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Pentylentetrazol Associated Changes of Hippocampal Neurons in Immature Rats

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Abstract: Using histochemical analysis, the NADPH-diaphorase, Fluoro-Jade B and bis-benzimide (Hoechst 33342) the effect of intraperitoneal administration of pentylentetrazol (PTZ) on hippocampal neurons was studied. 18-day-old male rats of the Wistar strain received PTZ (60 mg/kg) in one dose. The next day, the 19-day-old animals were transcardially perfused with 4% paraformaldehyde under deep thiopental anaesthesia. Cryostat sections were stained to identify NADPH-diaphorase positive neurons that were then quantified in the CA1 and CA3 areas of the hippocampus, in the dorsal and ventral blades of the dentate gyrus and in the hilus of the dentate gyrus. Combination of the Fluoro-Jade B and bis-benzimide (Hoechst 33342) staining was used in the same areas, to identify possible neurodegeneration. Number of NADPH-d positive neurons was higher after pentylentetrazol administration in CA1 and CA3 areas of the hippocampus and in the hilus of the dentate gyrus, compared to the control group which we consider as baseline. Morphological alterations (cell loss) in CA3 area of the hippocampus and in the hilus of the dentate gyrus only (evaluated by Hoechst 33342) were found in animals receiving PTZ; no FJ-B positive cells were found and we can conclude that neurons were destroyed by the PTZ insult.

Introduction

Human epilepsy is a symptom complex, which encompasses many different seizure types with different etiologies [1]. Many animal models of human epilepsy have been established in past decades. One of the most useful is pentylentetrazol induced various types of generalized seizures in rats [2].

It is well established that the administration of pentylentetrazol in high doses in rodents induces generalized tonic-clonic seizures. In rodent PTZ elicits two different types of motor seizures: minimal, predominantly clonic and major tonic-clonic generalized seizures. PTZ is a selective blocker of the chloride ionophore complex to the $GABA_A$ receptor, it has convulsant effects after repeated or single dose administration and it affects the GABAergic and the glutamatergic system in many brain areas including the hippocampus [3].

One of the possible mechanisms of brain injury is the activation of some enzymes as nitric oxide synthases, resulting in nitric oxide (NO) production. NO is produced from L-arginine (L-Arg) by nitric oxide synthase (NOS). There are at least three different forms of this enzyme [4], the endothelial (eNOS) that is responsible for cardiovascular actions, the inducible (iNOS) found originally in macrophages and involved mainly in immunological processes and the neuronal (nNOS). Neuronal NOS is a constitutive enzyme, which is expressed only by a few percent of neurons. The production of NO is a calmodulin- dependent process; therefore, it must be preceded by the elevation of intracellular Ca^{2+}-concentration [4]. It has been observed that nNOS produces NO almost exclusively after activation of N-methyl-D-aspartate (NMDA) receptors [6].

It is well known that nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) may correspond to NOS, and it is therefore suggested that neurons containing NADPH-d might be capable of producing NO [7].

The NADPH-diaphorase histochemical technique is based on the presence in certain neurons of an enzyme that can catalyze the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble, visible formazan [8]. Although the NADPH diaphorase activity has been well defined histochemically, the function of this enzyme has remained a mystery [8]. This relatively simply technique can be used to study the nitrergic system and NADPH-d histochemistry is a useful method to detect neurons producing the neuroactive molecule NO. Furthermore, some evidence suggests that NADPH-d activity would be directly related to NO synthesis [9].

The aim of our study was to observe the acute morphological changes in the hippocampus after systemic PTZ administration in convulsive doses. The second aim was to describe and compare the postnatal development of NOS activity (using histochemical method NADPH-diaphorase staining) in immature hippocampus (18-day-old rats) and to identify alterations of the nitrergic system after the administration of this excitotoxic substance, during the period of structural and functional maturation of CNS.

To evaluate the histological changes bis-benzimide (Hoechst 33342) and Fluro-Jade B dye were used.

Materials and Methods

Male rats 18-day-old of the Wistar strain of our own breed were used for the experiments. They were housed at a constant temperature (23°C) and relative humidity (60%) with a fixed 12 hr light/dark cycle and fed with food and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies. There were 12 animals in all the experimental groups, six animals in each group:

- 18-day-old animals received corresponding volumes of normal saline solution i.p.
- 18-day-old animals received one dose of pentylentetrazol (60 mg/kg) i.p.

For the histological analysis only animals, which manifested tonic-clonic seizures were used. Brains of four animals in each group were stained by NADPH-diaphorase method and brains of two animals in each group were stained by combination of Fluoro-Jade B and Hoechst staining. In each brain 25–30 sections were examined and quantified. Animals were studied the 19th day, one day after the drug administration. All rat pups were perfused under deep thiopental anaesthesia with 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.4. Brains were removed, postfixed for one hour in 4% buffered paraformaldehyde and then

submerged for 1h into 20 % sucrose for cryoprotection. Each brain was sliced in the frontal plane into 40 μm thick sections with a cryostat. Two different histochemical methods were used:

NADPH-diaphorase staining
For the NADPH-diaphorase staining the free-floating sections were placed in 0.1 M phosphate buffer and incubated in 0.1 M phosphate buffer containing 0.5 mg/ml β-NADPH (Sigma), 0.2 mg/ml Nitro blue tetrazolium (NBT, Sigma) and 0.3 % Triton (Sigma) for 4 h at 37 °C in thermostat. Following the reaction, sections were rinsed in 0.1 M phosphate buffer and kept at 8 °C for 16 h. The histochemically reacted sections were mounted on precleaned 0.5 % gelatine-coated microscope slides (Menzel-Gläser), air-dried and cover slipped with microscope cover glasses (Menzel-Gläser) using D.P.X. neutral mounting medium (Aldrich) [10]. NADPH-d positive neurons were then quantified in five regions of the hippocampal formation (each section was taken and evaluated) (Figure 1):

- In CA1 area of the hippocampus,
- In CA3 area of the hippocampus,
- In the hilus of the dentate gyrus,
- In the dorsal blade of the dentate gyrus,
- In the ventral blade of the dentate gyrus.

Hippocampus between the AP plane corresponding to 2.5 mm and 4.0 mm posterior to the bregma in adults was subjected to quantification of NADPH-d positive neurons under the light microscope Olympus Provis AX 70 with epifluorescence. For the statistical evaluation, ANOVA and the unpaired t-test (GraphPadPrism) were used (level of significance was set at $p<0.05$).

Combination of the Fluoro-Jade B (Histo-Chem Inc.) and bis-benzimide 33342 Hoechst (Sigma)
Fluoro-Jade B (FJB) is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration [11]. Hoechst 33342 staining was

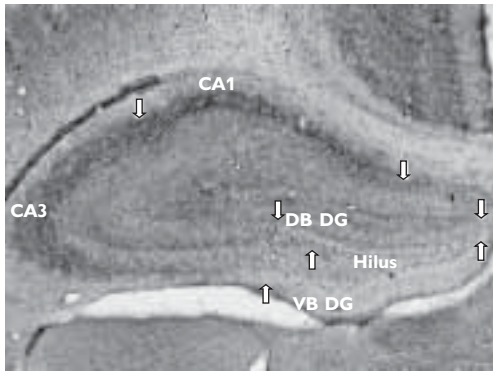

Figure 1 – Hippocampus – investigated areas of hippocampus: CA1 and CA3 areas of the hippocampus, Hilus of the dentate gyrus, DB DG – dorsal blade of the dentate gyrus, VB DG – ventral blades of the dentate gyrus. NADPH-d staining. Direct magnification 40×. The microphotograph was made using the microscope OLYMPUS AX70 Provis with digital camera OLYMPUS DP70.

used as an apoptotic marker, which detects apoptotic nuclei with condensed and/or fragmented DNA.

After cryostat sectioning free-floating slices were placed in 0.1 M phosphate buffer. Tissue sections were then mounted onto gelatinized slides and allowed to dry at room temperature. Slides were than placed in staining racks (one slide/slot for even staining) and immersed in 100% ethanol solution for 3 minutes, in 70% ethanol solution for 1 minute, in distilled water for 1 minute, in 0.01% potassium permanganate ($KMnO_4$) (Sigma) for 15 minutes with gentle shaking. Slides were washed in distilled water three times. Staining proceeded in dim place by immersing slides into 0.001% Fluoro-Jade B solution for 30 minutes with occasional gentle shaking [11]. After that slides were rinsed in the distilled water three times for 1 minute. Slides were then immersed in 0.01% Hoechst staining solution for 10 minutes and dehydrated (in ethanol series); cover-slipped using D.P.X. neutral mounting medium and allowed to dry. Fluoro-Jade B positive neurons were studied in the same hippocampal regions as those used for NADPH-d evaluation (each section was taken and evaluated). The tissue was examined using an epifluorescent microscope with blue (450–490 nm) excitation light.

Semiquantitative analysis of Fluoro-Jade B (FJB) positive cells was done by two independent experimenters in all slices. Each area of the hippocampus in the slice

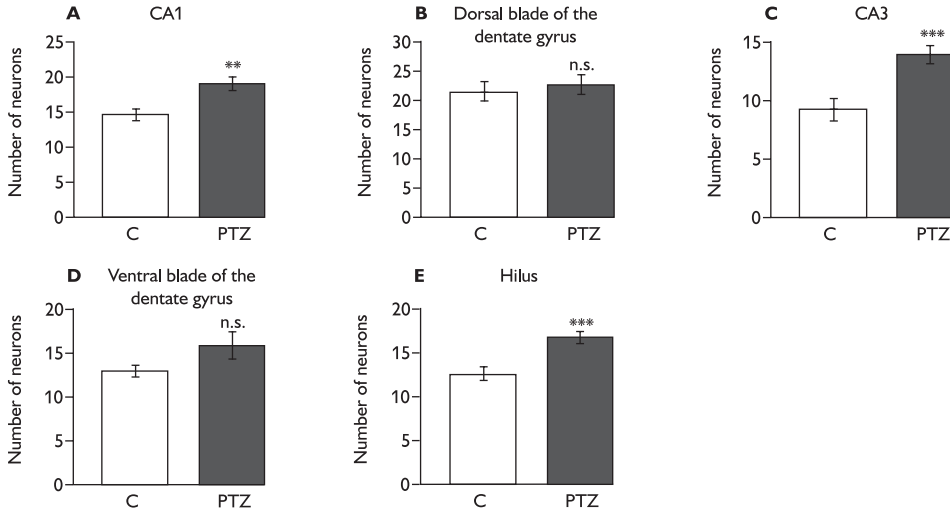

Figure 2 – (A) Number OF NADPH-d positive neurons in CA1 area of the hippocampus per section area, (B) Number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area, (C) Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (D) Number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (E) Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, C = control group received corresponding volumes of normal saline solution, PTZ = animals received one dose of pentylentetrazol. Mean ± S.E.M. Level of significance; $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*).

was evaluated by the scale: (0) no FJB positive cells in field of view, (+) sporadically present FJB cells in field of view, (++) groups of FJB positive cells in field of view (+++) massive alteration, the study area is completely undergoing degeneration [12].

Results

Pentylentetrazol treatment brought about higher number of nitrergic neurons in CA1 hippocampal area by 28% ($p < 0.01$), in CA3 area of the hippocampus by 48% ($p < 0.001$) and in the hilus of the dentate gyrus by 31% ($p < 0.001$). No changes in the number of nitrergic neurons in both of the blades of the dentate gyrus were detected (Figure 2).

Bis-benzimide dye revealed the morphological alterations (cell loss) only in CA3 area of the hippocampus and in the hilus of the dentate gyrus in animals receiving PTZ. CA1 area of the hippocampus and both blades of the dentate gyrus remained intact. Semiquantitative analysis did not detect FJ-B positive cells in CA1 and CA3 areas of the hippocampus and in both blades of the dentate gyrus, but in the hilus of the dentate gyrus FJ-B positive cells were sporadically found. We conclude that the neurons were impaired by PTZ insult (Table 1).

Discussion

In present study, we were observed morphological changes in immature hippocampus after PTZ administration. It is well known that this substance cause epileptic seizures and brain damage, acting on defined receptors groups and it is well documented that the consequences of status epilepticus in the developing brain differ from those of the mature brain [13, 14, 15, 16].

Previous studies in brain devoid of cerebellum have shown that reactive oxygen species have been implicated in PTZ-induced seizures and kindling [15]. More recently, it has been shown that PTZ administration at the convulsive (but not

Table 1 – Semiquantitative analyze of Fluoro-Jade B positive cells in experimental groups

	Exp. Group	
Area	Control	PTZ
CA1	0	0/+
CA3	0	0/+
Hilus	0	+
VB DG	0	0
DB DG	0	0

Control-control group received equal dose of normal saline solution. PTZ – pentylentetrazol treated rats (60 mg/kg). Each area of the hippocampus in the slice was evaluated by the scale: (0) no FJB positive cells in field of view, (+) sporadically present FJB cells in field of view, (++) groups of FJB positive cells in field of view (+++) massive alteration, the study area is completely undergoing degeneration

subconvulsive) dose caused significant increase in the whole brain fatty acid content but did not change the levels of the antioxidant enzymes superoxide dismutase and glutathione peroxidase in rat hippocampus [16].

In this experiment PTZ administration leads to the injury of CA3 area of the hippocampus. CA1 area of the hippocampus remained unaffected. This was detected by FJ-B dye. It is well documented that the CA3 area of the hippocampus is most likely the most vulnerable structure of the hippocampal formation [17, 18, 21] and epileptiform activity propagates from this area to CA1 area of the hippocampus via Schaeffer's collaterals. Some investigators [17, 18] speculated that the selective destruction of CA3 area of the hippocampus may paradoxically protect the area CA1. This hypothesis was sustained by our previous experiments where the other one excitotoxin kainic acid, which causes epileptic seizures, acting on non-NMDA receptors subfamily, has been applied once or repeatedly. The results showed that subconvulsive dose of this compound, applied six times, selectively extinct pyramidal cells of CA3 area of the hippocampus while neurons in CA1 area remained practically intact [18].

In this study the pentylentetrazol administration brought about higher numbers of NADPH-diaphorase positive neurons in CA3 and CA1 areas of the hippocampus and hilus of the dentate gyrus in the comparison with either group of control animals. The nitrergic system can be influenced by many insults. This fact has been reported repeatedly [22, 23, 24]. NADPH-d positive neurons were purposed to be relatively resistant to the injury caused by status epilepticus [19, 20], however, the findings related to vulnerability or this neuronal population survival after status epilepticus are still controversial. We can conclude that pentylentetrazol induced changes affecting the nitrergic system of the rat's hippocampus and that these changes are region specific. These results are in line with findings of other authors, where Fluoro-Jade B dye has been proposed as a useful agent to elucidate neuronal degeneration.

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