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Electrophysiological and Morphological Changes of Hippocampal CA1 Pyramidal Cells Following Ischemia in the Mongolian Gerbils

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ABSTRACT

Consequences of transient ischemia on the neuronal activity of hippocampal CA1 region were investigated in *Mongolian gerbil*. Ischemia was done for 5 min in two groups, such that in Group 1 it was done 1 week after implantation of recording micro-wire electrodes and in Group 2 immediately before electrode implantation. Pyramidal neurons of CA1 of anesthetized gerbils showed stable spontaneous activities before ischemia (5.21±0.54 Hz). Immediately after the initiation of ischemia, neurons in both groups showed initial cessation of spontaneous activity. Spike discharge reappeared about 5 min after recirculation. However neural activity was suppressed for 25 min of post-ischemia period. In both Group 1 and Group 2, some neurons (Type 1) showed full recovery of spontaneous activity by 6 h after ischemia and subsequent hyperactivity, but other neurons (Type 2) exhibited only partial recovery and subsequent deterioration of activity. Neural activity of Type 1 was maximally enhanced at post-ischemic 12 h & day 1 (Group 1: 97.31% and Group 2: 110.34%). Thereafter, CA1 activity was gradually decreased to below the pre-ischemia level (-29.17% at day 3 in Group 1 and -44.95% at day 4 in Group 2). Histological analysis indicated that in both hemispheres CA1 cell death was observed in Group 2 but it was noticed only at small region in Group 1. Animals with implantation surgery but without ischemia did not show any cell death. The results of this study suggest that functional loss of CA1 activity and morphological death following ischemia in chronically implanted *Mongolian gerbil* could not occur in parallel.

Key words: Transient ischemia, hippocampal CA1 pyramidal cells, temporal activity change

INTRODUCTION

Global ischemia is induced when the entire blood supply to the forebrain is substantially but transiently reduced for several minutes. The most frequent clinical examples of global ischemia occur following recovery from acute cardiac arrest or near-drowning. Animal models are produced by occluding the major arteries supplying the cerebral hemisphere (i.e., the carotids and vertebrals) for 5

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to 20 min (Ginsberg et al., 1989; Pulsinelli et al., 1989). The damage following transient forebrain ischemia is limited to the CA1 pyramidal neurons of the hippocampus, and to a lesser extent, certain neurons in subregions of the neocortex, thalamus and striatum (Lin et al., 1990; Pulsinelli et al., 1989; Smith et al., 1984). The reason(s) for the selective vulnerability of CA1 neurons following global ischemia is still not completely known. A second, fascinating characteristic of global ischemia is that no significant neurodegeneration is seen until several days after the ischemic perturbation, long after the precipitating crisis has apparently passed (Pulsinelli et al., 1991). Temporal pattern of events raises related questions which continue to evoke interest among investigators: the time of death; the degenerated pattern. One way of addressing these questions would be to compare the time of functional decline of the CA1 pyramidal to the time of morphologically verified neuronal death of the same neurons. Insight into the temporal relationship between these different events should, in turn, may help to refine hypothesis regarding the delayed cell death phenomenon. However, report of recovery of neuronal activity following the transient ischemia varies from several minutes (Imon et al., 1991; Suzuki et al., 1983) to several hours (Andine et al., 1991; Chang et al., 1989) and longer (Buzsaki et al., 1989; Urban et al., 1989). While some authors reported 'functional death' as early as post-ischemic day 2 (Suzuki et al., 1983), others observed gradual decline of function through day 3 (Chang et al., 1989; Urban et al., 1989). Some do not report loss of responsiveness till day 4 (Buzsaki et al., 1989). Thus, these studies do not permit one to make a generalization about the time of actual death of CA1 neurons following ischemia.

In this study, we recorded single unit activity of CA1 pyramidal neurons following global ischemia in Mongolian gerbil to determine the exact time of ischemia-induced activity change. Multi-channel electrode was used for the measurement of multiple single unit activity. Experimental animals were divided into two groups. In Group 1, gerbils were subject to ischemic surgery following implantation of recording electrode. In Group 2, they were subject to ischemic surgery, and then they were implanted with recording electrode. Histological verification of CA1 cell death was done for both groups. Temporal change of functional loss and histological cell death of CA1 were correlated.

MATERIALS AND METHODS

Experimental protocol

All animals were chronically implanted with electrodes. Gerbils in the Group 1 received implantation surgery, and then they received ischemic surgery. After implantation surgery, they were given 7 d of recovery period before experiment. We recorded neuronal activity in animal anesthetized with 10% chloral hydrate, for 30 min before ischemia. Neural activity was recorded at 8 specified time points (30 min before ischemia, 6 h, 12 h, day 1, day 2, day 3, day 4, day 6 following ischemia). Four gerbils served as nonischemic control. Gerbils in Group 2 were subject to ischemic surgery, and then to implantation surgery. We recorded neural activity at 8 specified time points as in Group 1. Five gerbils served as nonischemic controls. After the last recording session, animals were sacrificed and histological analysis on CA1 region was performed.

Experimental animals before surgery

Adult male or female Mongolian gerbil weighing 60～65 g were used in this study. All animals were succeeded in experimental animal center of Hallym University. The environment of breeding room was maintained at condition that temperature was 23± 2°C and relative humidity was 55±10%. Artificial lighting maintained 12 h per d. Animals were housed 5 per cage with food and water available *ad libitum*. The animals were transferred to laboratory one day before electrode implantation or ischemic surgery.

Implantation surgery

Animals were deeply anesthetized with i.p. injection of 10% chloral hydrate (400 mg/kg). They transferred to a stereotaxic apparatus and fixed as prone position. The skull surface was exposed and 6 holes were drilled through the skull. Five screws were turned in as anchors to the skull. The remainder hole was used for insertion of micro wire electrode. The tips of electrode was aimed at hippocampal CA1 region (AP=-1.5 mm from bregma,

ML=1.5 mm from midline, and 1.7 mm below dura). Recording electrode was a 8-channel multi-wire array (tungsten micro-wire, A-M systems, USA, 75 μm diameter, teflon-insulated). Each 8-channel array was consisted of two rows of four micro-wires (2×4 arrangements). Each row was separated about 100 μm, and each interval between adjacent micro-wire was also 100μm. After correct positioning of electrode, all of them were cemented altogether with dental resin to the pre-screwed anchors. After surgery, each rat was transferred to previously sterilized cage.

Forebrain ischemic surgery

Animals were placed under general anesthesia using 2.5% isoflurane in a mixture of 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck. Both common carotid arteries were isolated, freed of nerve fibers, and occluded using non-traumatic aneurysm clips. The complete interruption of blood flow (reperfusion) was observed directly under the microscope. Body temperature was monitored and maintained at 37± 0.5°C during surgery and throughout the immediate postoperative period until the animals recovered fully from anesthesia

Electrophysiological method

Neural recording was carried out from animals anesthetized with 10% chloral hydrate.

A head stage plug was used to connect the implanted electrodes to a preamplifier whose outputs were sent to a Multi-Neuronal Acquisition Processor (MNAP, Plexon Inc., Dallas, TX, USA) for online multi-channel spike sorting and data acquisition. A maximum of four extracellular single units per micro-wire and total maximum of up to 32 units per experiment could be discriminated in real time using time-voltage windows and a principal component-based spike sorting algorithm (Nicolelis et al., 1993). Autocorrelation histograms were also generated to verify the individuality of the single unit firing. Correct positioning of the electrode bundles was verified by histological examination under light microscope after sacrificing the animal. During the experiment, neural ensemble activities were stored in a PC for further electrophysiological analysis.

Histological analysis

All animals were anesthetized with urethane, and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4) after last recording session. Brains were removed, postfixed in the same fixative for 4 h, and brain tissues were cryoprotected by infusing with 30% sucrose overnight. Thereafter the tissues were frozen and sectioned with a cryostat at 10μm and consecutive sections were collected in six-well plates containing PBS. We investigated neuronal degeneration using cresyl violet and Fluoro-Jade staining method.

Cresyl Violet (CV) staining procedure

Cryo-sections were deparaffinized in two changes of xylene for 5 min each and then washed sequentially in a gradient of ethanol to water. The sections then were stained with 0.1% CV in a sodium acetate buffer for 30 min. The stained sections were dehydrated through an ethanol gradient, cleared, and coverslipped with permount.

Fluoro-Jade dye

Fluoro-Jade was obtained from Histo-Chem Inc., P.O.Box 183, Jefferson, AR 72079, USA. Fluoro-Jade is an anionic tribasic fluorescein derivative with a molecular weight of 445 daltons. It has an emission peak at 550 nm and excitation peaks at 362 and 390 nm, respectively. The dry powder was stable when stored in the dark in an air tight container. The 0.01% stock solution was stable for at least 2 months when stored in the refrigerator, in contrast to the working solution which was used the same day as prepared.

Fluor o-Jade staining procedure

Brain sections were mounted with distilled water onto gelatin coated slides and dried on a slide warmer at 45°C. The tissue was fully dried within 20 min at which time it was immersed in 100% ethyl alcohol for 3 min followed by a 1 min change in 70% alcohol and a 1 min change in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 15 min and were gently shaken on a rotating platform. This solution when kept in a sealed glass container remained usable for a period of about 1 week. The

slides were rinsed for 1 min in distilled water and were then transferred to the Fluoro-Jade staining solution where they were gently agitated for 30 min. A 0.01% stock solution of the dye was prepared by dissolving 10 mg Fluoro-Jade in 100 ml of distilled water. The 0.001% working solution of Fluoro-Jade was prepared by adding 10 ml of the stock Fluoro-Jade solution to 90 ml of 0.1% acetic acid in distilled water. After staining, the sections were rinsed with three 1 min changes of distilled water. Excess water was drained off, and the slides were rapidly air dried on a slide warmer or with a hot air gun. When dry, the slides were immersed in xylene and then coversliped with D.P.X. (Aldrich Chem. Co., Milwaukee, WI, USA) mounting media. Sections were examined with an epifluorescence microscope using a filter system suitable for visualizing fluorescein or FITC. The resulting slides are quite stable and require no special storage conditions or anti-quench agents. The potassium permanganate pretreatment further enhances the permanence of the preparation resulting in extremely slow fading, even under high magnification epifluorescent illumination.

RESULTS

Hippocampal CA1 neurons of control group (n=4) against Group 1 were recorded for 6 d (from the 8th day to the 13th day after implantation surgery). In this group, neural activity (in Hz, day 1: 6.75±0.7, day 2: 6.00±0.9, day 3: 5.27±0.8, day 4: 4.94±0.9, day 6: 5.77±1.0) was quite stable throughout the 6 d period (Fig. 1). Recording sites in the CA1 of the right hippocampus and the integrity of pyramidal neurons were verified with cresyl violet and fluoro-jade staining (Fig. 2).

In the Group 1, we compared CA1 spontaneous neural activities during pre- and post- (6 h, 12 h, day 1, day 2, day 3, day 4, day 6) ischemic periods (Fig. 3). There was a complete suppression of CA1 activity immediately after recirculation. A stable neu-

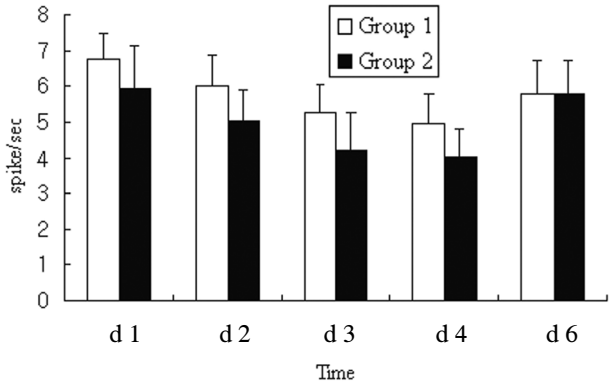

Fig. 1. Spontaneous hippocampal CA1 neuron activity at various time points in control group. Data obtained from 46 neurons in the CA1 region of the hippocampus in 4 gerbils. In Group 2 data were collected from 51 neurons of 5 gerbils. During 6 days, neuronal activity was stably maintained; statistical difference was not observed in reference to day 1.

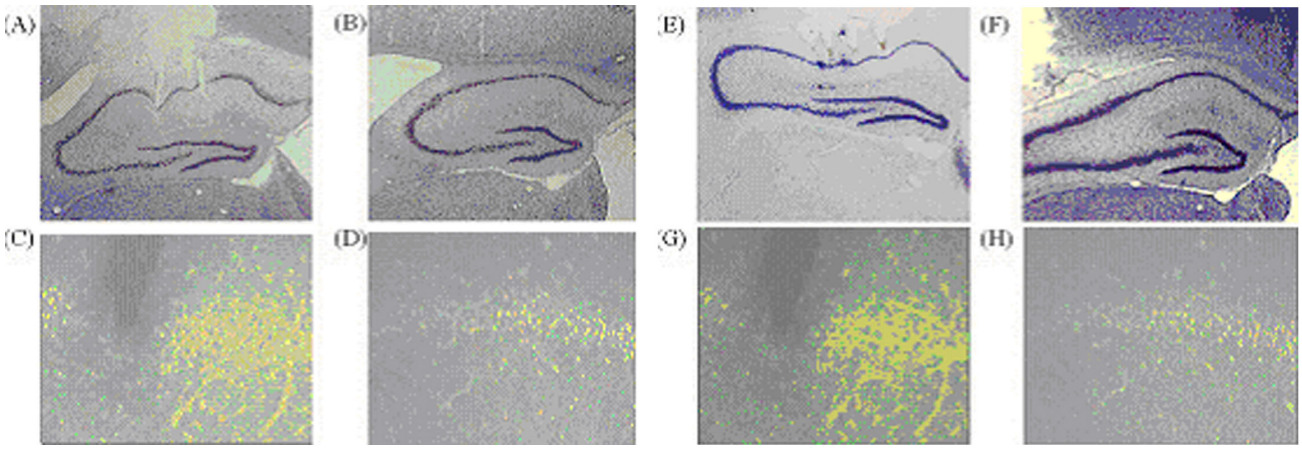

Fig. 2. Verification of recording sites and CA1 neurons in control group. (A)～(D) were the control group against Group 1, and (E)～(H) were against Group 2. The electrode was implanted in the right hemisphere (A), (E). Through cresyl violet staining, we confirmed that neuronal loss did not occur in both right (A), (E) and left (B), (F) hemispheres, and through fluoro-jade staining it was also reconfirmed (C, G: right, D, H: left hemispheres).

ral activity (6.73±0.06 Hz) before ischemia almost ceased (0.04 Hz) quickly right after initiation of ischemia and then even after recirculation (1.96 Hz). Spike discharge reappeared about 5 min after recirculation. However neural activity was till suppressed (-61%) for 25 min of post-ischemia period as compared with that of pre-ischemia state. Fifty seven % (Type 1) CA1 neurons of Group 1 showed gradual recovery by post-ischemia 6h. The Student's *t* test revealed no statistically difference (p=0.53) between pre-ischemia and 6 h after ischemia. The major activity change in the Type 1 neurons of hippocampal CA1 occurred between 12 h and 1 d following ischemia. In this period, neuronal activity was maximally enhanced by 177% in comparison with pre-ischemic state. However, at post ischemic day 2 it was similar to that of pre-ischemic condition, and then from 3 d following ischemia it was gradually decreased. Finally, at post-ischemia day 6 it was diminished to -76% in comparison with pre-ischemia state (Fig. 3). In Group 1, however, the rest of neurons (43%, n=53, Type 2) showed incomplete recovery of activity by 6 h after ischemia. The CA1 activity was gradually suppressed till post-ischemia day 6 without showing phasic hyperactivity (in Hz, pre: 6.14±0.7, 6 h 1: 3.04±0.8, 12 h: 1.26±0.4, d 1: 0.91±0.2, d 2: 0.37±0.1, d 3: 0.42±0.1, d 4: 0.23±0.1, d 6: 0.18 ±0.1). Histological analysis indicated no evidence of neuronal death in cresyl violet stained sections. However, fluoro-jade staining revealed a partial loss of CA1 neurons around middle region of hippocampal CA1 at post-ischemic day 7 (Fig. 4).

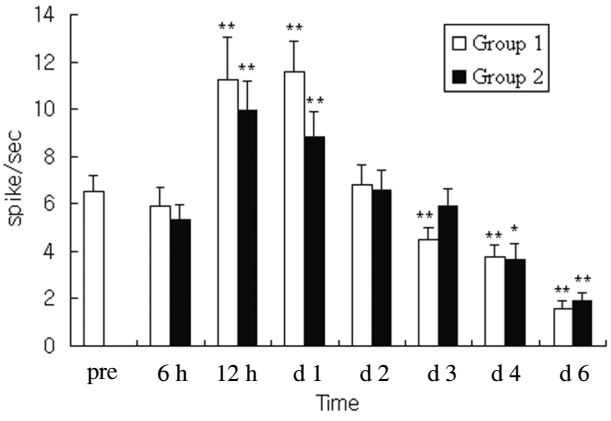

Fig. 3. Temporal changes of Type 1 CA1 neural activity following ischemia in Group 1 and Group 2. In Group 1 data were obtained from 70 neurons following ischemia in 12 gerbils (*p＜0.05, **p＜0.01 in reference to pre-ischemia), and in Group 2 data were collected from 105 neurons following ischemia in 10 gerbils (*p＜0.05, **p＜0.01, in reference to 6 h).

Animals (n=5) of control group against Group 2 were not given recovery period after the implantation surgery, and they were subject to the experimentation from the first day after surgery. Recording of CA1 neuronal activity (n=51) in the control group was done for 6 d. Spontaneous spike fre-

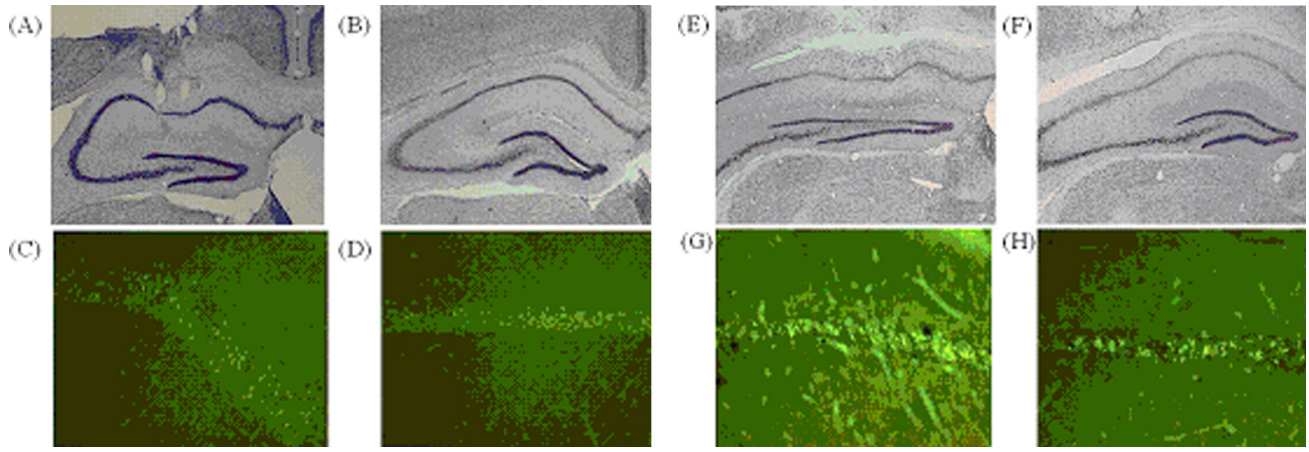

Fig. 4. Micrographs of the hippocampal CA1 area of the Group 1 and Group 2. (A)～(D) for Group 1, and (E)～(H) for Group 2. (A) (C) (E) (G) from right hemispheres with implanted electrode. (B) (D) (F) (H) from left hemispheres. In Group 1 (A) and (B) stained with cresyl violet indicated no apparent CA1 cell death, but (C) and (D) stained with fluoro-jade showed cell death around middle region of hippocampus at pot-ischemic day. In Group 2, the neuronal loss was seen in cresyl violet stained sections of both right (E) and left (F) hemispheres. This result was reconfirmed through fluoro-jade staining (G; right, H; left hemispheres.)

quency of the recorded neurons for 6 days was from 4.02±0.8 Hz to 5.96±1.2 Hz. The variation among each time point was small and statistical difference wasn't observed (Fig. 1). The histology of the hippocampal CA1 in the control animals was shown in Fig. 2. Recording sites and intact neurons of hippocampal CA1 were verified. These results were similar to the control group against Group 1.

In Group 2 temporal alteration of neuronal activity following ischemia had also two patterns as in Group 1. The Type 1 neurons showed post-ischemic hyperactivity period. In other words, the neural activity which was 5.34±0.61 Hz at 6 h after ischemia was enhanced to 9.95±1.25 Hz at 12 h after ischemia and this increase persisted by 1 day (8.48±1.07 Hz) after ischemia. After that, it became similar to that at the post-ischemia 6 h during day 2 to day 3 post-ischemia. From the day 4 post-ischemia, it was gradually diminished and reached to 1.84±0.43 Hz at day 6 post-ischemia (Fig. 3). Type 1 CA1 neurons were 70% (n=105) of the all recorded neurons in Group 2. Type 2 neurons (30%) in Group 2 did not show post- ischemic hyperactivity. Activity at 6hr following ischemia was very low (2.36±0.4 Hz) and it was further strongly decreased to 0.23±0.2 Hz by 12 h after ischemia. The decreased neuronal activity was maintained till 6 d after ischemia.

The recording sites and the ischemia-induced neuronal death were shown in Fig. 4. As can be seen in the cresyl violet stained sections, the electrode was located in hippocampal CA1 and the ischemia-induced neuronal death was verified in the CA1 area. This neuronal death was reconfirmed by fluoro-jade staining.

Taken together above results, the temporal change of CA1 neuronal activity following ischemia in Group 2 was similar to that of Group 1. However, results of histological analysis at 7 d after ischemia were quite different between Group 1 and Group 2. That is to say, neuronal loss following ischemia in Group 1 was seen just in small region, but in Group 2 it was observed in broad area of the hippocampal CA1.

DISCUSSION

The main findings of the present study are that a) the spontaneous activity of hippocampal CA1 neurons was completely suppressed immediately after ischemia and about 5 min after reperfusion it began to recover, b) according to the degree of neurophysiological recovery within 6 h after the ischemic insult, temporal alterations of ischemia-induced change of neuronal activity were divided into two types: Type 1 neurons had the pattern showing significant phasic enhancement of neuronal activity at 12 h and 1 day following ischemia, while Type 2 neurons had the pattern exhibiting only suppression of neural activity till post-ischemic day 6, c) ischemia study in chronically implanted gerbils showed difference of hippocampal CA1 histology between two groups (Group 1: ischemia after implantation, Group 2: implantation after ischemia).

In this study, the spontaneous activity of CA1 neurons was recorded for 25 min after ischemia, and it was completely suppressed. About 5 min after reperfusion neuronal activity begun to show and then it recovered gradually till 6 h after the ischemic insult. The temporal change of the spontaneous neuronal discharges after ischemia may be related to the temporal alteration of glutamate level. Akira and his colleagues showed that extracellular glutamate level was increased gradually after initiation of ischemia and it was increased to 18.7-fold at the end of ischemia. After recirculation, the increased glutamate level was recovered to almost pre-ischemic level by 5 min of recirculation. The accumulated glutamate in extracellular space has been proposed to produce an influx of sodium and chloride and the entry of these ions draws water and finally leads to extreme swelling (Rothman, 1985; Rothman et al., 1986).

The CA1 pyramidal cells regain their electrophysiological function during the post-ischemic recirculation preceding cell degeneration (Suzuki et al., 1983; Andine et al., 1988; Buzsaki et al., 1989; Chang et al., 1989). Using single unit recordings, Suzuki et al., (1983) reported a 3-fold hyperactivity from 7 to 24 h after 5 min of ischemia in the gerbil hippocampus. They did not find any activity during 2～3 days after ischemia. Chang et al., (1989), also using single-unit recording, reported a recovery to pre-ischemic activity 24 h after a 10-min ischemic insult in the rat, followed by a phase of hyperactivity on days 2 and 3. The authors concluded that

the cells probably did not die until 4～6 d after ischemia. In the present study ischemia- induced neuronal loss was confirmed. However, the neurons were divided into two types in terms of the presence or absence of hyperactivity period after ischemia. The Type 1 neurons with hyperactivity period were maximally active at 12 h and 1 d following ischemia, and from day 2 begun to diminish activity. These results are in agreement with above reports. However, there is another neuron type (Type 2, without showing hyperactivity period). At 6 h after ischemia Type 2 neurons didn't show recovery of neural activity to pre-ischemic level and at 12 h after ischemia neuronal activity was fairly low. Time-course study on morphological change of hippocampus following ischemia (Katsutoshi et al., 1990) showed that degeneration of CA1 neurons was not apparent till 6 h after ischemia, but it was seen at 24 h after ischemia. Most pyramidal cells of the CA1 region showed severe damage at 3 d after ischemia. When considered together with morphological change following ischemia, the data described in our experiment suggest that the delayed neuronal death of the Type 1 CA1 cells in the gerbil may be preceded by hyperactivity. However functional loss in Type 2 CA1 neurons was begun without hyperactivity period after reperfusion.

Many investigators have used chronically implanted animals (Peter et al., 1991; Katsutoshi et al., 1990; Buzsaki et al., 1989) and reported ischemia-induced changes of neural activity. However, these studies did not illustrate micrographs of hippocampal CA1 showing cell death and recording site, and merely described it in words. In our study, the electrophysiological results of two groups were very similar. However, histological graphs observed 7 d after ischemia showed difference between two groups. In Group 2 pyramidal cells in hippocampal CA1 were not stained with cresyl violet, but in Group 1 we could observe stained pyramidal cells. Causes of this discrepancy could be various. Firstly, to fix implanted electrode on the skull we used dental resin polymerized with eugenol, which has a neuroprotective effect against ischemia-induced neurotoxicity (Won et al., 1998). Secondly, astrocytes and microglia activated after implantation of micro-wire recording electrode (Schiffer et al., 1992) could protect ischemia response. However, since these two studies were done only with histological methods, the integrity or the deterioration of CA1 neuronal activity was not verified. The results of our study suggest that functional loss of CA1 activity may not be in parallel with morphological death following ischemia in chronically implanted *Mongolian gerbil*.

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