Naloxone-Precipitated Morphine Withdrawal Elicits Increases in c-fos mRNA Expression in Restricted Regions of the Infant Rat Brain

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ABSTRACT—This paper is the first report of a genetic index for morphine withdrawal in infant rats. We examined the effects of naloxone (2 mg/kg) on c-fos mRNA levels in brains of infant and adult rats following repeated treatment with morphine (20 mg/kg, once daily for 5 days). One hour after a single administration of naloxone (naloxone challenge), an increase in c-fos mRNA was observed in the olfactory bulb, hypothalamus and medulla oblongata of infant rats, and in the olfactory bulb and hypothalamus, but not in the medulla oblongata of adult rats. The c-fos mRNA levels returned to control levels 6 h after the naloxone challenge. The increase in c-fos mRNA levels was followed by body weight loss in both infant and adult rats. When MK-801, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, was co-administered along with morphine, it inhibited the naloxone-induced increases in c-fos mRNA levels in infant rats following repeated morphine administration. These results suggest that physical dependence develops in infant rats following repeated morphine administration and that the increment of c-fos mRNA levels is a useful indicator for naloxone-precipitated morphine withdrawal in infant as well as in adult rats.

Keywords: c-fos mRNA, Infant rat, MK801, Morphine withdrawal, Naloxone

Discontinuation of chronic exposure to opioids in the adult rat produces a complex set of behavioral and physiological changes including wet-dog shakes, teeth chattering, weight loss, and the expression of the immediate early gene, c-fos, in certain areas of the brain. The neonatal rat also demonstrates behavioral changes indicative of opioid withdrawal (1) as do human newborns who are passively exposed to opioids through maternal therapeutic use of methadone or illicit use of heroin (2). The study on morphine withdrawal in infant rat could provide important information on clinical treatment for morphine withdrawal in human newborns. However, there are few reports on opioid withdrawal in infant rat and the estimation of opiate withdrawal signs has been less established in infant rat. Moreover, the withdrawal signs in infant rats are distinct from those in adult rat (1). Therefore, an index distinct from the behavioral signs is required for studying morphine withdrawal in infant rat.

Neuronal expression of the immediate early gene, c-fos, has been used as a marker for increased neuronal activity and is presumed to be a mediator of neural plasticity (3). In morphine withdrawal, c-fos is expressed in certain brain regions of adult rats (4, 5). Furthermore, the N-methyl-D-aspartate (NMDA) receptor, which has a key role in neural plasticity in the CNS, is involved in c-fos expression during morphine withdrawal. It has been shown that pretreatment with the NMDA receptor antagonist MK-801 decreased some of the behavioral signs of morphine withdrawal (6) and inhibited c-fos mRNA expression during naloxone-precipitated morphine withdrawal in adult rats (7). Not only is there no information concerning c-fos mRNA expression level in infant rat brain during morphine withdrawal, there is likewise no information on whether any of these changes could be prevented by co-administration of an NMDA antagonist. The present study was performed to determine the effects of naloxone on c-fos mRNA levels in infant rat brain following repeated morphine administration, to compare these with the changes observed in the adult rat, and to measure the ability of MK-801 to attenuate any increases in c-fos mRNA observed in the infant animal.

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MATERIALS AND METHODS

Subjects
Male Sprague-Dawley rats (SLC, Osaka) weighing 250–310 g at the start of morphine treatment were used for morphine withdrawal experiments in adult rats. For the experiments in infant rat, pregnant Sprague-Dawley rats at day 16 of gestation were purchased and their offspring were used without regard to sex. All rats were housed in an animal room with controlled temperature (22–24°C), humidity (60–70%) and light-dark cycle (on 07:00–19:00). They were fed laboratory chow and water ad libitum. To determine the parturition day, housing cages were checked daily at 10:00 AM and 5:00 PM. Pups found at either time on that day were termed day 0 of age. Experiments in infant rats were started at day 2 of age. All animals received humane care in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Drug administration
Infant and adult rats were repeatedly injected with morphine hydrochloride (Takeda, Osaka) 20 mg/kg, s.c., once daily at 10:00 AM for 5 days to produce morphine dependence. Twenty-three hours after the last morphine injection, animals were given naloxone hydrochloride (Sigma, St. Louis, MO, USA) at 2 mg/kg, s.c. to precipitate morphine withdrawal. To examine the effect of an NMDA-receptor antagonist on c-fos levels in infant rats, infant animals were pretreated with (5R,10S)-(+)5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrochloride maleate (MK-801, 0.1 mg/kg, s.c.; Sigma) or saline 30 min prior to every morphine administration. All of the infant rats receiving repeated administration of morphine were alive until the collection of samples (n = 67).

RNA extraction and cDNA synthesis
At 1, 3 or 6 h after naloxone administration, rats were sacrificed by decapitation under ether anesthesia and the whole brain was removed. The olfactory bulb, hypothalamus, midbrain and medulla oblongata were dissected freehand. Total RNA was extracted from the tissues by a guanidine-phenol extraction method and purified with ethanol precipitation. The RNA was reverse-transcribed using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The cDNA (native cDNA) was diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) for the preparation of template in the polymerase chain reaction (PCR).

Competitive PCR
The location of the oligonucleotides of primer pairs in cloned sequences for native cDNA was as follows: c-fos, 939–958 and 1625–1644 (8); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 325–344 and 843–862 (9). We generated the competitor cDNA in two consecutive PCRs (10, 11). The competitor for c-fos and GAPDH contained sequences of respective primers used in competitive PCRs on both ends. The component of the PCR mixture, including a fixed amount of native cDNA (0.16 fg) and a dilution of competitor cDNA, was pursuant to AmpliTaq Gold protocols (Perkin Elmer, Branchburg, NJ, USA) for competitive PCR of c-fos and to Takara Taq protocols (Takara, Otsu) for competitive PCR of GAPDH. Four dilutions of competitor cDNA were added: 0.16, 1.0, 1.6 and 3.2 fg for c-fos PCR and 1.0, 3.2, 10.0 and 32.0 pg for GAPDH PCR. The competitive PCR was performed under the following conditions for c-fos PCR: an initial step at 95°C for 10 min, followed by 48 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s. For GAPDH PCR, 24 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. Under the above conditions of amplification and PCR cycles, we observed linearity of exponential amplification of competitor and native cDNA by PCR.

The PCR products of native cDNA and competitor cDNA were 706-bp- and 522-bp-long in c-fos PCR, respectively, and 538-bp- and 320-bp-long in GAPDH PCR, respectively. Aliquots of PCR products of native and competitor cDNA were electrophoresed on 1% agarose gels, visualized by ethidium bromide staining, and photographed using the Printgraph (ATTO, Tokyo). The fluorescent intensity of the product bands was analyzed by NIH image 1.60 (NIH, Bethesda, MD, USA). Finally, the ratio of the intensity of native PCR products to that of competitor PCR products was plotted using a logarithmic scale on the ordinate against the logarithmic dilutions of the competitor cDNA on the abscissa. The amount of c-fos and GAPDH cDNA was determined as the amounts applied to the lane in which a 1:1 ratio of PCR products was observed. Ratios were expressed as c-fos to GAPDH mRNA.

Statistical analyses
All data were expressed as means ± S.E.M. Differences between groups were examined for statistical significance using the Student-Newman-Keuls test.

RESULTS
Naloxone induced increases in c-fos mRNA levels 1 h after its administration in a discrete brain region of infant rats receiving repeated morphine (Fig. 1). Neither naloxone challenge itself nor repeated morphine itself influenced the c-fos mRNA levels in any brain region. In infant rats treated repeatedly with morphine, 1 h after naloxone administration, marked and significant increases in c-fos mRNA levels were observed.
levels were observed in the olfactory bulb, compared with saline administration. Smaller but still significant increases in c-fos mRNA levels were observed in the hypothalamus and the medulla oblongata, but no increases were seen in the midbrain. In adult rat brains, the changes in c-fos mRNA levels under the same drug treatment conditions were very similar to those observed in infant rat brain: the largest increase in c-fos mRNA was detected in the olfactory bulb, a smaller increase was observed in the hypothalamus, and no increase was seen in the midbrain (Fig. 2). In the adult animals, the small increase in c-fos mRNA in the medulla oblongata was not significant.

The c-fos mRNA levels in infant rat brain changed in a time-dependent manner (Fig. 3). The maximal increases in the three brain regions were observed 1 h after naloxone challenge, and they returned to basal levels 6 h after naloxone challenge. No significant change in midbrain c-fos mRNA levels was found at any time after naloxone challenge.

To elucidate whether NMDA receptor activation during morphine treatments participated in the naloxone-induced increment of c-fos mRNA levels in infant rats, we examined the effects of co-administration of MK-801 with morphine on c-fos mRNA levels after naloxone challenge in infant rats. Co-administration of MK-801 significantly suppressed the increment of c-fos mRNA levels in the olfactory bulb and hypothalamus 1 h after naloxone challenge, although c-fos mRNA levels in the medulla oblongata were not significantly affected by MK-801 (Fig. 4).
After naloxone challenge, body weight loss, an indicator of morphine withdrawal, occurred in adult rats following repeated morphine in a time-dependent fashion (Fig. 5).

**DISCUSSION**

In our previous study, naloxone challenge induced morphine withdrawal signs in adult rats receiving repeated treatment with morphine using the same dosing regimen as employed in the present study (12). These included significant decreases in body weight that occurred 6 h after naloxone challenge. In the present study, significant decreases in body weight were observed in both adult and infant rats 6 h after naloxone challenge. These results suggest that morphine dependence developed in infant rats by repeated administration of morphine with the dosing regimen employed in the present study.

Naloxone challenge markedly increased c-fos mRNA levels in olfactory bulbs of both infant and adult rats treated daily with morphine. This has not been reported before. Immunohistochemical study revealed that two isoforms of the /opioid receptor, MOR-1 and MOR-1B, are abundant in rat olfactory bulb (13). In addition, the olfactory bulb contains type VIII adenylate cyclase, whose upregulation in the locus coeruleus is believed to be closely related to morphine dependence and withdrawal (14, 15). Although these reports and our results let us focus attention to whether the olfactory bulb is involved in morphine withdrawal, it remains to be clarified.

Most of the previous studies revealed increments of c-fos mRNA and c-Fos protein levels in the nuclei of the midbrain, medulla oblongata and hypothalamus of the adult rat during morphine withdrawal (4, 5, 16, 17), except for one study that demonstrated that c-Fos protein-like immunoreactivity was restricted to the cerebral cortex (18). Although the studies described above were different in methodology from the present study, the increases in c-fos mRNA in medulla oblongata and hypothalamus in the present study are in agreement with the earlier data (4, 5, 16, 17). In contrast to these earlier studies, we failed to find the naloxone-induced increase in c-fos mRNA levels of midbrain in either infant or adult rats following repeated morphine administration. It is unlikely that this discrepancy was due to the difference in quantitative analysis method; the competitive RT-PCR technique used in the present study yields highly reliable quantitative analysis of mRNA and is a more sensitive method than the traditional procedures such as Northern blotting. The in situ hybridization analysis revealed that only a few nuclei of the mesencephalic structure in adult rats showed increments of c-fos mRNA levels in naloxone-precipitated withdrawal (16).
Therefore, future studies require an analysis with a higher level of neuroanatomical resolution, i.e. in situ hybridization, which would resolve the discrepancy in the c-fos mRNA expression in midbrain of infant and adult rats.

In adult rat, it has been reported that c-fos mRNA is expressed specifically during morphine withdrawal (4, 5, 16, 17). In the present study, naloxone-precipitated c-fos mRNA was expressed in the hypothalamus of infant rat repeatedly receiving morphine in a sensitive manner to MK-801, as observed in adult rat (7). These results suggest that c-fos mRNA expression may be a specific index for morphine withdrawal in infant rat. On the other hand, it was revealed that the time of the peak of naloxone-precipitated c-fos mRNA expression occurred before that of naloxone-precipitated body weight loss in infant rats. It is believed that c-Fos is one of the transcriptional factors and regulates the biological response. If c-Fos acts as a cue for morphine withdrawal, it is possible that the c-fos mRNA is expressed before morphine withdrawal-induced body weight loss. However, the mechanism of body weight loss after morphine withdrawal is still unclear.

Investigators have demonstrated that pretreatment with MK-801 suppressed some behavioral signs (6) and c-fos mRNA expression (7) induced by morphine withdrawal in adult rats. In the current study, concomitant administration of MK-801 with morphine significantly attenuated naloxone-induced increments of c-fos mRNA levels in the olfactory bulb and hypothalamus of infant rats. In the medulla oblongata, the increment of c-fos mRNA was not suppressed by the co-administration of MK-801 with morphine. Region-specific expression profiles of NMDA-receptor subunits could contribute to the regional differences in MK-801-induced prevention of increased c-fos mRNA expression. NR2D, an NMDA-receptor subunit, is predominantly expressed in the brainstem including the medulla oblongata. NMDA receptor consisting of NR2D has considerably lower affinity for MK801 than NMDA receptor consisting of the other NR2 subunits (19, 20). Therefore, it is possible that the NMDA receptor in the medulla oblongata is less sensitive to MK-801. Alternatively, the naloxone-induced increment of c-fos mRNA in medulla oblongata in infant rats following repeated morphine may be through a mechanism that does not involve NMDA receptor activation.

The blockade of NMDA receptor is reported to inhibit most of the behavioral signs induced by morphine withdrawal in adult rats. However, MK-801 was found to fail to reduce body weight loss in adult rats after morphine withdrawal (21), suggesting that the NMDA receptor may not be involved in body weight loss induced by morphine withdrawal.

In conclusion, we demonstrated that naloxone induced increases in c-fos mRNA in restricted brain regions in infant rats treated repeatedly with morphine. Those changes were similar to those observed in adult rats. Concomitant administration of MK-801 with morphine blocked the increment of c-fos mRNA in olfactory bulb and hypothalamus of infant rats following naloxone challenge. Furthermore, infant rat following naloxone challenge showed body weight loss, a reliable indicator of morphine withdrawal (22, 23). These results suggest that morphine withdrawal can occur in infant rats on postnatal day 7 and that increased c-fos mRNA expression is a useful indicator for morphine withdrawal in infant rat.

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