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Ischemia-induced changes in the electrical activity of the hippocampus

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Summary. Consequences of transient (15–20 min) ischemia on the neuronal activity of the dentate gyrus and hippocampal CA 1 region were investigated in chronically implanted Sprague-Dawley rats. Forebrain ischemia was produced by occlusion of the carotids for 15 or 20 min, following cauterization of the vertebral arteries. Following the release of the carotids, both spontaneous and evoked activity showed a steady but partial recovery, reaching a maximum 12 to 24 h after the ischemic insult. From this plateau, both the power of rhythmic slow activity recorded during walking and the power of slow delta activity obtained during alert immobility decreased monotonically, with large changes occuring between postischemic days 2 and 4. The changes in spontaneous activity were accompanied by a decrease and eventual disappearance of the Schaffer collateral evoked responses in CA 1. Perforant path volleys were less efficient in activating the granule cells following ischemia compared to baseline levels. This decreased responsiveness was paralleled by a relative impairment of paired pulse depression. Neurophysiological signs of spontaneous or evoked neuronal hyperexcitability were not observed at any time point during the 8 postischemic days. Neuronal damage in the CA 1 region varied from moderate to complete loss of pyramidal cells. In addition, degenerating neurons were also observed in the hilus of the dentate gyrus. These findings do not support the "overwork" version of the excitoxic hypothesis of delayed neuronal damage and indicate that the cause of ischemic cell death should be sought in factors other than neuronal hyperactivity.

Key words: Ischemia – Hippocampus – EEG – Evoked potentials – Chronic monitoring – Degeneration – Rats

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Introduction

The neuronal damage associated with transient global ischemia in both human and animal brain results in a relatively selective pattern of neuronal degeneration (Kirino 1982; Petito et al. 1987; Pulsinelli et al. 1982; Schmidt-Kastner and Hossmann 1988; Smith et al. 1984; Zola-Morgan et al. 1986). Rats exposed to forebrain ischemia of variable duration develop a relatively circumscribed degeneration in the majority of pyramidal neurons in the hippocampal CA 1 region and in other neurons in the striatum and neocortex (Pulsinelli and Brierly 1979). Neuronal degeneration in the hippocampus is not immediate but takes several days to reach complete ischemic necrosis.

The mechanisms of the delayed death of CA 1 pyramidal cells is not known. A currently dominant hypothesis, based on the following observations, suggests that the excitatory amino acids L-glutamate and/or aspartate are involved in the pathogenesis of ischemic damage (Benveniste et al. 1984; Johanson et al. 1986; Meldrum 1985; Olney 1983; Rothman 1984; Simon et al. 1984; Wieloch 1985). First, neurons in the CA 1 region possess a high density N-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptors (Monaghan and Cotman 1985). Second, the anticonvulsant MK-801, a non-competitive NMDA receptor antagonist (Wong et al. 1986), and other NMDA antagonists have been claimed to be protective against ischemic cell death (Gill et al. 1987; Prince and Feeser 1988; Simon et al. 1984; Swann et al. 1988). Third, ablation of the excitatory amino acid pathways, such as the perforant path input to the granule cells of the dentate gyrus and the mossy fiber projection to CA 3 pyramidal cells, was found to ameliorate ischemia-induced neuronal degeneration (Johanson et al. 1986; Onodera et al. 1986; Wieloch et al. 1985). Explicit in the 'excitotoxic'

hypothesis of delayed neuronal death is that excessive release of glutamate and/or aspartate from afferent terminals lead to overexcitation of CA 1 pyramidal cells via their NMDA receptors. The enhanced energy load due to hyperactivity would be the direct cause of the sequences of events leading to neuronal death. Pertinent to this hypothesis, increased postischemic firing of hippocampal and neocortical neurons was reported in the anesthetized gerbil (Suzuki et al. 1983).

A characteristic EEG pattern in the rat hippocampus during exploratory behaviors (walking, rearing, sniffing, head turning) is rhythmic slow activity (RSA or theta) (Vanderwolf 1969). The extracellular currents producing RSA derive from anatomically separated dipoles along the somadendritic axis of the pyramidal and granule cells (Bland and Whishaw 1976; Buzsaki et al. 1983; Green et al. 1960; Leung 1984; Winson 1974). The direct septohippocampal input modulates the activity of hippocampal interneurons (Freund and Antal 1988) which, in turn, rhythmically hyperpolarize the principal cells of the hippocampus. This rhythmic inhibition is coupled with excitation of the distal dendrites of pyramidal and granule cells via the perforant path input originating in the entorhinal cortex (Buzsaki et al. 1983, 1986). Since the main source of extracelluar RSA is the membrane fluctuation of the laminarly arranged large pyramidal cells, it is expected that ischemia-induced changes in these neurons can be monitored chronically by a quantitative evaluation of hippocampal RSA.

Another population index of hippocampal excitability is the responsiveness of hippocampal neurons to afferent activation. Stimulation of the perforant path input sequentially excites granule cells and CA 3 pyramidal cells which, in turn, may excite CA 1 pyramidal neurons via their Schaffer collaterals (Andersen et al. 1971). Because the size of the extracellularly recorded evoked potential depends mainly on the degree of postsynaptic depolarization and the number of neurons participating in the population event, monitoring the changes of the evoked activity is a useful neurophysiological tool for assessing the functional state of hippocampal neurons.

The present study was designed to examine the presumed hyperexcitability of hippocampal neurons and the time course of the functional alteration following ischemia produced by a four-vessel occlusion (Pulsinelli and Brierley 1979) in the rat. Since recent reports have dealt with the postischemic EEG changes occurring in the hippocampus during and immediately after ischemia (Freund et al. 1989) and from 8 days to a month after the ischemic insult (Monmaur et al. 1986), we investigated the spontaneous and evoked electrical activity during and immediately after recirculation, at three intervals during the first day and every day for up to a week.

Methods

Subjects and surgery

Fourteen adult female rats (220–250 g), of the Sprague-Dawley were used in this study. They were deeply anesthetized with a mixture (4 ml/kg) of ketamine (25 mg/ml), xylazine (1.3 mg/ml), and acepromazine (0.25 mg/ml) and placed in a stereotaxic instrument. Pairs of Polyimide-insulated 125-μm stainless-steel electrodes with 0.5 mm vertical tip separation were placed in the right and left angular bundles ($AP = -7.0$ mm from bregma, $L = 4.5$ mm from midline, and 3.5 mm below dura; bregma and lambda in the same horizontal plane) to stimulate the perforant path input to the hippocampus. Two pairs of recording electrodes with 1.0 mm vertical tip separation were lowered into the hippocampus bilaterally ($AP = -4.0$, $L = 2.6$, $V = 2.9$, deep electrode). The tips of the recording electrodes were aimed at the hilus and CA 1 region. After baseline recordings (see below) the rats were reanesthetized and the alar foramina were enlarged by drilling on each side to expose the vertebral arteries running in the foramina transversaria. The vessels were cauterized and split under direct visual control (Todd et al. 1986). Next, the carotid arteries were exposed bilaterally and a loop, made from a 0.4 mm atraumatic silicon tube, was formed around the arteries. When the skin was closed the end of the silicon tubes protruded between the edges of the skin (Farber et al. 1988). In 3 animals the vertebral arteries and carotids were not exposed and these rats served as 'no-ischemia' controls.

Recording and data processing

Baseline spontaneous and evoked hippocampal activity was recorded in 3 to 5 sessions in all 14 rats. The rat was placed into a Plexiglas observation chamber ($40 \times 20 \times 30$ cm). The chamber was fixed to a magnet and coil type stabilimeter to monitor the movement of the rat. Bioelectric activity was amplified and filtered (1–70 Hz for EEG, 1 Hz–5 kHz for evoked potentials), recorded on a polygraph, and stored on hard disc. The sampling frequency was 200 Hz for the EEG and 5 kHz for evoked potentials. Power of EEG was estimated for periods of 20 s by averaging five 4-s short-time spectra obtained during identical behaviors. Numerical values of power were obtained for the delta band (1–4 Hz) during immobility and for the theta/alpha band (6–9 Hz) during spontaneous walking. If the rat did not move spontaneously the tail was pinched lightly to induce movement.

The perforant path was stimulated by double pulses (0.1 ms at 0.1 Hz) with 25 ms interpulse intervals. The intensity was gradually increased until the dentate gyrus population spike in response to the conditioning pulse completely inhibited the population spike to the test pulse. This intensity was usually sufficient to also evoke a multisynaptic (dentate-CA 3-CA 1) response in the CA 1 region. Detailed input-output curves were constructed in 4 rats using several stimulus intensity levels. The amplitude of the population spike was measured on the printout as determined by the height between the peak and a line drawn

between the onset and termination of the population spike (see Fig. 5).

In the ischemia session the rat was gently restrained and the silicon tubes were gently pulled. Recording continued throughout the ischemic period (15 min, $n=5$; or 20 min, $n=6$) and during 2 h following the release of the carotids. Additional recording sessions followed between 3–5 h and 10–14 h after ischemia and every day up to 8 days.

Histology

Following the last recording session the rats were deeply anesthetized and perfused through the heart first with saline followed by a fixative containing 1% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. The brains were removed from the skull, sectioned on a Vibratome at 80 µm, and extensively washed in several changes of 0.1 M phosphate buffer. Alternate sections were mounted on gelatine-coated slides, stained for Nissl substance with cresyl violet, dehydrated, and embedded in a neutral medium. Another series of sections were silver-impregnated using the method developed by Gallyas et al. (1980).

Results

The daily variability of the EEG power and the amplitude of the evoked changes was less than 20 percent for both measures in the 'no-ischemia' control rats. The largest variability occurred in the delta band power during immobility, although all immobility samples were obtained while the rat was sitting still with head up and eyes open.

Four rats died during or following the occlusion of the carotids. In two additional animals the occlusion was incomplete as indicated by the survival of the electrical activity during the occlusion of the carotids and these rats were excluded from further analysis.

Occlusion of the carotids was typically followed by hippocampal rhythmic slow activity (RSA) with gradually decreasing amplitude. The spontaneous activity disappeared within 15 to 25 s. During this time the evoked potentials remained unaltered and began to decrease gradually 15–20 s after the disappearance of the EEG. Complete failure of the evoked responses occurred after 1 min. In animals with a recording electrode in the stratum radiatum of CA 1, the disappearance of the evoked response was reliably predicted by a transient polarity reversal of the trisynaptically evoked Schaffer collateral potential.

The time required for the recovery of the evoked potentials following the release of the carotids varied between 7 and 24 min in different animals and preceded the spontaneous EEG activity and the occurrence of the righting reflexes by 5 to 20 min.

At 1 h after the release of the carotids, the rat walked or crawled spontaneously. The hippocampal EEG activity did not have rhythmic components in the RSA band during movement. During immobility large amplitude, often rhythmic, slow waves at 0.5–1 Hz dominated the hippocampal record and were replced by very low amplitude activity during spontaneous or forced walking (Fig. 1). Evoked potentials in the dentate gyrus were reduced, and the low amplitude conditioning responses produced facilitation of the test responses, similar to paired pulse facilitation seen in intact rats. No responses could be elicited in the CA 1 region.

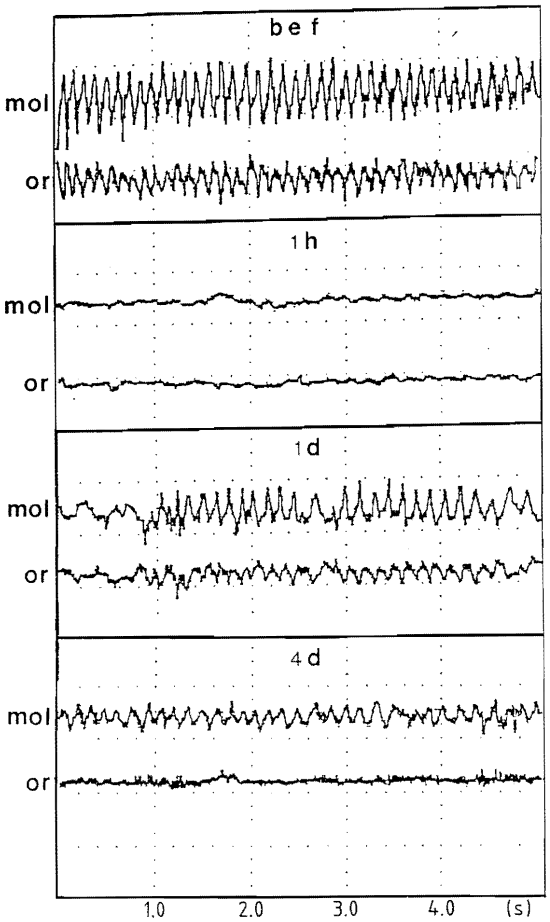

Fig. 1. Hippocampal RSA recorded from the molecular layer of the dentate gyrus (mol) and stratum oriens of CA 1 (or) during walking before and 1 h, 1 day and 4 days following 20 min forebrain ischemia. Note the complete disappearance of RSA from the CA 1 record and decreased amplitude in the dentate record at 4 days after the ischemic insult. Walking at 1 h was elicited by pinching the tail of the rat. Note lack of RSA at this time point. Calibration: 1 mV

Movement-related hippocampal RSA was present in all animals at 4 h after the release of the carotids. However, the frequency of RSA was slow (4–6 Hz), very low in amplitude and occurred

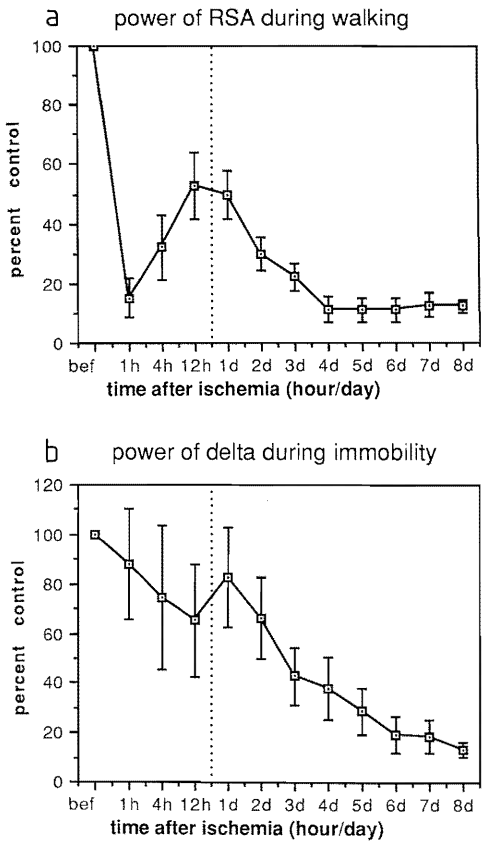

Fig. 2a, b. Power changes of EEG activity following ischemia. **a** Power of RSA at various postischemic time points. Vertical lines indicate standard errors of the means. Dotted line: change in scale from hours to days. **b** Changes in spectral power of delta EEG (1–4) recorded during alert immobility. Data in **a, b** were obtained from 8 hemispheres with ischemic neuronal damage in the CA 1 region of the hippocampus in 5 rats

mostly in bursts interspersed with irregular activity. By 12 h postischemia both EEG and evoked potentials appeared qualitatively normal in most rats. In most animals, however, the power of RSA during walking and the power of delta activity during immobility remained below the control values (Fig. 2). At the group level, however, the decrease of power of neither RSA nor delta differed significantly from pre-ischemic values. Similarly, the amplitude of the trisynaptically evoked response in CA 1 was smaller at this time point. This was the case even when high-intensity stimuli delivered to the perforant path evoked large amplitude population spikes in the dentate gyrus (Fig. 4). The electrical patterns have remained similar during the first 2 postischemic days. Most rats developed a typical walking pattern with stretched hindlegs.

The major changes in the hippocampal activity occurred between 2 and 4 days following forebrain ischemia (Figs. 2 and 3). In a given rat, the power of RSA typically showed a 3- to 4-fold decrease in 24 h (e.g. Fig. 3b). The absence of this sudden change in the group data of Fig. 2 is due to averaging. The decrease of RSA power was proportionally larger in records obtained from the shallow electrode in the CA 1 region (Fig. 1). Often, the peak in the RSA band was completely missing in the power spectra derived from the shallow electrode, while a diminished but obvious peak in the RSA band was present in the record derived from the deep electrode.

In two rats ischemic cell damage was confined to one hemisphere only, as indicated by the subsequent histological analysis. In these animals the electrophysiological changes occurred in an identical fashion in the two hemispheres during the first 4 h of postischemia. While the electrical activity returned to normal in the histologically intact hemisphere, the power of RSA during walking and the power of delta during immobility decreased significantly in the histologically affected hippocampus (Fig. 3).

Postischemic changes of the evoked responses in the dentate gyrus and CA 1 region are illustrated in Fig. 4. Parallel with the decrease of the power of RSA, the trisynaptically evoked response in CA 1 also diminished and was completely absent by day 4 to day 6, even when perforant path stimulation continued to evoke large population spikes in the dentate gyrus. Postischemic changes were also evident in the dentate evoked responses. Typically, the amplitude of the population spike in response to a given intensity of stimulus was smaller during the postischemic period (Fig. 5). However, the test response was facilitated even at higher intensities which were sufficient to evoke large population spikes to the conditioning stimulus. Comparison of the input-output curves, constructed before and after forebrain ischemia, revealed that a given amplitude population spike in response to the conditioning stimulus produced less suppression of the test response following ischemia (Fig. 5c).

Spontaneous or stimulation-induced seizures or large amplitude interictal spikes were not observed in any rat at any time point following ischemia. Stimulation of the perforant path always evoked a single population spike in the dentate gyrus and occasionally in the CA 1 region. None of the monitored electrophysiological indices suggested the presence of epileptic tendencies. Behavioral clonic seizure was observed in one rat which became adipsic and aphagic following the forebrain ischemia and died despite intragastric feeding.

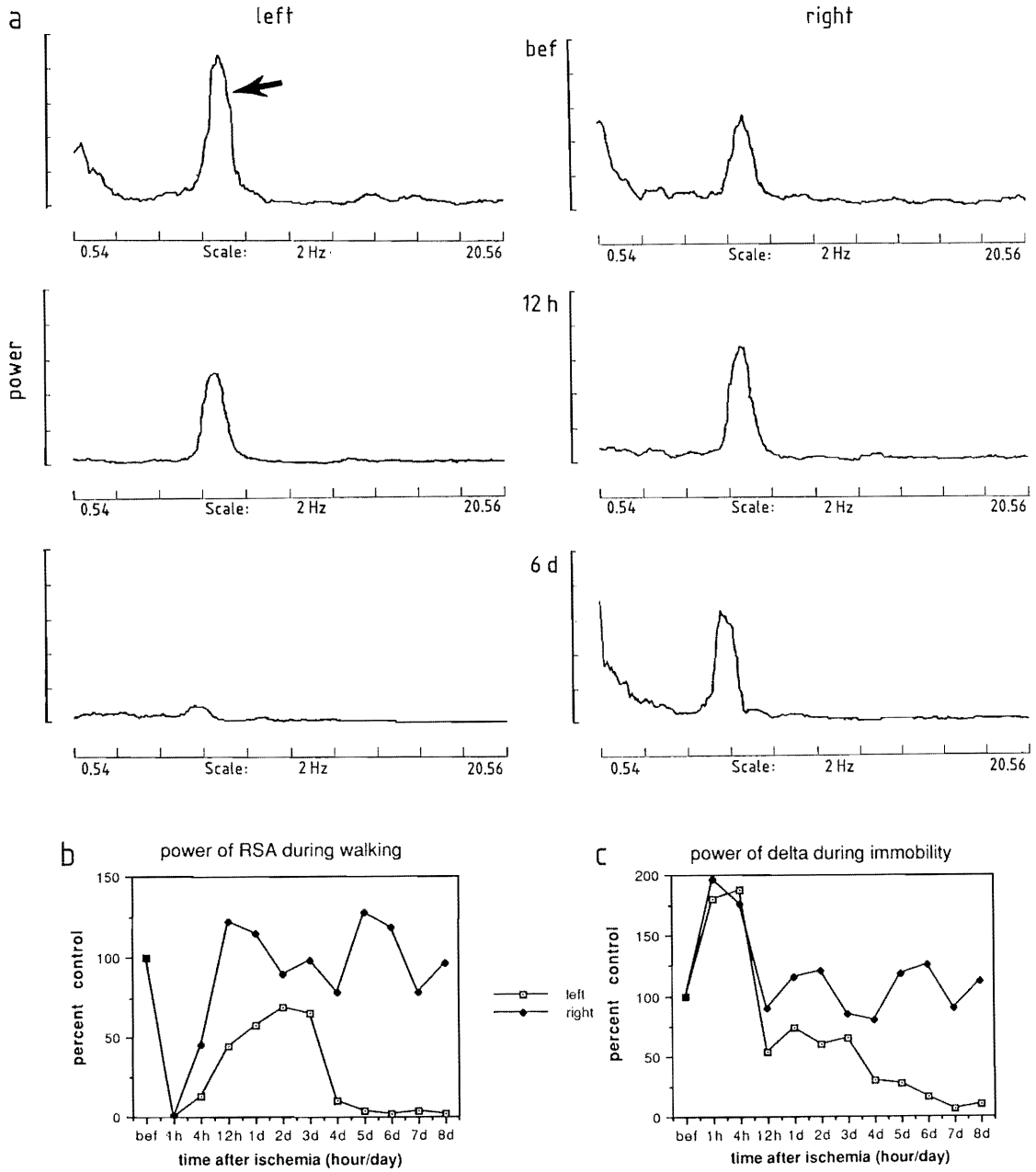

Fig. 3a–c. EEG changes in an animal with unilateral (left) damage of the CA 1 region. **a** Power spectra of EEG recorded during walking before (bef), 12 h and 6 days after forebrain ischemia. Arrow indicates power peak in the RSA band. Ordinate: arbitrary units. **b** Power changes of RSA at various postischemic time points in the two hippocampi. **c** Power of delta activity in the two hemispheres recorded at various postischemic time points recorded during alert immobility. Large amplitude slow waves at 1 to 2 Hz were present during immobility at 1 and 4 h after releasing the carotids in this animal, which were completely suppressed during movement

Histological findings

Degeneration neurons can be visualized by the silver impregnation technique of Gallyas et al. (1980), which allows a clear identification of damaged cells even in the early stages of degeneration. The general pattern of neuronal damage in the hippocampus was very similar in each of the five animals that survived for 7–8 days. Argyrophilic neurons were consistently found in the CA 1 region of the hippocampus and in the hilus of the dentate gyrus. There was, however, some variability in the degree of cell loss among the individual animals, and even between the two hemispheres of the same

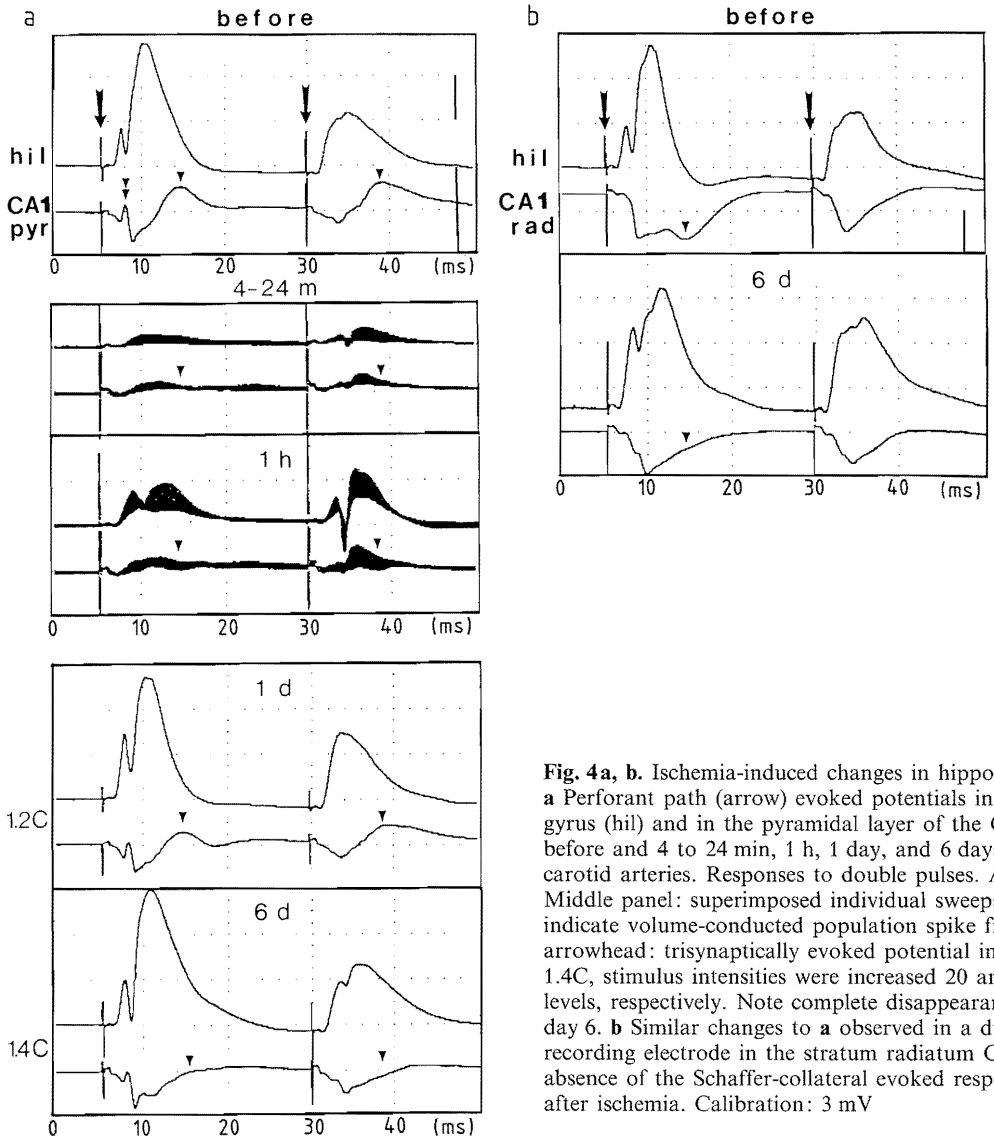

Fig. 4a, b. Ischemia-induced changes in hippocampal evoked potentials. **a** Perforant path (arrow) evoked potentials in the hilus of the dentate gyrus (hil) and in the pyramidal layer of the CA 1 region (CA 1 pyr) before and 4 to 24 min, 1 h, 1 day, and 6 days after the release of the carotid arteries. Responses to double pulses. Averages of 8 repetitions. Middle panel: superimposed individual sweeps. Double arrowheads indicate volume-conducted population spike from the dentate gyrus. Single arrowhead: trisynaptically evoked potential in the CA 1 region. 1.2C and 1.4C, stimulus intensities were increased 20 and 40 percent above control levels, respectively. Note complete disappearance of the CA 1 response by day 6. **b** Similar changes to **a** observed in a different animal with the recording electrode in the stratum radiatum CA 1 (CA 1 rad). Note absence of the Schaffer-collateral evoked response (arrowhead) by day 6 after ischemia. Calibration: 3 mV

animal in two rats. In two animals the damage was almost complete unilaterally, but was negligible in the contralateral hemisphere (Fig. 6). The ventral hippocampus was better preserved than the dorsal part in all animals. Large groups of intact pyramidal cells were often found in temporal portions of the CA 1 region, even when the septal pole was severely damaged. In the dorsal hippocampus the extent of degeneration of hilar cells correlated well with the degree of pyramidal cell loss in the CA 1 region. Argyrophilic neurons were not observed in strata radiatum, oriens and lacunosum moleculare of CA 1 and CA 3, or in the molecular layer of the dentate gyrus, suggesting that interneurons of these layers remained intact. Granule cells and CA 3 pyramidal cells did not show detectable changes with the present methods.

Discussion

The main findings of the present study are that a) partial neurophysiological recovery occurs in the hippocampus within 12 to 24 h after the ischemic insult, b) deterioration of the membrane function of CA 1 pyramidal neurons occurs relatively suddenly between 2 and 4 days postischemia, c) neuronal transmission is impaired also in the dentate gyrus, and d) these changes occur without significant enhancement of neuronal activity at any time point during the postischemic period.

The degree of the power reduction of RSA and the decrease of the amplitude of the CA 1 response correlated well with the eventual cell loss in the individual animals. These findings confirm and extend similar observations by Monmaur et al.

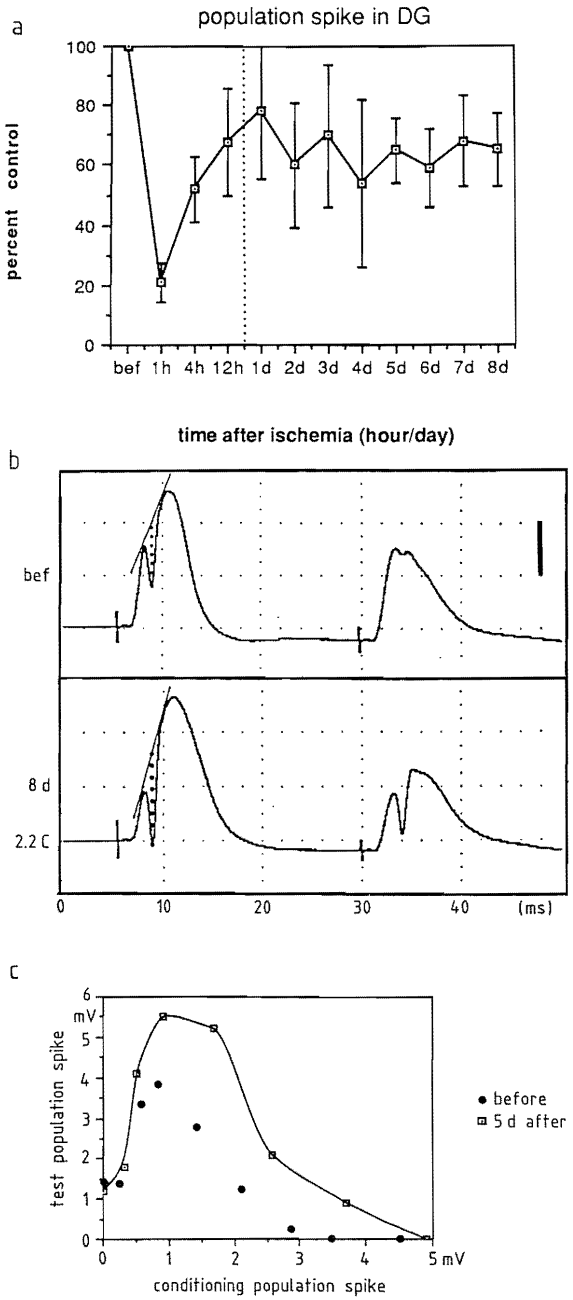

Fig. 5a–c. Postischemic changes of granule cell excitability. **a** Relative change of the amplitude of the population spike in the dentate gyrus in response to perforant path stimulation ($n=6$ hemispheres). The method of amplitude measurement is shown in **b**. **b** Averaged ($n=8$) evoked responses in the dentate gyrus before (bef) and 8 days after forebrain ischemia. 2.2C: 2.2 times increase of stimulus intensity above control level. Note suppression of the population spike to the test response before and impaired suppression of the test response after ischemia. Calibration: 3 mV. **c** Relationship between the amplitude of the population spike in response to the first (conditioning) stimulus and the amplitude of the population spike to the test stimulus before and 5 days after ischemia. Note that after ischemia a given amplitude of the population spike to the conditioning pulse produced less suppression of the test pulse-evoked population spike

(1986). Significant postischemic reduction of the power of delta activity during immobility indicates that these changes are not due to deterioration of specific afferent systems but reflect a global dysfunction of postsynaptic membrane activity in CA 1 pyramidal cells.

Reduction of the amplitude of the evoked population spikes in the dentate gyrus following ischemia indicates impaired neuronal transmission also in this region. This decreased excitability was accompanied by a relative increase in responsiveness as assessed by the double pulse stimulation experiments. Decreased responsiveness of granule cells to perforant path volleys, following a large population spike evoked by a conditioning stimulus, is usually interpreted to reflect activation of recurrently excited inhibitory interneurons whose enhanced discharge would prevent granule cell firing in response to a delayed test pulse (Lømo 1971). Reduction of the double pulse suppression in the dentate gyrus in ischemic animals therefore may indicate malfunctioning of the granule cell-interneuron-granule cell recurrent circuitry. The substantial loss of neurons in the hilar region of the dentate gyrus (see also Johanssen et al. 1987) is compatible with this suggestion. However, the loss of inhibitory neurons would also predict enlarged population spikes to the conditioning stimulus because inhibitory interneurons of the recurrent circuitry participate also in feed-forward inhibition (Buzsaki 1984). Possible explanations for the decreased responsiveness of granule cells to perforant path activation may involve impaired release of glutamate from the perforant path terminals and/or reduction in the number of perforant path axons stimulated due to ischemia-induced cell damage in layers II and III of the entorhinal cortex, the origin of the perforant path (Steward 1976). Compatible with the first possibility is the finding that the binding of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) to quisqualate receptors was significantly decreased in the dentate gyrus up to 4 weeks after the ischemic attack (Westerberg et al. 1988).

The excitotoxic hypothesis of delayed neuronal death

According to the currently dominant hypothesis, neurodegeneration that follows ischemia may result from an excessive release of glutamate at some level of the intrahippocampal circuitry which eventually leads to overstimulation of CA 1 pyramidal neurons via their NMDA receptors (Jorgensen and

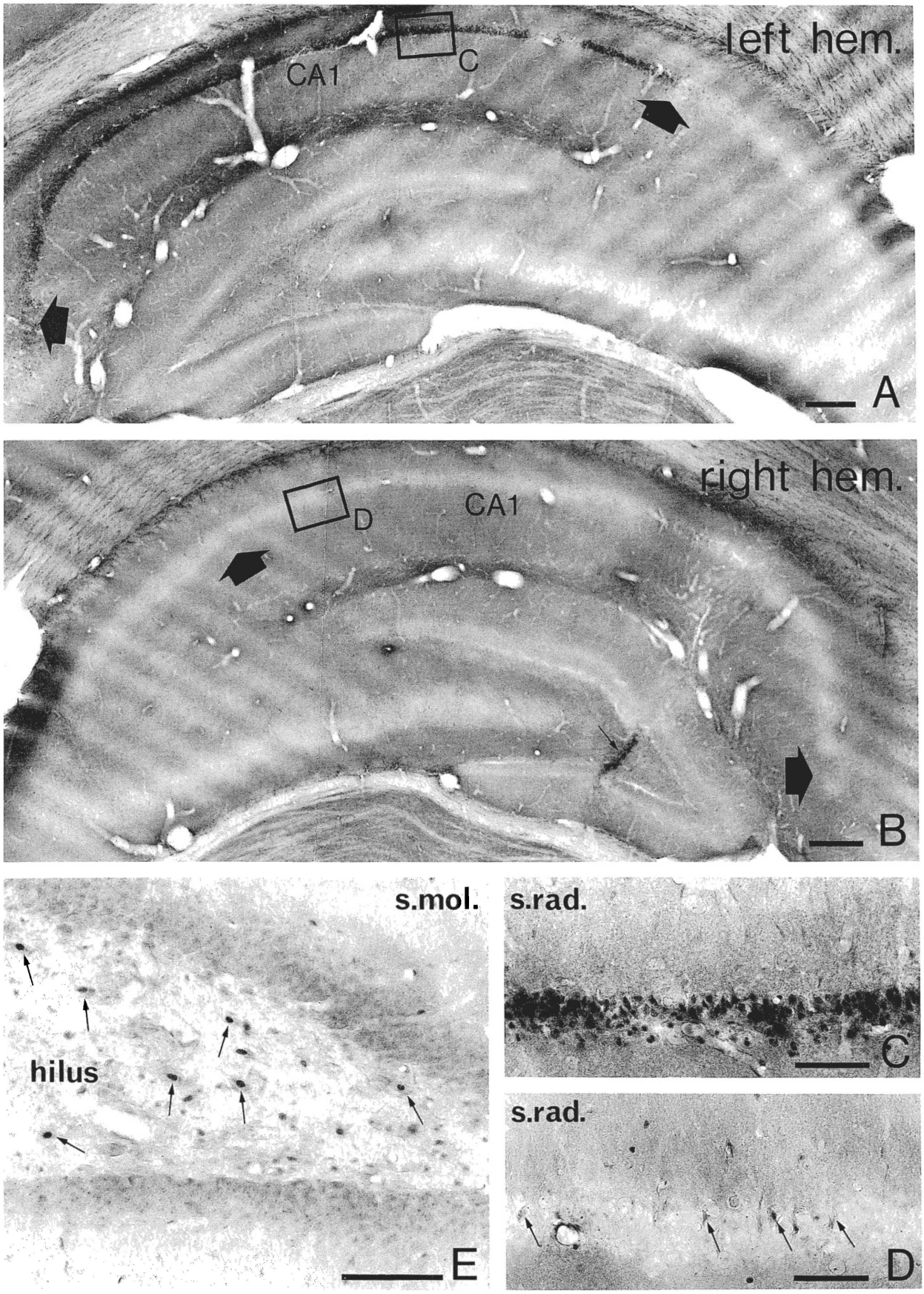

Fig. 6

Diemer 1982; Meldrum 1985; Rothman 1984; Rothman and Olney 1986; Swann et al. 1986).

Large and sufficiently long depolarization of the pyramidal cell appears to relieve a Mg^{2+}-block of the NMDA channel (Collingridge et al. 1983). Only when this channel is unblocked can it conduct in response to glutamate released into the synaptic cleft. Opening of the NMDA channels then would further accelerate the depolarization of the postsynaptic membrane which, in turn, would facilitate Ca^{2+} entry (MacDermott et al. 1986), swelling and energy depletion (Olney 1983; Siesjo 1981). Prolonged neuronal hyperactivity is thus an essential factor, if not the major cause of cell death in the excitotoxic models.

In our experiments we failed to confirm an important step in the suggested sequence of events in the excitotoxin chain. We found no evidence of substantially increased spontaneous or evoked neuronal activity in either the dentate gyrus or the CA 1 region. Stimulation of the perforant path input produced smaller responses after forebrain ischemia without a preceding hyperexcitable period. Similarly, evoked potentials in the CA 1 region were significantly smaller in response to a given size of population spike in the dentate gyrus at any time after the release of the carotids. The decrease of evoked activity occurred in a monotonic manner following the initial partial recovery. Similar changes were observed in the spontaneous EEG activity. No electrical signs indicated excessive neuronal activity that might have reflected "overwork" of individual neurons. On the contrary, the spontaneous and evoked changes were quite opposite to what is observed during neuronal hyperexcitability in epilepsy. In a chronic model of epilepsy in the rat, perforant path volleys evoked up to 25 mV population spikes in the dentate gyrus and large multiple population spikes in the CA 1 area (Buzsàki et al. 1989; Buzsàki and Gage 1989). In addition, interictal spikes with concurrent synchronous discharge of a large number of pyramidal cells were present for several months. Involvement of the NMDA receptors in chronic epilepsy has recently been suggested (Mody et al. 1987). In a stimulation model, Sloviter (1986) produced status epilepticus lasting for several hours, yet found cell loss in somatostatin-containing interneurons of the hilar region only. The CA 1 region was essentially preserved.

Based on the findings of the present experiments, we suggest that causes of the delayed death of hippocampal neurons following ischemic insults should be sought in factors other than overstimulation of neurons by excitatory amino acids. Clearly, further studies are needed, involving recording from individual neurons of the ischemic brain, to substantiate this claim. A modified version of the excitotoxic hypothesis, however, remains compatible with our electrophysiological findings. It is conceivable that the excessive Ca^{2+} entry following ischemia causes a slow death of the CA 1 pyramidal cells by interfering with cytoplasmic enzymes necessary for survival without significantly affecting the membrane function of these neurons.

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Fig. 6A–E. Demonstration of silver-impregnation of unilateral neuronal degeneration in the hippocampus of animal GF 24. The electrical changes in the same animal are shown in Fig. 3. In the left hemisphere **A** most of the pyramidal cells in the CA 1 region (framed area is shown at higher magnification in **C**) and some cells in the hilus of the dentate gyrus (arrows in **E**) are shrunken, and have dark argyrophylic nuclei. Granule cells in the dentate gyrus, pyramidal cells in the CA 3 region of this hemisphere and nearly all cells in all regions of the right hemisphere **B** appear to be intact. Even in the CA 1 region of the right hemisphere (framed area is shown at higher magnification in **D**) only a few degenerating neurons are visible (arrows). The electrode track is indicated by a small arrow in **B**. The two large arrows in **A**, **B** label the borders of the CA 1 region. Scales: **A, B, E** 200 μm; **C, D** 50 μm

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