Neuroscience 169 (2010) 388–394

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

M. JAFARIAN,[a,b] S. RAHIMI,[c] F. BEHNAM,[c] M. HOSSEINI,[d] H. HAGHIR,[a] B. SADEGHZADEH[b,e] AND A. GORJI[f]*

[a]*Department of Anatomy, Mashhad University of Medical Sciences, Mashhad, Iran*

[b]*Shefa Neuroscience Center, Tehran, Iran*

[c]*Department of Biology, Ferdowsi University of Mashhad, Mashhad, Iran*

[d]*Department of Physiology, University of Medical Sciences, Mashhad, Iran*

[e]*Razavi Research Center, Razavi Hospital, Mashhad, Iran*

[f]*Institüt für Physiologie I, Westfalische Wilhelms-Universität Münster, Münster, North Rhine-Westphalia, Germany*

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 μm 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

*Corresponding author. Tel: +49-251-8355564; fax: +49-251-8355551.
E-mail address: gorjial@uni-muenster.de (A. Gorji).
Abbreviation: SD, spreading depression.

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doi:10.1016/j.neuroscience.2010.04.062

(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 μl Hamilton syringe by a polyethylene tube. The 3 M KCL solution was injected in a total volume of 10 μl 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 μl 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/μm^3 in control rats, 892±87/μm^3 in sham rats, and 609±34/μm^3 in SD-treated rats. The volume-weighted mean volume of normal neurons in layer V of the temporal neocortex was 2167±108/μm^3 in control group,

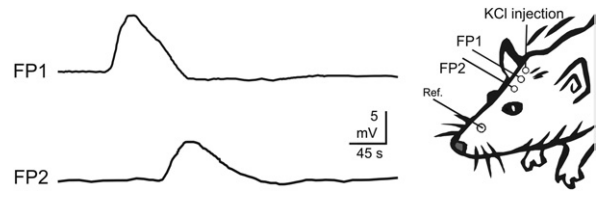

Fig. 1. Recordings of spreading depression after injection of KCl (3 mol/l) in juvenile rat brain. A stainless steel, 23-gauge guide cannula and two silver recording electrodes (2–3 mm apart) were implanted above the somatosensory neocortex and fixed with dental acrylic cement. Negative direct current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

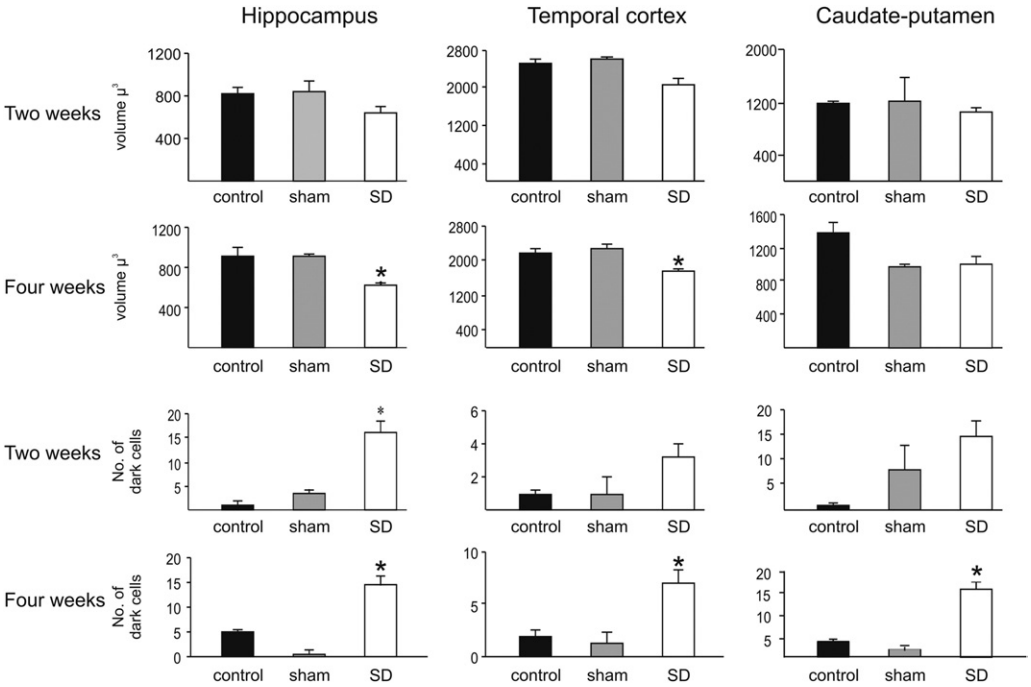

Fig. 2. The effect of spreading depression (SD) on the volume-weighted mean volume of normal neurons and the mean number of dark neurons in the hippocampal dentate gyrus, layer V of the temporal cortex, and the caudate-putamen. The volume-weighted mean volume of normal neurons (two upper rows) and the mean number of dark neurons (two lower rows) in the hippocampal dentate gyrus, layer V of the temporal cortex, and the caudate-putamen in control, sham, and SD treated juvenile rats after 2 or four weeks of repetitive SD induction. Note the significant reduction of the volume-weighted mean volume of normal neurons in the dentate gyrus of the hippocampus as well as V layer of the temporal cortex. After 4 weeks of induction of repetitive SD, the mean number of dark neurons was significantly higher compared with control and sham rats. * indicates significant difference in comparison to respective control and sham groups.

$2256\pm107/\mu m^3$ in sham group, and $1743\pm54/\mu m^3$ in SD group (Fig. 2). Induction of four SD-like waves within 4 weeks in the caudate–putamen did not change the volume-weighted mean volume of neurons among control, sham, and SD groups (Fig. 2).

Density of dark neurons

Dark neurons were identified by the neuronal shrinkage, cytoplasmic esoinophilia, nuclear pyknosis, and surrounding spongiosis. Density of dark neurons in the hippocampal dentate gyrus, ipsilateral to the hemisphere in which SD-like waves were evoked, was significantly increased after induction of two SD-like events in two consecutive weeks ($P<0.007$) in comparison to sham and control groups (Figs. 2 and 3). The mean number of dark neurons was 1.1 ± 0.3 in control rats, 3.6 ± 0.1 in sham rats, and 16 ± 2.2 in SD-treated rats. However, there was no significant difference in density of dark neurons either in the temporal cortex or in the caudate–putamen between these rats (Figs. 2 and 3).

Induction of four SD-like waves in four consecutive weeks significantly increased the number of dark neurons in all studied brain regions. Dark neurons in the dentate gyrus were increased to 14.2 ± 0.3 in comparison to control (5 ± 0.5) and sham (0.7 ± 0.6) rats ($P<0.001$). Dark neurons also increased in the temporal cortex to 7 ± 1.3 in comparison to control (1.9 ± 0.7) and sham (1.25 ± 0.1) animals ($P<0.002$). In the caudate–putamen, the number of dark neurons was also significantly higher in SD-treated rats (15 ± 1.6) in comparison to control (3.9 ± 1.5) and sham (1.6 ± 0.2) rats ($P<0.001$; Figs. 2 and 3).

Correlation between the volume-weighted mean volume of normal neurons and density of dark neuron have shown that decrease in the volume-weighted mean volume of normal neurons was accompanied with increase in the density of dark neurons in the hippocampal dentate gyrus ($r=0.837$, $P<0.01$) and the temporal cortex after induction of four SD-like events in four consecutive weeks ($r=0.831$, $P<0.05$). However, there were no significant correlations in all areas after induction of two SD in two consecutive weeks. No significant correlation was also found between the volume of normal neurons and the number of dark neurons in the caudate–putamen after induction of repetitive SD-like waves after 2 or 4 weeks (Fig. 4).

DISCUSSION

In the present study the effect of repetitive SD-like events on the volume-weighted mean volume of normal neurons as well as on the production of dark neurons were investigated. Our findings revealed a significant reduction of the volume-weighted mean volume of normal neurons in the dentate gyrus as well as in the temporal cortex after induction of repetitive SD-like fluctuations for 4 weeks in juvenile rats. Furthermore, our data indicate a significant enhancement of the density of dark neurons in the dentate gyrus,

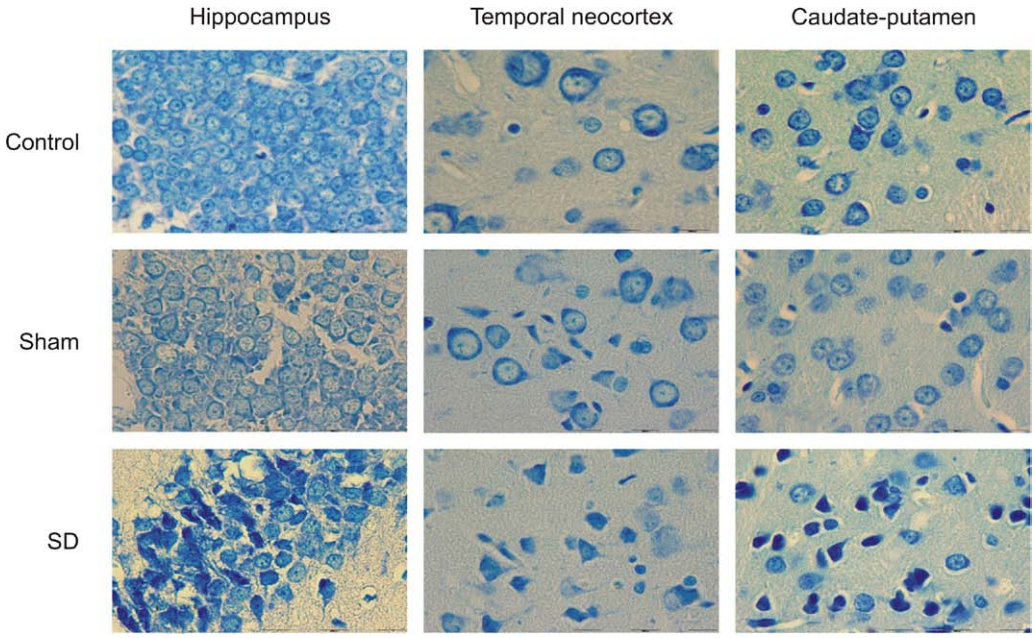

Fig. 3. Light-microscopic appearance of Toluidine Blue-stained dark neurons in 5 μm sections of the hippocampal dentate gyrus, the temporal cortex, and the caudate-putamen in juvenile rats. Dark neurons are present in the dentate gyrus of hippocampal tissues, Fifth layer of the temporal cortex, and the caudate-putamen following induction of four consecutive spreading depression (SD; lower row), in sham rats (middle row), and in control rats (upper row) in juvenile rat brain. Note the higher shrunken dark blue stained (basophilic) cell bodies in SD group.

the temporal cortex, and the caudate–putamen after repetitive SD-like waves. The changes observed in the volume of normal neuronal cells and the density of dark neurons after repetitive SD-like waves in the dentate gyrus and in the temporal cortex were statistically correlated.

Previous investigations indicate that SD waves are responsible for neuron death in pathological conditions (Dietrich et al., 1992; Dreier et al., 2007). However, the presented data point to the induction of cell damage by repetitive SD in normal brain tissue of juvenile rats. In line with our results, Pomper et al. (2006) have shown the presence of dark neurons in CA1, CA3, and polymorphic layers of the dentate gyrus after repetitive SD in juvenile rats. SD in brain tissue of adult rat did not lead to cellular

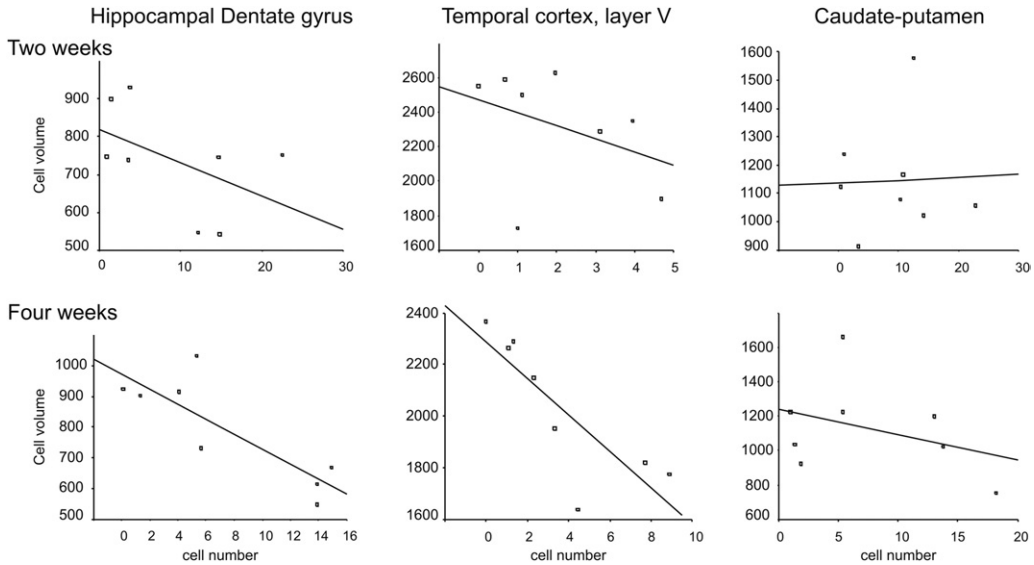

Fig. 4. The relation between the volume-weighted mean volume of normal neurons and density of dark neurons after repetitive SD in juvenile rat brain. A significant correlation between the volume-weighted mean volume of normal neurons and density of dark neurons in the hippocampal dentate gyrus (r=0.837, P<0.01) and in the temporal cortex (r=0.831, P<0.05) after induction of four consecutive sessions of SD was observed. However, there were no significant correlations in these regions after induction of two SD in two consecutive weeks. There was also no significant correlation between the volume of normal neurons and the number of dark neurons in the caudate-putamen after induction of repetitive SD after 2 or four weeks.

damage. Induction of SD in the adult rat brain cannot cause cell damage but has the potential to stimulate persistent neurogenesis evidenced by specific neuronal markers (Yanamoto et al., 2005). Evidences indicate that the brain of adult animals is more resistant to cell damage when compared to juvenile brain tissues. A higher neuronal damage has been reported in 3 weeks old rats than in 6 and 9 weeks old after hypoxic insults (Yager and Thornhill, 1997). Varied responses to SD as a good indicator of the state of the oxygen balance in the brain of rats in different ages have been noted (Mayevsky et al., 1982). SD caused an increase in neuronal oxygen utilization. Propagation of SD is accompanied with a large, passive efflux of potassium into the extracellular space. The pumping back of the potassium by the ATPase into the neurons is an energy-dependent process and increases the energy demand of the tissue. As a consequence, the mitochondria are activated in order to produce the extra ATP. In the normoxic brain, the increased blood flow will compensate for the extra oxygen and will keep the balance of oxygen in the normal range so that oxidation of NADH is observed. When oxygen delivery to the tissue is limited, an increase of the energy demand during SD will lead to an imbalance between supply and demand, resulting in a reduction of NADH in juvenile, but not adult, rat brain (Mayevsky et al., 1982).

Dark neurons are reported in clinical and experimental neuropathology from living neuronal tissues (Kherani and Auer, 2008). Dark neurons were first observed to occur in neurosurgical biopsies, but were not seen at autopsy. Because of the appearance of dark neurons after mechanical trauma to the brain prior to fixation, a mechanical stress force was hypothesized to produce these neurons (Cammermeyer, 1978; Ooigawa et al., 2006). However, dark neurons also appear under conditions where no trauma or mechanical forces are applied to the neuronal tissues. Several studies revealed that trauma is only one of the many processes for experimentally producing dark neurons (Løberg and Torvik, 1993; Ooigawa et al., 2006). It is concluded that neuronal trauma is not a prerequisite for the production of dark neurons, despite the fact that dark neurons can be produced by mechanical injuries. The effect of mechanical stimulation to cause both SD and dark neurons might be responsible for the association of neuronal trauma and dark neurons (Bures et al., 1974; Kherani and Auer, 2008). In perfusion-fixed brain tissue, dark neurons have been shown in epilepsy, ischemia, hypoglycaemia, exposure to excitatory amino acids, in addition to mechanical neuronal trauma (Ishida et al., 2004). SD is accompanied by massive redistribution of ions and water between extracellular and intracellular compartments. Dramatic changes of the size of neurons by redistribution of water may be the physical mechanism of the formation of dark neurons. In addition, the excitatory neurotransmitter glutamate essentially contributes to the initiation and propagation of SD (Kager et al., 2000; Smith et al., 2006). Cortical SD up-regulate the binding sites of glutamate *N*-methyl-D-aspartic acid (NMDA)-, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-, and kainate-receptors in rat neocortex, hippocampus, and striatum (Haghir et al., 2009; Menniti et al., 2000). Infusion of NMDA into neuronal tissues produces dark neurons (Dietrich et al., 1992) and some NMDA antagonists as well as AMPA receptor antagonist CNQX abolished formation of dark neurons (Kherani and Auer, 2008). Blockade of NMDA receptors also prevent SD initiation (Gorji et al., 2001). In the present study, subcortical regions are also affected by SD initiation in the neocortex. Several studies investigated the effect of cortical SD in subcortical areas (Wernsmann et al., 2006; Dehbandi et al., 2008; Haghir et al., 2009). These studies indicate that altered subcortical neural circuit function can be affected remote from SD propagation site by direct or indirect spread of SD.

The results of the present study also revealed that the volume of normal neurons after repetitive SD was significantly decreased in the hippocampus and temporal cortex. Concomitant occurrence of dark neurons and reduction of neuron size was reported (Petrasch-Parwez et al., 2007). There was a statistical correlation between changes in the volume-weighted mean volume of the neurons and formation of dark neurons. Decreases in the volume of normal neurons may initiate during massive metabolic and cellular alterations induced by repetitive SD. This may later lead to loss of cell activity and creation of dark neuron. SD has been shown to occur during aura phase of migraine attacks and was accompanied by ipsilateral scotoma (Hadjikhani et al., 2001). Using diffusion tensor magnetic resonance imaging, a reduction of the residual amount of normal white matter in cortical as well as subcortical regions was revealed in migraine patients. This finding indicates that damage of white matter in migraine is not confined to those areas that appear abnormal on dual-echo scans, but includes more subtle changes in normal appearing white matter (Rocca et al., 2003). In our study a reduction of the volume-weighted mean volume of normal neurons and an increased number of dark cells were observed in granular layer of the hippocampal dentate gyrus. It has been shown that hippocampal dentate gyrus damage is associated with spatial learning and memory deficits (Gold and Squire, 2005). Chronic migraine could cause a persistent, subtle central nervous system dysfunction. Previous studies reported that the performance of migraine patients was significantly lower on a variety of cognitive measures, including tests assessing attention, information processing, and memory (Mc Kendrick et al., 2006).

Our finding on reduction of the volume-weighted mean volume of normal neurons after repetitive SD is novel. Dark neurons represent a pathological process in the term of collapsed neurons (Ishida et al., 2004) and their formation may in some extent contribute to the pathogenesis of SD-related neurological disorders.

CONCLUSION

In conclusion, the result of the present study showed that repetitive SD-like events produces dark neurons and reduces the volume-weighted mean volume of normal neurons in cortex and subcortical structures of juvenile rat brain. This indicates SD potential to damage the neurons

in juvenile brain and may be important in pathophysiology of SD-related neurological disorders.

Acknowledgments—This study was supported by German Research Foundation (DFG/SFB-TR3-D10) and Mashhad University of Medical Sciences.

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(Accepted 25 April 2010)
(Available online 8 May 2010)