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# Post-ischemic Time-dependent Activity Changes of Hippocampal CA1 cells of the *Mongolian Gerbils*

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**Changes of single unit activity of CA1 hippocampus region were investigated in anesthetized *Mongolian gerbils* for six days following transient ischemia. Ischemia was produced immediately before the implantation of micro-wire recording electrodes. In control animals receiving pseudo-ischemic surgery, neither spontaneous neuronal activities (5.70±0.4 Hz) nor the number of recorded neurons per animal changed significantly for six days. Correlative firings among simultaneously recorded neurons were weak (correlation coefficient > 0.6) in the control animals. Animals subjected to ischemia exhibited a significant elevation of neural firing at post-ischemic 12 hr (9.95±0.9 Hz) and day 1 (8.48± 0.8 Hz), but a significant depression of activity at post-ischemic day 6 (1.84±0.3 Hz) when compared to the activities of non-ischemic control animal. Ischemia significantly (correlation coefficient < 0.6) increased correlative firings among simultaneously recorded neurons, which were prominent especially during post-ischemic days 1, 2 and 6. Although the numbers of spontaneously active neurons recorded from control group varied within normal range during the experimental period, those from ischemic group changed in post-ischemic time-dependent manner. Temporal changes of the number of cells recorded per animal between control group and ischemic group were also significantly different (p = 0.0084, t = 3.271, df = 10). Cresyl violet staining indicated significant loss of CA1 cells at post-ischemic day 7. Overall, we showed post-ischemic time-dependent, differential changes of three characteristics, including spontaneous activity, network relationship and excitability of CA1 cells, suggesting sustained neural functions. Thus, histological observation of CA1 cell death till post-ischemic day 7 may not represent actual neuronal death.**

**Key Words: Transient ischemia, Hippocampal CA1 cells, Temporal activity change, Correlative firing**

## INTRODUCTION

Animal models of global ischemia are produced by occluding the major arteries supplying the cerebral hemisphere (i.e., the carotids and vertebrals) for 5 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 certain neurons in subregions of the neocortex, thalamus and striatum to a lesser extent (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 still is not completely known. An interesting characteristic of global ischemia is that significant neurodegeneration is not seen until several days after ischemia (Pulsinelli et al. 1991). Temporal pattern of events raises related questions which continue to evoke interest among investigators: the time of death and the degenerated pattern. One way to address these questions would be to compare the time of functional decline of CA1 pyramidal neurons to the time of histological verification of neuronal death of the same neurons. Insight into the temporal relationship between these different events may eventually help to refine hypothesis regarding the delayed cell death phenomenon. Studies on the recovery of neuronal activity following the transient ischemia vary 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). On the other hand, some authors did not find any loss of responsiveness untill day 4 (Buzsaki et al., 1989). Thus, these studies make it almost impossible to generalize 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 anesthetized *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. Animals were subjected to ischemic surgery, and they then were implanted with recording electrode. CA1 cell death was histologically verified. Temporal change of spontaneous activity, correlative firing among simultaneously recorded units, number of spontaneously active neuron, and histological cell death of CA1 were analyzed.

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## METHODS

### *Experimental protocol*

Six gerbils were subjected to ischemic surgery, and then subsequently to a surgery for implantation of micro-wire electrodes. We recorded spontaneous neuronal activity of hippocampal CA1 in gerbils anesthetized with 10% chloral hydrate. Neural activities were recorded at seven specified time points (6 hr, 12 hr, day 1, day 2, day 3, day 4, and day 6) following ischemia. Four gerbils served as nonischemic control and were subjected to ischemia-like surgery without delivery of actual ischemia. Neural activities were also recorded at five specified time points (day 1, day 2, day 3, day 4, day 6) from the control animals. After the last recording session, animals were sacrificed and histological analysis of 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 in condition where temperature was 23±2°C and relative humidity was 55±10%. Artificial lighting was kept for 12 hrs per day. Animals were housed 5 per cage with food and water *ad libitum*. 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), and they were then 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 $\mu$m diameter, teflon-insulated). Each 8-channel array consisted of two rows of four micro-wires (2 × 4 arrangements). Each row was separated about 100 $\mu$m, and each interval between adjacent micro-wires was also 100 $\mu$m. After correct positioning of electrode, all of them were together cemented with dental resin to the pre-screwed anchors. After surgery, each animal 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 was confirmed by observing the central artery in the retinae using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. The restoration of blood flow (reperfusion) was observed directly using the ophthalmoscope. 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 and analysis of neural activity*

Neural recording was carried out with 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. Correlative firings were examined by constructing spike-triggered cross-correlation histograms (Johnson and Alloway, 1994; Jung and Shin, 2002), representing changes in the number of one neuron's discharges relative to other neuron's firing occurring at time zero. Time-locked discharges of a pair of neurons, called coincident events, appeared as peaks or valleys in the histogram, indicating that presumable synaptic interactions were represented as correlation coefficients (± 1). Statistical analysis was done with the unpaired Student's t-test. All results are presented as means ± SEM.

### *Histological analysis*

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

### *Cresyl Violet 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 were then stained with 0.1% cresyl violet in a sodium acetate buffer for 30 min. The stained sections were dehydrated through an ethanol gradient, cleared, and cover-slipped with permount.

## RESULTS

Control animals (n = 4) were subjected to experimentation from the first day after implantation surgery without recovery period. Recording of CA1 neuronal activity in the control group was done for 6 days. Spontaneous spike

frequency of the recorded neurons for 6 days was from 4.94 ±0.9 Hz to 6.76±0.7 Hz (mean value 5.70±0.4 Hz, Fig. 1). There was no significant alteration of neural firing rates during 6 days, indicating that pseudo-ischemic surgery and implantation surgery for micro-wire electrodes did not influence the CA1 neural activity. As shown in Fig. 2, simultaneously recorded CA1 units did not generally show any strong correlative firing patterns (correlation coefficient $< \pm 0.6$) during the 6 experimental days.

Fig. 3 illustrates changes of CA1 neuronal activities (n = 10) from 6 hr to 6 days after ischemic surgery from an animal. Ten neurons showed about 78% below the activity rate at 6 hr after ischemic surgery (an average of 1.23±0.4 Hz) compared to that of the control animals (5.70 ±0.4 Hz). Seven of the ten cells exhibited very low firing rates (in Hz, mean ± SEM; 6hr: 1.49±0.5, day1: 1.80±0.9, day 2: 0.19±0.1, day 3: 0.55±0.3, day 4: 0.17±0.1, day 6: 0.13± 0.1, $p < 0.05$ for 6hr vs. day 6), except 3 units which showed dramatic increases (6hr vs. day 6: 2271%, day 1: 0.63±0.5, day 6: 14.94±5.9 Hz) of activity from day 3 to day 6.

Correlation analysis on the 10 simultaneously recorded units indicated that most of the cells had quite low correlation coefficients (less than 0.6) between them (Fig. 4). However, some units exhibited fairly strong correlations (above 0.6) during certain days following ischemia, such as during days 1, 3 and 6. Three cells (Fig. 3) showing dramatic increase of firing (units 1, 2 and 3 in Fig. 4) did not show any ischemia-induced changes of correlative firings against other 7 units. At post-ischemic day 7, CA1 cells were very weakly stained with cresyl violet, indicating that most of the neurons were dead.

Ischemia induced significant elevation of neural firing at post-ischemic 12 hr (9.95±0.9 Hz) and day 1 (8.48±0.8 Hz), but significant depression of activity at post-ischemic day 6 (1.84±0.3 Hz) as compared to the activities of non-ischemic control animal (5.7±0.4 Hz). Within the experimental group, firings at post-ischemic 12 hr were significantly greater than those of other post-ischemic periods (6 hr: $p<0.001$, day 2: $p<0.01$, days 3, 4 and 6: $p<0.001$), except day 1.

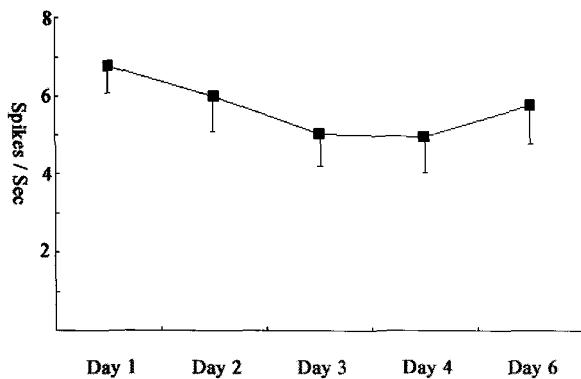


**Fig. 1.** Averaged spontaneous activities of CA1 hippocampal neurons at various time points from non-ischemic control animals. These data were collected from 4 gerbils, except day 6 (n = 2). For 6 days, activity was stable, and recorded neuron numbers were (day 1, 43; day 2, 44; day 3, 45; day 4, 40, and day 6, 20).

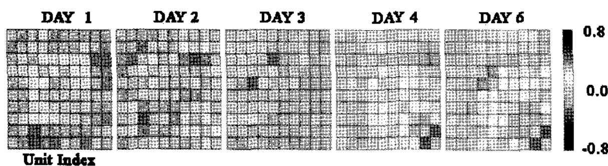


**Fig. 2.** Weak correlative firings of 10 simultaneously recorded CA1 units in a non-ischemic control animal. Simultaneously recorded units (n = 11) did not show strong correlative firings (correlation coefficient less than ± 0.6) among them during 6 days. X and Y axes stand for unit index (unit 1 to 10 from right to left for X axis, unit 1 to 10 from bottom to top of the Y axis). Vertical color bar represents scales of correlation coefficients from -0.8 to 0.8.

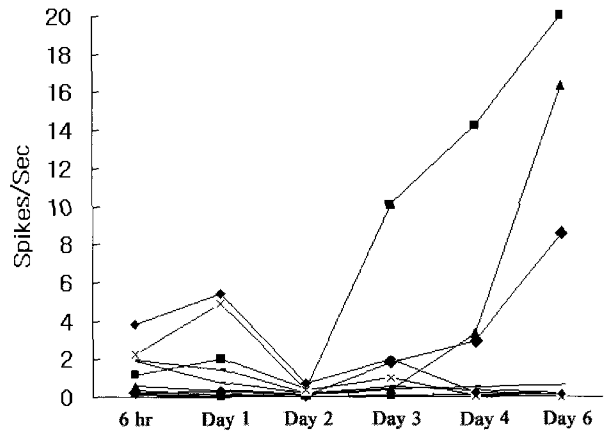


**Fig. 3.** Temporal changes of CA1 neural activities from an animal following ischemia. Data were collected from 10 simultaneously recorded neurons in a gerbil.

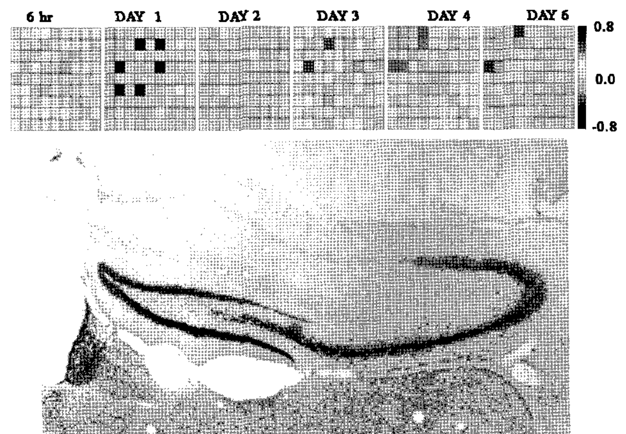


**Fig. 4.** Changes of correlative firings among simultaneously recorded units following ischemia and a histological section showing cell loss in CA1 region of the same animal. Upper panel: Data were collected from 10 neurons following ischemia in a gerbil, which showed neural activity changes shown as in Fig. 3. X and Y axes stand for unit index (unit 1 to 10 from right to left for X axis, and unit 1 to 10 from bottom to top of the Y axis). Vertical color bar represents scales of correlation coefficients from -0.8 to 0.8. Lowe panel: histological verification of the CA1 cell death using cresyl violet staining at post-ischemic day 7.

Firings at post-ischemic day 1 were also significantly higher than those of other periods, except those of 12 hr and day 2. Activities at post-ischemic day 6 were significantly suppressed compared with those of other periods, except those of day 4. The numbers of cells recorded from control group varied within normal range between day 1 and day 6, however, those of cells recorded from ischemic group were changed time-dependently. Temporal changes of the number of cells recorded per animal between control and ischemic groups were also significantly different ($p = 0.0084$, $t = 3.271$, $df = 10$).

Fig. 5 shows post-ischemic changes of correlative firing properties among simultaneously recorded CA1 units from 5 gerbils (A-E). In animal A, at post-ischemic day 1, 2 and day 6, strong increases of correlative firings (correlation coefficients more than±0.6) were detected among some cells. Increases of correlative firing properties were observed at post-ischemic days 2 and 6 in animals B and at post-ischemic days 2 and 3 in animal C. In animals D and E, augmentations of correlative firings showed early onset and were more frequently observed throughout the whole experimental period.

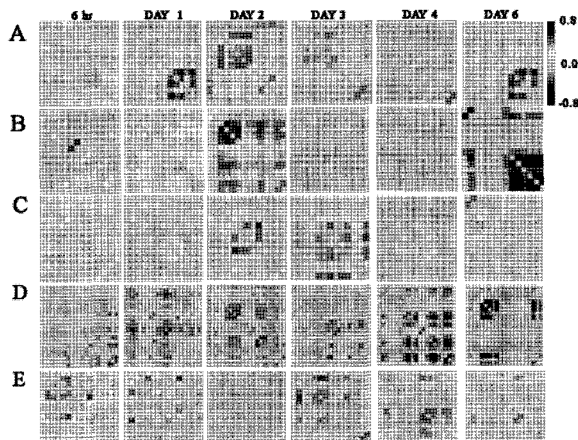


**Fig. 5.** Temporal changes of correlation coefficients of simultaneously recorded CA1 units after ischemia (from 6 hr to day 6) from 5 animals (A-E). X and Y axes stand for unit index as shown in Fig. 4. Vertical color bar represents scales of correlation coefficients from -0.8 to 0.8.

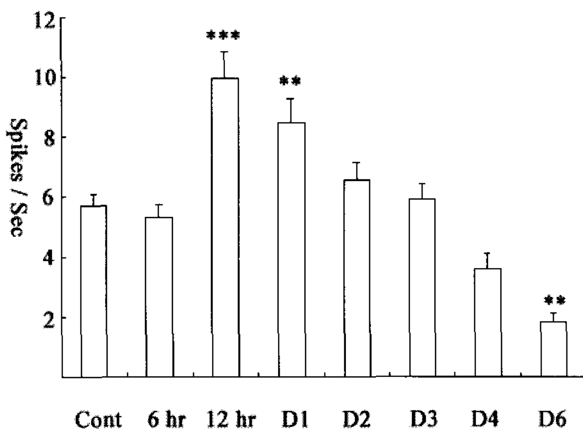


**Fig. 6.** Overall averaged temporal changes of CA1 neural activity following ischemia. Data were collected from 6 gerbils (** $p < 0.01$, *** $p < 0.001$, in reference to non-ischemic control animals (Cont)). Recorded neuron numbers (Cont: 192, 6 hr: 132, 12 hr: 76, day 1: 146, day 2: 142, day 3: 142, day 4: 134, day 6: 50). D stands for the post-ischemic day.

## DISCUSSION

The main findings of the present study are a) ischemia induced strong increase of spontaneous activity of hippocampal CA1 neurons at post-ischemic 12 hr and day 1, but significant suppression at post-ischemic day 6, b) ischemia significantly increased correlative firings among simultaneously recorded neurons, which were prominent especially during post-ischemic days 1, 2 and 6, c) the numbers of spontaneously active neurons changed in post-ischemic time-dependent manner, d) ischemia resulted in loss of the CA1 cells at post-ischemic day 6, and e) ischemia induced differential temporal changes of spontaneous activity, network relationship and excitability of CA1 cells.

Although, in the present study, ischemia-induced neuronal loss was confirmed with histological analysis at post-ischemic day 7, CA1 neurons were still showing suppressed spontaneous activities (-68.25% compared to control group, Fig. 6). Furthermore, number of active neurons simultaneously recorded through implanted micro-wire bundle electrode was 8.33 per ischemic animal, and this was not quite lower than that in control group (10.6), suggesting that many neurons assumed to be dead in histological analysis might still be functioning even if in a lowered activity mode. This contradicts to a previous single unit recordings in gerbil, in which Suzuki et al. (1983) reported no activity during 2~3 days after ischemia. In the present study, significant correlative firings among some of the simultaneously recorded neurons in 4 of 6 gerbils even at post-ischemic day 6 (Fig. 5) suggest that they were able to communicate with neighboring cells through presumably functional synapses although neuron's spontaneous firing was quite lowered. However, we do not presently know the actual function of the heightened correlative firings at post-ischemic day 6. It could possibly be related to either a compensatory function for cell survival or a pathological process near to cell death.

In the current study, ischemia resulted in dramatic increase of spontaneous activity at 12 hr (+ 74.56% above the control group) and day 1 (+ 48.77% above the control) following ischemia. This partly agrees with a previous study, showing 3-fold hyperactivity from 7 to 24 hr after 5 min of ischemia (Suzuki et al., 1983), since we observed only a recovery of spontaneous activity from initial quiescence to a control level at 6 hr post-ischemia. In rat, Chang et al. (1989) reported a recovery to pre-ischemic activity 24hr after a 10-min ischemic insult, followed by a phase of hyperactivity on days 2 and 3. In our study, cell numbers showing spontaneous activity per animal were 22 at 6 hr, 12.67 at 12 hr, 24.33 at day 1, 23.67 at day 2, 23.67 at day 3 and 22.33 at day 4. Most of these numbers were more than twice the cell numbers of the control group (10.6), except at post-ischemic 12 hr. This suggests that CA1 cells were already in hyper-excitable state at post-ischemic 6 hr and many quiescent cells before ischemia might generate spontaneous activities at normal firing rate. Reduction of the number of spontaneously active cells at post-ischemic

12 hr is quite interesting, although they showed the strongest firing rates compared to those of other post-ischemic periods. This could be related to the occurrence of actual cell death around this time period. Temporal analysis of the morphological change of hippocampus following ischemia showed that degeneration of CA1 neurons was not apparent untill 6 hr after ischemia, and seen at 24 hr after ischemia (Katsutoshi et al., 1990). Most pyramidal cells of the CA1 region appeared to be severely damaged at 3 days after ischemia. Thus, the current result of gradual decrease of spontaneous firing rates from post-ischemic 12 hr to day 6 appears to be more in parallel with the time-course of the morphological changes. However, stable numbers of active cells at each post-ischemic period were not in accordance with previous histological studies, although changes of active cell number of ischemic animal were significantly different those of control group.

Several 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. Our study is different from previous studies; whereas they recorded a unit in a time, we used micro-wire bundle electrode, and therefore, we were able to simultaneously record many single from each animal. Our method is advantageous in terms of ability to count active units at a specified post-ischemic time and to carry out correlation analysis among simultaneously recorded neurons (Jung and Shin, 2002). In the current study, ischemia resulted in significant elevation of correlative firings among some of the cells adjacent to the recording electrode in the CA1. This suggests that some of the CA1 cells fire more frequently at a given time and they may share a common driving source, which is different from the heterogeneous network characteristic of non-ischemic control animal. Although the occurrence of significantly heightened correlative firings (correlation coefficient $> 0.6$) was dispersed throughout post-ischemic time period (except post-ischemic 6 hr, Fig. 5), the strong correlative firings (correlation coefficient $>$ 0.4) among many adjacent neurons were found from post-ischemic days 1 to 3. These periods coincides with the gradual declining of the spontaneous firing rates of CA1 cells (Fig. 6) and the cell loss previously reported in histological studies. Very strong correlative firings among small portion of simultaneously recorded neurons observed at post-ischemic day 6 often appeared to belong to different cells in comparison to those observed during post-ischemic days 1 to 3, suggesting that correlative firings at around post-ischemic day 2 might be differentially involved in the pathological processes following ischemia.

Overall, the results of this study, using simultaneous many single unit recording method, showed that post-ischemic temporal changes of spontaneous activity, correlative firing relationship, and excitability of cells were differentially expressed in anesthetized *Mongolian gerbils*. All of the three parameters which reflect the functionality of CA1 cells suggest absence of total loss of CA1 cells, as reported frequently in histological studies following ischemia.

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