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# THE EFFECT OF REPETITIVE SPREADING DEPRESSION ON NEURONAL DAMAGE IN JUVENILE RAT BRAIN

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**Abstract—Spreading depression (SD) is pronounced depolarization of neurons and glia that travels slowly across brain tissue followed by massive redistribution of ions between intra- and extracellular compartments. There is a relationship between SD and some neurological disorders. In the present study the effects of repetitive SD on neuronal damage in cortical and subcortical regions of juvenile rat brain were investigated. The animals were anesthetized and the electrodes as well as cannula were implanted over the brain. SD-like event was induced by KCl injection. The brains were removed after 2 or 4 weeks after induction of 2 or 4 SD-like waves (with interval of 1 week), respectively. Normal saline was injected instead of KCl in sham group. For stereological study, paraffin-embedded brains were cut in 5 $\mu$m sections. The sections were stained with Toluidine Blue to measure the volume-weighted mean volume of normal neurons and the numerical density of dark neurons. The volume-weighted mean volume of normal neurons in the granular layer of the dentate gyrus and layer V of the temporal cortex in SD group were significantly decreased after four repetitive SD. Furthermore, densities of dark neurons in the granular layer of the dentate gyrus (after 2 weeks), the caudate–putamen, and layer V of the temporal cortex (after 4 weeks) were significantly increased in SD group. Repetitive cortical SD in juvenile rats may cause neuronal damage in cortical and subcortical areas of the brain. This may important in pathophysiology of SD-related neurological disorders.** 

**Key words: cortical spreading depression, cell injury, dark neuron, hippocampus, neocortex, caudate–putamen.**

Spreading depression (SD) is a pronounced depolarization of neurons and glial cells that spreads slowly across the neocortex (Leao, 1944). This depolarization is associated with a depression of the neuronal bioelectrical activity for a period of minutes (Bures et al., 1974). It has been well documented that SD is accompanied by ionic, metabolic and hemodynamic changes and occurs in several regions of the brain such as the neocortex, the hippocampus, the thalamus, the caudate–putamen, etc. (Bures et al., 1974). There are reasons to believe that SD is involved in some neurological disorders including migraine, cerebrovascular diseases, brain injury, epilepsy, transient global amnesia and spinal cord disorders (Gorji, 2001).

In contrast to SD under normal conditions, SD causes neuronal damage under pathologic conditions (Busch et al., 1996; Takano et al., 1996; Obeidat et al., 1998). During ischaemia neurons depolarize and release the neurotransmitter glutamate, which accumulates extracellularly and binds to postsynaptic receptors. This initiates a sequence of events thought to culminate in immediate and delayed neuronal death (Dietrich et al., 1992; Wiggins et al., 2003; Dreier et al., 2007). SD in normal conditions stimulates the transformation of normal astrocytes into reactive species in adult animal brain (Kraig et al., 1991). However, some recent findings challenge the concept that SD is ever completely innocuous in normal condition. Pomper et al. (2006) have shown that in slice cultures of metabolically competent normally oxygenated but immature nervous tissue, repeated SD episodes do cause the death of neurons and glial cells. However, it should be noted that hippocampal slice cultures used by Pomper et al. (2006) may have a higher number of glutamatergic synapses and these could be more vulnerable to glutamate-mediated oxidative cell injury during induction of SD (Hoffmann et al., 2006). It is suggested that if the role of repeated SD in the death of neurons will be confirmed in intact juvenile brains, this finding may be relevant for the pathogenesis of neurological conditions of infants (Somjen, 2006). Dark neurons, cells with acidophilic cytoplasm and pyknotic nucleus, are traditionally known to represent a typical morphological change of injured neurons following many kinds of insults (Ooigawa et al., 2006). Since dark neurons show massive shrinkage and abnormal basophilia, they can be clearly distinguished from normal neurons (Johnson, 1975; Kövesdi et al., 2007). Using stereological methods to evaluate dark neurons as well as the volume-weighted mean volume of normal neurons, the aim of the present study was to investigate the possible role of repetitive cortical SD in production of cell damage in cortical and subcortical structures of juvenile rat brain.

## EXPERIMENTAL PROCEDURES

Forty juvenile male Wistar rats (20–35 days; 45–110 g) were housed individually under controlled environmental conditions

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*Abbreviation:* SD, spreading depression.

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(12-h light/dark cycle) with food and water available *ad libitum* for 7 days before being used in the experiments. The experiments were approved by the Mashhad University of Medical Science Committee on Animal Research. The animals were anaesthetized with ketamine (Sigma; 150 mg/kg ip) and Xylazine (Rompun®, Bayer; 0.1 mg/kg ip) and the head of each rat was placed in a stereotaxic instrument (Stolting Instruments, USA). Stainless steel, 23-gauge guide cannula and two silver recording electrodes (2–3 mm apart) were implanted above the somatosensory neocortex and fixed with dental acrylic cement. A stylet was placed into the guide cannula to allow it to maintain patency. The scalp was sutured closed and the animals were returned to their home cages and allowed to recover. The rats were kept for 1 week in well-ventilated box in an air conditioned room to recover from surgery before the beginning of the experimental protocol. The animals were anaesthetized by pentobarbital (Sigma; 60 mg/kg ip), the stylet was withdrawn from the guide cannula and a 27-gauge injection needle was inserted. The injection needle was attached to a 10 $\mu$l Hamilton syringe by a polyethylene tube. The 3 M KCL solution was injected in a total volume of 10 $\mu$l during 60 s. The injection needle was retained in the guide cannula for an additional 60 s after injection to facilitate diffusion of the drugs. In sham rats, 10 $\mu$l of Ringer solution was injected. SD induction carried out two or four times (with an interval of 1 week). During injection of KCl or Ringer solution and at least 60 min after the injections, all rats were under anesthesia. EEG was continuously recorded in anesthetized rats for 60 min after injection of KCl or Ringer solution.

### Tissue preparation

After two or four weeks of the first KCl or Ringer injection, rats were decapitated and the brains were removed. Before decapitation, the animals were given a deep anaesthesia with chloral hydrate (350 mg/kg) and perfused transcardially by 200 ml of saline and then 600 ml of 4% paraformaldhyde. The brains were removed and fixed in 4% paraformaldhyde solution for at least 1 week and then was processed for histological study. Coronal uniform random sections were cut through ipsilateral site to the hemisphere in which SD was evoked. Ten pairs of successive sections were selected by random systemic sampling from each animal and stained by Toluidine Blue. Different areas were studied under light microscopic coupled to camera (BX51, Olympus, Japan), and images were taken under objective lens (X 100; Olympus, Japan). The magnification was calculated by an objective micrometer.

### Stereological methods and physical dissector

The volume-weighted mean volume of normal neurons was calculated directly by point-sampled intercept on 10 uniform systematic randomly sampled microscopic fields (Kempermann et al., 1997; Mandarim-de-Lacerda, 2003). The volume-weighted mean volume of neurons was estimated in the following three areas: (i) the granule cell layer of the dentate gyrus, (ii) pyramidal layer (layer V) of the temporal cortex (iii) the caudate–putamen. The volume-weighted mean volume of neuronal cells was determined by the point sampled intercept method (Gundersen et al., 1988). A lattice of test points on lines was superimposed randomly on to the traced nuclear profiles in each particular field. Nuclei of neurons were marked and two isotropic lines from randomly selected directions were centred on that and superimposed over the neuron. The intersection of each line with the outer surface of the neuronal soma was marked. These lines produced point-sampled intercepts whose length were measured, cubed and then the mean multiplied by $\pi/3$, and finally averaged over all intercepts to give an estimate of the volume weighted mean neuronal volume.

For quantitative analysis of dark neuron the physical dissector method was carried out. We had 10 pairs of sections, with 5 mm distance apart, for each brain. The first section of each pair was designated as the reference and the second one as the look-up section. On each pair of sections at least 10 microscopic fields were selected by uniform systematic random sampling in every area of interest. Using unbiased frame and physical dissector counting rule, the counting of dark neurons in each field was carried out (Braendgaard and Gundersen, 1986).

Data were expressed as mean±SEM. Statistical analysis was carried out by using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The criterion for statistical significance was $P<0.05$. Correlation between density of dark neurons and the volume of neurons was analyzed by Pearson test.

## RESULTS

Injection of KCl induced negative DC deflections followed by positive waves in all tested animals. The mean amplitude and the mean duration of the first neocortical SD-like wave were 14.2±2.3 mV of 144±31 s, respectively. The velocity of propagation of DC negative deflection between the recording electrodes was 3.2±0.3 mm/min. The amplitude and duration as well as speed of propagation of SD-like deflection induced in following weeks were not significantly differed from the characteristic features of the first SD-like event (amplitude of 13.8±1.9 mV, duration of 136±35 s, and velocity of 2.9±0.3 mm/min; Fig. 1).

### The volume-weighted mean volume of normal neurons

The volume-weighted mean volume of normal neurons was investigated in different brain regions in ipsilateral hemisphere in which SD-like waves were evoked. There was no significant difference in the volume-weighted mean volume of normal neurons observed in the dentate gyrus, the temporal cortex, and the caudate–putamen after induction of SD-like fluctuation for two consecutive weeks compared with sham and control rats (Fig. 2). However, induction of four SD-like events within 4 weeks significantly decreased the volume-weighted mean volume of normal neurons in the granular layer of the dentate gyrus as well as in the fifth pyramidal layer of temporal cortex (layer V; $P<0.03$). The volume-weighted mean volume of normal neurons in the granular layer of the dentate gyrus was 913±11/$\mu$m$^3$ in control rats, 892±87/$\mu$m$^3$ in sham rats, and 609±34/$\mu$m$^3$ in SD-treated rats. The volume-weighted mean volume of normal neurons in layer V of the temporal neocortex was 2167±108/$\mu$m$^3$ in control group,

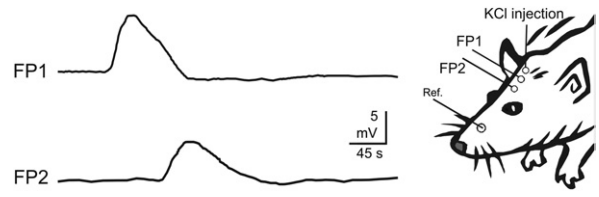


**Fig. 1.** Recordings of spreading depression after injection of KCl (3 mol/l) in juvenile rat brain. A stainless steel, 23-gauge guide cannula and two silver recording electrodes (2–3 mm apart) were implanted above the somatosensory neocortex and fixed with dental acrylic cement. Negative direct current fluctuation was recorded from somatosensory cortex of juvenile rats.