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Delayed neuronal death and delayed neuronal recovery in the human brain following global ischemia

Markus Horn and Wolfgang Schlote

Neurologisches Institut (Edinger-Institut) der Universität Frankfurt am Main, Deutschordenstrasse 46, W-6000 Frankfurt a.M., Federal Republic of Germany

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Summary. The understanding of delayed hippocampal death as a therapeutic window for post-ischemic treatment of the brain has led to numerous investigations focusing upon underlying cellular mechanisms and pharmacological potentials in gerbils and rats. Nevertheless, studies on the occurrence of delayed neuronal death in the human brain have been singular and dealt with only small files of patients. To complement these limited data, in the present study 26 adult patients with a history of a single cardiac arrest were included. Following successful resuscitation, individual survival ranged from less than 1 h to 186 days ($\bar{x}$ = 11 days). The severity of the resultant ischemic injury in hippocampus CA1, among Purkinje cells, or in frontal neocortex, respectively, was quantified by direct counting of necrotic neurons. Additionally, hippocampal specimens were immunostained for neuron-specific enolase. The data obtained demonstrate the occurrence of delayed neuronal death in human hippocampus and, in a minor form, in cerebellar Purkinje cells. This is in contrasts to the immediate manifestation of ischemic neuronal necrosis in the neocortex. Unlike previous findings in experimental animals and in humans, the delay of CA1 cell death could be defined as lasting about 7 days following cardiac arrest. Moreover, the immunohistochemical results indicate delayed neuronal recovery in CA1, which in the time course reciprocally corresponds to delayed manifestation of hippocampal neuronal death. Interpretation of the results must consider the lack of information about the exact individual duration of cardiac arrest and resuscitation, as well as missing data concerning pre-ischemic physiological variables.

Key words: Selective vulnerability – Delayed neuronal death – Delayed neuronal recovery – Cardiac arrest – Humans

Correspondence to: M. Horn (address see above)

It is basic knowledge that CA1 pyramidal cells show some special features in manifestation of ischemic neuronal death and, therefore, differ from other neuronal sites of the brain. Apart from selective vulnerability, which originally was reported by Spielmeyer [23], Ito et al. [6] emphasized that the progression in manifestation of definite ischemic cell death in CA1 depends on the magnitude of the preceding ischemia. They found a rather slow development of neuronal necrosis following brief cerebral ischemia, while longer-lasting ischemic periods resulted in a more rapid manifestation of lesions ("maturation phenomenon"). In 1982, Kirino [7] demonstrated that CA1 damage in the gerbil hippocampus takes place with a delay of about 48 h after brief forebrain ischemia and is completed at day 4 following the ischemic insult [7]. These results also have been confirmed in the rat model of four-vessel occlusion [8].

Meanwhile, numerous investigations were initiated to elucidate the characteristics of underlying cellular mechanisms in gerbil and rat models. Current data substantiate that cell processes determining the fate of CA1 neurons – delayed death or recovery and survival – are related to the first 40 min of post-ischemic reperfusion and, thus, underline the particular clinical importance of delayed neuronal death as a therapeutic window for post-ischemic treatment [12]. Actually, the phenomenon of delayed neuronal death in experimental gerbils and rats provides a practicable model for evaluating neuroprotective effects of pharmacological agents [10].

Nevertheless, studies on the presence of a similar occurrence in the human hippocampus are rare. Obviously this is because of the great variety and insufficient documentation of clinical parameters prior to, during, and after cardiac arrest which represents the most frequent cause of global cerebral ischemia in the clinical setting. To our knowledge, therere is only one relevant cliniconeuropathological investigation, performed by Petito et al. [17], on humans showing different survival times following resuscitation after cardio-

Table 1. Cases with survival intervals ranging from less than 1 h to 186 days following resuscitation from cardiac arrest ($n = 26$)

Age/sex	Etiology of cardiac arrest[a]	Persistent post-arrest neurological state	Post-arrest survival	Ultimate cause of death[a]	Brain weight[b]
50 y/♂	Respiratory distress	Coma	< 1 h	Ventricular fibrillation	1590 g
55 y/♂	Myocardial infarction	Coma	1 h	Ventricular fibrillation	1525 g
40 y/♀	Suicidal drug poisoning	Coma	5 h	Cardiovascular failure	1290 g
64 y/♀	Myocardial infarction	Coma	12 h	Cardiovascular failure	1200 g
62 y/♂	Cardiogenic shock	Somnolence	15 h	Cardiovascular failure	1235 g
68 y/♀	Myocardial infarction	Without deficit	18 h	Cardiovascular failure	915 g
62 y/♂	Hyperkalemia	Brain stem syndrome	24 h	Cardiovascular failure	1200 g
60 y/♂	Myocardial infarction	Without deficit	3 d	Cardiac reinfarction	1210 g
47 y/♂	Congestive cardio-myopathy	Brain stem syndrome	4 d	Cardiovascular failure	1450 g
22 y/♂	Suicidal drug poisoning	Coma	4.5 d	Central respir. arrest	1660 g
82 y/♀	Hematemesis/aspiration	Coma	6 d	Cardiovascular failure	1365 g
63 y/♀	Incident during surgery	Coma	7 d	Pneumonia	1225 g
77 y/♀	Myocardial infarction	Coma	9 d	Cardiovascular failure	1200 g
51 y/♀	Myocardial infarction	Persist. vegetative state	13 d	DIC[c]	1415 g
50 y/♂	Hypertroph. cardio-myopathy	Persist. vegetative state	15 d	Bradyarrhythmia	1175 g
66 y/♂	Myocardial infarction	Without deficit	17 d	Pulmonary embolism	1390 g
59 y/♂	Myocardial infarction	Without deficit	22 d	Cardiovascular failure	1250 g
69 y/♀	Incident during surgery	Coma, tetraplegia	28 d	Pulmonary embolism	1150 g
54 y/♂	Low-output-syndrome	Brain stem syndrome	30 d	Cardiovascular failure	1360 g
50 y/♀	Myocardial infarction	Persist. vegetative state	32 d	Tachyarrhythmia	1300 g
56 y/♂	Myocardial infarction	Spastic tetraparesis	51 d	Pneumonia, sepsis	1180 g
71 y/♀	Myocardial infarction	Persist. vegetative state	80 d	Pneumonia	895 g
77 y/♀	Myocardial infarction	Without deficit	115 d	Cardiac reinfarction	1100 g
78 y/♂	Myocardial infarction	Persist. vegetative state	150 d	Pneumonia, cachexia	1420 g
60 y/♂	Anaphylaxis	Persist. vegetative state	180 d	Pneumonia	1150 g
32 y/♂	Suicidal drug poisoning	Persist. vegetative state	186 d	Central respir. arrest	1110 g
$\bar{x} \pm s$: 58.7 ± 14.0			$\bar{x}$ = 14.3 d $\tilde{x}$ = 11 d		$\bar{x} \pm s$: 1268 ± 181 g

[a] As indicated by clinical and/or autopsy findings
[b] Post fixation
[c] Disseminated intravascular coagulopathy

respiratory arrest. By semiquantitative assessment of neuronal necrosis in cases with a survival of 18 h or less, and 24 h or more, respectively, the authors were able to find a significant difference in the post-ischemic increase of the histological ischemic grade merely in the hippocampus. The aim of the present study on autopsy material was to complement existing data on delayed neuronal death in the human brain, and to define the delay of definite ischemic damage in CA1 using a different methodological approach.

Materials and methods

In this study, 26 adult patients with a history of a single and clearly documented cardiac arrest caused by an extracerebral event were included. In all cases cardiac arrest had been terminated by successful cardiopulmonary resuscitation leading to restoration of spontaneous circulation. Resulting post-arrest survival intervals ranged from less than 1 h to 186 days. In 17 cases, the etiology of cardiac arrest was myocardial infarction or otherwise caused cardiogenic shock. The remainder also revealed a non-cerebral history of cardio-respiratory failure. Patients with pre-existent neurological deficits or primary involvement of the CNS as well as all kinds of central cardiopulmonary arrest were excluded (Table 1).

Immediately following autopsy, brains were immersion-fixed in buffered formalin 4% for at least 3 weeks prior to macroscopic sectioning and paraffin embedding. In addition to routine histological diagnostics, serial microscopic sections (7 μm) from hippocampus, cerebellum, and frontal neocortex (sulcus) were stained with hematoxylin-eosin, cresyl violet, or Klüver-Barrera stain (cresyl violet/luxol fast blue MBS). In cases with post-arrest survival $\leq$ 15 days quantitative neuropathological evaluation of regional definite ischemic neuronal damage was performed by direct counting of necrotic cells among at least 300 CA1 neurons, Purkinje cells, or neocortical neurons under high-power light microscopy. Diagnosis of ischemic neuronal necrosis was based on cytological features of homogenizing cell change consisting of eosinophilia or dark luxol fast blue staining of neuronal cytoplasm, cellular and nuclear pyknosis, disappearance of the nuclear-cytoplasmic border, and disintegration of both nucleus and nucleolus [2, 9]. Earlier stages of ischemic cell alteration have not been taken into consideration. In cases with a post-arrest survival of more than 15 days, the extent of ischemic neuronal damage was estimated as neuronal loss when compared to normal controls. A semiquantitative scale was used, and results were expressed as percentages.

Additionally, immunohistochemistry using a monoclonal antibody raised against neuron-specific enolase (γ, γNSE, Camon) was performed in hippocampal specimens of ischemic patients and of two non-ischemic controls by an avidin-biotin-complex technique (streptavidin-alkaline phosphatase complex, Camon). All sections were counterstained with hemalum. Throughout the whole procedure all specimens were incubated simultaneously in a specially designed immunostaining cuvette[1] providing the opportunity for qualitative comparison of NSE expression in CA1 neurons.

Results

Quantitative evaluation of regional ischemic neuronal death

Neuropathological analysis of the brains of subjects with a post-arrest survival of 1 h or less indicated various kinds of ischemic cell alteration but no neuronal necrosis. In neocortical and hippocampal CA1 neurons, early histological changes consisted of mild shrinkage of cell bodies and nuclei, and in homogenizing of cytoplasm. The latter showed enhanced staining with acidophilic agents. At the same time a reduction in staining intensity and fading-out of cytoplasm were visualized in Purkinje cells.

The earliest manifestation of ischemic neuronal necrosis corresponding to the above-listed cytological criteria was observed by 5 h following cardiac arrest in cortical layers 3, 5 and 6; whereas, hippocampal CA1 and Purkinje cell layer still revealed no definite ischemic cell damage. In these selective vulnerable areas the first necrotic neurons were not detected before 12 h following resuscitation from cardiac arrest (Fig. 1a–i).

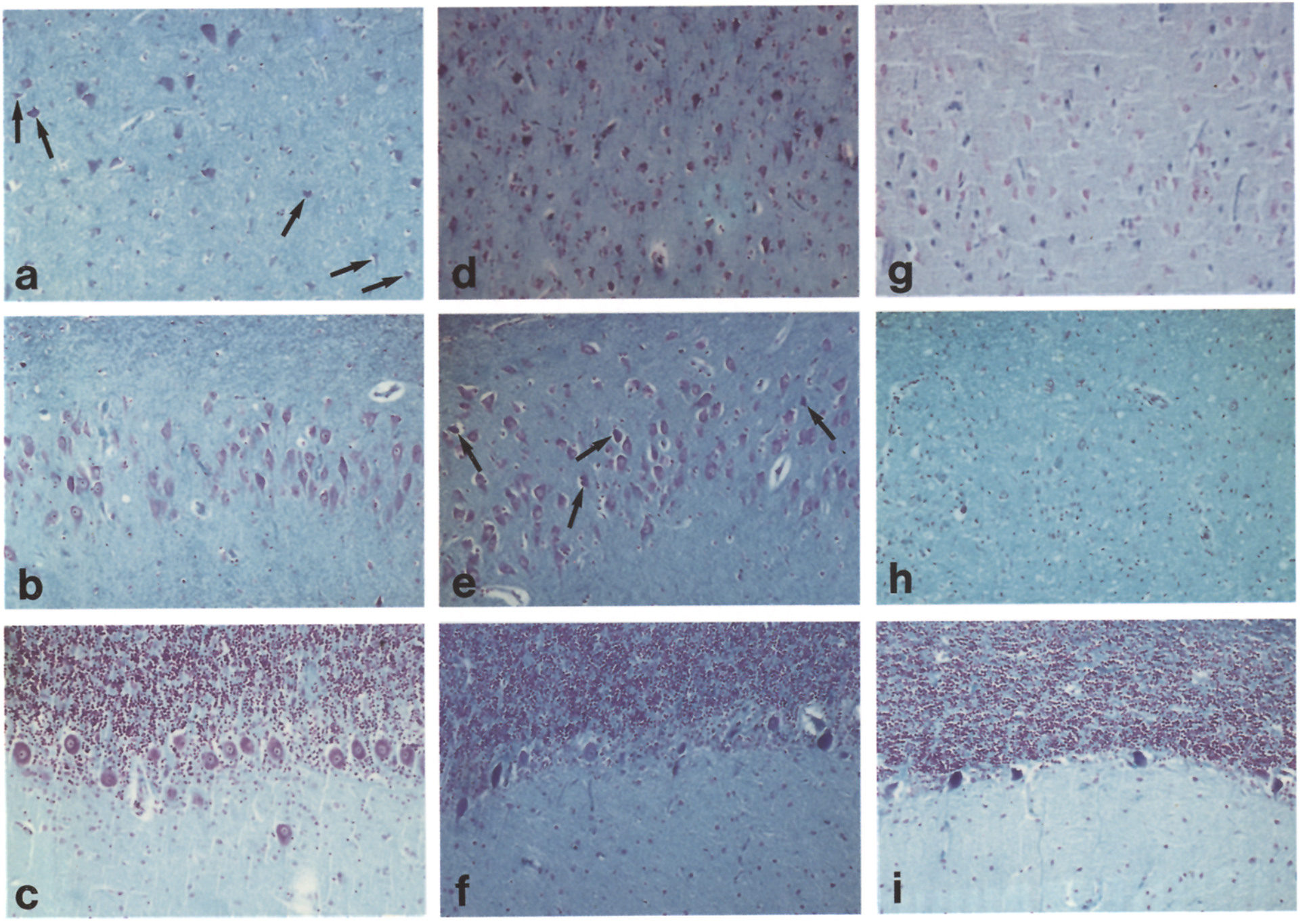
The proportion of neuronal necrosis steadily increased with advancing post-arrest survival in the neocortex and the cerebellar cortex. On the other hand, among hippocampal CA1 neurons the rate of cell death was minor between 12 h and 3 days of survival. A rapid increase was visible between day 4 and 13 of post-arrest survival (Fig. 2a). The extent of hippocampal damage exceeded the amount of cellular necrosis in both the neocortex and cerebellar cortex. This relationship remained stable in all cases with more than a 6-day survival following cardiac arrest (Fig. 2b).

Since the individual duration of cardiac arrest was not exactly reconstructable, direct quantitative comparison of cases appears inadmissible. Thus, interpretation of the results should be based on the individual pattern of regional ischemic injury. Therefore, in each case quotients were defined considering the density of neuronal necrosis in hippocampal CA1 or among Purkinje cells in relation to the results found in the neocortex. A ratio of 1.0 indicates equal quantities of necrotic neurons in compared areas. By the use of these two ratios it was possible to rate the delay in progression of hippocampal and cerebellar neuronal death when compared to immediate neocortical cell death.

The data obtained clearly indicate that CA1 pyramidal cell death occurs minimally until 4 days following global ischemia (cardiac arrest) but afterwards develops rapidly, exceeding the extent of neocortical neuronal injury at about 5 days post-arrest. Finally, it seems to be completed not earlier than 7 days following the ischemic insult (Fig. 3a).

Within the Purkinje cell layer neuronal death takes place significantly earlier and more gradually than in hippocampus CA1. Nervertheless, even in this area final morphological manifestation of neuronal injury seems to occur following a more or less "silent" time intervall, i.e., a delay of a few days. In one case with a 4-day post-arrest survival, for the first time the ratio for neuronal necrosis within the cerebellar cortex passes the value of 1.0 and, thereby, exceeds the quantity of necrotic neocortical cells (Fig. 3b).

[1] Designed and produced by W. Hirschberger, Frankfurt/Main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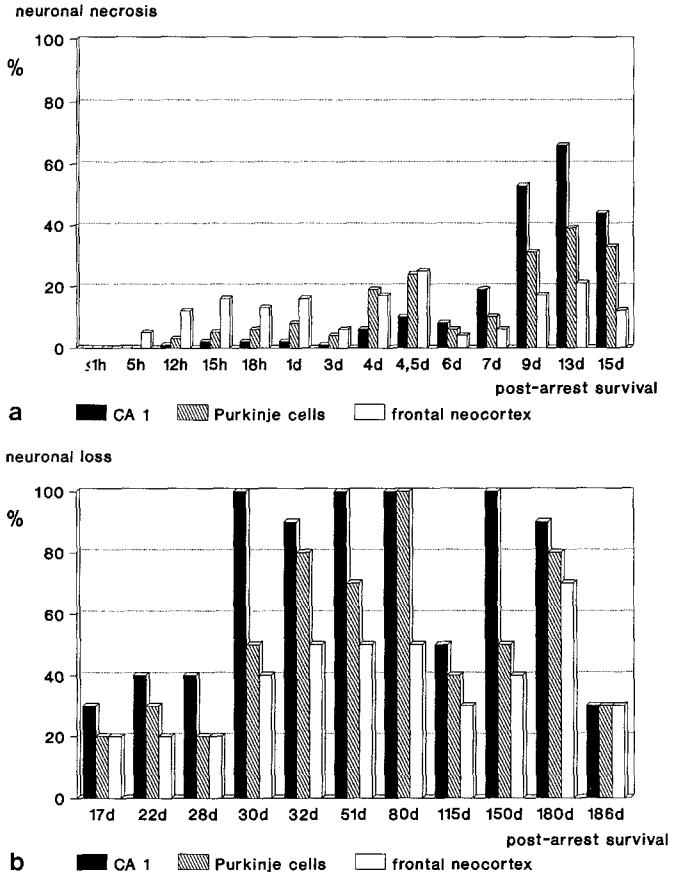


Fig. 2. Proportional amount of neuronal necrosis in patients with post-arrest survival intervals of ≤ 1 h to 15 days (**a**) and of neuronal loss by survival of 17 days to 186 days (**b**), respectively

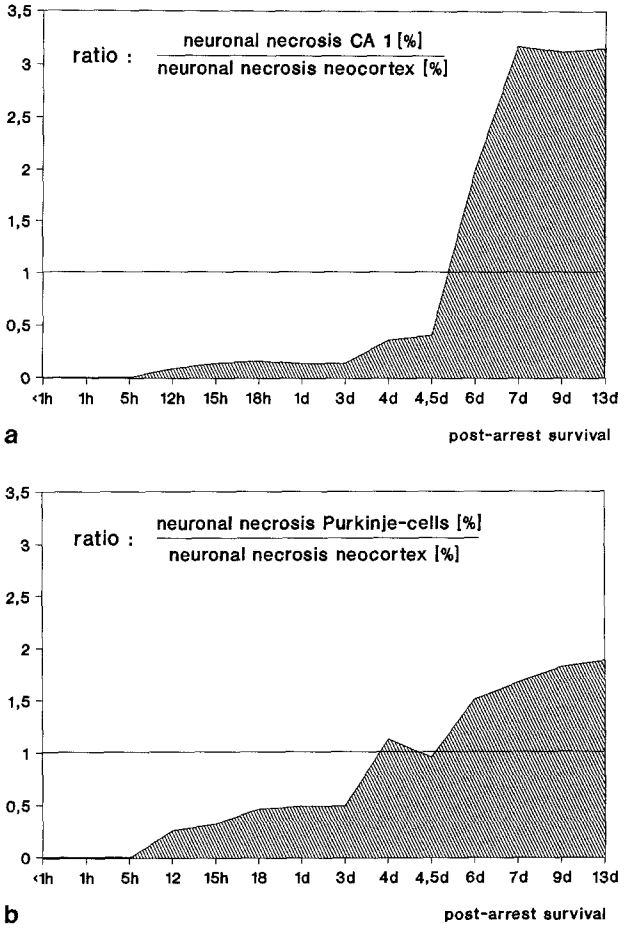


Fig. 3. Manifestation of definite neuronal damage in hippocampal CA1 pyramidal cells compared to neocortical neurons (**a**), and in Purkinje cells compared to neocortical neurons (**b**), respectively. A ratio of 1.0 indicates equal quantities of necrotic neurons in compared areas

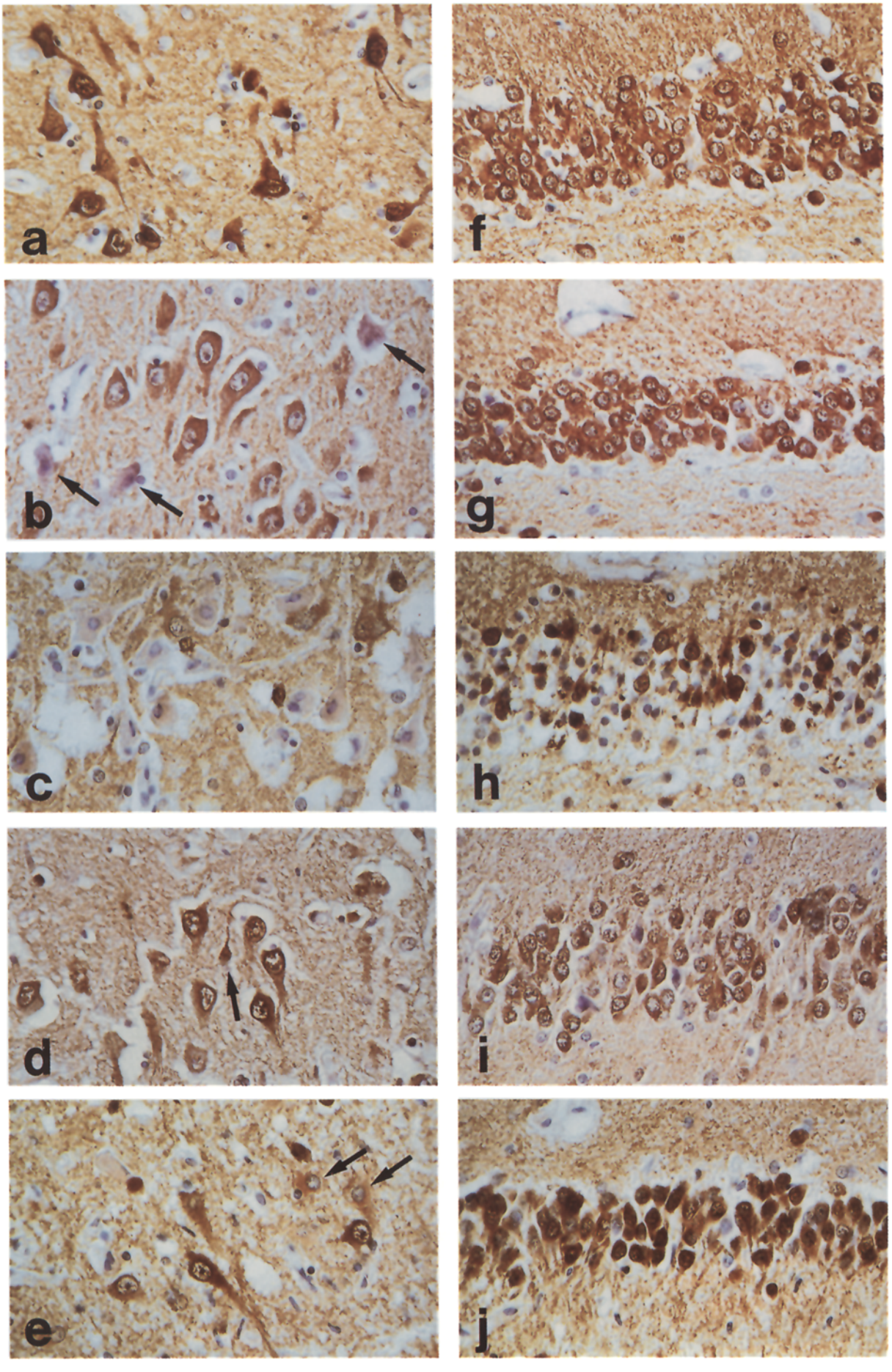
Findings by NSE immunostaining in hippocampus CA1

Specimens which had been simultaneously incubated during immunocytochemistry were examined with regard to the intensity of NSE immunostaining in histologically viable hippocampal CA1 neurons. Resistant granule cells of the dentate gyrus within each specimen provided an interior reference of immunoreactivity. Compared to controls which showed a distinct and homogeneous antibody labelling of nerve cell bodies and processes, a slight increase of immunoreactivity for NSE in neurons, dendrites, and neuropil was visible in cases with 1-h post-arrest survival. On the other hand, starting 5 h following cardiac arrest, CA1 pyramidal cells revealed a progressive loss of immunoreactivity, and the majority of CA1 neurons were negative between 4 days and 6 days post-arrest. Simultaneously, a sharp decrease of background labelling became obvious. Incipient restoration of normal immunostaining with anti-NSE in CA1 neurons was visualized not earlier than 7 days post-arrest. Labelling intensity of unaffected neurons, cell processes, and background neuropil comparable to controls was confirmed in cases with post-arrest intervals of 13 days and more. Afterwards, immunoreactivity did not change significantly with advancing survival until 186 days. Necrotic neurons were always detected as condensed, dark-staining debris (Fig. 4a–j).

Fig. 1a–i. Changes in the topistic pattern of ischemic neuronal damage by increasing post-arrest survival. **a,d,g** Frontal neocortex; **b,e,h** hippocampus CA1; **c,f,i** cerebellar cortex). **a–c** Five hours post-arrest survival: singular neocortical neurons show criteria of ischemic necrosis (*arrows*); whereas, hippocampal CA1 and Purkinje cell layer reveal absence of irreversible ischemic cell damage. Some CA1 neurons stain slightly more intensely with luxol fast blue, while nuclear membranes and nucleoli are well preserved. **d–f** Twelve hours post-arrest survival: increasing frequency of neuronal disintegration in neocortical location. Earliest manifestation of definite ischemic damage in hippocampus CA1 (arrows) and of Purkinje cells. **g–i** Thirteen days post-arrest survival: numerous necrotic neurons in scattered distribution in the neocortex. The great majority of CA1 pyramidal cells and most Purkinje cells are definitely damaged. **a–i** Paraffin sections, cresyl violet/luxol fast blue MBS, × 150

Discussion

The duration of cerebral ischemia itself, systemic as well as regional physiological and metabolical parameters prior to and due to ischemia, and pathophysiological cascades during post-ischemic reperfusion are known to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determine the final neuronal outcome [4, 22]. In contrast to the experimental setting providing minute monitoring of all relevant parameters, the validity of neuropathological post-mortem investigation of human global cerebral ischemia is restricted by several inevitable methodological limits.

Cardiac arrest, i.e., asystole or ventricular fibrillation, causes immediate break-down of systemic and cerebrovascular circulation [5]. During standard cardiopulmonary resuscitation global cerebral blood flow (CBF) is usually less than 30 % of pre-arrest values; whereas at least 20 % of normal CBF are necessary to maintain cell viability [3, 24]. Therefore, resuscitation performance represents a period of critical cerebral perfusion, i.e., critical oxygen supply to the neuronal tissue, which lasts until sufficient spontaneous circulation is restored. With regard to continuing metabolic disturbances, cardiopulmonary resuscitation has to be considered as a prolongation of preceding cerebral ischemia due to cardiac arrest.

In the present study individual pre-arrest physiological variables, systemic or regional blood flow levels during cardiac arrest and subsequent cardiopulmonary resuscitation were mostly not available. In none of the cases did clinical documentation permit the calculation of the exact duration of cardiac arrest. However, since in the great majority of cases included in this study cardiac arrest had occurred inside intensive care units, it is assumed than mean duration of the whole ischemic period, i.e., cardiac arrest plus cardiopulmonary resuscitation, did not exceed 10–15 min. In all cases diagnosis of cardiac arrest followed unequivocal clinical and electrocardiographical criteria, and cardiopulmonary resuscitation was initiated without delay. Post-arrest neurological deficits and survival were clearly indicated by the clinical records.

Fig. 4a–j. Ischemia-induced changes in neuron-specific enolase (NSE) immunoreactivity in the human hippocampus. All specimens were incubated simultaneously under identical conditions (**a–e** hippocampal CA1 neurons; **f–i** granule cells of the dentate gyrus). **a,f** Non-ischemic control: normal NSE immunoreactivity of neuronal cytoplasm, of cell processes, and of surrounding neuropil in CA1 and dentate gyrus. **b,g** Post-arrest survival for 12 h: morphologically altered pyramidal cells reveal complete loss of NSE immunoreactivity (*arrows*); whereas most preserved CA1 neurons and granule cells stain regularly. Additionally, a sharp decrease of background labelling becomes visible. **c,h** Post-arrest survival for 4.5 days: NSE immunostaining is negative in the majority of CA1 neurons, showing an absence of irreversible changes, as well as in a smaller number of granule cells. Histologically, the neuropil in both the CA1 subfield and hilus is of spongy appearance and stains weakly. **d,i** Post-arrest survival for 7 days: reappearance of NSE immunoreactivity in CA1 pyramidal cells. Labelling intensity of surrounding neuropil is still markedly reduced. Some granule cells remain negative for NSE. Necrotic neurons appear as dark staining debris (*arrow*). **e,j** Post-arrest survival for 13 days: complete restoration of normal NSE immunoreactivity in residual CA1 neurons, granule cells, and neuropil. Note the presence of reactive astrocytes which also stain positive for NSE (*arrows*) and of NSE-negative activated microglia. **a–j** Paraffin sections, avidin-biotin-complex technique, couterstaining with hemalum, × 200

The lack of reconstructability of individual arrest duration is a crucial point, especially in studying post-ischemic phenomenons like delayed neuronal death. This is because, at least in animals, the severity of ischemia influences both the progression (maturation phenomenon [6]) and the extent (selective vulnerability [4, 18]) of the final neuronal lesion. Nevertheless, since in the human brain the occurrence of delayed neuronal death has not yet been proven sufficiently and because of its outstanding therapeutic relevance, cliniconeuropathological studies are of basic importance.

With regard to the primary aim of the current investigation, the most remarkable finding was that even in the human hippocampus manifestation of definite neuronal injury is completed with a post-ischemic delay of several days. From the methodological approach employed, it is obvious that most *CA1 neurons* are not definitely damaged prior to 4 days following cardiac arrest. Subsequently, rapid progression of neuronal necrosis continues until it comes to an end 7 days post-arrest. Thus, the delay of post-ischemic death is longer lasting in the human hippocampus than that described in gerbil and rat studies. These results do not contradict but complement the findings elaborated by Pulsinelli et al. [17], who defined the delay of post-ischemic hippocampal death as lasting about 4 days. In the first place their results were based on material from patients with post-arrest survival intervals ranging from 1 h to 5 days. Further two cases in their study died 46 days or 180 days post-arrest, respectively. Thus, the post-ischemic interval of day 5 and 9, which our results suggest to be most relevant, had not been under consideration.

Within the *neocortex*, an earliest manifestation of cell death was visible as soon as 5 h post-arrest. In contrast to the hippocampal situation, during the first days of post-ischemic reperfusion, neocortical necrosis failed to show a significant increase, thereby demonstrating that ischemic cortical cell death is a rapidly starting and terminating process.

On the other hand, in some respects the velocity of definite post-ischemic *Purkinje cell* changes resembled hippocampal CA1 neurons. Remarkably, complete manifestation of ischemic cerebellar injury seems to have a delay of about 4 days and, therefore, is not as extended as it is in hippocampus. Additionally, the belated increase of neuronal necrosis is less pronounced in the cerebellar cortex than in CA1. Nevertheless, the post-ischemic behavior of Purkinje cells should be regarded as at least a minor form of delayed neuronal death. Our results show some correlation with recent experimental findings in dogs by Sato et al. [21], in so far as these authors described a "two-phase" damage of Purkinje cells following global ischemia with final neuronal desintegration 2–3 days after the onset of recirculation.

Since both hippocampal and cerebellar neurons are known to range at the top of the rank order of hypoxic/ischemic vulnerability [18], the question arises if there is any causal relationship explaining the coincidence of selective vulnerability and delayed neuronal

death in humans. The phenomenon of excitotoxicity as a consequence of enhanced pre-synaptic liberation and/or restricted post-synaptic uptake of excitatory amino acids (EAA) is claimed to be decisively involved in molecular mechanisms underlying ischemic neuronal injury [11, 20]. There is considerable evidence suggesting a major role for glutamate as a neurotoxic agent during post-ischemic reperfusion since the *N*-methyl-D-aspartate subtype of glutamate receptors is present in high density in CA1 pyramidal cells [15]. Purkinje cells themselves contain γ-aminobutyric acid, but their excitatory inputs by both parallel fibers of the granule cells and climbing fibers are operated by EAA receptors [19]. Autoradiographic data on mice identified glutamate as the neurotransmitter of the parallel fiber-Purkinje cell synapse, which quantitatively is the predominant synapse in the cerebellar cortex [16]. Presumably, delayed morphological manifestation of ischemic cell death in both CA1 neurons and Purkinje cells is linked to glutamate-mediated neurotransmission. This concept has already been proposed for molecular mechanisms related to selective vulnerability [1, 11].

In another analytical approach in the present study, the immunohistochemical reactivity of NSE in morphologically well-preserved CA1 neurons has been investigated. NSE represents the γ,γ isoenzyme of the dimeric protein enolase which is a soluble enzyme of the glycolytical pathway. Physiologically, NSE is present in nerve cell bodies and dendrites, and in cells of the amine precursor uptake and decarboxylation cell system [13]. In the state of functional or structural defects of the neuronal membrane, e.g., as a result of ischemia, the cytoplasmic enzyme is considered to be liberated into the extracellular space [14]. The changes in intensity of NSE immunostaining found here mainly consisted of a sharp decrease and, finally, of a complete loss of neuronal immunoreactivity during the first days of reperfusion. Reappearance of normal cytoplasmic immunostaining was visualized not earlier than 7 days post-arrest. This observation seems to reflect an aspect of delayed neuronal recovery, which in the time course reciprocally corresponds to the delayed manifestation of neuronal death in CA1. By contrast, following unilateral carotis occlusion Matsumoto et al. [14] did not mention notable changes with hippocampal NSE immunoreactivity between 3 days and more than 1 week after the insult. However, their approach included 30-min regional ischemia and was performed in the gerbil which may contribute to differences in findings.

In conclusion, the results presented indicate the occurrence of *delayed neuronal death* in the human brain also and support as well as supplement previously published data. Final neuronal injury among Purkinje cells and in hippocampus CA1 seems to be completed not earlier than 4 days or 7 days, respectively, following global cerebral ischemia caused by cardiac arrest. In contrast, ischemic neocortical damage is already accomplished within the 1st day of reperfusion. Moreover, for the first time immunohistochemical data demonstrate the presence of *delayed neuronal recovery* in human hippocampus CA1. Corresponding to therapeutic implications derived from rat and gerbil experiments, the current findings strongly point to the justification of subsequent treatment of cerebral ischemia in humans.

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