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# Mediation of *N*-methyl-D-aspartate on Neuropeptide Y Expression Induced by Morphine in Mouse Cerebellum

**Gee-youn Kwon and Soo-kyung Kim**

Department of Pharmacology, School of Medicine, Keimyung University, Daegu 700–712, Korea

The existence of opioid receptors in mammalian cerebellum except human, has not been clearly understood. In the present study, we found that NPY was inducible by morphine in the mouse cerebellar granular and Purkinje cell layers. We performed in situ RT-PCR and immunohistochemistry to characterize the NPY expression. The increase of NPY gene expression by morphine (30 mg/kg, i.p.) was inhibited by pretreatment with not only naloxone (100 mg/kg, i.p.) but also a noncompetitive NMDA antagonist, MK-801 (0.3 mg/kg, i.p.). The competitive NMDA antagonist, AP-5 (0.9 mg/kg, i.p.) slightly attenuated the increased NPY expression by morphine. Also, the finding similar to morphine was shown by NMDA (70 mg/kg, i.p.) treatment. Our results indicate that NPY was inducible by morphine and this might reflect activation of NMDA receptors in granule cells that relay mossy fiber inputs to Purkinje cells *via* parallel fibers.

Key Words: NPY, Morphine, NMDA, Cerebellum, Mouse

## INTRODUCTION

The distribution of opioid receptors is characterized by interspecies differences among mammals including mouse, rat, guinea pig, rabbit, and human. The presence of opioid receptors in cerebellum has not been defined in mammals (Maurer et al, 1983; Zagon et al, 1990; Minami & Satoh, 1995). Recently, however, Schadrack et al (1999) reported the existence of opioid receptors in human cerebellum by employing PET, mRNA expression, and autoradiography. The opioidergic mechanisms play important roles in nociception, reinforcement and reward, neuroendocrine regulation, motor control, learning and memory. Thus, alterations of opioidergic system have been implicated in the pathophysiology of the disorders like Parkinson's disease (Henry & Brotchie, 1996), Gilles de la Tourette syndrome (Haber et al, 1986), Alzheimer's disease (Hiller et al, 1987; Barg et al, 1993), schizophrenia (Berger et al, 1981), and depression (Gabilondo et al, 1995).

Neuropeptide Y (NPY) is an endogenous neuroactive peptide known to be associated with many physiological processes such as stimulation of food intake, regulation of hormone synthesis and release, circadian rhythm, body temperature, and memory processing (Dumont et al, 1992; Heilig & Widerlov, 1995). The findings that NPY system may mediate and/or modulate some effects of mu opioids have been reported (Hua et al, 1991; Broqua et al, 1996; Woldbye & Madsen, 1998). The naloxone-precipitated withdrawal symptoms were reduced by intracerebral ventricular administration of NPY (Woldbye & Madsen, 1998), and intrathecal administration of NPY produced antinociceptive effects in the hot plate test in rats and the acetic acid writhing test in mice, and these effects were not antagonized by the opioid antagonist, naloxone (Hua et al, 1991; Broqua et al, 1996). Also, the opioid antagonist naloxone blocked memory processing and discriminative stimulus effects of NPY (Cleary et al, 1994; O'Hare et al, 1998). However, Picker et al (1999) reported that discriminative and antinociceptive effects of morphine were independent of an NPYergic pathway.

Recently, cerebellar functions have been recognized

Corresponding to: Soo-kyung Kim, Department of Pharmacology, School of Medicine, Keimyung University, 194 Dongsan-dong, Chung-gu, Daegu 700-712, Korea. (Tel) 82-53-250-7476, (Fax) 82-53-250-7016, (E-mail) green@dsmc.or.kr

not only in motor control, but also in cognitive and emotional processes (Thach et al, 1992; Desmond et al, 1998). The control of cerebellar functions depends on the synapse formation among the distinct types of cerebellar neurons (Altman & Bayer, 1997). The granule cells are the most abundant cerebellar neurons, and they are excitatory interneurons that relay mossy fiber input to Purkinje cells *via* parallel fibers and control the activity of Purkinje cells. We observed significant increase of NPY expression in cerebellar granular and Purkinje cell layers by morphine. These findings are new and very interesting, because the existence of opioid receptors has not been shown in cerebellum except human brain. The purpose of this study was to determine the NPY expressed by morphine in mouse cerebellum, and to assess its mechanism related with activation of *N*-methyl-D-aspartate (NMDA) receptor.

## METHODS

### *Materials*

Morphine was purchased from Jeil Pharmaceutical Company (South Korea). NMDA, naloxone, and AP-5 were obtained from Sigma Chemical Company (St. Louis, MO, USA). MK-801 was purchased from RBI (Natick, MA, USA). All other reagents were of analytical grade and purchased from various commercial sources.

### *Animal treatment and preparation of brain sections*

Adult male ICR mice, weighing 25~35 g, were used in this study. Mice were housed in cages on a constant 12-h light/12-h dark cycle and food and water *ad libitum*. Experimental mice were divided into four groups: control (saline injected) group, morphine (30 mg/kg, i.p.) treated group, NMDA (70 mg/kg, i.p.) treated group, and pretreatment groups of MK-801 (0.3 mg/kg, i.p.), AP-5 (0.3 mg/kg and 0.9 mg/kg, i.p.), or naloxone (100 mg/kg, i.p.). MK-801 and AP-5 were given 1 h prior to morphine treatment. Average 4~6 animals were used in each group. All the experimental drugs were given in a volume of 0.1 ml/10 g body weight. To harvest the whole brain, animals were anesthetized with pentobarbital sodium and killed by transcardial perfusion with phosphate-buffered saline (PBS) containing 2 units/ml heparin. For in situ RT-PCR, brains were removed, snap-frozen in isopentane (−70°C), and cryosectioned at 10 $\mu$M thickness. For immunohistochemistry, brains were removed, fixed in 10% phosphate-buffered formalin (PBF), and were sectioned at 7 $\mu$M thickness. All animal experiments were performed in accordance with protocols approved by the Committee on Animal Care and Experimentation at School of Medicine, Keimyung University.

### *In situ RT-PCR*

To localize the intracellular expression site of NPY mRNA, in situ RT-PCR was used for mRNA amplification according to the method described by Ausubel et al (1995) with slight modification. In brief, in order to remove genomic DNA, frozen sections were treated with RNase-free DNase I (2.5 U/section: Promega, USA) for 3 h at 37°C, followed by heating at 94°C for 2 min. In situ reverse transcription (RT) was carried out in a solution containing 5 $\mu$l of 5X RT buffer, 5 $\mu$l of 10 mM dNTPs, 100 *p*mol of oligo $(dT)_{16}$, 20 units of RNasin (Promega, USA), 100 units of M-MLV reverse transcriptase (Promega, USA) and DEPC-treated RNase-free deionized water in a total volume of 25 $\mu$l. Sections were covered with plastic cover slips and incubated for 1 h at 42°C in a thermocycler Omin-Gene (Hybaid, UK). After RT reaction, sections were washed with deionized water and dehydrated with absolute ethanol. Slide seal for in situ PCR (Takara, Japan) was placed around the section on the slide and covered with PCR mixture. In situ PCR was carried out in a solution containing 2.5 $\mu$l of 10X buffer, 2.5 $\mu$l of 10X PCR digoxigenin labeling mix (Boehringer Mannheim, Germany), 25 *p*mol of sense and antisense primers, 2.5 units of Taq DNA polymerase and deionized water in a total volume of 25 $\mu$l. PCR oligonucleotide primers for NPY mRNA were 5′-ATGCTAGGTAACAAACG-3′ and 5′-TCACCACATGGAAGGGT-3′. Cycling conditions were as follows: denaturation at 94°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 30 s, total 27 cycles.

### *Immunological detection of in situ PCR products*

In situ PCR products were visualized using Digoxigenin detection kit (Boehringer Mannheim, Germany). After removal of slide seal, unincorporated dig dUTP was washed off with a solution of 2X SSC,

0.1% SDS for 10 min twice at room temperature, and for 10 min twice at 45°C with 0.1% SSC, 0.1% SDS, and 10% BSA. Slides were then incubated for 30 min with a solution containing 0.5% blocking reagent, 100 mM Tris-HCl (pH 7.5), and 150 mM NaCl. The amplified products were then detected by color development at room temperature by incubation with 1 : 200 dilution of anti-digoxigenin alkaline phosphatase Fab fragments in blocking solution for 30 min. Free Fab fragments were removed with a washing solution containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ for 3 min. Slides were incubated for 5~10 min at room temperature in substrate solution containing NBT/BCIP, and signals were detected using an optical microscope equipped with SPOT-JR Image Analyzer System (Diagnostic Instruments, Inc. USA).

*Immunohistochemistry*

Animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital, and underwent transcardiac perfusion with heparinized PBS, followed by 10% PBF. Whole brain was removed and fixed in 10% PBF for 2~3 days. The fixed tissues were hydrated, embedded in paraffin, sectioned at 7 $\mu$M thickness, and mounted onto 3-aminopropyltriethoxysilane-coated slides (Sigma, St. Louis, MO, USA). Coronal sections were deparaffinized in xylene, hydrated in a graded series of ethanol (100, 95, 80, and 70%) and PBS. Immunohistochemistry was performed using the avidin-biotin complex technique with 3,3-diaminobenzidine (DAB) as the chromogen. Sections were incubated for 30 min in methanol containing 0.3% $H_2O_2$ to block endoge-

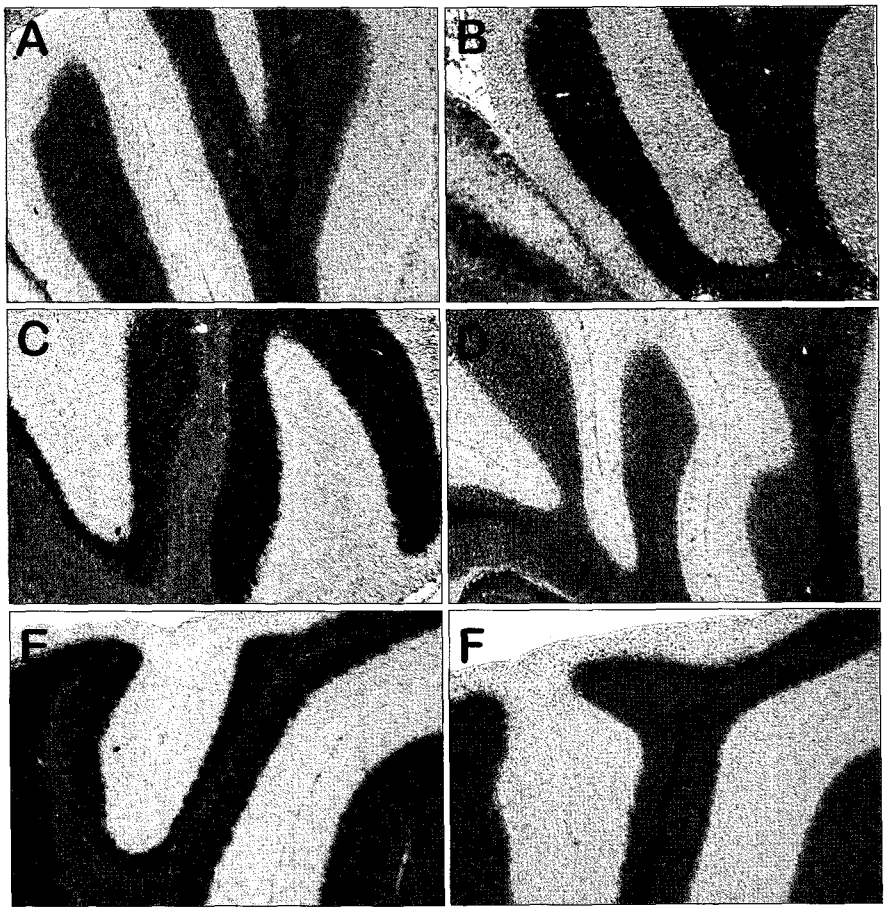


**Fig. 1.** NPY mRNA expression analyzed by in situ RT-PCR in mouse cerebellum. A, control (saline only); B, 2 days after morphine injection (30 mg/kg); C, 2 days after NMDA (70 mg/kg); D, MK-801 (0.3 mg/kg)+morphine; E, AP-5 (0.3 mg/kg)+morphine; F, AP-5 (0.9 mg/kg)+morphine. The figures are the images representing at least three independent experiments.

nous peroxidase activity. Then the tissue sections were washed in PBS and antigen retrieval was done by microwave irradiation (370 W, 10 min in 0.01 M citrate buffer, pH 6.0). After two PBS washes, the sections were incubated with 1 : 500 dilution of the primary antibody, anti-sheep NPY antiserum (Chemicon Int. Co.) for 1 h at 37°C. Immunolabeling was detected using a biotinylated universal immunoglobulins followed by visualization with an streptavidin peroxidase kit (DAKO LSAB kit) and DAB staining. The sections were counter stained in hematoxylin, and mounted with Canada balsam.

*H & E stain*

Coronal sections were deparaffinized in xylene, hydrated in a graded series of ethanol (100, 95, 80, and 70%) and water. Subsequently, the tissues were stained with hematoxylin & eosin.

## RESULTS

*Effects of morphine and NMDA on NPY expression in the cerebellum*

To examine the NPY expression induced by morphine, we performed in situ RT-PCR on frozen sections and immunohistochemistry on paraffin block sections of the mouse cerebellum. The prominent increase of NPY mRNA expression was observed by morphine injection in the granular layer (Fig. 1B). To

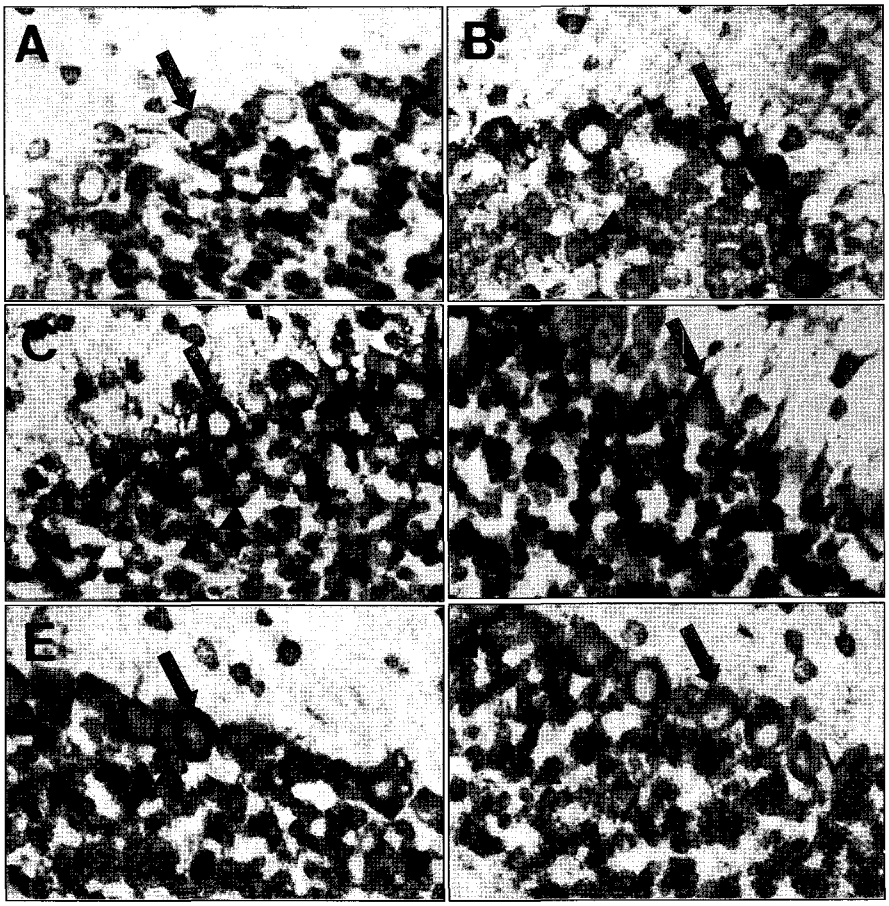


**Fig. 2.** Immunohistochemical localization of NPY in mouse cerebellum. A, control (saline only); B, 2 days after morphine; C, 2 days after NMDA; D, MK-801 (0.3 mg/kg)+morphine. E, AP-5 (0.3 mg/kg)+morphine; F, AP-5 (0.9 mg/kg)+morphine. Arrows indicate Purkinje cells, and arrowheads represent granule cells. The figures are the images representing at least three independent experiments (original magnification ×40).

further confirm the increased expression of NPY, we immunolabeled the mouse cerebellar slices with anti-sheep NPY antiserum, and found that NPY immunoreactivity was also increased in the granular layer and the large Purkinje cells which are characteristic of the cerebellum (Fig. 2B). Golgi type II cells of the granular layer were also positively stained with NPY antiserum.

The level of NPY expression was also elevated by NMDA treatment (70 mg/kg), evidenced by both RT-PCR and immunohistochemistry (Fig. 1C, 2C), and the degree of NMDA-induced increase of NPY expression was similar to those of morphine treatment.

*Effects of MK-801 and AP-5 on the morphine-induced NPY expression in the cerebellum*

Pretreatment with a non-competitive NMDA antagonist, MK-801 (0.3 mg/kg), significantly inhibited both mRNA and protein levels of morphine-induced NPY expression (Fig. 1D, 2D). The inhibition of the morphine-induced NPY expression by a competitive NMDA antagonist, AP-5 was observed at high dose of 0.9 mg/kg, and the degree of blockade in both mRNA (Fig. 1E, F) and protein level (Fig. 2E, F) was not as typical as that of MK-801.

*Effect of naloxone on the morphine-induced NPY expression in the cerebellum*

The morphine-induced NPY immunoreactivity was inhibited by naloxone in the cerebellar granular and Purkinje cell layers (Fig. 3).

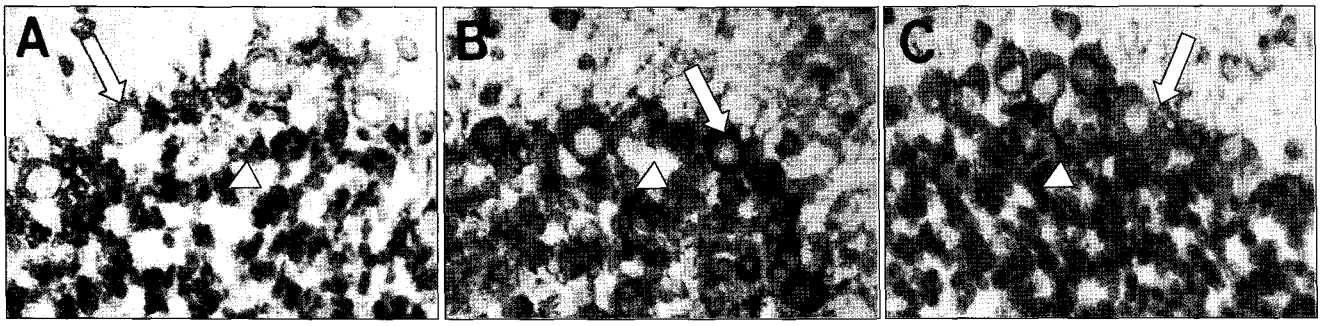


**Fig. 3.** Effect of naloxone on the morphine-induced NPY expression in mouse cerebellum. A, control (saline only); B, 2 days after morphine (30 mg/kg); C, naloxone (100 mg/kg) + morphine. Arrows indicate Purkinje cells, and arrowheads represent granule cells. The figures are the images representing at least three independent experiments (original magnification ×40).

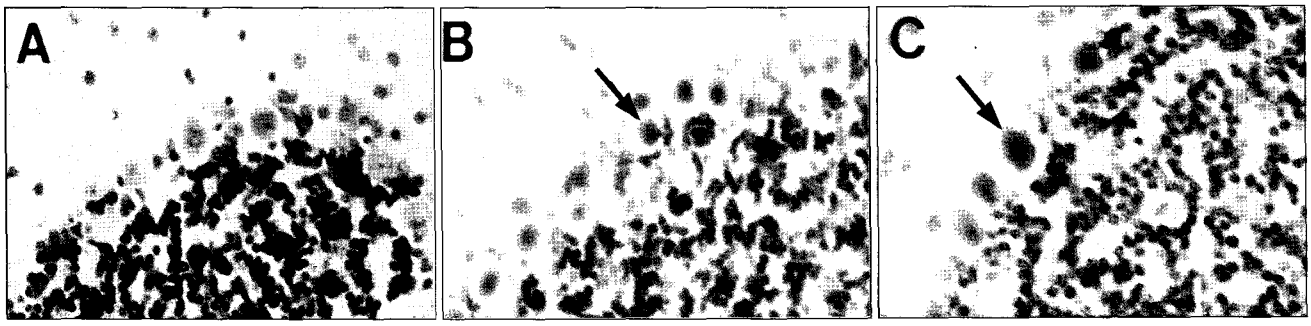


**Fig. 4.** Microphotographs of hematoxylin-eosin stained sections of the mouse cerebellum. A, normal cerebellar cortex from control animals (treated with saline); B, cerebellar sections of morphine treated (30 mg/kg, i.p., 2 days after a single injection) mice; C, cerebellar sections of NMDA treated (70 mg/kg, i.p, 2 days after a single injection) mice. Arrows indicate pyknotic nuclei in the Purkinje cell layer. The figures are the images representing at least three independent experiments (original magnification ×40).

*Morphological changes induced by morphine and NMDA*

Histological examination of the mouse cerebellum indicated a marked pyknotic changes in the granular and Purkinje cell layers in the animals treated with morphine or NMDA compared to saline-treated controls (Fig. 4).

## DISCUSSION

The data obtained in this study clearly showed that the administration of morphine increased NPY expression in mouse cerebellum, especially in the granular layer. We did not observe any significant expression of NPY in the molecular layers, which represent the outer region of the cerebellar cortex. The opioid receptors of the cerebellum have not been well defined in mammals except for human including mouse, rat, guinea pig, and rabbit (Maurer et al, 1983; Zagon et al, 1990; Minami & Satoh, 1995).

In the present study, we observed that NPY expression was inducible by morphine in the mouse cerebellum. In this novel *in vivo* studies, high levels of cerebellar NPY expression in both mRNA and protein levels were found to be localized in the granular and Purkinje cell layers. At present, it is rather difficult to state that the NPY expression was a response due to a direct action on the opioid receptors, since the existence of opioid receptors in the mouse cerebellum is not yet established. Therefore, the NPY expression observed in our system can be explained as an indirect action of opioid receptors which are present in the cerebrum. We suggest that the neuronal signaling of morphine on the cerebral opioid receptors was transmitted to the cerebellar granule cells *via* the inferior olive of the brain stem, and these signalings could promote the release of NPY. Alternatively, there might exist an opioidergic pathway in the mouse cerebellum.

We also observed the increase of NPY expression in the cerebellar granular and Purkinje cell layers two days after a single injection of NMDA (70 mg/kg, i.p.). Interestingly, the NPY expressions induced by morphine and NMDA were similar in their intensity and localization in the mouse cerebellum. The increased NPY expression in both mRNA and protein levels induced by morphine administration was readily reversed by the NMDA antagonist, MK-801 (Fig. 1D, 2D) and the opioid antagonist, naloxone (Fig. 3). One feasible explanation for this phenomenon is that NPY release might be inter-regulated with NMDA receptors in the brain. We speculated that the NPY expression observed in the granular and Purkinje cell layer might represent glutamatergic NMDA stimulation mediated by cerebellar input *via* mossy fibers; activation of NMDA receptors in granule cells triggered the signaling pathways for NPY expression. The cerebellar granule cells are excitatory interneurons that relay mossy fiber input to Purkinje cells *via* parallel fibers. The cerebellar granule cells form synapses with their input, the terminals of the mossy fibers and Golgi cells in the granular layer. All mossy fiber input into the cerebellum represents the glutamatergic excitatory pathway. Other fibers, which come exclusively from the inferior olive of the brain stem, contact with Purkinje cells of the cerebellar cortex. Schwarzer et al (1998) reported that NPY was synthesized in granule cells/mossy fibers and released during epileptic seizure through Y2 receptor. Marked increase of NPY has been reported in animal models of ischemia, epilepsy, and noxious stimuli (Castagne et al, 1987; Schwarzer et al, 1996). Further studies are in need to characterize physiological functions of the NPY upon morphine administration as well as excitotoxic stimulation in this system. In the CNS, the understanding of physiological roles played by NPY might provide a way toward pharmacological intervention that can be employed in many disorders of the brain.

The results obtained in the present study provide a new finding that morphine administration results in the increase of NPY expression at both mRNA and protein levels in the cerebellar granular and Purkinje cell layers. The similar finding due to morphine was also shown by NMDA injection. The increase of NPY by morphine was attenuated not only by naloxone pretreatment, also by MK-801 or AP-5 pretreatment. We suggest that the expression of NPY was due to a response generated by NMDA activation of granule cells *via* mossy fibers, since the existence of opioid receptors has not yet been proved in the mouse cerebellum.

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