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Quantitation of Neuronal Loss Induced by Status Epilepticus and the Effects of Nitric Oxide

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Abstract: Neuronal loss in the hippocampal CA1 and CA3 fields due to lithium-pilocarpine-induced status epilepticus (SE) and the effects of a nitric oxide (NO) substrate, L-Arginine and a NO synthase inhibitor, N^w-nitro-L-Arginine methyl ester (L-NAME) on SE-induced brain damage were determined using the neuron counting procedure in rats. Rats were implanted with four chronic, epidural screw electrodes in order to obtain electrocortical recordings. SE was determined from the electrocortical recordings. After decapitation, the brains were removed, and the hippocampal sections were obtained and stained with hematoxylin and eosin. An analysis of variance (ANOVA) and Student's t- test were used for statistical purposes. The normal pyramidal neuron numbers of rats which had exhibited SE were significantly lower than those of rats that had not displayed SE. Both L-Arginine and L-NAME increased the neuronal loss induced by SE. We concluded that lithium-pilocarpine-induced SE leads to neuronal loss in the hippocampal CA1 and CA3 fields. L-Arginine and L-NAME, which were expected to show opposite actions, acted in a similar manner at the given doses.

Key Words: Lithium-pilocarpine, Status epilepticus, L-Arginine, L-NAME, Neuron loss.

Introduction

Temporal lobe epilepsy (TLE) is a common neurological disorder which is frequently drug-resistant. A number of studies have focused on the pathophysiological mechanisms underlying this disease. The common neuropathological lesion is hippocampal sclerosis, characterized by neuronal loss and gliosis in the hippocampus (1). It has been reported that a severe convulsion in early childhood may be responsible for the characteristic neuronal loss in hippocampal sclerosis (2). The most vulnerable neurons in the hippocampus are CA1 and CA3 pyramidal cells and dentate hilar neurons, whereas CA2 neurons and dentate gyrus granule cells are relatively resistant (3).

It has been suggested that the cellular pathology associated with sustained seizure activity is due to seizure-mediated release and inefficient reuptake of excitatory amino acids (EAA), namely glutamate and aspartate (4). Excessive stimulation of N-methyl-D-aspartate (NMDA)-type EAA receptors is related to the induction and maintenance of the epileptic condition and also epileptic brain damage (5,6). It has been shown that a noncompetetive NMDA antagonist, ketamine, protects neurons from status epilepticus (SE)-induced damage (7).

It is now well known that nitric oxide (NO) mediates some of the physiological and pathological effects of NMDA receptor stimulation. NO is a free radical gas, which serves as a neuronal retrograde messenger in the central nervous system (CNS) (8-10). It is synthesized from L-Arginine by calcium/calmodulin-dependent NO synthase in association with the activation of NMDA receptors (8,10,11). When these receptors are activated, cytosolic Ca^{2+} increases due to the influx of large amounts of Ca^{2+} through the receptor-associated ion channels; this Ca^{2+} combines with calmodulin and activates NO synthase (12).

The aim of this study was to quantitate the neuronal loss due to SE induced by systemic injection of pilocarpine in lithium-pretreated rats and to investigate the effects of a NO substrate, L-Arginine, and a NO synthase inhibitor, N^w-nitro-L-Arginine methyl ester (L-NAME), on SE-induced brain damage.

Material and Methods

Male Wistar albino rats (Uludağ University, Experimental Animals Breeding and Research Centre, Bursa, Turkey) weighing 240-340 g were used. The rats were kept in a temperature-controlled room (18-22 °C) and were given food and water ad libitum.

Under Thiopental Sodium (Penthotal Sodium-Abott) anesthesia, the rats were implanted with four chronic, epidural screw electrodes in order to obtain electrocortical recordings. The electrodes were implanted bilaterally over the fronto-parietal cortices (±2 mm behind the bregma and ±2 mm lateral to the midline) and an additional electrode on the nasal bone served as a reference electrode. The rats were allowed to recover from surgery over seven days. SE was induced by systemic administration of LiCl (3 mEq/kg, i.p.), which was followed 24 hours later by a pilocarpine HCl injection (45 mg/kg, i.p.). L-Arginine (300 mg/kg, i.p.) or L-NAME (20 mg/kg, i.p.) was given 30 minutes before the pilocarpine. Each group consisted of 15 rats. All the rats were observed electrocortically and behaviourally for three hours after the pilocarpine injection. SE was determined from electrocortical recordings. In addition, electrodes were implanted in four rats and these rats received saline in order to form the sham-control group. Four other rats were used as naives and none of the interventions were applied to these rats.

All drugs were purchased from Sigma Chemical Co., St Louis. LiCl was dissolved in distilled water; pilocarpine, L-Arginine and L-NAME were dissolved in saline at a volume of 0.2 ml.

At the end of the experiments, the rats that had survived were placed individually in cages and allowed to live for 12 days. Then they were decapitated, and the brains were rapidly removed and frozen with CO_2 gas. Because of the high mortality observed in the L-NAME-applied rats (all rats that underwent SE in this group died within129±15 minutes after the pilocarpine injection), these rats were decapitated whenever a consistent flat line was observed on the electrocorticogram.

To obtain neuron counts from both the anterior and posterior hippocampus,12 µm coronal cryostat sections were obtained in the levels corresponding to bregma 2.8 and 5.6 of the Paxinos and Watson (13) atlas. From each level, two sections were obtained and stained with hematoxylin and eosin. In each section, undamaged pyramidal neurons of the hippocampal CA1 and CA3 fields were counted using an ocular grid at a total magnification of 400x. Only neurons with a visible nucleus and/or a complete cell contour were counted (14). Neurons touching the upper or left borders were included and those touching the bottom or right borders were rejected. For each hippocampal zone, neurons in five squares with an area of 2300 μm^2 were counted from four independent fields. The cell counts were corrected by Abercrombie's formula (15) and the average areal density of neurons in mm^2 for each hippocampal field in each section was then calculated. The neuron counts from each section in the same level were then averaged. In addition, since there was no statistically significant difference in terms of neuron counts between two hemispheres or between two levels, all the values for each hippocampal field were averaged for each rat and these values were used for statistical analysis.

Statistical analysis: For each rat, the neuron numbers in the anterior and posterior hippocampus and right and left hemispheres were compared with the Student's t test (unpaired). Comparisons of the neuron counts of all the groups, including SE-positive (+) and SE-negative (-) rats, were performed with an analysis of variance (ANOVA).

Results

The average pyramidal neuron numbers in the hippocampal CA1 and CA3 fields of the naives, sham-control group, control group, L-Arginine group and L-NAME group rats are shown in the Table. There was no statistically significant difference between the neuron counts of the naives, sham-control group and the control group SE (-) rats. In the control group, the neuron numbers in both hippocampal fields in the SE (+) rats were significantly lower than those in the SE (-) rats ($p<0.001$). The pyramidal neurons of the CA1 and CA3 fields were also lost due to SE in both the L-Arginine and L-NAME groups ($p<0.001$ for each group).

When compared to the neuron counts of the SE (-) rats of the control group, the neuron numbers in both fields of the SE (-) rats in the other two groups did not differ significantly. On the other hand, the neuron counts of the SE (+) rats of all the groups significantly decreased with respect to those of the control group SE (-) rats ($p<0.001$). A characteristic view of the neuronal damage was observed in the light microscopic analysis of the hippocampi of the SE (+) rats, with acidophilic neurons, as well as gliosis (Figures 1 and 2).

An interesting result regarding the effects of L-Arginine and L-NAME was that the neuron loss induced by SE was significantly greater in both hippocampal fields in

rats that were treated with either of these drugs ($p<0.001$). There was no significant difference between the L-Arginine and L-NAME groups with regard to the neuron counts of SE (+) rats.

Discussion

In this study, we induced SE by injection of a low-dose cholinergic muscarinic agonist, pilocarpine, in LiCl-pretreated rats. The neuronal damage observed in SE

Table. The average undamaged pyramidal neuron numbers in CA1 and CA3 fields of naives, sham-control, control, L-Arginine and L-NAME groups.

Groups	Neuron Numbers in CA1 Field ($\bar{x}$ ± SE / mm^2)	Neuron Numbers in CA3 Field ($\bar{x}$ ± SE / mm^2)
Naives (n=4)	1097.95 ± 29.01	706.27 ± 20.78
Sham-control group (n=4)	1208.85 ± 25.74	748.53 ± 15.45
Control group SE (-) (n=3)	1092.08 ± 53.90	703.00 ± 17.65
Control group SE (+) (n=4)	600.68 ± 46.99 *	426.75 ± 11.61 **
L-Arginine group SE (-) (n=4)	1227.57 ± 34.79	799.36 ± 18.28
L-Arginine group SE (+) (n=4)	185.92 ± 40.51*ψ	192.21 ± 43.79 **α
L-NAME group SE (-) (n=4)	1234.04 ± 44.70	775.94 ± 25.24
L-NAME group SE (+) (n=4)	130.31 ± 28.67 *δ	115.13 ± 19.73 **π

* $p<0.001$ vs control group SE (-) rats for CA1 field.
** $p<0.001$ vs control group SE (-) rats for CA3 field.
ψ $p<0.001$ vs L-Arginine group SE (-) rats for CA1 field.
α $p<0.001$ vs L-Arginine group SE (-) rats for CA3 field.
δ $p<0.001$ vs L-NAME group SE (-) rats for CA1 field.
π $p<0.001$ vs L-NAME group SE (-) rats for CA3 field.

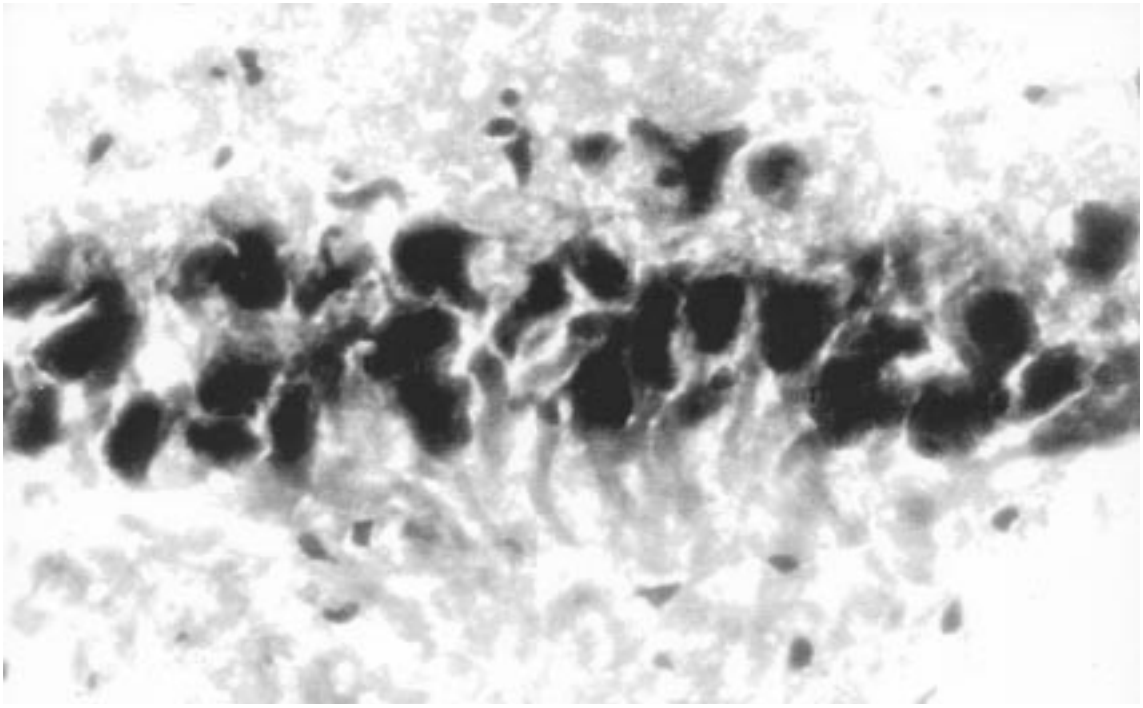

Figure 1. The normal appearance of the large pyramidal neurons of the hippocampal CA3 field. Sections were obtained from a normal rat. H&E, x40. (npn: normal pyramidal neuron).

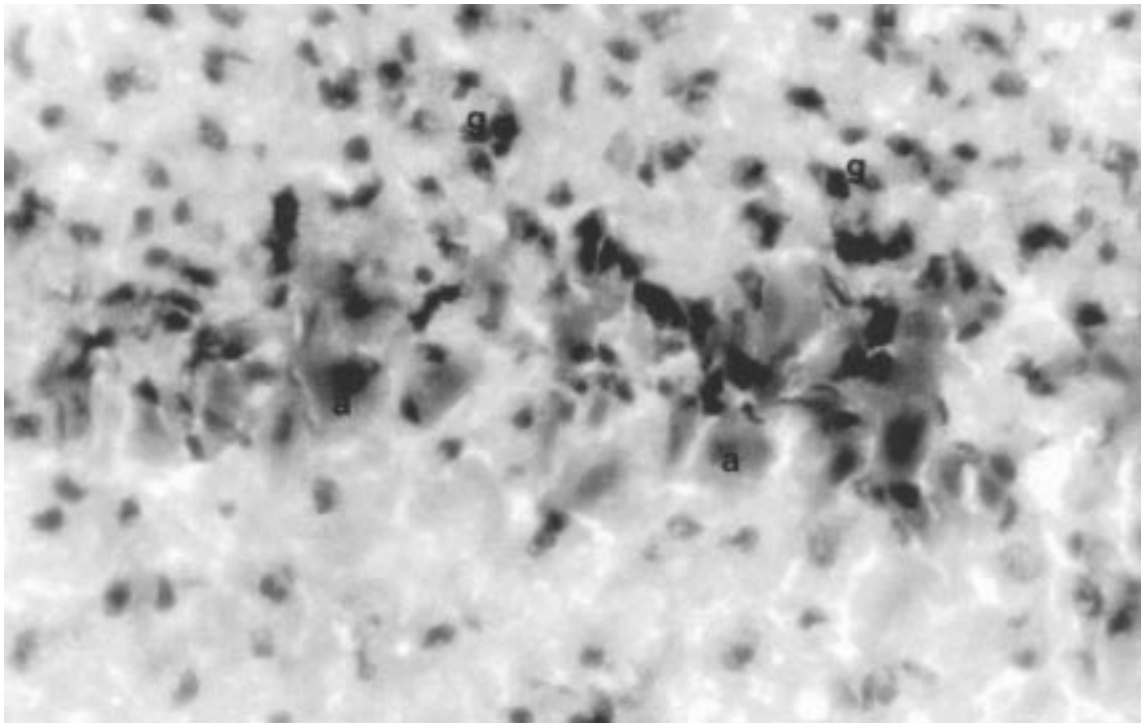

Figure 2. Microphotograph of the CA3 field of a rat that had displayed SE and was sacrificed on the twelfth day. Note the acidophilic neurons and gliosis. H&E, 40. (a: acidophilic neurons, g: gliosis).

induced by high-dose pilocarpine or lithium-pilocarpine have been shown to mimic the neuropathological features of human TLE (16-19). It has been reported that acetylcholine itself is not neurotoxic, since intraventricular injection of high doses of acetylcholine does not produce direct glial or neurotoxic effects, while intraventricular injections of glutamate and aspartate reproduce the characteristic morphological changes observed in human epileptics (5). Secondary activation of glutamatergic and aspartergic neurons due to hyperstimulation of cholinoceptive neurons may explain the neuronal damage observed in cholinergic seizures (4).

In this study, we found no significant difference concerning the neuron numbers between the naives, sham control group and control group SE (-) rats. However, when SE was well established, the pyramidal neuron numbers were significantly reduced ($p<0.001$). This implies that a severe seizure activity is necessary to induce characteristic neuron loss in this model of epilepsy. These findings are consistent with quantitative studies performed in rats (18) and in humans (2).

There have been a number of studies investigating the role of NO in the pathogenesis of epileptic seizures, but the effect of NO on seizure-induced brain damage has not been widely studied yet. Bagetta et al. (6) reported that intracerebroventricular microinfusion of L-NAME significantly reduces the seizures and brain damage induced by systemic injection of tacrine, an acetylcholinesterase inhibitor, in LiCl-pretreated rats. In our study, both L-Arginine and L-NAME significantly worsened the neuron loss due to lithium-pilocarpine-induced SE. The contradictory results may be due to the different routes of drug injection.

It is quite interesting that both a NO substrate and a NO synthase inhibitor increased the neuronal loss induced by SE, but it is difficult to explain these results. The dosage of these drugs may be the major point for their neuroprotective or neurodestructive actions, since NO is known to exert both of these effects, depending on several mechanisms. Stimulation of NMDA receptors results in the formation of both NO and superoxide anions (20). The interaction of NO with superoxide anions yields peroxynitrite anions that become protonated to peroxynitrous acid in aqueous solutions, which spontaneously decomposes into cytotoxic hydroxyl radicals (21,22). This may be responsible for the neurotoxic effects of NO. On the other hand, NO not only

activates guanylate cyclase and leads to an increase in cGMP in target cells in response to NMDA receptor stimulation, but it may also exert a negative feedback effect on its own synthesis by inhibiting a redox modulatory site on the NMDA receptor-channel complex (23,24). Such an inhibitory mechanism may inhibit excessive stimulation of NMDA receptors and prevent neurotoxicity. In addition, the antioxidant property of NO through its interaction with superoxide anions may account for its neuroprotective effect (25).

As a result, we conclude that SE induced by lithium-pilocarpine leads to significant neuron loss in the hippocampal CA1 and CA3 fields, which we determined quantitatively. NO exerts contradictory effects on SE-induced neuron loss in this model of epilepsy, which should be investigated by further dosage studies.

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