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Content of mRNA for NMDA Glutamate Receptor Subunits in the Frontal Cortex and Striatum of Rats after Morphine Withdrawal Is Related to the Degree of Abstinence

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We studied the expression of mRNA for genes, whose protein products regulate the glutamate/NO/cGMP signal cascade in the frontal cortex, striatum, midbrain, and hippocampus of rats with various degrees of spontaneous morphine withdrawal syndrome. The concentration of Grin2a mRNA (subunit of the NMDA glutamate receptor) in the frontal cortex increased mainly in animals with severe abstinence. By contrast, the expression of mRNA for the Grin2b subunit in the striatum decreased in animals with mild abstinence. Variations in the content of mRNA for other products of the cascade (isoforms of NO-dependent guanylate cyclase and cGMP-dependent protein kinase) after morphine withdrawal were not related to the degree of abstinence. Our results suggest that the region-specific expression of mRNA for certain subunits of the NMDA glutamate receptor after morphine withdrawal can determine the degree of abstinence.

Key Words: *mRNA; signal cascade of glutamate/nitric oxide/cyclic guanosine monophosphate; brain areas; rats; spontaneous morphine withdrawal syndrome*

The formation of the dependence on psychoactive drugs (*e.g.*, morphine) is characterized by individual variations, which can be determined by the specific features of the nervous system at the molecular, structural, and functional levels. The nature of these features is poorly understood. Previous studies have demonstrated the importance of evaluation of individual variability of processes underlying the formation of the dependences [7]. Changes in glutamatergic neurotransmission in the mesocorticolimbic structures of the brain can determine the formation of addiction. Among a variety of intracellular molecular cascades, the NO/cGMP signal cascade triggered by NMDA glutamate receptors plays an important role under these conditions [2,8,13]. Moreover, functional activity of this system can determine predisposition to dependences [10]. Specific variations in the expression of some genes in certain areas of the brain constitute the mechanism for structural and functional changes, which are observed during pathological dependence [6]. There are no data on the relationship between variations in the expression of mRNA for genes, whose protein

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products regulate the glutamate/NO/cGMP cascade, and specific features of morphine dependence.

Here we studied the expression of mRNA for the main components of the glutamate/NO/cGMP cascade in brain areas of rats with various degrees of morphine withdrawal syndrome.

MATERIALS AND METHODS

Experiments were performed on 37 male Wistar rats weighing 180-230 g. The animals were maintained under an artificial light/dark cycle and had free access to water and food. The rats were divided into the treatment group (subchronic administration of morphine followed by its withdrawal; n=24) and control group (n=13). Morphine in increasing doses of 10-100 mg/kg (3 ml/kg) was injected intraperitoneally at 8:00 and 20:00 for 6 days to induce the formation of physical dependence [11]. Control animals received an equivalent volume of isotonic NaCl (3 ml/kg). The spontaneous withdrawal syndrome that serves as an indirect criterion for the degree of dependence was evaluated 38 h after the last injection of morphine. The rats were tested in the open field for 5 min. The severity of withdrawal syndrome was estimated from the specific motor (wet-dog shaking, jumping activity, writhing, chewing, teeth scratching, and forelimb shaking) and autonomic symptoms (diarrhea, ptosis, rhinorrhea, piloerection, dyspnea, squeak in touching, and aggressiveness) [11]. When possible, the symptoms were analyzed quantitatively and expressed in points (depending on the specificity). The severity of withdrawal syndrome was expressed as the sum of points. The animals were decapitated 2 h after testing. The brain was removed. The frontal cortex, striatum, midbrain, and hippocampus were isolated and immediately frozen in liquid nitrogen.

We studied the expression of the following genes encoding the main proteins of the glutamate/NO/cGMP signal cascade: NMDA glutamate receptor subunits (Grin1, Grin2a, Grin2b, Grin2c, and Grin2d), neuronal NO synthase (Nos1), NO-dependent soluble guanylate cyclase isoforms (Gucya1 and Gucyb1), and cGMP-dependent protein kinase isoforms (Prkg1 and Prkg2). The relative content of mRNA was measured by the method of PCR after reverse transcription and real-time recording of the amplification product (real-time RT-PCR). Total RNA was isolated by extraction with the system of acid guanidine isothiocyanate/phenol/chloroform [1]. Reverse transcription was conducted using a kit for the synthesis of complementary DNA with Moloney murine leukemia virus (M-MLV) reverse transcriptase and random hexamers (as primers) according to the manufacturer's recommendations (Sileks). Real-time PCR was performed in the presence of specific synthetic oligonucleotides (as primers) with a kit, which contained Taq DNA polymerase with enzyme inhibitory antibodies and fluorescence intercalating dye EVA GREEN, according to the manufacturer"s recommendations (Sintol). An ANK-32 (Institute of Analytical Instrument Making and N. E. Bauman Moscow State Technical University) was used for real-time PCR.

The amount of specific mRNA was estimated by recording of the threshold cycle and further comparative analysis. The concentration of actin mRNA served as a reference gene ($2^{-\Delta\Delta Ct}$) [5].

The results were analyzed by Statistica 6.0 software (StatSoft, Inc.). The significance of differences between several independent samples was evaluated with the analysis of variance by ranks (Kruskal–Wallis test). The significance of differences between two independent samples was estimated by nonparametric Mann–Whitney U test. The data are presented as the arithmetic means and standard deviations (M±SD).

RESULTS

To reveal a possible relationship between the expression of specific mRNA and degree of morphine dependence, the treated animals were divided into 3 subgroups depending on the severity of spontaneous withdrawal syndrome. We took into account that the degree of abstinence serves as an indirect criterion for physical dependence. Group 1 consisted of 6 rats with mild withdrawal syndrome. The severity of withdrawal syndrome in these specimens (*i.e.*, sum of points for the motor and autonomic symptoms of abstinence) was 6.50±1.22. Group 2 rats (n=12) and group 3 animals (n=6) had moderate (12.58±2.47 points) and severe morphine withdrawal syndromes (27.67±7.09 points), respectively.

The Kruskal–Wallis test for several independent samples (control group and treatment group with various degrees of abstinence) revealed some differences in the expression of specific mRNA in the frontal cortex (Table 1) and striatum (Table 2). However, the amount of mRNA in the midbrain and hippocampus remained unchanged under these conditions (data not shown).

The Mann–Whitney U test for two independent samples showed that the concentration of Grin2a mRNA in the frontal cortex increases in animals with moderate and severe withdrawal syndromes (as compared to the control; Table 1). Moreover, the concentration of mRNA for Grin2b and Prkg2 in rats with moderate abstinence was higher than in control specimens (Table 1).

By contrast, the concentration of Grin2b mRNA in the striatum of animals with mild and moder-

TABLE 1. Relative Content of mRNA for Key Components of the Glutamate/NO/cGMP Signal Cascade in the Frontal Cortex of Rats with Various Degrees of Morphine Withdrawal Syndrome (*M*±*SD*)

Gene	Relative content of mRNA ($2^{-\Delta\Delta Ct}$)				Kruskal–Wallis test: H (3, *N*=37); *p*
	control (*n*=13)	mild abstinence (*n*=6)	moderate abstinence (*n*=12)	severe abstinence (*n*=12)	
NMDA glutamate receptor subunits					
Grin1	1.07±0.41	1.20±0.36	1.29±0.37	1.40±0.39	H=2.750; *p*=0.432
Grin2a	1.03±0.27	1.20±0.40	1.47±0.29**	1.77±0.42**	**H=13.946; *p*=0.003**
Grin2b	1.03±0.26	1.38±0.42	1.62±0.29***	1.66±0.59	**H=12.931; *p*=0.005**
Grin2c	1.07±0.43	1.28±0.36	1.48±0.51	1.47±0.53	H=4.852; *p*=0.183
Grin2d	1.04±0.31	1.05±0.29	1.19±0.28	1.65±0.94	H=3.936; *p*=0.269
Neuronal NO synthase					
Nos1	1.05±0.38	1.46±0.51	1.21±0.35	1.60±0.75	H=3.981; *p*=0.263
Soluble guanylate cyclase isoforms					
Gucya1	1.03±0.27	0.86±0.50	0.96±0.19	0.98±0.25	H=1.126; *p*=0.771
Gucyb1	1.04±0.27	1.01±0.25	1.12±0.18	1.19±0.14	H=1.838; *p*=0.607
cGMP-dependent protein kinase isoforms					
Prkg1	1.07±0.45	1.57±0.83	1.38±0.46	1.68±0.46	H=7.226; *p*=0.065
Prkg2	1.03±0.24	1.88±1.16	1.37±0.18**	1.46±0.76	**H=9.006; *p*=0.029**

Note. **$p<0.005$ and ***$p<0.0005$ in comparison with the control. Here and in Table 2: results of Kruskal–Wallis test at $p<0.05$ are shown in bold.

ate abstinence was much lower than in the control (Table 2). Besides this, the concentration of mRNA for Gucya1 and Gucyb1 in rats with various degrees of withdrawal syndrome was lower than in control specimens (Table 2).

Previous studies showed that activation of NMDA receptor-mediated glutamate signaling serves as one of the mechanisms for behavioral disturbances during abstinence [14]. It can be suggested that the greater is this signaling, the higher is the degree of abstinence (*i.e.*, stronger dependence). Our results are consistent with these data.

The striatum is under the excitatory afferent glutamatergic influence of the prefrontal cortex [9]. An increase in glutamate level in the nucleus accumbens of the striatum during morphine withdrawal depends on the influence of the prefrontal cortex. For example, damage to the medial prefrontal cortex is accompanied by a less significant increase in the content of glutamate [3]. These data suggest that the stronger is the glutamatergic influence of the neocortex on the striatum, the higher is the degree of abstinence disorders. Our experiments showed that the concentration of Grin2a mRNA increases in the frontal cortex of animals with severe abstinence. These changes can precede the activation of glutamatergic neurotransmission in the striatum and, therefore, an increase in the degree of withdrawal-related behavioral alterations.

Induced and spontaneous morphine withdrawal is accompanied by an increase in the concentration of excitatory amino acids (glutamate and aspartate) in the intercellular space of the nucleus accumbens [3,12]. It can be suggested that the observed reduction of Grin2b mRNA expression in the striatum of animals with mild abstinence is directed toward the decrease in glutamatergic neurotransmission (*i.e.*, alleviation of withdrawal symptoms). Our suggestion is confirmed by the results of previous studies [4]. Published data show that mice knockout for the NMDA glutamate subunit Grin2a are characterized by low degree of naloxone-induced withdrawal syndrome. The transfer of Grin2a gene cDNA into the nucleus accumbens of knockout mice produces the opposite effect [4].

We conclude that the observed increase in Grin2a mRNA expression in the frontal cortex can increase the severity of withdrawal symptoms. By contrast, a

TABLE 2. Relative Content of mRNA for Key Components of the Glutamate/NO/cGMP Signal Cascade in the Striatum of Rats with Various Degrees of Morphine Withdrawal Syndrome (*M*±*D*)

Gene	Relative content of mRNA ($2^{-\Delta\Delta Ct}$)				Kruskal–Wallis test: H (3, *N*=37); *p*
	control (*n*=13)	mild abstinence (*n*=6)	moderate abstinence (*n*=12)	severe abstinence (*n*=12)	
NMDA glutamate receptor subunits					
Grin1	1.01±0.37	0.73±0.27	0.73±0.15	0.71±0.15	H=5.309; *p*=0.151
Grin2a	1.06±0.38	0.83±0.14	0.92±0.22	1.05±0.39	H=1.530; *p*=0.675
Grin2b	1.05±0.35	0.62±0.28*	0.61±0.26**	0.70±0.29	**H=11.567; *p*=0.009**
Grin2c	1.05±0.34	0.84±0.24	1.03±0.46	0.87±0.30	H=2.023; *p*=0.568
Grin2d	1.06±0.40	0.69±0.20	0.74±0.25	0.98±0.36	H=7.544; *p*=0.056
Neuronal NO synthase					
Nos1	1.05±0.37	0.82±0.26	1.00±0.33	1.09±0.38	H=2.595; p=0.458
Soluble guanylate cyclase isoforms					
Gucya1	1.04±0.29	0.64±0.11*	0.71±0.28*	0.62±0.24*	**H=12.987; *p*=0.005**
Gucyb1	1.02±0.21	0.76±0.18*	0.81±0.20*	0.74±0.17*	**H=8.996; *p*=0.029**
cGMP-dependent protein kinase isoforms					
Prkg1	1.09±0.45	0.63±0.17	0.75±0.26	0.74±0.12	H=7.370; *p*=0.061
Prkg2	1.04±0.30	0.93±0.21	1.06±0.32	1.08±0.32	H=0.735; *p*=0.865

Note. *$p<0.05$ and **$p<0.005$ in comparison with the control (*U* test).

decrease in Grin2b mRNA in the striatum is followed by the alleviation of withdrawal-related symptoms.

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