PHYSIOLOGY

Cycloheximide Prevents Inhibition of Expression of Immediate Early Gene *c-fos* in Paraventricular Nuclei of Rat Hypothalamus Produced by Delta Sleep-Inducing Peptide

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We studied expression of the immediate early gene *c-fos* in hypothalamic paraventricular nuclei in rats with different prognostic resistance to emotional stress receiving delta-sleep-inducing peptide after intracerebroventricular administration of cycloheximide. Delta-sleep-inducing peptide inhibited expression of the *c-fos* gene in rats receiving intracerebroventricular injection of physiological saline. Pretreatment with the protein synthesis blocker cycloheximide abolished changes produced by delta-sleep-inducing peptide.

Key Words: *c-fos; open field; emotional stress; cycloheximide*

Previous studies showed that delta-sleep-inducing peptide (DSIP) improves the resistance to stress in rats [2]. Intensive expression of the immediate early gene *c-fos* in hypothalamic paraventricular nuclei is a primary response to emotional stress [4,7]. Intraperitoneal injection of DSIP reduces the stress-induced expression of *c-fos* in the hypothalamic paraventricular nucleus [8].

DSIP blocks neuronal response in the hypothalamic paraventricular nuclei to microiontophoretic application of glutamate. Published data show that the content of excitatory neurotransmitters (*e.g.* glutamate) increases under stress conditions, which contributes to the development of neurotoxicity [5]. The effects of glutamate on the genetic apparatus in brain neurons are mediated by protein and peptide factors, including activator protein-1 (AP-1) transcriptional factor [9].

Here we studied whether protein synthesis blocker cycloheximide prevents the effect of DSIP on the expression of the immediate early gene *c-fos* in hypothalamic paraventricular nuclei in rats.

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MATERIALS AND METHODS

Experiments were performed on 50 male Wistar rats weighing 250±10 g, kept in a vivarium with free access to food and water. After 1-week adaptation animal behavior in an open-field was analyzed. The prognostic resistance of animals to emotional stress was estimated by the coefficient [1]. Fifteen rats prognostically resistant to emotional stress demonstrated high locomotor activity and short latency of the first movement and entrance into the center, while 15 rats prognostically predisposed to emotional stress showed low locomotor activity and long latencies.

Cannulas were implanted into the lateral cerebral ventricles under barbamyl anesthesia (10 mg/rat). The control group included 5 rats prognostically resistant and 5 rats prognostically predisposed to stress. The controls received intraperitoneal injections of physiological saline 30 min after intracerebroventricular administration of 10 μl physiological saline. Five re-

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sistant and 5 predisposed rats received intraperitoneal injections of DSIP in a dose of 60 nmol/kg 30 min after intracerebroventricular administration of 10 μl physiological saline. Other resistant and predisposed rats received intraperitoneal injections of DSIP in a dose of 60 nmol/kg 30 min after intracerebroventricular administration of cycloheximide (10 μg in 10 μl physiological saline).

The rats were decapitated 1 h after intraperitoneal administration of physiological saline or DSIP. The experiments were approved by the Ethical Committee of the P. K. Anokhin Institute of Normal Physiology. The brain was removed and frozen in isopentanol at -45°C. Frontal brain sections (20 μ) were obtained on a freezing microtome at -18°C. The cells containing Fos protein (immediate early gene *c-fos* expression product) were identified immunohistochemically.

Brain sections were fixed in 4% paraformaldehyde (Sigma) and washed in phosphate buffer. The slides were incubated with primary antibodies against protein Fos (rabbit polyclonal antibodies, Santa Crus Biotechnology, dilution 1:2000) in a Shandon laboratory chamber, washed in phosphate buffered saline, treated with biotinylated antibodies (Vectastain Elite ABC Kit, Vector) and avidin-biotin complex (ABC), and incubated with 3,3-diaminobenzidine (Amersham) and H_2O_2 for 10 min. The stained sections were dehydrated in alcohols, placed in toluene, and covered with coverslips using Depex resin (Baxter).

Images were analyzed under an Olympus microscope equipped with a Nikon video camera coupled to a computer. Fos-positive cells were counted in the parvocellular part of the hypothalamic paraventricular nuclei (0.07 mm^2, 0.23×0.31 mm). The total number of cells on serial sections of this brain structure was determined.

Automatic selection of Fos-positive cells and their counting were performed by the blind method using Image-Pro Plus system. Brown to black cells were considered as immunopositive.

Brain structures were identified using Stereotactic Atlas of Rat Brain [6]. The significance of differences was evaluated by multifactor analysis of variance (Statistica 5.0 software).

RESULTS

The number of Fos-positive cells in hypothalamic paraventricular nuclei was high in control rats. It should be emphasized that in rats prognostically resistant to emotional stress the count of cells expressing *c-fos* 2-fold surpassed that in stress-predisposed animals (Table 1). These results show that the number of immunoreactive cells in the hypothalamic paraventricular nuclei in stress-resistant rats was significantly higher than in animals prognostically predisposed to emotional stress.

In rats resistant to emotional stress and receiving DSIP the count of *c-fos*-expressing cells was lower than in control animals ($p<0.001$, Table 1).

Cycloheximide markedly increased the number of *c-fos*-expressing cells in rats of both groups (Table 1).

Our findings suggest that intracerebroventricular administration of physiological saline is a stress factor for rats. Our previous studies showed that intraperitoneal injection of DSIP inhibits stress-induced expression of *c-fos* in the hypothalamic paraventricular nuclei. This effect was most pronounced in animals prognostically resistant to emotional stress. The count of Fos-immunopositive cells in the hypothalamic paraventricular nuclei tended to decrease in animals prognostically predisposed to emotional stress and receiving DSIP. Our results are consistent with published data that intraperitoneal administration of DSIP inhibits expression of protooncogene *c-fos* in rat hypothalamic paraventricular nuclei induced by immobilization [8].

Intracerebroventricular pretreatment with cycloheximide blocked the inhibition of *c-fos* expression produced by DSIP. In rats prognostically resistant and predisposed to emotional stress and receiving intracerebroventricular injections of cycloheximide before intraperitoneal administration of DSIP the intensity of *c-fos* expression far surpassed that in animals receiving only DSIP. The count of Fos-immunoreactive cells in rats receiving DSIP after pretreatment with cycloheximide did not differ from that in control animals injected with physiological saline.

Our experiments showed that the inhibitory effect of DSIP on the expression of immediate early genes

TABLE 1. Count of Fos-Immunoreactive Cells in the Parvocellular Part of Hypothalamic Paraventricular Nuclei in Rats with Different Resistance to Emotional Stress ($M\pm m$, $n=5$)

Experimental conditions	Resistant	Predisposed
Physiological saline intracerebroventricularly and intraperitoneally, control	31.8±2.9	15.5±2.5*
Physiological saline intracerebroventricularly and DSIP intraperitoneally	15.1±2.1*	8.8±2.6
Cycloheximide intracerebroventricularly and DSIP intraperitoneally	40.8±6.9^{++}	35.8±5.0^{+}

Note. *$p<0.001$ compared to control resistant rats; $^{+}p<0.0001$ and $^{++}p<0.001$ compared to DSIP and intracerebroventricular administration of physiological saline.

is blocked by cycloheximide. It can be hypothesized that DSIP modulates functional activity of the genetic apparatus in cells. This is probably associated with modulation of some protein transcriptional factors transducing signals from membrane NMDA receptors to cell nuclei, in particular AP-1. The production of this factor is suppressed by protein synthesis inhibitor cycloheximide [9].

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