Specificity of Action of Epileptogenic Agents in Diazepam-Tolerant Rats

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The development of tolerance in rats by repeated intraperitoneal injection of 0.5 mg/kg diazepam increased the sensitivity of the frontal cortex to the epileptogenic effect of NMDA and kainic acid. The median effective dose of NMDA was shown to decrease under these conditions. Intracerebroventricular injection of NMDA in these doses induced clonic seizures and tonic extension of the forelimbs.

Key Words: *tolerance; benzodiazepines; seizure activity; excitatory amino acids*

The development of diazepam tolerance is associated with changes in functional activity of GABA-A benzodiazepine receptors (absence of the increase in their affinity typical of seizures) [7]. Moreover, benzodiazepine tolerance is characterized by dysfunction of the endogenous excitatory amino acid system. Some previous studies revealed potentiation of the effect of glutamate and NMDA receptor agonists [2,4], while others demonstrated their decreased effectiveness [5].

Here we studied the sensitivity of brain structures in diazepam-tolerant rats to epileptogenic agents with various mechanisms of neurotropic action.

MATERIALS AND METHODS

Acute experiments were performed on male Wistar rats weighing 180-250 g. The tolerance of animals was induced by intraperitoneal injection of diazepam (Gedeon Richter) in a dose of 0.5 mg/kg for 4 weeks [1]. Seizure activity was tested by the end of each week. Pentylenetetrazole was injected intraperitoneally in a dose of 40 mg/kg. We estimated the number of animals with generalized tonic-clonic seizures. The increase in the number of animals with generalized seizures after treatment with diazepam reflects the development of tolerance (Table 1). Control animals received an equivalent volume of 0.9% NaCl.

Twenty-four hours after the last injection of diazepam, tracheostomy was performed under ether anesthesia and artificial pulmonary ventilation was started. The animals were fixed in a SEZh-3 stereotactic device. d-Tubocurarine (Orion) was injected intraperitoneally in a dose of 2 mg/kg to cause muscle relaxation. Soft tissues of the head and ear holder-compressed areas were infiltrated with 0.5% novocain solution. The skull was trephined. The dura mater was dissected. The frontal cortex was exposed. Focal epileptiform activity (EPA) was induced by application of a filter paper (2×2 mm) soaked with convulsant solutions onto the cerebral cortex. Electrical activity was recorded unipolarly. The reference electrode was fixed to the nasal bones of the skull. Bioelectric signals were recorded on a computer electroencephalograph (DX system). The following solutions of convulsants were prepared *ex tempore*: benzylpenicillin sodium salt (10,000 U/ml), NMDA (10.0 mg/ml, Sigma), kainic acid (2.0 mg/ml, Sigma), and strychnine nitrate (30.0 mg/ml).

The power of focal EPA was expressed in arbitrary units. It was calculated as the product of the mean frequency of spike potential generation (Hz) and mean amplitude of spike potentials (mV) over a 1-min epoch of EEG recording. The duration of focal EPA was evaluated as the period between the first and last

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spike potentials. Only animals without ictal discharges were taken into account.

NMDA was injected into the lateral cerebral ventricle through a cannula. The cannula was implanted according to stereotaxic coordinates (AP=0.8; L=1.5; H=3.5) [3] and fixed to the surface of the skull with a fast-hardening plastic (Noracryl). The experiment was performed on day 14 after surgery. NMDA was dissolved in 0.9% NaCl and administered (10 μl) for 10-15 sec via a Hamilton microsyringe (SGE). After microinjection, the animals were maintained in a plastic chamber and observed for 30 min.

The effective doses of NMDA (ED_{16}, ED_{50}, ED_{84}, and ED_{100}) causing clonic seizures and tonic extension of the forelimbs (TEFL) [6] in 16, 50, 84, and 100% animals, respectively, were evaluated by the probit analysis.

The results were analyzed by ANOVA and Newman—Keuls test.

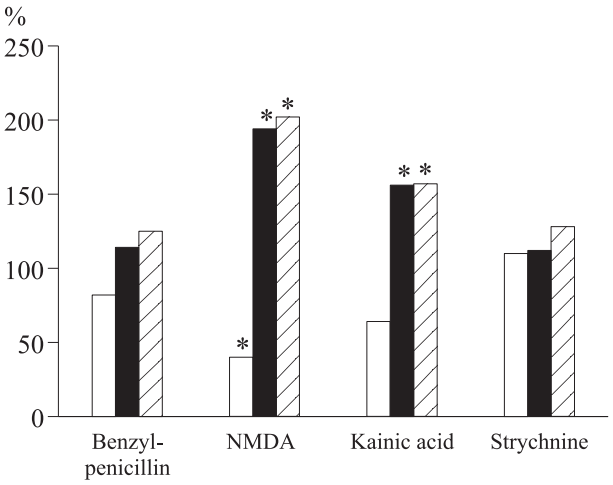

Fig. 1. Dynamics of focal EPA due to application of epileptogenic agents to the frontal cortex of diazepam-tolerant rats. Light bars, latency; dark bars, power of EPA; shaded bars, duration of EPA. *p<0.05 compared to the control. Control, 100%.

RESULTS

Application of penicillin solution to the frontal cortex was followed by the appearance of spike discharges. The latency of discharges in control animals was 7.5±0.6 min. At the peak of activity, the power of focal EPA was 59.4±4.5 arb. units and its duration was 73.7±10.2 min. The latency of focal EPA in diazepam-tolerant rats was shorter than in controls by 17.3%. However, the power and duration of EPA in diazepam-tolerant animals were higher than in control rats (by 13.5 and 25.5%, respectively; p>0.05; Fig. 1). Application of NMDA solution was followed by generation of spike potentials in diazepam-tolerant rats. The latency of this effect in diazepam-tolerant animals was by 60% shorter than in controls (p<0.05). The frequency and amplitude of discharges were shown to increase progressively over 3-10 min. Ictal discharges were found in 3 of 8 animals. Before the appearance of ictal discharges, the power of focal activity in diazepam-tolerant rats was 1.94-fold higher than in controls (p<0.05). The duration of focal EPA in diazepam-tolerant animals was 2 times higher than in control rats (p<0.05). Application of kainic acid was followed by the first spike discharges. Their latency was by 35.7% shorter than in the control (p>0.05). Ictal discharges in 2 of 7 rats were revealed on minutes 15 and 21, respectively. The duration of focal EPA in animals of the treatment group exceeded that in controls (by 56.9%, p<0.05). The latency of spike discharges after application of strychnine to the frontal cortex was 9.6% greater compared to the control. The power and duration of focal EPA in treated rats were higher than in controls by 12.5 and 28%, respectively (p>0.05; Fig. 1).

In control animals, intracerebroventricular injection of NMDA in a dose of 0.5 μg was followed by swift runs, exophthalmos, and increased tone of the tail 7-30 sec after microinjection. Over the next 15-40 sec, clonic seizures and fast runs were revealed in 3 of 10 rats. The estimated ED_{50} of NMDA inducing clonic seizures was 0.69 μg (Fig. 2). Injection of

TABLE 1. Effect of Long-Term Treatment with Diazepam on Pentylenetetrazole-Induced Generalized Tonic-Clonic Seizures (40 mg/kg) in Rats

Group	Ratio of rats with seizures (4-5-point severity) to the total number of animals per group					
	single administration of preparations	period of administration, weeks				
		1	2	3	4	5
Control (physiological saline+ pentylenetetrazole, 40 mg/kg)	15/15	14/15	12/12	10/11	10/10	8/8
Diazepam (0.5 mg/kg)	8/15*	6/15*	10/15	9/14	7/10	5/7

Note. *p<0.05 compared to the control (Fisher's exact test for fourfold table).

NMDA in a low dose (0.5 μg) to diazepam-tolerant rats was followed by short-term freezing (5-15 sec postinjection). This reaction was accompanied by high amplitude and rate of respiration, exophthalmos, and increased tone of the tail. Clonic seizures, jumps, and fast running in 90% animals were observed over the next 10-30 sec. The estimated ED_{50} of NMDA inducing clonic seizures was 0.30 μg (2.3-fold lower than in control animals; $p<0.05$; Fig. 2). ED_{100} of NMDA in these rats was 2.18 times lower than in controls ($p<0.05$, Fig. 1).

Jumps and fast running of intact rats were revealed 15-50 sec after administration of NMDA in a dose of 5.0 μg. TEFL was observed in 2 of 10 rats on the 5th and 8th minutes after treatment. ED_{50} of NMDA inducing TEFL was 11.36 μg, which surpassed ED_{50} of NMDA inducing clonic seizures by 16.5 times (Fig. 3). In diazepam-tolerant animals, administration of NMDA in a dose of 5.0 μg was followed by jumps and swift runs 5-20 sec after treatment. These rats were characterized by vocalization, exophthalmos, increased tone of the tail, and rotational movements. Extension of forelimbs and fingers were found 1-5 min after NMDA injection and lasted 1-5 sec. The observed changes were followed by persistent flexion of the forelimbs, extension and flexion of the hindlimbs, and loss of equilibrium. Repeated seizures and TEFL did not cause animal death. ED_{50} of NMDA inducing TEFL was 2.66 μg, *i.e.* 4.27-fold lower than in intact rats ($p<0.05$; Fig. 3). ED_{100} of NMDA in these animals was 4.41 times lower than in intact animals ($p<0.05$, Fig. 1).

Our results show that an epileptogenic effect of receptor agonists for excitatory amino acids (NMDA and kainic acid) on the frontal cortex is particularly pronounced in diazepam-tolerant animals. However, the epileptogenic effect of benzylpenicillin and strychnine nitrate remains unchanged in these specimens. Hyperactivity of the endogenous system of excitatory amino acids in diazepam-tolerant rats probably contributes to the development of tolerance (*e.g.*, decrease in antiepileptic activity of diazepam).

In freely moving animals, the sensitivity to TEFL increases more significantly than the sensitivity to clonic seizures. Therefore, these effects are due to generation of NMDA-induced seizure discharges [6]. The effect of spatial summation is realized in the neuronal network and probably plays an important role in the pathogenesis of high seizure activity. Our results are consistent with published data that function of NMDA receptors on the isolated neuron remains unchanged under conditions of benzodiazepine tolerance [5].

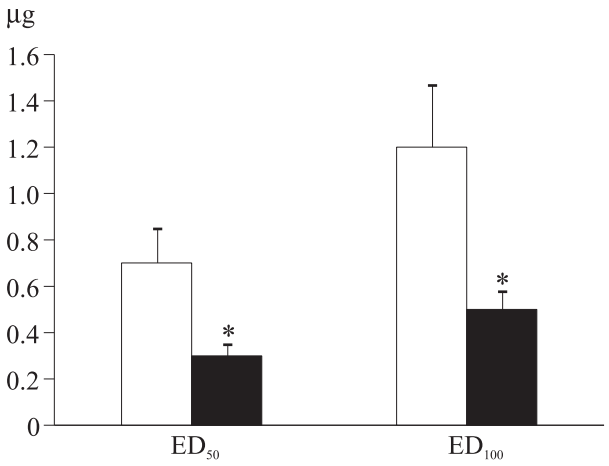

Fig. 2. Median effective doses of NMDA causing clonic seizures. Intracerebroventricular injection of NMDA during the development of diazepam tolerance. Abscissa: doses of NMDA inducing clonic seizures in 50 and 100% animals. Light bars, intact rats; dark bars, diazepam-tolerant rats. Here and in Fig. 3: *$p<0.05$ compared to intact animals.

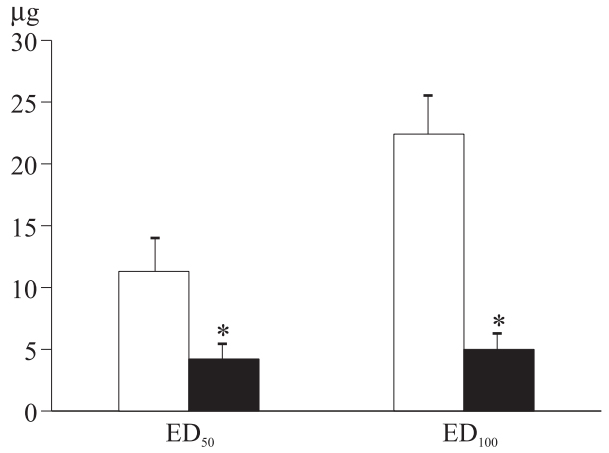

Fig. 3. Median effective doses of NMDA causing TEFL. Intracerebroventricular injection of NMDA during the development of diazepam tolerance. Abscissa: doses of NMDA inducing TEFL in 50 and 100% animals.

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