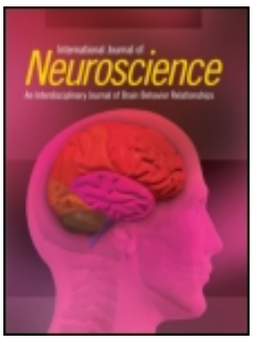

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IMMUNOCYTOCHEMISTRY OF NEURON SPECIFIC ENOLASE (NSE) IN THE RAT BRAIN AFTER SINGLE AND REPEATED EPILEPTIC SEIZURES

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The aim of this study was to investigate neuron-specific enolase (NSE) immunoreactivity of the different brain regions after pentylenetetrazol (PTZ)- induced epileptic seizures in rats. Light microscopic examinations provided evidences for changes of neuronal activity after single and repeated seizures. The number of NSE (+) cells was well correlated with Nissl staining. The results suggest that NSE immunoreactivity may be a valuable marker for determination of the number of metabolically active neurons in different brain regions after single and repeated experimental seizures.

Keywords epileptic seizure, neuron specific enolase, nissl, pentylenetetrazol, rat brain

INTRODUCTION

Epilepsy is a disorder of the central nervous system characterized by recurrent and sudden increase in electrical activity. Metabolic studies have shown that oxygen availability, glucose utilization, and blood flow all increase dramatically during epileptic seizures. It is also known that epileptic activity may induce some molecular and structural changes in the different brain regions (Ingvar & Siejo, 1983; Siesjo et al., 1986; Oztas et al., 2001).

Enolase is glyoclytic enzyme that converts 2-phosphoglycrate to phosphoenol pyruvate. It has three immunologically distinct subunit; α, β, and γ. The γ form is found primarily in the cytoplasm and process of neurons, which is referred to as neuron-specific enolase (NSE). NSE is a sensitive marker of neuronal damage in several central nervous system (CNS) diseases including epilepsy (Schmechel et al., 1978; Nogami et al., 1998a; Nogami et al., 1998b; Rodriquez-Nunez et al., 2000). Changes in membrane integrity as a result of neuronal injury can cause leakage of protein such as NSE from cytosol into extracellular space. Increased NSE in serum (sNSE) and in cerebrospinal fluid (cNSE) have been observed in animal model of traumatic and ischemic brain injury, cerebral hypoxia, and epileptic seizures (Hay et al., 1984; Persson et al., 1988; Hatfield & McKernan 1992; Barone et al., 1993; Brandel et al., 1999; Steinhoff et al., 1999). sNSE levels are also reported to increase in epileptic activity due to increased blood-brain barrier (BBB) permeability. Elevation of

sNSE after SE correlated with overall histologic evidence for damage (Jacobi & Reiber, 1988; DeGiorgio et al., 1996; Sankar et al., 1997; Correale et al., 1998; Büttner et al., 1999; DeGiorgio et al., 1999; Schreiber et al., 1999). Although sNSE is not sensitive enough to detect neuronal damage, cNSE seems to be a reliable parameter for assessing neurological insult in patients (Lima et al., 2004). Although multiple reports have documented elevation in NSE levels following neuronal injury in various neurological disorders, little is known about the localization of NSE in different brain regions after chemically induced acute and chronic seizures. Therefore, the present work was designed to investigate changes in NSE immunoreactivity in different brain regions including the cerebral cortex, thalamus, hypothalamus, and hippocampus in the single- and repeated PTZ-induced generalized tonic-clonic seizures in rats.

METHODS

Animals and Seizure Induction

Male Wistar rats weighing 220–260 g, were housed under standard conditions (12 h light-dark cycle), with free access to water and food *ad libitum*. The study was approved by the Institutional Ethics Committee. Animals were divided into three groups: (1) Control group ($n = 7$); (2) Single dose PTZ-treated group ($n = 7$); (3) Repeated doses PTZ-treated group ($n = 7$). In the single dose PTZ-treated group, rats were injected intraperitoneally (i.p.) with 55 mg/kg PTZ (Sigma Chemical Co) and observed for behavioral epileptic activity. One hour later, the animals were sacrificed by transcardial perfusion with 0.9% saline solution, followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, under ether anesthesia. Fixed brains were removed and postfixed in the same fixative overnight. The brains were embedded in paraffin. The animals in the repeated doses of PTZ-treated group were given 55 mg/kg PTZ i.p. on alternate days for six times and then the seizure activity was observed during each seizure period. After the last injection on the sixth day, similar procedure was applied as in the single dose PTZ-treated group. For the control group, saline solution was applied instead of PTZ.

Histology and Immunocytochemistry

Serial coronal sections (5 μm) of the cortex, thalamus, hypothalamus, and hippocampus were cut from brain blocks. Paraffin sections were stained with Cresyl Fast Violet (CFV). CFV binds very strongly to the RNA in the neuron's rough endoplasmic reticulum (Chan & Lowe, 2002). NSE is a

major neuronal protein that catalyzes the interconversion of 2 phosphoglycerate and phosphopyruvate. Immunocytochemistry was performed using the avidin-biotin-peroxidase method. The rehydrated sections were pretreated with 3% hydrogen peroxide for 10 min to abolish endogenous peroxidase activity. Sections were then washed in phosphate buffered saline (PBS)-Triton X (Tx) 100. To eliminate the nonspecific binding, sections were pretreated with normal rabbit serum. The sections were then incubated with anti-NSE primary antibodies (Zymed, Carlton Court, San Francisco) for 24 h at 4°C in a humidified chamber. Following washing in PBS-Tx, biotinylated anti IgG secondary antibodies were applied for 15 min at room temperature. Samples were washed with PBS-Tx and Streptavidin-peroxidase conjugate was applied to the sections for 15 min at room temperature. Following washing in Tris, 0.6% hydrogen peroxide and 0.02% diaminobenzidine (DAB) was applied 5 min at room temp. As control, the primary antibody was omitted and replaced with non-immune serum. Immunoreactivity of NSE was examined by light microscope (BX50F-3; Olympus, Tokyo, Japan).

Statistical Analysis

The number of NSE immunoreactive cells was quantified in 0.5 mm^2 of the rat brains with an X40 objective using an ocular micrometer system (Olympus). NSE (+) cells were counted in selected sections independently each brain regions including the cortex, thalamus, hypothalamus and hippocampus by each of three investigators, who were blinded to the group. NSE immunoreactive cells were counted in ten sections ($n = 10$) for each brain region except hypothalamus ($n = 9$) in the repeated dose PTZ-treated group. Similar levels of brain sections were maintained in all groups according to atlas of Paxinos between plate 23 and 41 (Paxinos & Watson, 1994). The data obtained from NSE (+) neurons were statistically analyzed using the SPSS statistical software package. The means of NSE (+) neurons in three groups were compared using an ANOVA. Values are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was determined as $p < .05$ using Mann-Whitney U-test.

RESULTS

Physiological Data

Fifty-five mg/kg PTZ induced generalized tonic-clonic seizures in all animals. Following i.p. injections, generalized seizures started with the clonus of the

facial and the forelimb muscles, and continued with the neck and tail extensions, loss of straightening reflex with tonic flexion-extention, wild running and usually with extented clonic activities.

Histologic Findings

Different brain regions were examined for neuronal rER and NSE immunoreactivity in the control and PTZ-treated groups using light microscopy. In the control brains, the observed morphology was as follow; nuclei of the neurons were huge in comparison with those of surrounding glial cells; DNA in the nucleus and nucleoli had similar staining properties; dispersed chromatin and prominent nucleoli reflect a high level of protein synthesis. The extensive cytoplasm was basophilic due to extensive rRNA. Nissl method stained RNA, identifying the rER (Nissl substance) as purple blue (violet) material giving the neuronal cytoplasm a granular appearance (Figure 1A, D, G, J).

A slight increase in Nissl staining was observed in the neurons of the cortex, thalamus, hypothalamus, and hippocampus of the single dose PTZ group rat brains comparing to the control group (Figure 1B, E, H, K). However, slight decrease in the amount of nissl staining was noticed in III-VI layer of the cortex in the repeated dose PTZ-treated group (Figure 1C).

The NSE immunoreactivity was largely expressed in the brains of the control and the seizing animals. This immunoreactivity was observed to be robust in the neuronal perikarya and dendrites. Representative coronal sections of NSE (+) cells depicting the cortex, thalamus, hypothalamus, and the hippocampus of the control, single and repeated dose PTZ-treated group are shown in Figure 2. The number of NSE (+) cells from the cortex, thalamus, hypothalamus, and hippocampus of all the groups are shown in Table 1.

In the cerebral cortex, no statistical significant difference was observed in the number of NSE (+) neurons in the single (B, E, H, K) and repeated (C, F, I, L) dose PTZ-treated groups compared to the control group (A, D, G, J), respectively. On the other hand, although a slight decrease in the NSE (+) immunoreactivity in the cortex of the repeated doses PTZ-treated group was noticed compared to the control group (Figure 2A–C; Table 1), a significant increase in the number of NSE (+) cortical neurons was observed in the single dose PTZ-treated group (Figure 2B) compared to the repeated doses PTZ-treated group (Figure 2C) ($F = 2.57$; $df = 2$; $p < .05$).

In the thalamus, the number of the neuron showing NSE immunoreactivity was significantly ($F = 4.68$; $df = 2$; $p < .05$) increased in the repeated doses PTZ-treated group compared to the control group (Figure 2 D, F and Table 1).

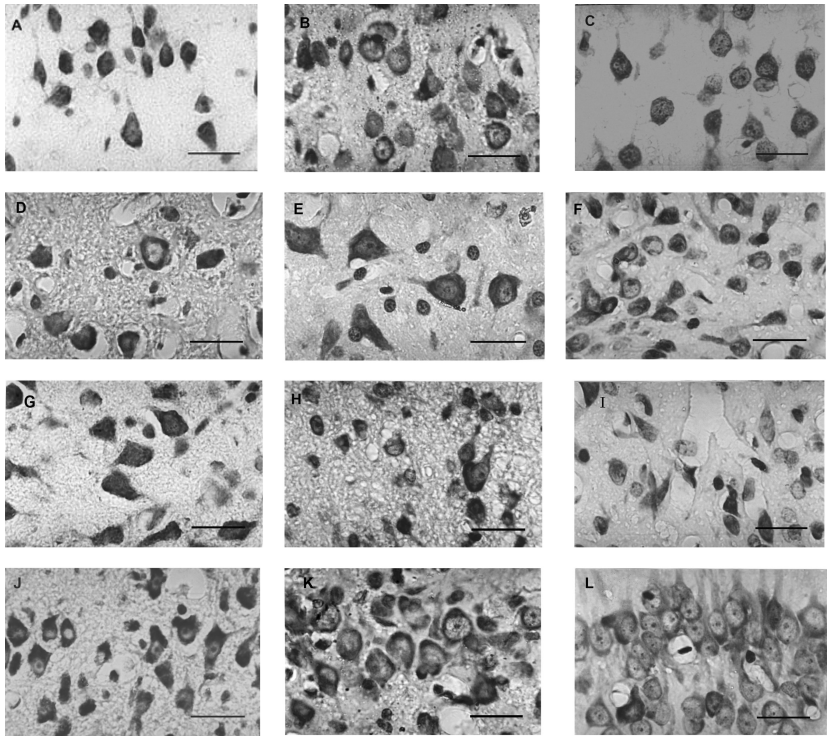

Figure 1. Nissl staining of cerebral cortex (A, B, C), thalamus (D, E, F), hypothalamus (G, H, I), and hippocampus (J, K, L) of the control (A, D, G, J), single dose (B, E, H, K) and repeated doses (C, F, I, L) PTZ-treated group, respectively. Bar, 25 μm.

Although there was a significant ($F = 13.05$; $df = 2$; $p < .001$) increase in the number of NSE (+) hypothalamic neurons of the single dose PTZ-treated group (Figure 2H) compared to the control group (Figure 2G), a significant ($p = .001$) decrease in the number of NSE (+) hypothalamic neurons was detected in the repeated doses PTZ-treated group compared to the single dose PTZ-treated group (Figure 2H, I and Table 1).

In the hippocampus, no statistical significant difference was observed in the number of NSE (+) neurons in the single and repeated PTZ-treated groups. A slight increase of NSE immunoreactivity was seen in the hippocampus of the single dose PTZ-treated group (Figure 2K) compared to the control (Figure 2J) and repeated doses PTZ-treated group (Figure 2L) rats.

Table 1. Number of NSE (+) neurons in the cortex, thalamus, hypothalamus, and hippocampus in the control and epileptic brains following single dose and repeated doses PTZ-induced seizures

Brain regions	Cortex[a]	Thalamus[b]	Hypothalamus[c]	Hippocampus[d]
Groups		Mean ± S.E.M (N)		
Control group	414.00 ± 51.41	224.00 ± 14.86	124.50 ± 6.91	231.30 ± 23.14
Single dose PTZ-treated group	480.10 ± 32.01*	315.70 ± 33.13	251.70 ± 28.42**	263.30 ± 10.97
Repeated doses PTZ-treated group	359.70 ± 23.76	362.20 ± 43.00**	144.66 ± 14.52***	235.10 ± 29.30

*$p < .05$, compared with the repeted doses PTZ-treated group;
**$p < .05$ compared with the control group;
***$p < .05$ compared with the single dose PTZ-treated group.
NSE immunoreactive neurons were counted in 0.5 mm^2 of brain tissues.

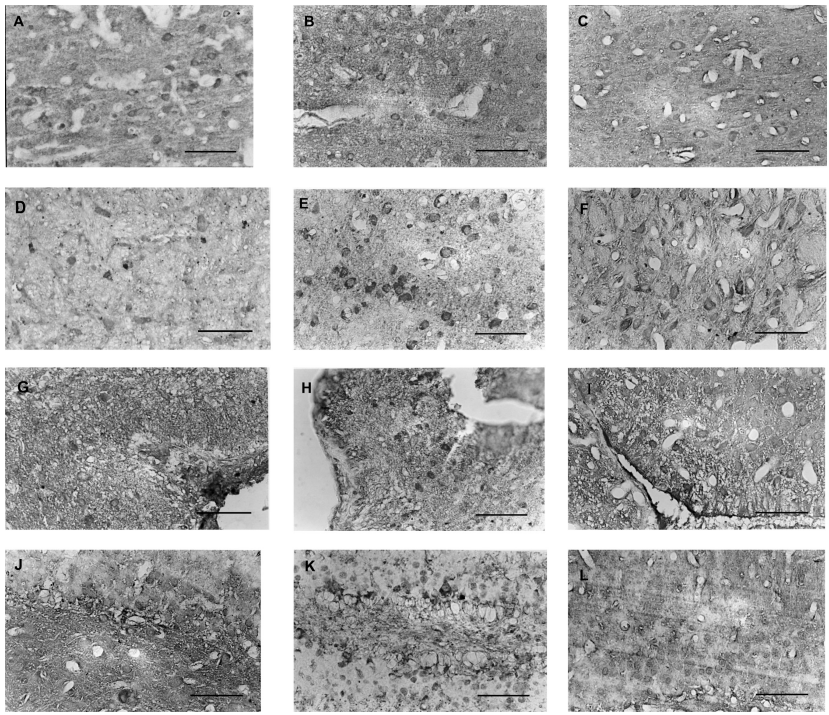

Figure 2. NSE immunostaining of cerebral cortex (A, B, C), thalamus (D, E, F), hypothalamus (G, H, I), and hippocampus (J, K, L) of the control, single dose, and repeated doses PTZ-treated group, respectively. Bar, 100 μm.

DISCUSSION

NSE Immunoreactive Neurons

The results of the present study demonstrated the presence of NSE-immunoreactivity and Nissl staining in neurons of the different brain regions after PTZ-induced seizures. Although NSE (+) neurons significantly increased in the hypothalamic regions of the single dose PTZ-treated group, NSE (+) neurons were found to be increased only in the thalamic region of the repeated doses PTZ-treated group compared to the control group. In addition, NSE (+) neurons were found to be slightly increased in the cortex, thalamus and hippocampus in the single dose PTZ-treated group compared to the control group.

Seizure-Associated Brain Damage and NSE Immunostaining

Several studies have demonstrated that seizure-associated brain damage was initiated by the release of excitatory amino acid neurotransmitters from excessively firing presynaptic terminals in ultimately neurotoxic concentrations. Additionally, pannecrosis through excessive focal tissue acidosis may also contribute to the neurotoxicity processes (Sloviter & Dempster, 1985; Auer & Siejö, 1988). Interictal energetic deficiency in the epileptogenic hippocampus could contribute to impaired glutamate reuptake and glutamate-glutamine cycling that resulting in persistently increased extracellular glutamate. Increased lactate production together with poor lactate and glucose utilization were the cause of worsening of energy metabolism, which then produced glial and neuronal toxicity. It has been reported that in the epileptogenic hippocampus of patients with temporal lobe epilepsy the level of glutamate were increased (Cavus et al., 2005) and glial glutamine synthetase is down regulated (vander Hel et al., 2005). In this study, the number of NSE (+) neurons that was observed to be increased in investigated brain regions after single dose PTZ-induced seizure indicates that these regions become more active metabolically. These regions seems to be compatible with those that shown to have increased metabolic activity during epileptic seizures in previous studies (Siesjo et al., 1986; Rasmussen et al., 1994). NSE immunostaining in the brain is useful for evaluating brain damage from various cases. This also supports the notion that NSE immunoreactivity is spared in less necrotic neurons (Nogami et al., 1998a). The present authors' findings, which showed an increase in NSE immunoreactivity and nissl staining in the single dose PTZ-treated group, were correlated with the results of Nogami et al. (1998a). In another study that

quantified NSE (+) neurons in the frontal cortex and hippocampus of the rat brain after systemic administration of kainic acid, while the concentrations of NSE remained unaffected in the frontal cortex, NSE levels were found to be significantly decreased in the hippocampus (Ding et al., 2000). These results are in accordance with the present findings. In the present study, although the number of NSE (+) neurons in the cortex and hippocampus increased in the single dose PTZ-treated group, it was found to be decreased in the repeated doses PTZ-treated group. Neuronal damage from epileptic insults occurs predominantly in cortical lamina III and IV where thalamocortical afferents terminate, suggesting a transneuronal effect in producing cortical neuronal necrosis in SE (Auer & Siejö, 1988). In accordance with this study, in repeated doses PTZ-treated group, the present authors observed also a slight decrease in NSE (+) immunoreactivity and nissl amount in the cortical and hypothalamic neurons. It is well known that in comparison with the other brain regions, the hippocampal neurons appear to be more vulnerable to the excitatory damage caused by seizures (van Bogaert, 2001). In the present study, no statistically significant difference obtained in the hippocampus after repeated PTZ-induced seizures and the number of NSE (+) neurons in these regions were close to the control values. Pavlova et al. (2005) stated that the rats developed tolerance to PTZ kindled seizures, showing oxidative stress and neurodegeneration in hippocampal region. On the other hand, in the present study, the intensity of NSE staining in the hippocampal neurons was found to be decreased after repeated PTZ-induced seizures. Considering to the results of both study, it can be suggested that oxidative damage of neurons resulting in neurodegeneration in hippocampus was not directly related to the recurrence of convulsive activity.

Correlation between Severity of Nissl Staining and NSE Immunostaining

According to the present results, anti-NSE immunostaining might reflect the cellular damage of neurons during the antemortem period and could add further information about the integrity of neurons, which could be helpful determining the injured brain areas besides the morphological change of neurons assessed by CFV. Nissl substance can usually be seen in neuronal bodies stained with basophilic dyes and consist of rER and associated ribosomes. The ribosomes contain RNA and are the sites of protein synthesis. It imparts a light violet color to the rER. This stain gives a diffused coloration when the rER is less

and spread out, and imparts a granular appearance when the rER is abundant (Young & Health, 2000). Although neurons metabolically are highly active, the Nissl substance is often very prominent (Crossman & Neary, 2000). In this study the Nissl bodies were especially abundant in the perikarya of the cortical, thalamic, hypothalamic, and hippocampal neurons in the control and single PTZ-treated group, but the severity of Nissl staining was less in the repeated PTZ-treated group.

The results also indicated that a significant increase in the number of NSE (+) neurons in the thalamus after repeated PTZ seizures comparing to the control group. This may be related with increase metabolically activity of the thalamic neurons after epileptic seizures. The increase in the number of NSE (+) cells in the brain were in parallel to the other studies that reported an increase in the serum levels of NSE following epileptic seizures (Sankar et al., 1997). NSE is a good indicator of neuronal damage. The level of sNSE and cNSE are increased in brain diseases such as anoxic brain injury and stroke (Hasegawa et al., 2002). sNSE levels are reported to reach maximum levels between 1 and 6 h postictally (Tumani et al., 1999). It appears that this enzyme located in the neurons passes to the serum with the destruction of the BBB and reaches peak levels for a certain amount of time. The reversible opening of the BBB has already been shown during the PTZ-induced epileptic seizure (Sahin et al., 2003).

As a result, while the number of NSE (+) neuron were increased in all investigated brain region of the single dose PTZ-treated group, the same increase was noted only in the thalamic region of repeated dose PTZ. Additionally low NSE immunoreactivity was seen in the cortex, hypothalamus, and hippocampus of the repeated doses PTZ-treated group. These findings suggest that some adaptive changes may develop in the CNS after repeated seizures. On the other hand, low immunoreactivity in the brain regions could be reflecting the lower metabolic state of damaged neurons (Nogami et al., 1998). Cellular and molecular mechanisms related with the metabolic changes that are observed following epileptic seizures may be responsible from the brain damage.

In conclusion, NSE immunoreactivity may be a valuable marker for determining the number of metabolically active neurons and the regions where these changes take place after single and repeated seizures. New studies investigating the neuronal activity changes during the epileptic seizure including modulation of gene expression will give new insight for future projection.

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