Dynamics of NADPH Diaphorase Activity in Raphe Neurons during Chronic Treatment with Opiates

I. V. Dyuizen, P. A. Motavkin, and V. V. Shorin

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Systemic administration of diacetylmorphine considerably reduced the number of NADPH diaphorase-positive (NO-synthesizing) neurons in rat brain raphe nuclei. This effect was blocked by naloxone. In animals with the withdrawal syndrome NO-ergic activity in raphe neurons increased and surpassed the normal.

Key Words: *NADPH diaphorase; diacetylmorphine; raphe nuclei*

It is now established that nitric oxide (NO) modulates the analgetic and narcotizing effects of opiates. The inhibitory analysis showed that suppression of NO• synthesis potentiates the analgetic effect of morphine [2], prevents the development of tolerance [2], and attenuates the symptoms of abstinence [13]. However, the topography of NO-ergic targets for opiates in the central nervous system (CNS) remains unclear.

Neuromodulatory activity of NO is associated with its ability to affect the synthesis and synaptic release of various transmitters [6]. Here we studied NO-ergic functions of neurons in the raphe nucleus, the main source of serotonin (5-hydroxytryptamine) in CNS [7]. The dynamics of NO• synthesis during long-term treatment with opiate receptor ligand diacetylmorphine (DAM) followed by its withdrawal was studied using histochemical reaction for NADPH diaphorase (NADPH-D).

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 280 g. The animals daily received increasing doses of DAM (0.05-0.5 mg/kg intravenously) for 7-14 days. The animals were divided into groups (5 rats per group) according to the treatment duration (group M7 and M14 rats received DAM for 7 and 14 days, respectively) and the duration of withdrawal syndrome after 2-week opiate treatment (4, 7, and 14 days in groups M14+4, M14+7, and M14+14, respectively). For modeling acute withdrawal (group M14+N) specific μ-, σ-, and κ-opiate receptor antagonist naloxone was injected subcutaneously in a dose of 0.1 mg/kg [3]. Intact animals served as the control.

NO•-synthesizing neurons were visualized using a histochemical reaction for NADPH-D. The rats intraperitoneally anesthetized with sodium thiopental were perfused with paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.2) through the ascending aorta. The brain was removed and fixed at 4°C for 2 h. The fixative was washed out with 0.1 M phosphate buffer containing 15% sucrose at 4°C. Cryostat sections (50 μ) were processed as described elsewhere [14]. The number of NADPH-D-positive neurons in the central portion of studied nuclei and their ratio to the total number of Nissl stained nerve cells were evaluated.

The topography of raphe nuclei was identified as proposed by K. Fuxe *et al*. [7], who classifies 9 serotoninergic centers in the brain of vertebrates (B1-B9). The caudal group of raphe nuclei (B1-B4) includes *nucl. raphe pallidus* (RPa), *nucl. raphe obscurus* (ROb), *nucl. raphe magnus* (RMg), and *nucl. raphe pontis* (RPn). The rostral group (B5-B9) consists of *median raphe nucl.* (RMn), *caudal linear nucl.* (CLi), and *dorsal raphe nucl.* (DR), which is divided into ventro-

Laboratory of Pharmacology, Institute of Sea Biology, Far-Eastern Division of the Russian Academy of Sciences, Vladivostok; Department of Histology, Vladivostok State Medical University. ***Address for correspondence:*** iduizen@yahoo.com. Dyuizen I. V.

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medial (DRVM), dorsomedial (DRDM), lateral (DRL), and caudal regions (DRC) [8].

RESULTS

The symptoms of narcotic intoxication were observed over 1 h after DAM injection and included behavioral depression, sedation, akinesia, and stereotypic gnawing [1]. Behavioral reactions typical of abstinence were found in rats during acute (naloxone-induced) or chronic DAM withdrawal: ptosis, gibbous posture, diarrhea, and wet dog shakes.

Histochemical reaction revealed neurons with high NADPH-D (NO synthase) activity in the studied raphe nuclei. The number of NADPH-D-positive neurons in various nuclei was different. This parameter was minimum in RMg, RMn, and CLi nuclei (no more than 7.2-9.4% positive cells). ROb and RPn nuclei contained 32.4 and 48.8% NADPH-D-containing neurons, respectively. The histochemical reaction was most intensive in large multipolar DR cells (Fig. 1, *a*), 80% of which can synthesize NO.

The absolute number of NO-ergic neurons in the studied raphe nuclei decreased after long-term treatment with DAM (Table 1, Fig. 1, *b*). The most pronounced changes were found in rostral nuclei after 1-week morphinization: the count of NO-positive neurons decreased by 48-82%. The caudal serotoninergic RPa and ROb nuclei underwent less pronounced changes. In these nuclei only 9-12% neurons lost their ability to synthesize NO.

Single treatment with naloxone against the background of chronic intoxication was accompanied by a considerable increase in histochemical activity of neurons. Their count in ROb, RMg, RPn, and RMn nuclei approached the control (Table 1).

NADPH-D activity in raphe nucleus neurons progressively increased during chronic DAM withdrawal. The count of NO-producing cells in the studied nuclei (except RMg and RPn) returned to normal within 1 week. However, by the end of week 2 after DAM withdrawal we observed expression of enzyme activity, which was most pronounced in the rostral and dorsal raphe nuclei retaining their NO-ergic capacity during long-term narcotization. The count of NADPH-D-positive cells in these regions increased by several times during chronic DAM withdrawal.

Thus, long-term treatment with DAM in minimum addictive doses [1] markedly suppresses NO-ergic activity in raphe neurons. The inhibition of $NO^{\bullet}$ synthesis is realized through the opiate-induced blockade of Ca^{2+}-channels, decrease in intracellular Ca^{2+} concentration, and inactivation of Ca^{2+}-calmodulin-dependent NO synthase [15]. It can not be excluded that the effects of DAM are mediated through desensitization of NMDA-glutamate receptors [9] playing a key role in NO-ergic activity of nerve cells [11].

DAM-induced changes in NADPH-D activity are primarily related to activation of opiate neuroreceptors. The μ- and σ-receptor antagonist DAM [4] most significantly decreases NADPH-D activity in the raphe nuclei, whose neurons express a considerable number of μ-opiate receptors [5]. Specific localization of various receptors in the studied brain regions probably determines different NO-ergic reactions to naloxone.

Chronic withdrawal of opiates is accompanied by induction of $NO^{\bullet}$ synthesis in raphe neurons. It was hypothesized that activation of $NO^{\bullet}$ synthesis during opiate withdrawal is associated with metabolic and neurotransmitter changes in the brain after long-term narcotization [10]. NO-ergic neuromodulation affects the synthesis and release of various neurotransmitters, including serotonin [6]. Since most NO synthase-positive neurons in the dorsal raphe nucleus produce 5-hydroxytryptamine (70-90%) [8], it can be assumed that the morphine-induced serotoninergic imbalance is related to changes in $NO^{\bullet}$ synthesis [12].

TABLE 1. Mean Number of NADPH-D-Expressing Neurons in Rat Brain Raphe Nuclei under Normal Conditions, after Long-Term Treatment with DAM, and after Its Acute or Chronic Withdrawal (*M*±*m*)

Nucleus	Intact	M7	M14	M14+4	M14+7	M14+14	M14+N
ROb	19.00±0.84	14.90±0.91*	13.80±0.62*	19.50±1.34^{+}	16.20±0.61^{+}	12.4±1.1	13.20±0.91
RMg	11.0±0.5	5.7±0.3*	4.00±0.16*	11.20±0.47^{+}	10.10±0.55^{+}	9.70±0.48^{+}	12.60±0.51^{+}
RPn	30.30±0.94	5.40±0.31*	5.10±0.21*	7.60±0.41^{+}	12.90±0.58^{+}	39.0±1.8^{+}	23.50±0.69^{+}
RMn	36.10±0.45	14.10±0.18*	8.60±0.92*	18.90±0.15^{+}	19.1±0.5^{+}	38.80±1.46^{+}	27.2±1.9^{+}
DRVM	8.80±0.63	3.40±0.24*	3.30±0.21*	5.10±0.25^{+}	7.70±0.16^{+}	10.80±0.51^{+}	5.30±0.42^{+}
DRDM	18.00±0.63	3.90±0.19*	3.80±0.91*	8.20±0.25^{+}	12.20±0.77^{+}	37.3±2.1^{+}	9.90±0.57^{+}
DRL	23.10±1.32	7.00±0.35*	6.00±0.32*	10.60±1.35^{+}	21.80±1.85^{+}	47.9±2.7^{+}	11.60±0.46^{+}
DRC	7.40±0.41	2.00±0.14*	2.70±0.17*	3.20±0.17^{+}	5.50±0.25^{+}	12.50±4.37^{+}	5.10±0.14^{+}

Note. $p<0.05$: *compared to normal; $^{+}$compared to M14 group.

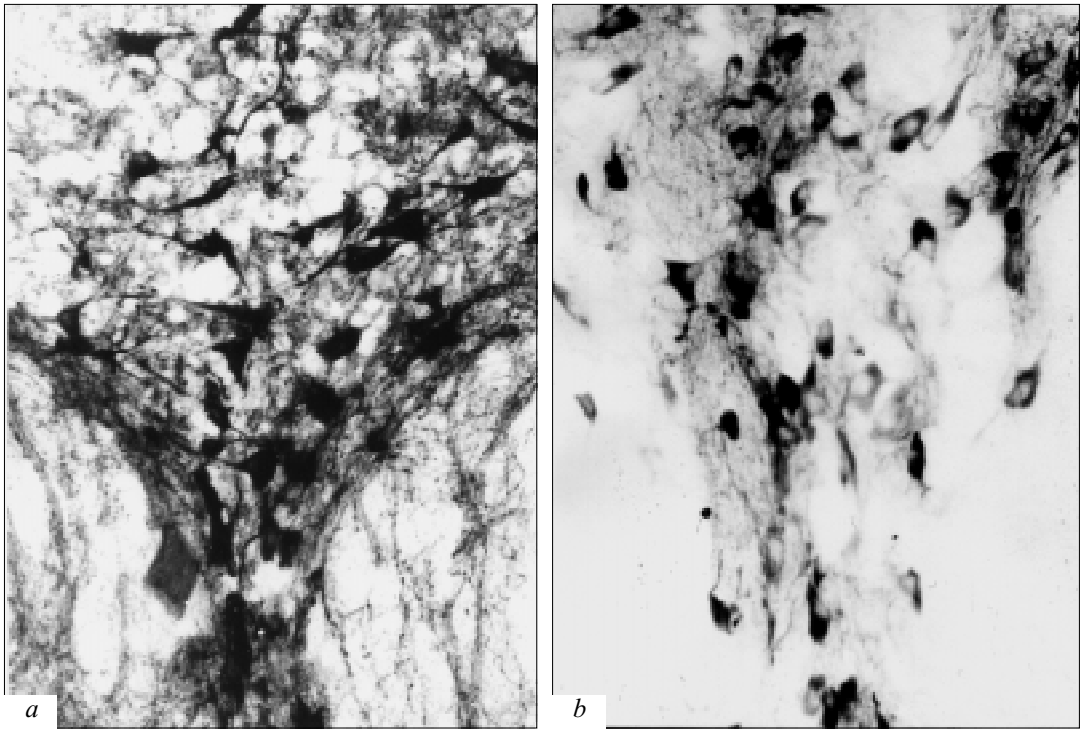

Fig. 1. Distribution of NADPH diaphorase in dorsal rephe nucleus neurons in intact animals (*a*) and after long-term treatment with diacetylmorphine (*b*), ×400. Method of S. R. Vinsent *et al*. [14].

Our findings suggest that long-term treatment with opiates followed by their withdrawal changes the NO-ergic neuromodulation in brain regions responsible for the formation of drug tolerance and dependence [10]. The regulation of this neuromodulatory system by selective inhibitors of neuronal NO synthase holds much promise for the correction of behavioral and emotional disorders in drug addicts.

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