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The use of spreading depression waves for acute and long-term monitoring of the penumbra zone of focal ischemic damage in rats

(anoxic depolarization/middle cerebral artery occlusion/slow potentials)

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ABSTRACT **Slow potential recording was used for long-term monitoring of the penumbra zone surrounding an ischemic region produced by middle cerebral artery (MCA) occlusion in adult hooded rats (n = 32). Four capillary electrodes (E1–E4) were chronically implanted at 2-mm intervals from AP −3, L 2 (E1) to AP 0, L 5 (E4). Spontaneous or evoked slow potential waves of spreading depression (SD) were recorded during and 4 h after a 1-h MCA occlusion and at 2- to 3-day intervals afterward for 3 weeks. Duration of the initial focal ischemic depolarization was maximal at E4 and decreased with distance from the focus. SD waves in the penumbra zone were high at E1 and E2, low and prolonged at E3, and almost absent at E4. Amplitude of elicited SD waves was further reduced 3 days later and slowly increased in the following week. Cortical areas displaying marked reduction of SD waves in the first days after MCA occlusion either remained low or showed substantial (60%) recovery, the probability of which decreased with the duration of the initial focal ischemic depolarization and increased with the distance from the focus. It is concluded that the outcome of ischemia monitored by long-term SD recovery in the perifocal region can be partly predicted from the acute signs of MCA occlusion.**

Research into Leao spreading depression (SD) (1), a self-propagating neurohumoral reaction mediated by release of potassium ions and excitotoxic amino acids from depolarized areas of cerebral cortex, has always been tightly related to studies of electrophysiological manifestations of brain anoxia or ischemia (2, 3). The anoxic depolarization (AD) (4) is elicited by slow accumulation of K^+ escaping from neurons after failure of active transport. When the extracellular K^+ concentration attains 10–12 mM, opening of voltage-dependent channels and liberation of transmitters from depolarized synaptic terminals (5) suddenly increases potassium release (6) and the extracellular K^+ concentration raises within a few seconds to 70 mM or more. The depolarization is not followed by repolarization, which is prevented by an inadequate supply of energy.

Focal ischemia produced by occlusion of the middle cerebral artery (MCA) elicits depolarization of the ischemic cortex. The high extracellular K^+ concentration in the focus initiates diffusion of K^+ into the adjacent normally perfused cortex and triggers SD waves propagating from the rim of the focus to the surrounding intact cortex during the early stages of focal ischemia (7–13). Their role in the development of ischemic damage is not fully understood but the prevailing opinion is that they facilitate the spread of the ischemic damage.

Anoxia can also affect ongoing SD waves, mainly by interfering with the metabolic repolarization phase of the SD wave. A typical manifestation is prolonged duration of the slow potential wave from 30 s to several minutes (14). Changing shape of SD waves propagating over the cerebral cortex reveals areas with reduced perfusion. For chronic focal ischemia, which has caused severe reduction of neuronal density in particular brain regions, SD may stop when the density of active elements drops below the level required for interactive propagation (15). Both situations can be implemented after occlusion of a cerebral artery that has caused infarction of a central zone, reduced cellular density in the peri-infarct zone, and hypoperfusion of a wide peripheral zone of otherwise intact cortex. An SD wave triggered in intact cortex and propagating toward the ischemic focus gradually becomes more and more prolonged as it enters the hypoperfused region. Its amplitude may drop in the partially damaged cortex and the SD wave will disappear completely in the infarcted tissue.

The purpose of the present study was to use the above phenomena for examination of focal ischemia in rats during the acute phase immediately after MCA occlusion and during the delayed degenerative and reparative processes taking place in the following days and weeks. It was hoped that SD may prove a useful tool for assessment of the efficacy of allegedly neuroprotective drugs recommended for clinical treatment of stroke.

METHODS

Animals. Three-month-old male Long Evans rats (n = 32), obtained from the breeding colony of the Institute of Physiology, were used. They were housed in individual cages with free access to food and water in an animal room with constant temperature and a natural light cycle. All experiments were in accordance with the National Institutes of Health Principles of laboratory animal care.

Surgery. Under deep pentobarbital anesthesia (50 mg/kg), the skull was exposed over the left hemisphere up to the orbit rostrally and to the mastoid bulge ventrally. Four 0.6-mm trephine openings were made at the following stereotaxic coordinates according to the atlas by Fifkova and Marsala (16): AP −3, L 2; AP −2, L 3; AP −1, L 4; AP 0, L 5. Short (10 mm) capillary electrodes with a tip diameter of 10–30 μm and a broad-end diameter of 1.5 mm filled with physiological saline were inserted 0.7–1 mm below cortical surface and fixed to the skull with anchoring screws and acrylate. An additional trephine opening over the occipital cortex served for eliciting SD by application of mechanical or chemical stimuli (Fig. 1).

Slow Potential Recording. The anesthetized animal was fixed in the stereotaxic instrument and the saline in the capillaries was connected by saline-soaked cotton threads with calomel half-cells in the input of a four-channel symmetric DC amplifier with a gain of 1000, a 90-dB suppression of in-phase signals, a 1-G input impedance, and a 1-pA bias current. A

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Abbreviations: AD, anoxic depolarization; E, electrode; FID, focal ischemic depolarization; MCA, middle cerebral artery; SD, spreading depression.

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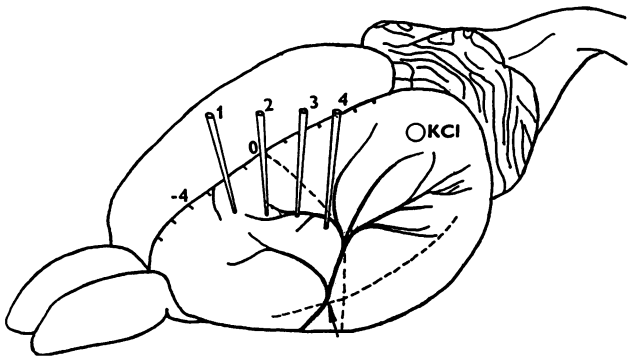

FIG. 1. Diagram of a brain showing the site of MCA occlusion (arrow), the location of the four intracortical capillary electrodes (labeled 1 to 4), and the site of the KCl injection (open circle). A 0 marks the bregma, the dashed lines mark the coronal plane and the rhinal sulcus. Note the millimeter scale along the sagittal sulcus.

saline-soaked cotton wick applied on the exposed muscle or skin and connected to another calomel half-cell served as reference. The outputs of the amplifiers were fed into a multiplexer and a 12-bit ADC on the I/O board of a standard PC that sampled each channel at 100-ms intervals. The recording was displayed on-line on the PC monitor and stored on a floppy disk for further off-line analysis. Throughout the experiment the rat's colonic temperature was maintained at 36°C by means of a servocontrolled heater.

MCA Occlusion. After implantation of the capillary electrodes, the head of the animal was tilted 45° around the rostrocaudal axis and fixed in this position by the nose clamp and adapted ear bars. A circular trephine opening 3 mm in diameter was made in the temporal bone immediately behind the orbit. The center of the opening was about 1 mm rostrally from bregma. MCA was cautiously exposed under dissecting microscope. A miniature forceps fixed in the electrode holder of the stereotaxic instrument was placed above the MCA at the rhinal sulcus and inserted into the surface layer of cerebral cortex. The fine jaws of the forceps were then slowly closed with a screw until the MCA was fully occluded. After an interval of 60 min, the clamp was released, the forceps was removed, and recording was continued for several more hours. After the conclusion of the first recording session, the scalp was sutured around the acrylate implant, the capillaries were closed with a rubber cap, and the rat was placed into a cage by itself. The animals survived well and showed only transient signs of focal lesion (impairment of contralateral forelimb placing and ipsilateral or contralateral turning) on subsequent days. On the recording days, the animal was anesthetized with pentobarbital, fixed in the head holder of the stereotaxic apparatus, and connected with saline-soaked threads to calomel cell electrodes. The session began with 10-min baseline recording followed by injection of 1 μl of 5% KCl into the occipital cortex. Two or three SD waves were elicited at 10- to 15-min intervals and propagation of the slow potential to electrodes (E) 4 to E1 was monitored. The animal was then returned to its home cage and observed until full recovery. After completion of the experiments (usually 2–3 weeks after MCA occlusion), the animal was deeply anesthetized with pentobarbital and intracardially perfused with saline followed by 10% (vol/vol) formalin. The brain was cautiously removed from the skull and stored in formalin. The 30-μm sections cut with a freezing microtome were stained with cresyl violet and evaluated for signs of ischemic damage in the areas affected by MCA occlusion.

RESULTS

Fig. 2 illustrates MCA occlusion eliciting severe focal ischemia followed by a good recovery. After the control SD wave produced by KCl injection from occipital cortex, piercing the cortical surface around the MCA with microforceps elicited another SD wave, which after some delay reached all recording electrodes. Occlusion of MCA induced after a 2-min delay focal ischemic depolarization (FID, a negative slow potential shift lasting more than 5 min), which started at E4 and propagated gradually to E1. Its duration was longest at E4, where repolarization started only after the clamp had been released. At E3 and E2, reperfusion through opened collaterals terminated ischemic depolarization after 40 and 20 min, respectively, whereas only a protracted SD wave was seen in the first minute after occlusion at E1. A series of spontaneous SD waves appeared at about 8-min intervals at E1 and propagated with reduced amplitude to E2 and E3. After their generation ceased, two SD waves were elicited by KCl injection. They reached a normal amplitude at E1 and E2, were almost normal at E3, but were considerably reduced (to 30%) at E4. Further development of the ischemic focus was reflected by changed properties of the evoked SD waves. On day 3, they

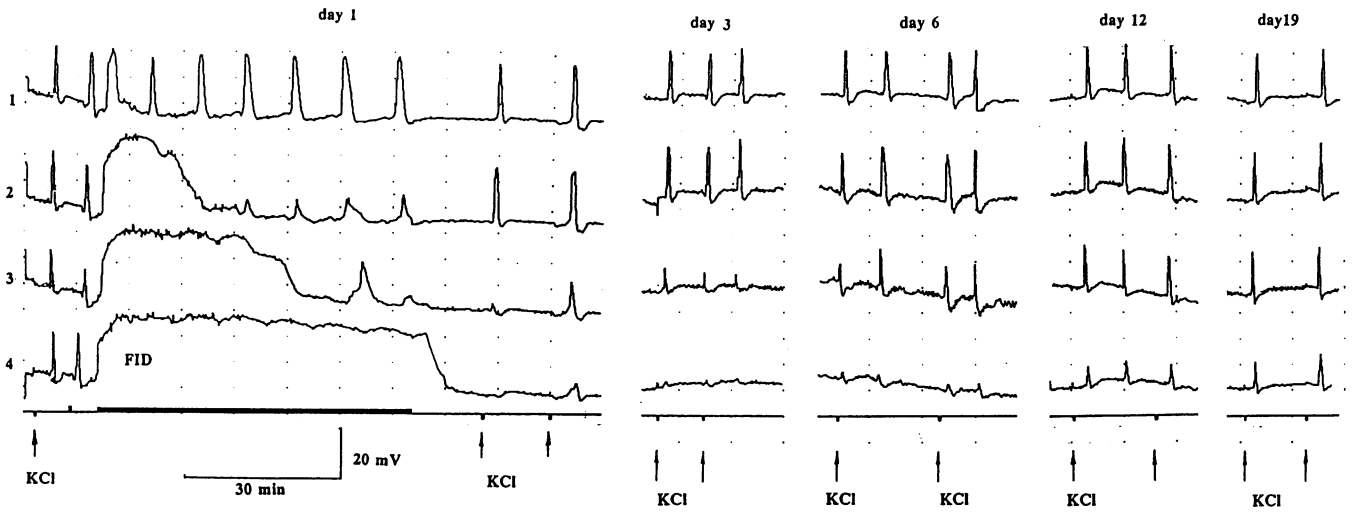

FIG. 2. Severe form of ischemia with good recovery. Traces 1–4 show slow potential recording from E1 to E4 on day 1 (acute concomitants of MCA occlusion) and on days 3, 6, 12, and 19 (chronic recording). The arrow and downward deflection of the marker line denote microinjections of 1 μl of 5% KCl into the occipital cortex. The upward deflection of the marker line on day 1 indicates the application of the clamp and the duration of the subsequent MCA occlusion (for details see text). Calibration: 30 min and 20 mV.

were normal only at E1 and E2 but were severely reduced or absent at E3 and E4. SD waves started to penetrate more regularly to E3 on day 5 and reached normal shape on day 7. Gradual recovery at E4 started on day 7 and reached 80% of the preischemic value on day 11 when the sequence of the elicited SD waves suggested analogous propagation conditions as before ischemia. Increased SD susceptibility was indicated by generation of repetitive SD waves during the first week after ischemia.

Statistical evaluation of the above experiments yielded results illustrated in Figs. 3–7.

Fig. 3 shows the overall distribution of mean FID durations at the four electrodes. The longest FIDs were observed at E4; their duration decreased in a geometric progression at E3 and E2 and was practically indistinguishable from a protracted SD wave at E1. This means that the depolarized zone extended in the first 20 min after MCA occlusion up to E2 but shrank gradually in spite of continued occlusion to the radius corresponding to E4. A one-way ANOVA showed a significant effect of electrode position [$F(3,99) = 31.1$; $P < 0.001$]. Newman–Keuls multiple comparisons revealed significant differences for E1 vs. E3, and E4 and E2 vs. E4, and E3 vs. E4, at $P < 0.01$.

Fig. 4 shows the incidence of spontaneous SD waves in 30 rats in which at least one such wave (not including the first SD evoked by MCA occlusion) appeared at E1 and E2. Out of the 131 waves observed, about 75% were generated in the first hour after MCA occlusion. Their incidence dropped abruptly to 20% and 5% in the second and third hours, respectively, and no spontaneous SD waves were observed later. High SD susceptibility of the perifocal region was manifested by generation of repetitive SD waves after a single KCl injection. Incidence of such additional SD waves was again highest in the first 2 h after MCA occlusion.

Postischemic development of the amplitude of evoked SD waves at E1 to E4 is shown in Fig. 5. The day 1 values reflect the situation 4 h after the 1-h MCA occlusion. Note the increased SD amplitude at E1 on day 2 corresponding to further decrease of SD amplitude at E4 and E3. The minimum SD amplitude was observed on days 3 and 4. Subsequent recovery is significant at E4 but seems to reach an asymptotic level from day 11. SD amplitude at E4 was lower than at E3 during the first 9 days after ischemia but not on days 10–15. Paired t test indicated significant difference of SD amplitude at E4 between day 4 and days 9–15.

Fig. 6 shows different arrangement of the same data: the records were classified irrespective of the electrode position as no decrease (nd; $n = 51$), full recovery (fr; $n = 19$), partial recovery (pr; $n = 37$), and no recovery (nr; $n = 18$). For each class, average values of SD amplitude were established at the

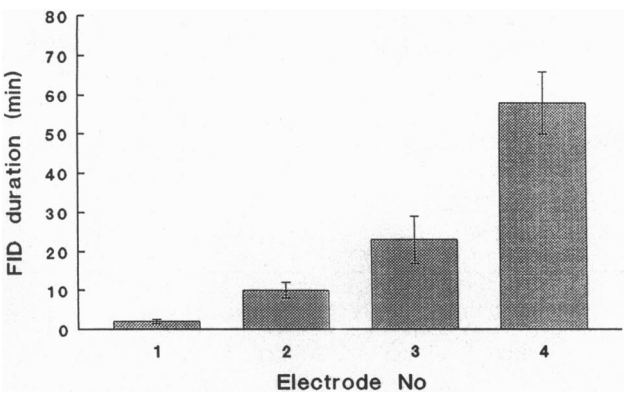

FIG. 3. FID duration at E1 to E4 is inversely related to the distance from the focus. Individual bars are based on 26–31 FIDs. Data are the mean ± SEM.

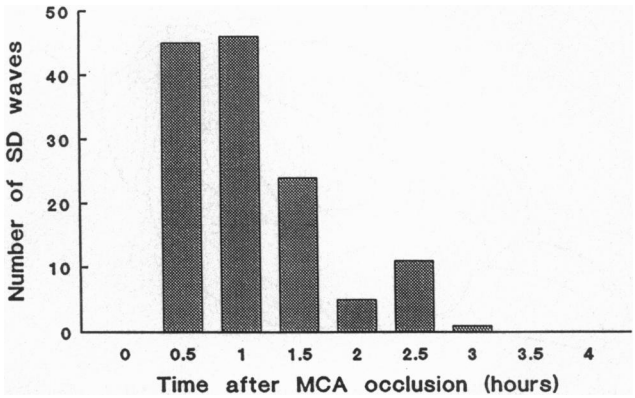

FIG. 4. Incidence of spontaneous SD waves recorded at 30-min intervals after MCA occlusion. The histogram is based on data obtained from 30 rats.

respective minima and maxima of the individual records and plotted at the corresponding mean postischemic delays. The partial recovery and no recovery classes reached the same postischemic minimum but showed sharply divergent development afterward.

Fig. 7 illustrates the inverse relationship between the initial FID duration and final recovery expressed by the amplitude of SD waves recorded from the same electrodes. Pearson correlation of the underlying data ($n = 54$) yielded $r = -0.76$. The relative SD amplitude (%) was dependent on FID duration (min) by using the following equation: SD = 97.9 − (0.921 × FID). The electrophysiological results were in good agreement with histological findings (to be published elsewhere).

DISCUSSION

The present report addresses several problems in which the common denominator is the slow potential change generated by massive depolarization of neurons and glia cells due to excessive accumulation of K^+ in the extracellular space and concomitant intracellular accumulation of Na^+ and Cl^- (2, 15, 17–19). The striking disruption of the homeostasis of brain microenvironment is manifested by two closely related but nevertheless different phenomena, SD (1) and AD (4).

AD and FID. Attempts to use AD for assessing the effect of drugs protecting the brain against ischemic damage date back to the 1950s when it was demonstrated that AD onset was advanced by hypoglycemia and delayed by hyperglycemia, hypothermia, and local treatment of cerebral cortex with $MgCl_2$ (20, 21). AD latency is not increased by *N*-methyl-D-

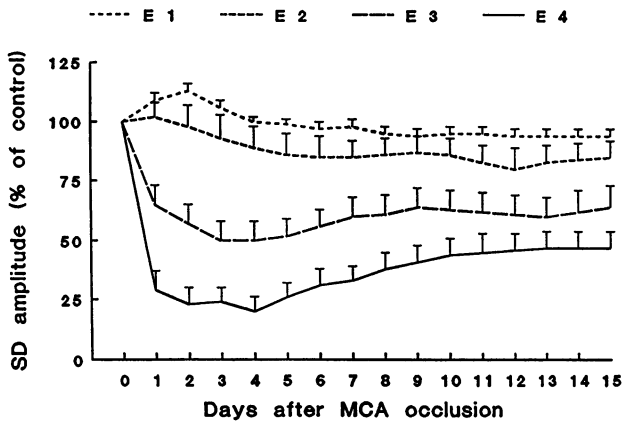

FIG. 5. Postischemic changes of relative SD amplitude (mean ± SEM) at E1 to E4 during 15 days after MCA occlusion. The day-1 values reflect the situation 4 h after MCA occlusion.

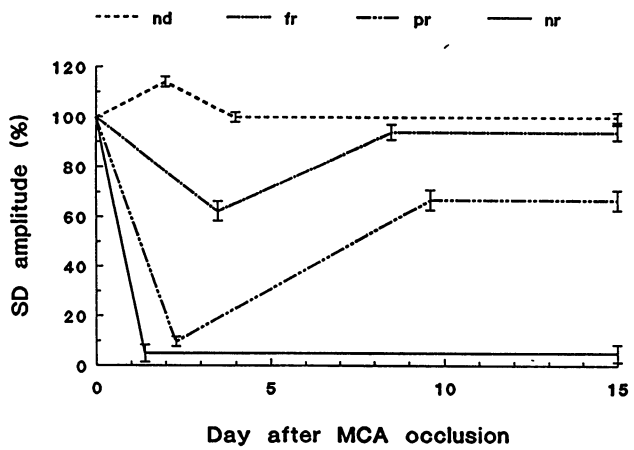

FIG. 6. Postischemic changes of relative SD amplitude (mean ± SEM) observed during 15 days after MCA occlusion in four types of reactions characterized by time and amplitude of the maximum SD decrease (increase) and subsequent maximum recovery in cortical areas showing no decrease (nd), full recovery (fr), partial recovery (pr), and no recovery (nr).

aspartate receptor antagonists (22–24) at dosages that block SD propagation (25–28). The relationship between AD duration and subsequent recovery is less well investigated. For FID, which can be reversed by reperfusion, the negative potential gradually decreases as a sign of disappearance of the generating dipoles. The present data suggest that FID duration is directly related to incidence and severity of postischemic damage and can serve, therefore, as a useful, albeit not fully reliable predictor of the anticipated damage.

Spontaneous SD Waves. The intact brain is well protected against spontaneous generation of SD waves, the initiation of which requires depolarization of a minimum volume of the susceptible brain tissue (about 1 mm^3). When the active substances released from this critical volume reach a concentration sufficient for depolarization of adjacent neurons, a self-propagating SD wave is triggered. The ischemic focus elicited by MCA occlusion exceeds the critical volume of depolarization and its appearance is, therefore, accompanied by an SD wave propagating from the focus into the intact brain. In the subsequent minutes and hours, further SD waves can be generated from the boundary of the focus provided that the chemical gradient is steep enough to support sufficiently intensive diffusion of active substances into the intact cortex. An SD wave evoked from a single point at the periphery of the focus propagates away from it but may turn around and enter the penumbra zone in a different sector of the focus. Such SD waves last significantly longer than those occurring in intact

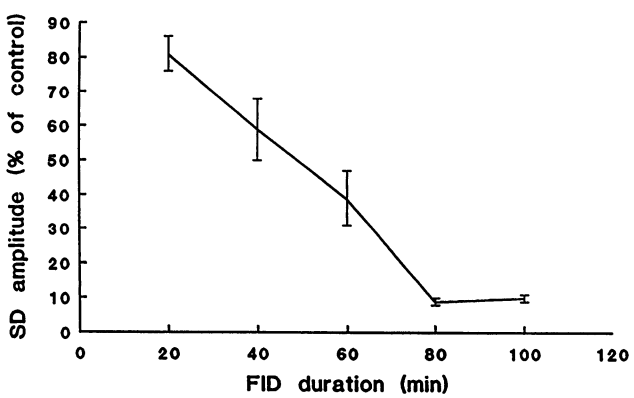

FIG. 7. Negative correlation between the duration of the initial FID and relative SD amplitude (mean ± SEM) recorded at the same electrode 15 days after ischemia.

brain (7, 18) and can be potentially dangerous because they are accompanied by additional release of glutamate and influx of calcium into the neurons. Whereas repeated SD waves in the intact cortex do not elicit any signs of metabolical (29) or morphological (18) damage, their deleterious effects can be potentiated by hypoxic or hypoglycemic conditions in the penumbra zone. The assumption that SD episodes can enlarge the ischemic lesion received indirect support by studies indicating that treatment with *N*-methyl-D-aspartate receptor antagonists reduces the infarct size (30) and decreases the neuronal loss in the periinfarct region (7).

The present experiments show that the generation of SD waves was limited to an ≈2-h period after ischemia, followed by a shorter interval of increased SD susceptibility manifested by generation of multiple SD waves after a single triggering stimulus. All these signs of SD facilitation disappear 3–4 h after the onset of focal ischemia. No clear relationship was found between the incidence of spontaneous SD waves and the probability of long-term recovery.

SD Penetration into the Ischemic Cortex. Although SD in the intact cortex can only be evoked by abnormal stimuli, its amplitude and duration reflect the efficiency of the homeostatic mechanisms and indicate the normal morphological properties of the susceptible tissue. In this respect, SD can be used to assess the functional state of the cerebral cortex. This approach was first used by Van Harreveld and Stamm (31), who exposed rabbits 3 days before SD recording to reversible ischemization of the forebrain. A 35-min ischemia caused local disappearance of EEG activity and heavy neuronal damage. Although the loss of neurons was almost complete, neuroglia was not seriously affected. This did not disprove the possible participation of glia in SD propagation since glial reaction could persist in the ischemic brain in some hidden way. To examine this possibility, Hull and van Harreveld (32) limited the ischemization to an oblong strip of parietal cortex separating the frontal and occipital lobes by a 5-mm zone completely free of neurons. Although normal SD could be elicited rostrally and caudally from the damaged strip, SD propagation across the asphyxiated region was interrupted. The blockade also occurred when the asphyxiation only caused a marked decrease of neuron density.

In the present study, attenuation or complete blockade of SD was observed in the cortical regions repolarized after a FID episode. This indicates that the cortex was not fully recovered, because it could not support propagation of spontaneous or elicited SD waves. Repolarization combined with SD blockade can be due to increased active transport during reperfusion, postocclusion decrease of neuronal excitability, or increase of interneuronal distance due to development of edema (33, 34).

Predictive Value of the Phenomena Studied. Testing of drugs used for treatment of stroke patients should employ a standard model providing sufficiently uniform initial conditions for comparative evaluation. This is not easy for MCA occlusion in which the inherent variability is illustrated by the wide scatter of some critical parameters measured in our acute experiments as follows: range of FID duration from 10 min to 120 min; range of spontaneous SD incidence from 0 to 10; range of postischemic SD amplitude from 0% to 110%. Obviously animals showing no or weak suppression of SD waves 4 h after MCA occlusion are not suitable for testing drugs that should ameliorate their condition. Only the rats showing an at least 30-min FID and clear reduction of SD amplitude at least at one electrode (usually E4 or E3) can be positively influenced by postischemic treatment. Incidence of spontaneous SD waves should be taken into account as an auxilliary parameter.

Even strict observance of the above criteria does not guarantee uniformity of the further consequences of the focal ischemia. The chronic experiments showed that ischemic regions displaying clear SD suppression in the acute phase may

either remain suppressed or attain striking recovery during the next 2 or 3 weeks. Limited predictability applies also to cases showing partial SD suppression in the early postischemic period: in about 50% of rats in which SD amplitude was acutely reduced to 20–60% of the control value, SD blockade deepened in the subsequent 2–4 days. Even the maximal SD blockade reached in the above cases several days after MCA occlusion does not preclude substantial recovery. This is in good agreement with the finding (35) that the infarct volume in mice is larger 3 days than 7 days after MCA occlusion and further decreases during the following 2 weeks. Similar results stress the importance of therapeutic interventions aimed at late stages of postischemic recovery, which are critical for success of clinical applications.

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