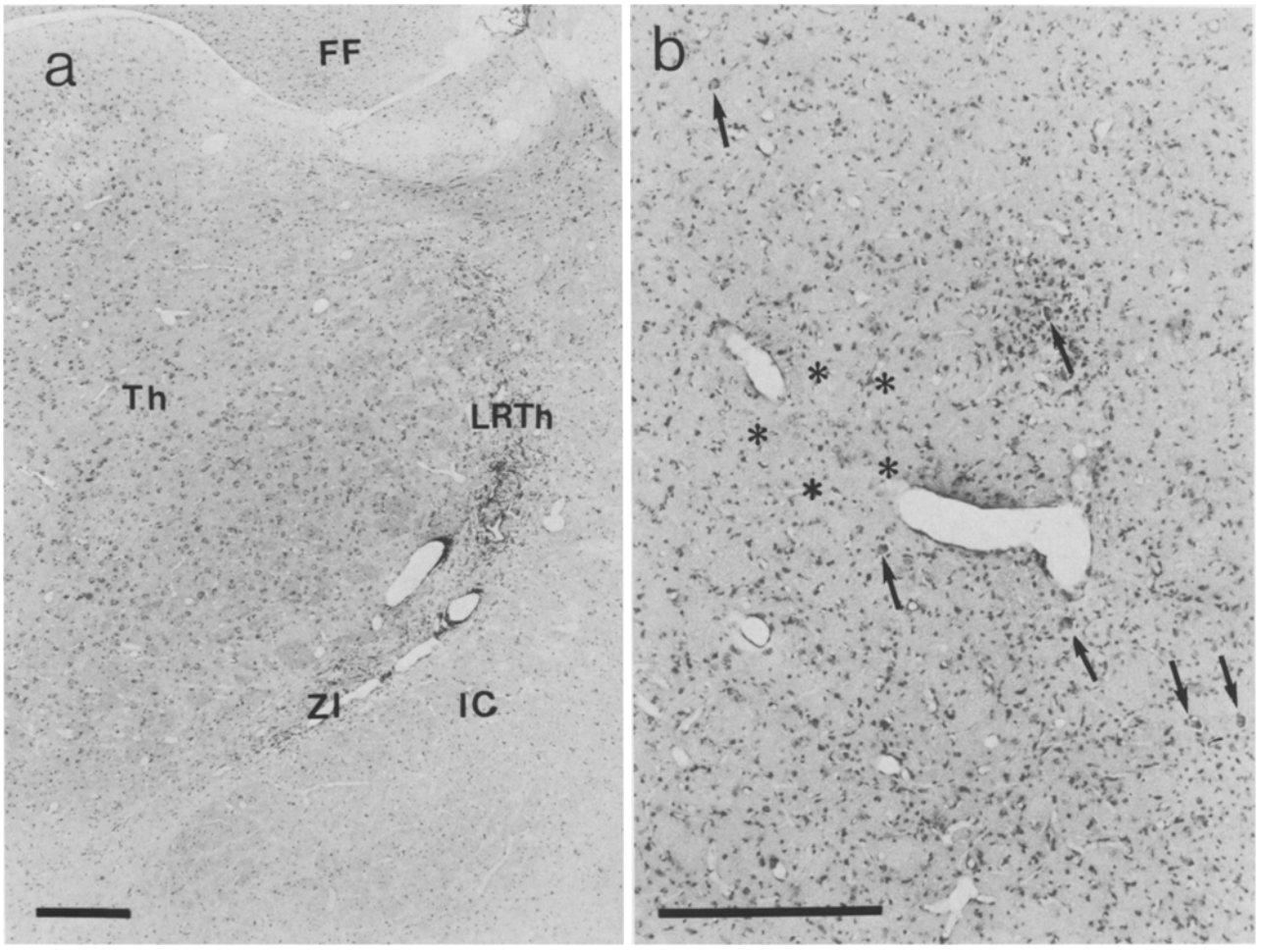

Fig. 5a. Thalamus (*Th*), and surrounding fimbria-fornix (*FF*) and internal capsule (*IC*). The characteristic pattern of damage is present in the lateral reticular nucleus of the thalamus (*LRTh*), seen as a curvilinear infiltrate of macrophages corresponding to the neuronatomical boundaries of the nucleus. Damage extended into the zona incerta (*ZI*) in some animals. Eight minutes of ischemia, 1 week survival. The caudate nucleus (**b**), shows patchy and demarcate involvement. Large neurons (*arrows*) tend to be spared, and one is seen surviving even in the patch of damage rendered hypercellular due to macrophage infiltration. A patch of spared tissue with intact neurons is seen in the center (*asterisks*). Ten minutes of ischemia, 1 week survival. Acid fuchsin/cresyl violet. Bars = 400 μm

the cerebral cortex was recruited in five animals of this group. Scattered acidophilic neurons (3 – 55) were seen, mostly in the middle cortical laminae III – V. In this group, no dentate cells were affected and only one had a few acidophilic CA3 cells. The distribution of subiculum and CA1 damage is illustrated in Fig. 3a. As observed, cell necrosis was predominately seen in levels 2 – 5. CA4 was affected in all animals (Fig. 4a).

A characteristic pattern of thalamic damage became apparent in four of six animals of this group, with selective affectation of the lateral reticular nucleus (Paxinos and Watson 1982) of the thalamus. This was visible as a distinctly demarcated curvilinear area of acidophilic neuronal necrosis in the lateral portion of the thalamus abutting the white matter of the internal capsule, the lesion becoming especially distinctive when accompanied by dense macrophage infiltration (Fig. 5a). Damage often extended into the contiguous zona incerta, but never caudally into the reticular formation proper.

Ischemia 6 min. Neocortical damage appeared in all animals of this group. However, this varied between the extremes of 1 and 1,500 cells per section. Necrotic cells were predominately located in cortical layers III, IV, and V. A bilaminar pattern, affecting layers III and V, with relative sparing of layer IV, was looked for, but was only rarely seen. Damage predominated over the superolateral convexities of the hemispheres, between the territories of supply of the anterior and middle cerebral arteries.

Only one animal had sparse necrosis of a few dentate granule cells but two had CA3 damage, moderate in one (0.2% – 3%) and marked in the other

(6% – 49%). The latter animal was that having the densest neocortical damage. This animal also showed acidophilia of thalamic neurons and Purkinje cells of the cerebellar vermis. All animals had some necrotic CA4 cells and grave damage of the subiculum and CA1 sectors, with 60% – 100% affectation in levels 2 – 5. The CA1 damage was less pronounced in levels 6 and 7 (the middle and temporal portions of the hippocampus), but CA4 damage extended more uniformly into the temporal end of the hippocampus.

The amygdaloid nuclei were affected in one of six animals, and the substantia nigra, pars reticularis in one. Thalamic damage was seen in all animals in the lateral reticular nucleus, in a pattern similar to that seen after 4 minutes. In addition, scattered selective neuronal necrosis was seen in the main nuclear groups.

Ischemia 8 min. Mild to moderate neocortical damage was observed in all animals, mostly in the middle layers III – V. Infarction was not seen.

Mild CA3 damage was seen in three animals, and mild dentate damage in only two. Damage to subiculum and to the CA1 and CA4 sectors resembled that in the 6-min group. No striking gradients were seen along the CA1 pyramidal cell band, although occasional patches of affected and unaffected neurons, roughly 200 μm in length, were seen (Fig. 4b). Damage to CA4 did not recruit the majority of pyramidal cells in the hilus, even when all of the CA1 cells were affected in the same hippocampus.

Two animals in this group had strictly unilateral hippocampal lesions although both had bilateral neocortical damage (the number of dead cells on the two sides were 9 and 12, and 300 and 150, respectively.

In this group, acidophilic neurons were observed in the septal nucleus. However, it should be mentioned that occasional pyknotic neurons were observed in the 2, 4, and 6 min groups.

Two animals had striatal damage. This affected close to 100% of the neurons in the dorsolateral portion of the caudoputamen in one, and only a few cells in the other. Affected and unaffected areas showed sharp demarcations, with patchy and often irregular geographic interdigitations of normal and affected tissue (Fig. 5b).

Infarcts were seen in the substantia nigra, pars reticularis, in two animals. Damage was seen in the thalamus in five animals, in the amygdala in two, the entorhinal cortex in four, and the septal nuclei in three of six animals.

Ischemia 10 min. Neocortical damage in this group ($n = 8$) took on two distinctive patterns. The first occurred over the superolateral convexity in the zone between the territory of drainage of the anterior and middle cerebral arteries. Selective neuronal necrosis was seen in the middle layers (III – V) of the cortex (Fig. 6a). Infarction developed in the zone between the territory of drainage of the anterior and middle cerebral arteries, tapering posteriorly as the territory of the posterior cerebral artery was approached (Fig. 7a). It also involved the middle cortical laminae, sparing layer I even in the most severely affected animals (Fig. 6b). The neocortex adjacent to infarcts showed selective neuronal necrosis in the middle cortical laminae. Thus, both the intracortical localization of infarcts, and their distribution over the hemisphere constitute an augmentation of the pattern of selective neuronal necrosis seen after the shorter doses of insult.

The second pattern of neocortical damage, seen in the most severely affected animals, consisted of patchy 300 – 500 μm parasagittal "microinfarcts" (no cavitation was seen) in the cingulate cortex and paramedial portion of the cerebral convexity (Fig. 7b). These patchy "microinfarcts", strictly speaking areas of dense selective neuronal necrosis, alternated from side to side as one progressed from anterior to posterior, and were often related to effluent veins draining the cortex. In contrast to the arterial pattern of cortical damage, this pattern extended into the parasagittal occipital lobes where damage in the arterial pattern disappeared. Hence this parasagittal distribution was interpreted as a venous pattern.

In the hippocampus, slit-like cavitation of the pyramidal layer was observed, but cavitation of the stratum radiatum or stratum oriens was never seen, even in the most severely affected animals. Moderate affectation of even the relatively resistant dentate granule cells was observed in four animals and CA3 damage in all but one. The distribution of CA1 to CA4 damage is illustrated in Fig. 3b.

The caudoputamen was heavily involved in all but one animal (see below). Infarcts were seen in the substantia nigra in one animal, and damage was present in the entorhinal cortex in six, the septal nuclei in six, the thalamus in seven, and the amygdala in three out of eight animals.

Density of Neuronal Damage vs Duration of Ischemia. For three brain structures, cell counts were performed. Table 3 gives percentage figures for CA1 damage, averaged from levels 2 – 5. As observed, 10 min of ischemia caused virtually complete necrosis of the CA1 sector. However, extensive CA1 damage was also observed after shorter ischemic periods (note the 4- and 6-min groups).

For the cerebral cortex, the number of acidophilic cells are given in Table 4. In the 6 and 8 min groups, extensive damage was observed in only two animals

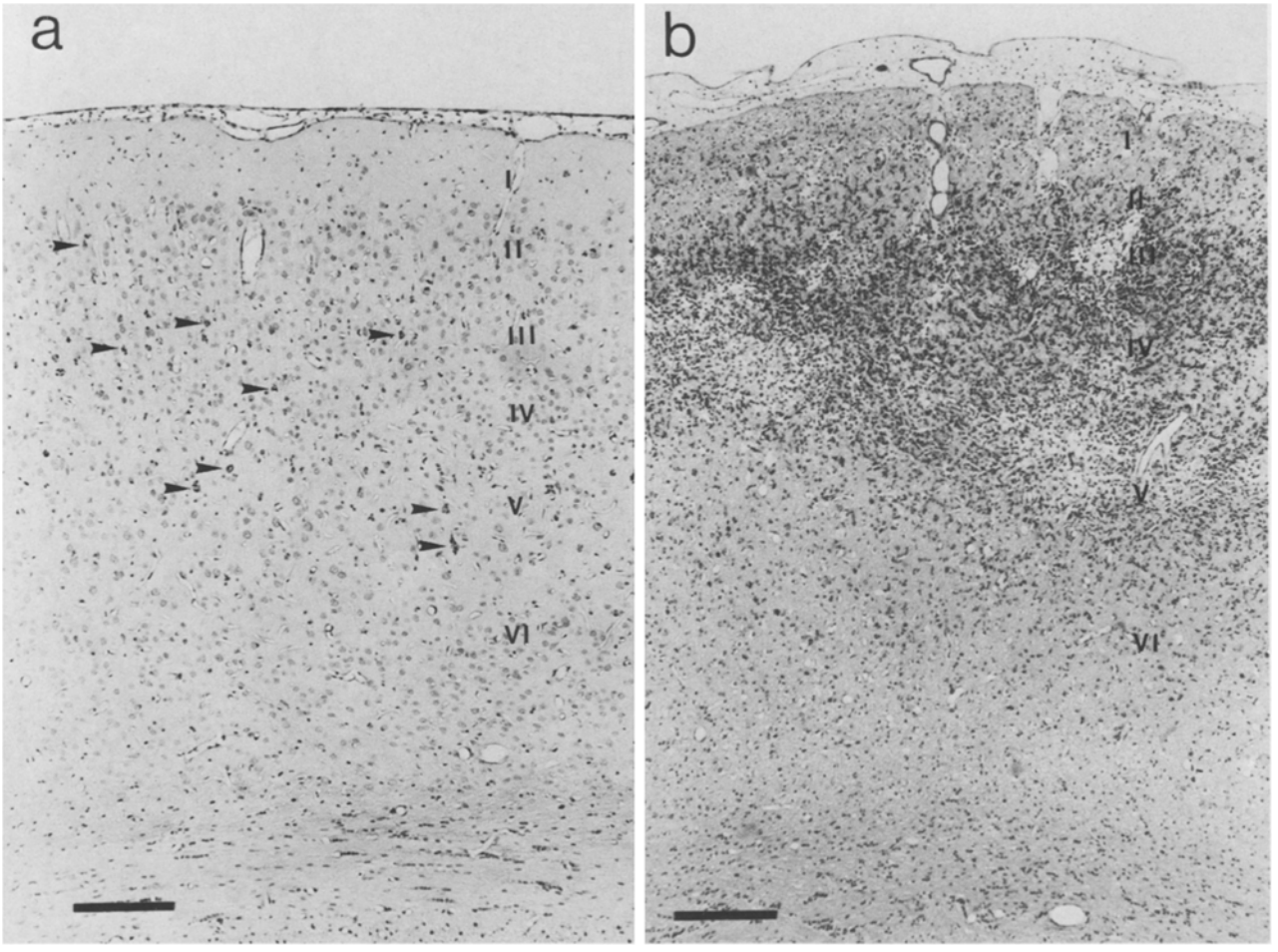

Fig. 6a, b. Cerebral cortex showing selective neuronal necrosis affecting laminae III–V, 1 week and 10 min of forebrain ischemia (**a**). Necrotic neurons demonstrate acidophilia and are rendered dark and punctate in this photomicrograph (*arrowheads*). One week after 10 min of ischemia, some animals show infarction (**b**) also centered on layers III–V. Adjacent areas show tapering damage until a pattern resembling that in the left picture is reached. Acid fuchsin/cresyl violet. Bars = 200 μm

of each, but after 10 min gross neocortical damage was more prevalent, three animals showing infarction.

Damage to small and medium sized neurons in the caudoputamen, evaluated from two microscopic fields in the lateral portion of the nucleus, showed some unusual characteristics. Thus, no animal showed damage following 2–6 min of ischemia. Second, once damage was incurred (8–10 min of ischemia) it tended to occur in all or none fashion, with 13 or 18 affected hemispheres showing more than 85% affectation in the portions sampled for counting.

Rank Order of Vulnerability. To illustrate the susceptibility of the different structures to ischemia we calculated the number of animals in which each structure showed unequivocal damage, excluding those in which less than five dead neurons were found in a given structure. As Fig. 8 shows, the rank order (with decreasing susceptibility) was CA4 > subiculum plus CA1 > neocortex (middle laminae) > CA3 > caudoputamen. Three additional features should be emphasized. First, although CA4 damage was observed with the shortest ischemic periods, and although the number of affected cells increased with increasing duration of ischemia, only part of the CA4 cell population was damaged. Second, damage to dentate granule cells was infrequent, and the damage remained moderate even in the most severely affected cases. Third, in the most caudal hippocampal levels some CA1 cells were spared even after 10 min of ischemia, notably in the ventral parts of the hippocampal formation.

Discussion

The present results have given quantitative information on the brain cell damage incurred by short periods

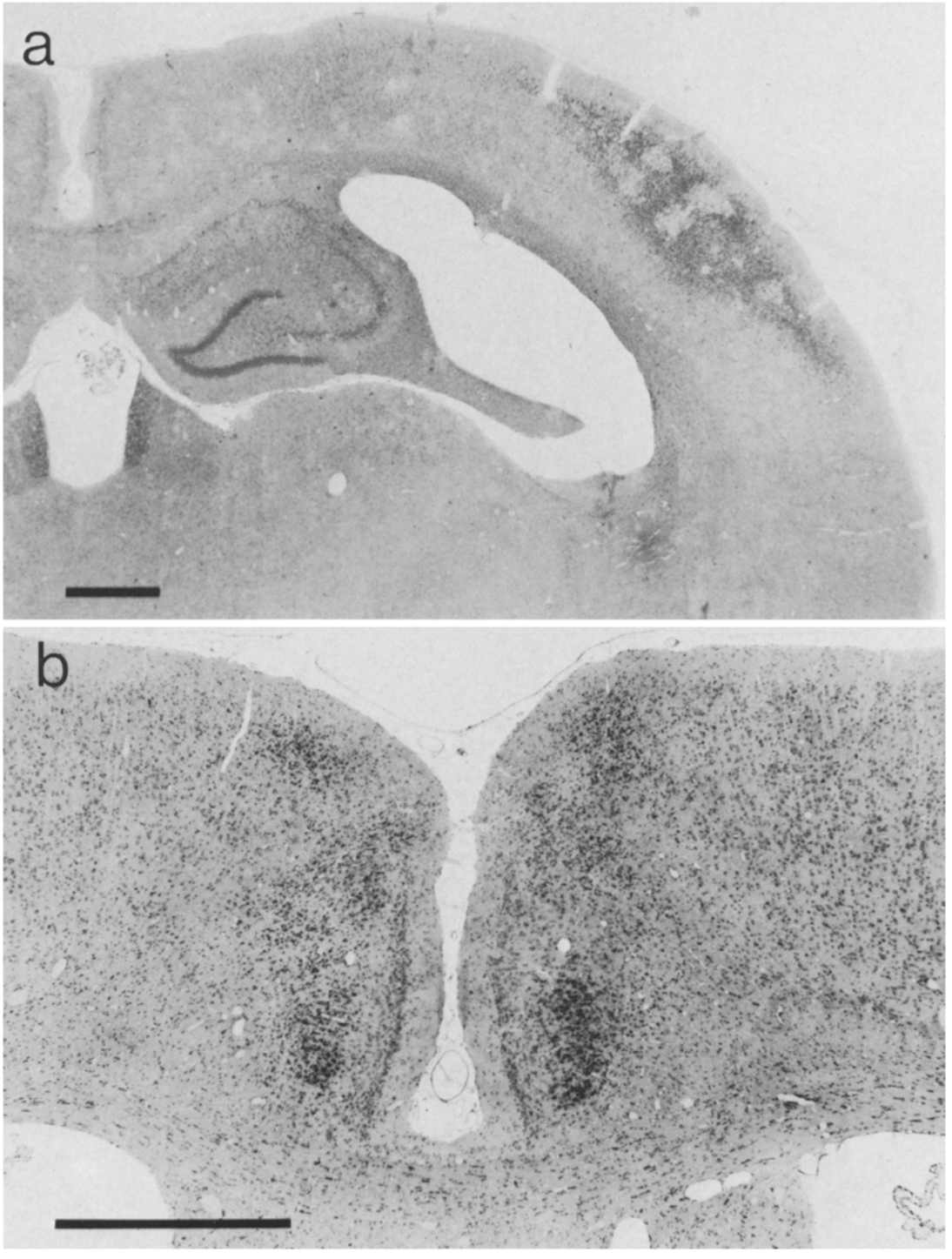

Fig. 7. Forebrain (**a**) showing hypercellular infarction with early and patchy central cavitation, located over the superolateral convexity of the hemisphere. There is moderate hydrocephalus of the lateral ventricle. Ten min of ischemia, 1 week survival. Cingulate cortex (**b**), showing patchy hypercellular microinfarcts parasagittally. These were interpreted as venous, due to obstruction of the sagittal venous drainage by postischemic edema at sometime in the recovery period. Ten minutes of ischemia, 1 week survival. Acid fuchsin/cresyl violet. Bars = 1 mm

Table 3. Density of acidophilic cells in hippocampal CA1 sector (integrated from levels 2–5) in per cent of total cell populations. Individual animals, left and right hemispheres

2 min Ischemia		4 min Ischemia		6 min Ischemia		8 min Ischemia		10 min Ischemia	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
0.2	0	6	1	78	67	73	86	96	82
0	7	92	94	97	95	0	88	98	98
0	0	12	20	89	83	92	94	96	96
0	0.6	8	6	61	90	65	35	94	95
0	0	17	87	98	98	0	97	97	96
0	0	40	40	96	0	95	95	98	98
								98	99
								95	97

Table 4. Density of cortical neuronal necrosis after 2–10 min of ischemia. Number of acidophilic neurons per section in left and right hemispheres

2 min Ischemia		4 min Ischemia		6 min Ischemia		8 min Ischemia		10 min Ischemia	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
0	0	3	0	10	2	0	2	3,500[a]	3,500[a]
0	0	3	0	96	68	9	12	3,000[a]	4,000[a]
0	0	0	0	8	14	12	9	30	57
0	0	22	18	0	1	26	1	25	18
0	0	30	25	1,150	350	300	152	150	450
0	0	17	6	230	60	850	900	5,000[a]	1,100
								63	29
								167	135

[a] Denotes infarction

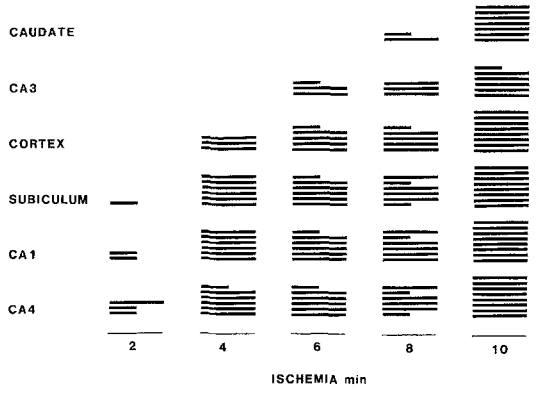

Fig. 8. Recruitment of neuronal damage in six brain regions with increasing duration of ischemia (2–10 min). *Long bars* denote bilateral damage with more than five acidophilic neurons per hemisphere. *Short bars* denote unilateral damage. $n = 6$ in each group except the 10 min group were $n = 8$

of forebrain ischemia. This information concerns the shortest periods of ischemia giving unequivocal brain damage, the rank order of vulnerability of brain structures, and the influence of density of ischemia on the distribution and nature of damage incurred.

Revival Times

As discussed in the introduction, experiments on gerbils have shown that 5 min of bilateral carotid artery ligation gives extensive necrosis of CA1 (and CA4) cells in the hippocampus (Kirino 1982), while 10 min of forebrain ischemia in the rat causes neuronal damage in most, but not all animals (Pulsinelli et al. 1982a). Clearly, the present results reveal an even greater susceptibility since they demonstrate necrosis of subiculum-CA1 cells in a few animals subjected to 2–3 min of ischemia, and consistent damage after 4–5 min of ischemia (Kirino and Sano 1984a, b). These results, obtained in rats, are thus akin to those reported in the gerbil but differ from those reported by Pulsinelli et al. (1982a). However, they extend those of Kirino (1982) and Suzuki et al. (1983a) in showing that subiculum-CA1-CA4 damage may be incurred even after even shorter periods of ischemia. In fact, if the true ischemic period corresponds to that during which pressure was held at 50 mm Hg the results imply that 2–2.5 min of ischemia may cause some pyramidal cell damage in the hippocampus. Of perhaps

greater interest is the finding that 6–8 min of ischemia gave neocortical damage in most, and 10 min in all animals studied. Furthermore, after 10 min of ischemia such damage encompassed laminar necrosis and circumscript venous or arterial infarcts in some of the animals. Further investigation will be required to determine whether delayed neuronal necrosis occurred after a morphologic "free interval".

It can be excluded that the marked susceptibility noted was due to deficient recirculation of the brain upon removal of the carotid clasps, and re-infusion of the shed blood. Thus, previous results have demonstrated a pronounced hyperemia, with no evidence of locally deficient reperfusion, 5 min after start of recirculation (Kågström et al. 1983 b). Furthermore, the present experiments demonstrate hyperemia already after 1–1.5 min of recirculation, with only neocortical areas showing somewhat delayed recirculation. It should also be recalled that postischemic hypotension or hypoxia did not occur, nor did we notice seizure activity in any of the animals included in the series.

2. *Rank Order of Vulnerability*

This was similar, but not identical, to that described in many previous studies, including that of Pulsinelli et al. (1982a). Thus, our results demonstrate that CA4 pyramidal cells were as susceptible as CA1 and subiculum neurons, and that CA3 pyramids were less susceptible than neocortical cells. The least susceptible hippocampal cells were dentate granule cells. It was an unexpected finding that the small to medium sized neurons in the caudoputamen were affected first after 8–10 min of ischemia, and then suffered massive damage ("all-or-none" behavior). We can offer no explanation for this, nor can we explain why cells in the dorsolateral portion of the caudoputamen are more susceptible than those in central or ventromedial positions, although this may be related to the blood supply. The geographic, demarcated borders of damaged tissue, and the patchy caudate involvement have been observed by others in ischemia (DeGirolami et al. 1984), are in contrast to the gradients of tissue damage seen in this nucleus in hypoglycemia (Auer et al. 1984b).

Also unexpected was the consistent damage in the thalamic reticular mucleus, conforming to its neuroanatomic borders. This suggests a neuroanatomic rather than a vascular basis for this selective vulnerability. Neurons of the reticular nucleus of the thalamus are mostly GABAergic (Houser et al. 1980), and are inhibited by acetylcholine (Ben-Ari et al. 1976). GABAergic neurons have been shown to selectively degenerate following hypoxia (Sloper et al. 1980) and ischemia (Francis and Pulsinelli 1982) with a decrease in glutamic acid decarboxylase activity (Bowen et al. 1978; Francis and Pulsinelli 1982).

Ten minutes of ischemia caused not only dense neuronal necrosis in the neocortex but also circumscript infarction in "venous" and "arterial" patterns. Clearly, after this period of ischemia tissue damage was severe enough to cause destruction of glial and endothelial cells as well. Since the present model leaves the vertebral arteries patent at a blood pressure of 50 mm Hg, and since the rat circle of Willis has patent posterior communicating arteries, a trickle of flow through the circle of Willis would be expected. The area exposed to the greatest density of ischemia would therefore be the zone between the territories of supply of the anterior and middle cerebral arteries ("borderzone"), explaining the arterial pattern of infarcts seen. The parasagittal venous pattern, however, suggestive of superior sagittal sinus obstruction, would require obstruction of venous outflow. Low cerebral blood flow rates were seen parasagittally at 90 min recovery following 30 min ischemia in one model of ischemia in cats (Ginsberg et al. 1978), and ischemic cell change was reported to be enhanced in this region (Ginsberg et al. 1979). The most likely cause for venous obstruction in the present model is postischemic edema (Garcia and Kamijyo 1974; Harrison et al. 1975; Katzman et al. 1977; Myers 1979; Ito et al. 1979; White et al. 1979), since this pattern was seen only in the more severely damaged animals. Increased outflow resistannce from the microcirculation (Hudetz et al. 1982) due to postischemic edema and increased intracranial pressure could cause secondary damage in a venous pattern in the recovery period by obstructing the parasagittal draining veins of the cortex.

Occasionally, infarction developed in the pars reticularis of the substantia nigra, but not in other regions. Neocortical involvement in general, and infarction in particular, have been associated with hyperglycemia (Meyers 1979). There is no evidence, though, that hyperglycemia contributed to neocortical damage in the present series since all animals were starved overnight, and preischemic plasma glucose concentrations were usually below 12 mol $\cdot$ ml^{-1}.

After 4–10 min ischemia, some animals had unequivocal damage to thalamic neurons, and after 6 min some showed cerebellar damage localized to the vermis. Since parts of the thalamus suffer dense ischemia in the present model neuronal damage is to be expected, once the revival times of the neurons has been surpassed. Cerebellar damage is, at first sight, more surprising but seems explicable in terms of intracerebellar variations in ischemic blood flow rates (see below).

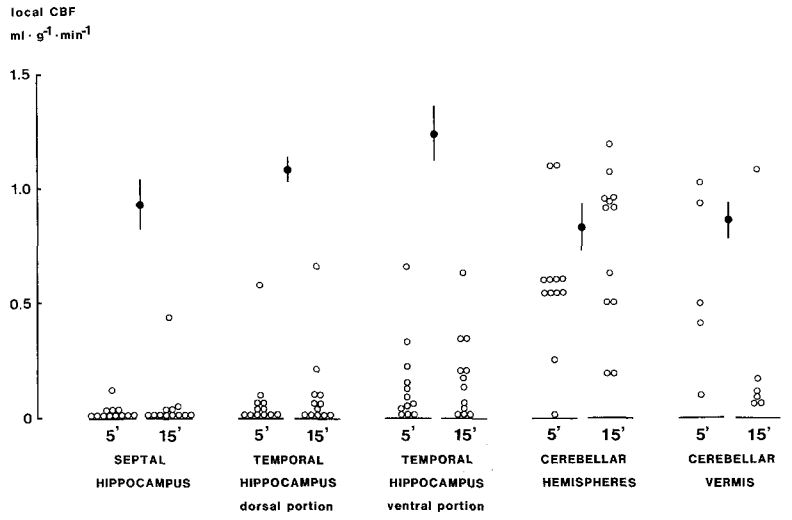

Fig. 9. Local CBF during ischemia (5 and 15 min duration) in hippocampus and cerebellum. The results are taken from Smith et al. (1984), but the autoradiographic films were re-examined to provide data on the hippocampus along its septotemporal axis and on the cerebellar vermis. *Open circles* denote flow rates averaged over one hemisphere (except vermis). *Filled circles* give control flow rates (± SD)

Relationship Between Density of Ischemia and Brain Damage

Three findings demand an explanation: (a) the unilateral damage incurred in some animals, (b) the gradient of damage within the hippocampus, with lesions decreasing in the septotemporal direction, and (c) the involvement of the cerebellum. Tentatively, all can be explained in terms of variations in ischemic flow rates. For example, our previous data show that, in occasional animals, flow rates are partly maintained on one side of the brain (Smith et al. 1984).

The variations in density of cell damage within the hippocampal formation, and the involvement of the cerebellar vermis, prompted us to re-examine the autoradiograms of the previous study (Smith et al. 1984). Now, instead of measuring flow rates in septal hippocampus, we also analyzed the mid and temporal hippocampus; in addition, CBF was measured in cerebellar vermis. As Fig. 9 shows, CBF was higher in temporal than in septal hippocampus, and higher in the ventral than in the dorsal portion. Clearly, the density of ischemia is less pronounced, and variable, in the temporal portion of the hippocampus, possibly explaining why CA1 damage tends to be less pronounced in these parts. Analyses of flow rates in cerebellar vermis likewise offer a circulatory explanation for the occasional appearance of cerebellar damage since, at least with prolonged ischemia, most animals had CBF values of less than 20% of control.

The Purkinje cells of the cerebellum, and to some extent also the basket cells, are known to be susceptible to ischemic insults (Grenell 1946; Hirsch and Müller 1962; Diemer and Siemkowicz 1981). Since models of forebrain ischemia usually spare the cerebellum they are less suited to reveal cerebellar pathology. However, Pulsinelli et al. (1982a) reported an occasional damage to cerebellar cells in the border zone between the superior and posterior inferior cerebellar arteries in a four vessel occlusion model.

The cerebellar vermal infarcts in the present study always occurred in the most severely damaged animals with the venous pattern of parasagittal "micro-infarcts". Hence, an alternative explanation for the vermal infarcts may be secondary venous obstruction due to postischemic edema in the recovery period.

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