# Expression of mRNA for Corticotropin-Releasing Hormone and Vasopressin in the Hypothalamus and Amygdala of Rats after Administration of Narcogenic

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Male Wistar rats received intraperitoneal injections of physiological saline (control), phenamine, fentanyl, ethanol, sodium ethaminal, or dexamethasone in increasing concentrations for 4 days. Forced administration of these drugs provided gradual load of the organism and prevented the development of tolerance. Such approach is extensively used for the development of drug addiction or several manifestations of this state. Expression of corticotropin-releasing hormone mRNA in the amygdala was maximum after administration of dexamethasone (0.46 arb. units *vs*. β-actin), but was much lower in experiments with sodium ethaminal and fentanyl (0.07 and 0.037 arb. units, respectively). In the hypothalamus, enhanced mRNA expression was observed after injection of sodium ethaminal, ethanol, and fentanyl (0.8, 0.37, and 0.039 arb. units, respectively). Phenamine did not increase mRNA expression in the amygdala and hypothalamus. Expression of vasopressin mRNA was not detectable in brain structures of animals from various groups. Our results indicate that the hypothalamic reinforcement system provides a similar response to narcogens, whereas the extended amygdala includes elements of both reinforcement and stress reactivity.

**Key Words:** *corticotropin-releasing hormone; vasopressin; stress; brain limbic brain structures; mRNA expression*

Corticotropin-releasing hormone (CRH) is a major inductor of the stress response in CNS [5,11,13]. Intracerebroventricular administration of CRH to animals is followed by the stress response, which persists for 40-60 min [7,11]. CRH is involved in the mechanisms of reinforcement [1,2,4,10], memory [7], anxiety, fear, and depressiveness [1,7,9]. Moreover, the effects of pharmacological agents on these mechanisms are realized via CRH [2,4,8,9, 11]. CRH treatment during early ontogeny produces delayed effects [8,9]. Previous studies showed that the effects of CRH are associated with activation of the stress/antistress system. This system is mainly presented by limbic structures of the brain, including the hypothalamus and amygdala. Labeled ligand binding assay and *in situ* mRNA hybridization showed that expression of CRH-$R_1$ receptor is high in the neocortex (particularly in the prefrontal and entorhinal cortex), olfactory brain structures, amygdaloid complex, hippocampus, cerebellum, and sensory relay nuclei. At the same time, CRH-$R_2$ receptors are located primarily in subfornical structures (ventromedial hypothalamic nucleus, lateral septum, stria terminalis nuclei, and

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several amygdaloid nuclei), but not in the cortex [7]. The main functions of CRH receptors were evaluated in experiments with CRH receptor antagonist. CRH-$R_1$ receptors regulate ACTH secretion and state of anxiety. CRH-$R_2$ receptors are involved in the regulation of food and sexual behavior, cardiovascular activity, and reproductive function [11]. However, little is known about the role of CRH receptors in the mechanisms of reinforcement and memory.

Our previous experiments showed that the central nucleus of the amygdale, a component of the extended amygdala system, modulates the action of pharmacological agents with narcogenic activity [2,4,6,10]. It should be emphasized that the amygdala triggers the hypothalamic dopaminergic mechanisms of reinforcement [5,10]. Here we studied the expression of mRNA for CRH and arginyl 8-vasopressin in the hypothalamus and amygdala of rats after forced administration of pharmacological narcogenic agents in increasing concentrations.

## MATERIALS AND METHODS

Experiments were performed on 69 male Wistar rats weighing 180-200 g and obtained from the Rappolovo nursery (Russian Academy of Medical Sciences, Leningrad region). The animals were divided into groups. The following agents in increasing concentrations were injected intraperitoneally for 4 days: physiological saline, control (0.1, 0.2, 0.4, and 0.8 ml/rat; psychomotor stimulator phenamine (0.5, 1.0, 2.0, and 4.0 mg/kg); narcotic analgetic fentanyl (0.00625, 0.0125, 0.025, and 0.05 mg/kg); ethanol (0.5, 1.0, 2.0, and 4.0 g/kg); barbiturate soporific sodium ethaminal (2.5, 5, 10, and 20 mg/kg); and synthetic glucocorticoid dexamethasone (0.5, 1.0, 2.0, and 4.0 mg/kg). The forced regimen of drug administration suggested 2-fold increasing the dose of the test substance on each day of administration (4 injections). This type of drug treatment provides gradual load of the organism and prevents the development of tolerance. This approach is extensively used for the development of drug dependence or several manifestations of this state.

The animals were maintained in a vivarium at 22±2°C and inverted light/dark conditions (8.00-20.00). They had free access to water and food. The rats were decapitated 2 h after the last injection, the brain was removed, the hypothalamus and amygdala were isolated on ice. The samples were frozen and stored at -70°C until biochemical studies.

Expression of mRNA for CRH and arginyl 8-vasopressin in the hypothalamus and amygdala of rats was studied by reverse transcription assay and polymerase chain reaction (PCR). Total mRNA was isolated by the standard method with guanidine thiocyanate (Promega) [12].

Reverse transcription was conducted with 1 μl priming oligo-dT primers (Promega), nucleotide triphosphates (final concentration 1.25 mmol, Sileks M), 0.5 μl ribonuclease inhibitor (Promega), and 1 μl reversed transcriptase M-MLV (Promega). Each reaction was performed with 2 μg sample. The total volume of the reaction mixture was brought to 20 μl with diethyl pyrocarbonate-treated deionized water.

In PCR, products of the reverse transcription reaction (2 μl) were added to the reaction mixture containing 2.5 μl 10-fold buffer, nucleotide triphosphates (final concentration 0.8 mmol), specific (+) primer (25 pmol), specific (-) primer (25 pmol), Taq DNA polymerase (1 μl), and $MgCl_2$ (specific concentration for each pair of primers, Table 1). The final volume was brought to 25 μl with deionized water. PCR was conducted in a Techne amplifier under the following temperature conditions: 1 min at 94°C, 1 min at annealing temperature; and 1.2 min at 72°C. The annealing temperature was determined for each pair of primers (Table 1).

Specific primers were selected from nucleotide sequences of rat mRNA and DNA (European Molecular Data Bank) using Primer-Master 1.0 software. The sequence of primers and conditions of PCR (concentration of magnesium ions and number of

**TABLE 1.** Parameters of PCR

| Parameter | Primer | Sequence | Annealing temperature, °C | [Mg] | Number of cycles | Size of fragment |
|---|---|---|---|---|---|---|
| CRH | + | 5'aggtacctcgcagaacaa3' | 56.9 | 2 | 32 | 249 |
| | — | 3'actaggcgtacccacttc5' | | | | |
| Arginyl 8-vasopressin | + | 5'gccacatccgacatggag3' | 57.3 | 2 | 38 | 271 |
| | — | 3'gctcacagctctcccaaa5' | | | | |
| β-Actin | + | 5'gaagatcctgaccgagcgtg3' | 59 | 2 | 30 | 347 |
| | — | 3'gagatacggttgtgtcacga5' | | | | |

reaction cycles) are shown in Table 1. β-Actin mRNA was used as the internal standard in the reverse transcription reaction.

PCR products were studied by electrophoresis in 1.5% agarose gel stained with ethidium bromide to visualize mRNA. The gels were photographed using a Canon digital camera (Power Shot S30) in transmitted ultraviolet light. The experiment was performed using a Vilber Lourmat transilluminator.

Densitometry of electrophoretic bands was performed with SCNImage software. The concentration of mRNA for CRH and vasopressin was normalized to β-actin mRNA. The data were expressed as the ratio of these values.

The results were analyzed by nonparametric tests (Mann—Whitney *U* test and paired comparison test).

## RESULTS

CRH mRNA expression in the amygdala was maximum after administration of dexamethasone (0.46 arb. units *vs*. β-actin, $p<0.01$), but was much lower in experiments with sodium ethaminal (0.07, $p<0.05$) and fentanyl (0.037 arb. units, Table 2). In the hypothalamus, mRNA expression increased after injection of sodium ethaminal (0.8 arb. units, $p<0.001$), ethanol (0.37 arb. units, $p<0.01$), and fentanyl (0.039 arb. units). Phenamine did not increase mRNA expression in the amygdala and hypothalamus (Fig. 1).

As regards vasopressin, the conditions for PCR with positive samples were selected in 30 cycles. However, arginyl 8-vasopressin mRNA expression was undetectable in brain structures of animals from various groups. The number of cycles was successively increased to 38. Arginyl 8-vasopressin mRNA was detected only in the hypothalamus of control animals. However, this number of cycles did not correspond to the exponential phase. Hence, electrophoresis also revealed only nonspecific changes.

Our results indicate that forced administration of pharmacological agents with narcogenic activity does not activate the expression of arginyl 8-vasopressin mRNA in rat hypothalamus and amygdale, but selectively activated of CRH mRNA expression in the hypothalamus (sodium ethaminal>>ethanol> fentanyl) and amygdala (dexamethasone>>sodium ethaminal>fentanyl).

It is not surprising that dexamethasone increases CRH mRNA expression in the amygdala. The amygdala plays a greater role in the reinforcing effect of narcogens than the hypothalamus [2,6,10]. Our recent studies showed that phenamine (1 mg/kg), morphine (1 mg/kg), and sodium ethaminal (5 mg/kg) differ in their capacity to activate self-stimulation of the lateral hypothalamus in rats (by 18-37%) [2,4,6,10]. Nonselective CRH receptor antagonist astressin (1 μg/μl) administered into the amygdala and paraventricular hypothalamus inhibited self-stimulation by 55 and 17%, respectively. Blockade of extrahypothalamic CRH receptors (central amygdaloid nucleus) with astressin modified the effect of various narcogens on self-stimulation of the lateral hypothalamus. Sodium ethaminal and, to a lesser extent, phenamine produced a psychoactivating effect under these conditions. Moderate stimulatory effect of morphine was transformed to depressant activity. Leu-enkephalin produced a strong depressant effect and potentiated the action of astressin. Astressin-induced blockade of CRH receptors in the paraventricular hypothalamus slightly modified the effect of narcogens on self-stimulation of the lateral hypothalamus. Phenamine, morphine, and sodium ethaminal retained psychoactivating actvity. Leu-enkephalin had no effect on the depressant action of astressin. It was hypothesized that the amygdaloid CRH system produces an activating effect on the hypothalamic reinforcement system. Astressin potentiates the inhibitory effect of leu-enkephalin on brain self-stimulation, which is probably associated with transient inhibition of hypothalamic activation by the central amygdaloid nucleus. Hence, astressin-induced blockade of CRH receptors in the amygdala and, particularly, in the hypothalamus abolishes or significantly decreases the reinforcing effect of morphine, sodium ethaminal, and leu-enkephalin (as differentiated from phenamine) [6,10]. The central amygdaloid nucleus (major part of the extended amygdala) probably modulates reinforcing activity of the lateral hypothalamus, which is related to function of extrahypothalamic CRH-containing neurons.

**TABLE 2.** Effect of Narcogens on CRH mRNA Expression in the Amygdala and Hypothalamus of Rats

| Drug | Activity relative to β-actin, arb. units | |
|---|---|---|
| | amygdala | hypothalamus |
| Physiological saline (control) | 0 | 0 |
| Phenamine | 0 | 0 |
| Fentanyl | 0.037 | 0.039 |
| Ethanol | 0 | 0.37** |
| Sodium ethaminal | 0.07* | 0.8*** |
| Dexamethasone | 0.46** | 0 |

**Note.** *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to the control.


**Fig. 1.** Effect of narcogens on expression of CRH mRNA in the amygdala (*I*) and hypothalamus of rats (*II*). Electrophoretic visualization of PCR products. Physiological saline (control, *1*); phenamine (*2*); fentanyl (*3*); ethanol (*4*); sodium ethaminal (*5*); and dexamethasone (*6*).

However, we found that sodium ethaminal increases CRH mRNA expression in the hypothalamus and amygdala. Moreover, the expression of CRH mRNA in the hypothalamus was enhanced after administration of ethanol. These drugs belong to a group of hypnotic and sedative narcogens. Therefore, they have similar modulatory effects on the cerebral mechanisms of reinforcement. CRH mRNA expression in the amygdala increased slightly after administration of sodium ethaminal and fentanyl (0.070-0.037 arb. units *vs*. β-actin) and was considered as relatively nonspecific. However, only fentanyl induced the nonspecific expression of CRH mRNA in the hypothalamus (0.039 arb. units). CRH mRNA expression in the hypothalamus was high after treatment with sodium ethaminal and ethanol (0.8 and 0.37 arb. units, respectively). Probably, the hypothalamic mechanisms of reinforcement are less associated with stress-limiting systems of the brain (CRH—ACTH). However, the reinforcing mechanisms of the extended amygdala strongly depend on exogenous and endogenous stress [5,6,9]. The hypothalamus exhibits a similar response to narcogens and, particularly, to sedative drugs (sodium ethaminal, fentanyl, and ethanol). By contrast, the extended amygdala includes elements for reinforcement and stress reactivity. Synthetic glucocorticoid dexamethasone induces the stress response and triggers the reinforcing mechanisms, which are not related to or depend slightly on the function of the hypothalamus.

Our findings explain the absence of mRNA expression for arginyl 8-vasopressin in the hypothalamus and amygdala. This neuropeptide has little role in the mechanisms of reinforcement, but is involved in memory function [3,11]. Limbic brain structures are involved in these processes and modulate the emotional component of memory [5,11].

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