Effect of Antibodies to Glutamate on Retention of Conditioned Passive Avoidance Response in Rats with Ischemic Injury of the Prefrontal Cortex

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Experiments were performed on rats with bilateral photothrombosis of vessels in the prefrontal cortex. Intranasal administration of antibodies to glutamate (1 h after ischemia of the brain cortex) improved retention of conditioned passive avoidance response, which was elicited before ischemic damage. The content of antibodies to glutamate in the serum of rats increased significantly on day 8 after bilateral photochemical thrombosis of vessels in the prefrontal cortex compared to that in the control.

Key Words: *photothrombosis; prefrontal cortex; learning; memory; antibodies to glutamate*

The number of patients with permanent dysfunction of CNS after cerebral stroke progressively increases. Therefore, the search for new methods of therapy and rehabilitation is an urgent problem. Experimental study of this disorder holds promise for evaluation of pathological changes and search of new therapeutic agents. Under conditions of neuropathology (*e.g.*, due to ischemic or traumatic injury of the brain), changes in glutamatergic neurotransmission are an important mechanism for neuronal death. The excessive release and impaired reuptake of glutamate leading to an increase in intracellular calcium concentration produce an excitotoxic effect and determine neuronal death during cerebral ischemia [2]. The formation of antiglutamate antibodies (Glu-AB) in response to its excessive release in CNS is a mechanism of immune defense from the toxic effect of glutamate on neurons. Recent studies showed that neurodegenerative damages to the brain are accompanied by Glu-AB production [3-5]. In light of this, it is interesting to study the formation of Glu-AB during cerebral ischemic injury and evaluation of the role of these antibodies under conditions of brain damage. Bilateral ischemic injury to the prefrontal cortex in rats was used as an experimental model of cerebral ischemic injury [6]. In rats, the anterior part of the prefrontal neocortex and the hippocampus are known to play a role in integrative activity of the brain associated with learning and memory. Damage to these regions leads to severe cognitive dysfunction, including the loss of conditioned passive avoidance response (CPAR) [6,7].

This work was designed to study the production of Glu-AB during focal ischemia of the prefrontal cortex in rats. We evaluated the effect of intranasal administration of Glu-AB on CPAR retention.

MATERIALS AND METHODS

Experiments were performed on 60 male outbred rats weighing 200-220 g obtained from the nursery of the Institute of General Pathology and Pathophysiology. The animals were maintained in a vivarium at the 12/12-h light-dark cycle and had free access to food and water.

Our study was conducted in accordance to the Directives of European Community Council (86/609/

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EEC) on protection of animals used for experimental and other scientific purposes.

The study was conducted in 2 series. In series I, production of Glu-AB in blood serum from rats was studied 1 h, 4 days, and 8 days after bilateral ischemic infarction of the prefrontal cortex. In series II, the animals were divided into the following five groups: group 1 (n=9), intact rats; group 2 (n=9), sham-operated rats; group 3 (n=10), rats with bilateral ischemic infarction of the prefrontal cortex, intranasal administration of 7 μl distilled water 1 h after surgery; group 4 (n=10), intranasal administration of aqueous solution of Glu-AB in a dose of 250 μg/kg (similar scheme of treatment); and group 5 (n=10), administration of aqueous solution of rabbit γ-globulin from intact animals (the same dose, similar scheme of treatment).

Bilateral focal ischemic infarction of the prefrontal cortex (Fr1 and Fr2 fields) [8] was induced by the method of photochemical thrombosis [11] (Fig. 1). The animals were intraperitoneally anesthetized with 300 mg/kg chloral hydrate. Photosensitizing dye Bengal rose (40 mg/kg; Sigma) was injected intravenously. The rat was fixed in a stereotaxis. The periosteum was separated after a longitudinal skin incision. A special device for irradiation consisted of a cold light source (halogen lamp, 250 W) and fiber-optic light guide

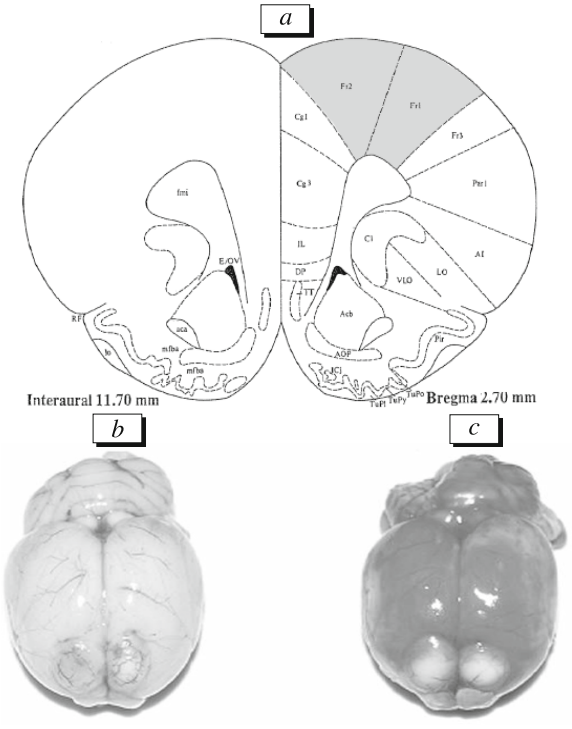

Fig. 1. Bilateral ischemic infarction of the prefrontal cortex in rats with photoinduced thrombosis. Prefrontal region of the brain (areas Fr1 and Fr2) on the stereotaxic scheme of the coronary section (2.12 mm rostral to bregma) [13] (*a*); native rat brain on day 8 after surgery (*b*); rat brain on day 8 after surgery, staining with a vital dye triphenyltetrazolium chloride (*c*).

(inner diameter 3 mm). The fiber-optic light guide was positioned at a distance of 1 mm from the cranial surface (2 mm rostral to bregma, 2 mm lateral to the sagittal suture). The cranial surface was bilaterally irradiated with cold light at 560 nm for 15 min (250-W lamp). Sham-operated rats were subjected to the same manipulations except administration of Bengal rose.

The concentration of Glu-AB in blood serum was measured by ELISA on 96-well polystyrene plates. The plates were sensitized with the test antigen. The conjugate of glutamate and BSA was used as the test antigen. It was synthesized by the standard method with modifications using a bifunctional reagent glutaraldehyde [9]. The test antigen (100 μl) in a final concentration of 0.3 μg/well was put in wells (Costar). Incubation was performed at 4°C for 18 h. The plates were washed 3-4 times with physiological saline and 0.05% Tween 20. Serum samples were incubated in 0.05 M phosphate buffered saline (pH 7.4) and 0.05% Tween 20 (100 μl) at 37°C for 1 h. The study was conducted with 10-fold dilutions (initial dilution 1:10). After incubation, the plates were washed and treated with horseradish peroxidase-labeled secondary antibodies to rat IgG (dilution 1:2000). The plates were washed after 1-h incubation. A substrate mixture (100 μl) of 10 ml 0.2 M $Na_2HPO_4 \times 2H_2O$, 10 ml 0.1 M citric acid, 8 mg *o*-phenylenediamine (Sigma), and 8 μl 33% H_2O_2 was added to wells. Incubation was performed in darkness at room temperature for 1 h. The reaction was stopped by addition of 6 N H_2SO_4. The content of antibodies in each well was estimated from the optical density of blood serum at 495 nm (Mini-reader, Dynatech) and expressed in arb. units. We calculated the ratio of the optical density of blood serum from each rat to the mean optical density of serum samples from control animals. Antibodies were concluded to be present in blood serum when this ratio exceeded 1.

The titer of Glu-AB was 1:1000. The γ-globulin fraction was isolated from serum samples of immunized and intact rabbits (over-precipitation with ammonium sulfate), lyophilized, and stored at 4°C.

CPAR was trained as described elsewhere [1]. The response latency was estimated as the time from the start of this test to the moment when the rat passed the hole between the light and dark compartments of a chamber. On day 1 of training, the rat was placed in an illuminated compartment (100-W lamp). The rat examined this area and then moved to the dark compartment (latency before training). The door was closed, and the rat remained in this compartment for 5 min. The procedure was repeated after 1 h and during this session the rat was immediately removed from the dark compartment. On day 2, the procedure was performed 2 times at a 1-h interval. When the rat repeatedly entered the dark compartment, the door was

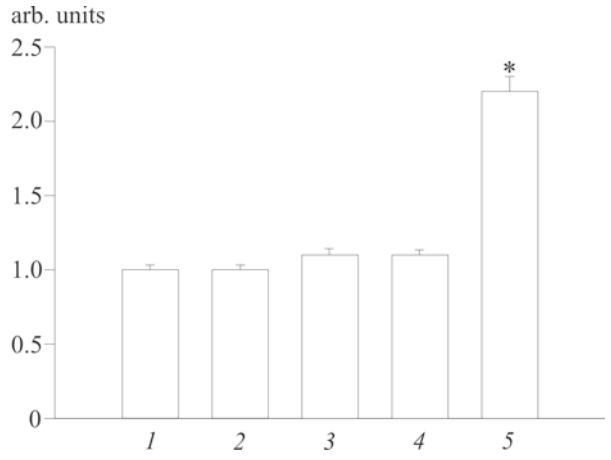

Fig. 2. Glu-AB level in rats with bilateral photothrombosis of the prefrontal cortex. Intact rats (*1*); sham operation (*2*); 1 h after ischemic injury (*3*); day 4 after ischemic injury (*4*); day 8 after ischemic injury (*5*). Here and in Fig. 3: **p*<0.05 compared to intact rats.

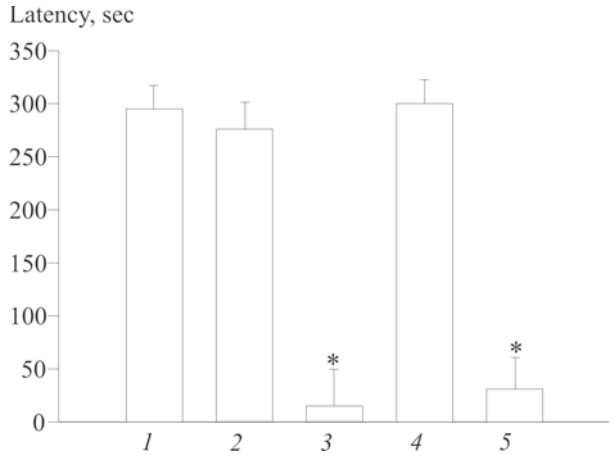

Fig. 3. Effect of intranasal administration of Glu-AB on CPAR retention in rats with bilateral photothrombosis of the prefrontal cortex. Intact rats (*1*); sham operation (*2*); ischemic injury (*3*); intranasal administration of Glu-AB+ischemic injury (*4*); intranasal administration of γ-globulin+ischemic injury (*5*).

closed. Electric current (1.3 mA, 50 Hz) was delivered via a metal-grid floor for 5 sec. The criterion of CPAR learning was transition latency >300 sec. The animals with lower latency were excluded from further observations. The antiamnestic effect of Glu-AB was studied 8 days after induction of cortical infarction.

The data were subjected to a nonparametric analysis of variance (Kruskall–Wallis test) and intragroup comparison (Mann–Whitney *U* test).

RESULTS

Previous studies showed [6] that bilateral photochemically induced thrombosis of blood vessels in the prefrontal cortex of rats is followed by the development of focal ischemia. It spreads over the width of the cortex and is separated from the surrounding normal tissue. Glutamate content was shown to be elevated in the area of ischemic injury [7]. Cortical damage is accompanied by the loss of CPAR.

The amount of Glu-AB in serum samples from rats with focal ischemia of the prefrontal cortex was measured 1 h, 4 days, and 8 days after photothrombosis. Glu-AB level in treated animals was compared with that in sham-operated rats and intact specimens. Significant intergroup differences were found ($H(4,N=31)=17.54946$, $p=0.0015$; Fig. 2). The content of Glu-AB did not differ in serum samples from intact and sham-operated animals (Fig. 2). Glu-AB content in rats 1 h after photochemical injury of the prefrontal cortex did not differ from that in control animals. The level of Glu-AB in blood serum from rats with bilateral photochemical thrombosis of blood vessels in the prefrontal cortex remained unchanged on day 4 after treatment, but increased sharply by the 8th day. It was associated with immune response to enhanced production of glutamate during ischemic injury. This reaction manifested in increased production of antibodies. We studied the effect of Glu-AB on CPAR retention, which was elicited before the incidence of cortical ischemia. Glu-AB were administered intranasally, which resulted in the rapid transport of antibodies to the brain [10]. Glu-AB were administered in the acute period of glutamate excitotoxicity (1 h after photothrombosis). The level of endogenous antibodies is extremely low during this period. Intranasal administration of Glu-AB (250 μg/kg) 1 h after surgery was shown to improve the retention of CPAR in rats with bilateral photothrombosis of the prefrontal cortex. On day 8 after ischemic injury, CPAR retention in animals of the treatment group did not differ from that in intact control specimens. Intranasal administration of γ-globulin from intact rabbits had no effect on the loss of CPAR, which was elicited before ischemia (Fig. 3).

Our results show that the production of Glu-AB in rats is elevated on day 8 after bilateral ischemic injury of the prefrontal cortex. Intranasal administration of Glu-AB 1 h after surgery has an antiamnestic effect on rats with ischemic injury of the prefrontal cortex. We conclude that Glu-AB produce a strong protective effect under conditions of neurodegenerative changes in cognitive function of the brain. This effect is probably associated with the reduction of glutamate overproduction and prevention of neuronal death.

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