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Changes of μ-opioid receptors and GABA in visual cortex of chronic morphine treated rats

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Abstract

Electrophysiological and biochemical studies have indicated that GABAergic modulation is involved in the opioid-induced altering of response properties of visual cortical cells and impairing of short-term synaptic plasticity in the geniculo-cortical visual pathway. The aim of the current study was to examine whether there were changes in the localization and density of μ-opioid receptor subtype (MOR1) and GABA in the visual cortex of morphine-dependent and abstinent rats. Immunofluorescence histochemical method was applied to display MOR1 and GABA distribution. We found that MOR1-like immunoreactive neurons were significantly lowered in layer I–VI of visual cortex of rats sacrificed immediately after the last injection (defined as morphine-dependent (MD)) than saline-control group. In rats sacrificed just before the last injection (defined as morphine-abstinent (MA)), the density of μ-opioid receptor was higher than that in dependent group in layer I–V neurons of visual cortex, but remained lower than those in control group. Three hours after the last morphine injection (defined as 3 h after morphine-abstinent (3 h)), MOR1-like immunoreactive neurons in layer I and layer IV of visual cortex were still significantly lower than control. As to GABA-like immunoreactive neurons, they were significantly decreased in abstinent group compared to dependent group. These results provide morphological evidence that opioid-induced altering of response properties of visual cortical cells might be due to the change of visual cortical GABAergic system induced by opioid via μ-opioid receptor, which result in disinhibition of visual cortex projection neurons and their abnormal firing.

Keywords: Immunofluorescence histochemistry; μ-Opioid receptor; GABAergic neuron; Visual cortex; Rat

Changes of endogenous opioid peptides system have been considered to constitute an important mechanism of opioid dependence. Repeated use of addictive drugs leads to multiple adaptive neuronal responses. Increasingly, morphine-like drugs decreased visual sensitivity in humans, impacted visual discrimination performance in rats and affected cortical potentials evoked from optic chiasm stimulation in cats [5]. Recent work in our lab also showed that chronic morphine exposure influences the response properties of cortical neurons in cats and impaired short-term synaptic plasticity in the geniculo-cortical visual pathway of rats [20]. It has been proposed that the modulation of morphine on GABAergic system could play a key role in their results.

Neuroanatomy study indicated that the pyramidal cells of morphine treated rats showed a significant decrease in the total dendritic length and the dendritic spine density of dendritic arborization of layer III in the primary visual cortex in rats [12]. It may contribute to synaptic plasticity and modify synaptic efficacy by altering fast synaptic neurotransmission [9] or the local chemical environment [18]. Our previous neurochemical research showed that GABA content and the activity of its synthetase-glutamic acid decarboxylase in the visual cortex of morphine-abstinent rats were significantly decreased. Other researches also shown that GABAergic synaptic transmission is influenced by opiates [4,6,11]. It has been suggested that the visual system is subject to opiate modulation. Although opioid receptors express extensively in the visual system of rats, it is unknown whether and how chronic opioid expo-

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sure affects response modulation in visual cortical neurons of rats.

Our previous work on rats and cats indicated the chronic morphine exposure could impair the inhibition on visual cortical cells [7]. However, this phenomenon still lacks morphological evidence. Therefore, it is critical to investigate the effects of chronic morphine exposure on the distribution pattern of μ-opioid receptor and GABA in visual cortex. The current study was undertaken to examine the expression of μ-opioid receptor and GABA in the visual cortex of morphine-dependent and morphine-abstinent rats at different time intervals by using immunofluorescence histochemical staining for μ-opioid receptor and GABA.

Thirty Male Sprague Dawley (200–230 g) rats were obtained from the Laboratory Animal Center, An'hui Medical University (Hefei, China). Rats were housed in groups and maintained with food and water ad libitum on a 12 h light/dark cycle. Fifteen rats were injected with morphine (10 mg/kg) (SC injection) twice per day at 12 h intervals for 10 days as described previously [17,19]. Fifteen rats were treated similarly with the normal saline (NS) instead of morphine. Of the 15 morphine-treated rats, 5 were sacrificed immediately after the last injection, 5 sacrificed just before the last injection and the last 5 rats were sacrificed 3 h after the last injection. Of the 15 saline-control rats, they were sacrificed timely as above. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The animals were deeply anaesthetized with urethane and 0.9% NaCl was perfused through the ascending aorta till the liver became pale, followed immediately by 0.1 M phosphate-buffered saline (PBS; pH7.4) containing 4% (w/v) paraformaldehyde and 0.5% (w/v) glutaraldehyde. After perfusion, the skull was opened and the visual cortex was dissected out. After further fixation for 20–24 h in the perfusing fixative, the samples were washed in PBS, dehydrated in ethanol, transparentized with xylene and embedded in paraffin. Consecutive coronal sections 8 μm in thickness were cut and mounted for histological staining and immunostaining on poly-L-lysine-coated microscope slides. The sections were placed into three different dishes according to their numerical order while cutting (e.g., sections 1, 3, 5 to dish 1, sections 2, 4, 6 to dish 2, sections 3, 6, 9 to dish 3, respectively). All sections were washed carefully with 0.1 M PBS.

All of the sections were deparaffinized in xylene, hydrated through a graded series of ethanol and washed for 5 min in distilled water. Sections in the first and second dish were treated at room temperature for 10 min with 10% goat serum to suppress background staining. For μ-opioid receptors, sections in the first dish were incubated sequentially with: (1) guinea pig anti-μ-opioid receptor subtype (MOR1) polyclonal antibody (AB1774, 1:8000 dilution; Chemicon) for 48–72 h at 4 °C; (2) Cy3 conjugated secondary antibody (1:800 dilution, Chemicon) for 1 h at 37 °C. For GABA, sections in the second dish were incubated sequentially with: (1) rabbit anti-GABA serum (A2052, 1:400 dilution; Sigma, St. Louis, MO) for 48–72 h at 4 °C; (2) Goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC; F6005; 1:700 dilution; Sigma) for 1 h at 37 °C. The sections were rinsed at least three times in 0.1M PBS (pH7.4) after each incubation, lasting over 10 min every time. Then, the sections were air dried and cover-slipped with a mixture of 50% (v/v) glycerin and 0.1M PBS. Finally, the sections were observed with a fluorescence microscope (IX-70; Olympus, Tokyo, Japan) under appropriate filters for green-emitting FITC and for red-emitting, respectively. In the control experiments, the primary antibodies were omitted or replaced with PBS. No immunofluorescence histochemical staining for the omitted or replaced antibodies was detected. The sections in the third dish were processed for Nissl staining to identify the visual cortex according to Paxinos and Watson [16].

Quantitative measurement was done by a person blind to experimental groups. At 400×, according to the Nissl stained sections and the rat brain atlas, the numbers of GABA-like and MOR-like immunopositive neurons in visual cortex were counted in a calibrator (50 μm × 50 μm) and the density (cells/mm^2) was calculated. The criterion for acceptance as a neuron was the clear differentiation from background staining and a profile of soma.

All the data were expressed as mean ± S.E.M. The significance of the difference between the groups was evaluated by a two-way analysis of variance (ANOVA), and a P-value of <0.05 was considered significant.

MOR1-like immunoreactive non-pyramidal neurons were distributed diffusely throughout layers I–VI of rats' visual cortex. MOR1-like immunoreactive neuronal cell bodies were round, fusiform, ovoid or triangular in shape (Fig. 1). The diameters of MOR1-like immunoreactive neuronal cell bodies were about 10–20 μm. There was significant group and time differences in layer IV in the density of MOR1 between experiment and control groups ($F[1, 24] = 4.55$, $P < 0.05$; $F[2, 24] = 4.01$, $P < 0.05$). In morphine-dependent group, MOR1-like immunoreactive neurons significantly decreased in layer VI than that in saline-control (Table 1). Three hours after last morphine injection, MOR1-like immunoreactive neurons were counted predominantly more than morphine-dependent groups. In morphine-abstinent rats, MOR1-like immunoreactive neurons were notably more than morphine-dependent ones but still fewer then saline-control. In conclusion, morphine-dependence leads to decreased MOR1 expression while morphine-abstinence increases density of MOR1-like immunoreactive neurons.

GABA-like immunoreactive labeling was usually observed in the perikary of non-pyramidal neurons and their processes. Immunopositive labeling of GABA-like immunoreactive neurons was predominantly located underneath the cytomembrane of the perikarya and their processes. GABA-like immunoreactive neurons had similar morphological features to the MOR-like immunoreactive neurons, i.e., GABA-like immunoreactive neuronal cell bodies were also round, fusiform ovoid or triangular in shape. The diameters of GABA-like immunoreactive neuronal cell bodies were the same as the MOR-like immunoreactive neurons (about 10–20 μm) (Fig. 2).

GABA-like immunoreactive neuronal cell bodies were located scattered through all layers of visual cortex, but their

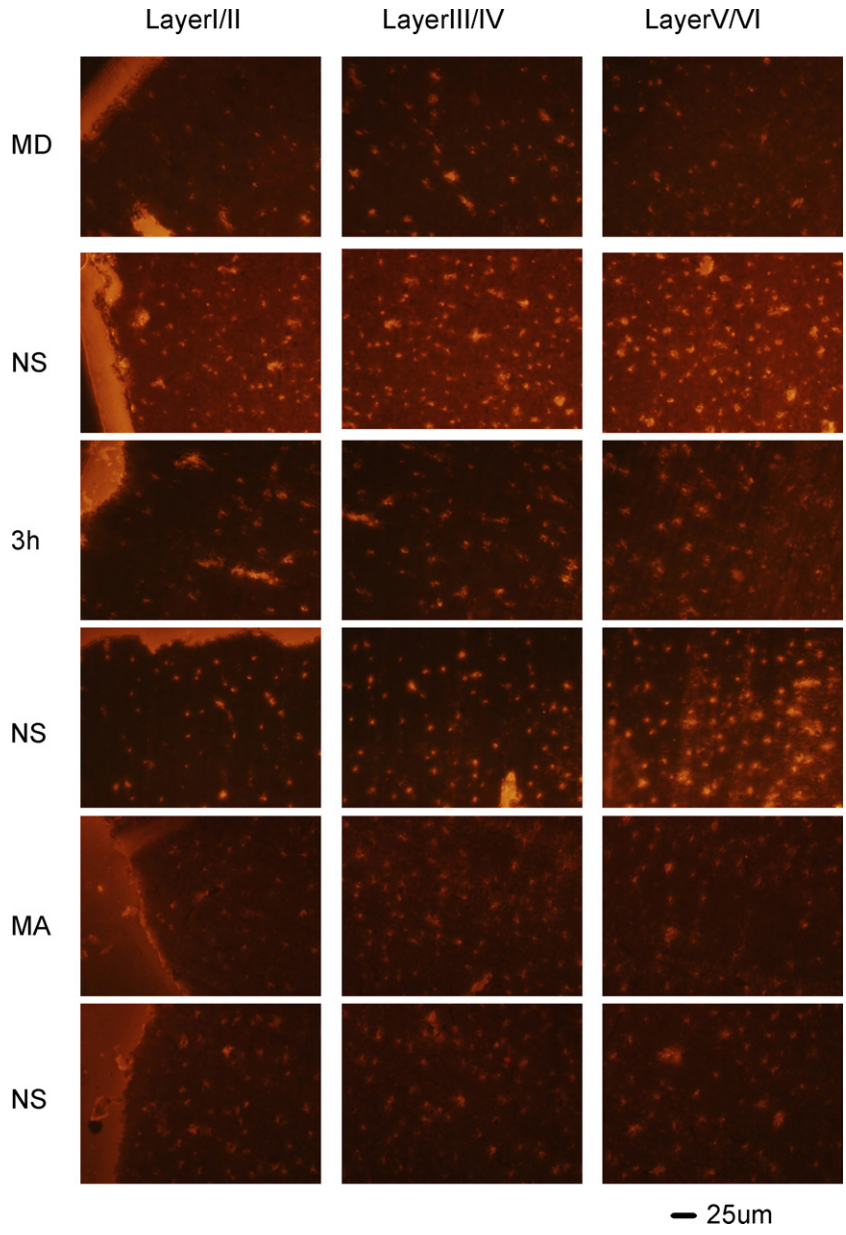

Fig. 1. MOR1-LI scatters through layers I–VI in the visual cortex. Morphine-dependent visual cortical neurons (MD), morphine-abstinent neurons (MA) and 3 h after morphine-abstinent neurons (3 h) were stained with polyclonal guinea pig antibody against MORs. NS represents normal control. Layer I/II (left), layer III/IV (center), layer V/VI (right) represent different layers of visual cortex I–VI. Scale bar = 25 μm.

Table 1
Numerical densities (of neurons/mm^2) of MOR1-immunoreactive neurons in the visual cortex

Cortical layer	MA	Control of MA	MD	Control of MD	3 h	Control of 3 h
I	3365.36 ± 111.14	3656.64 ± 357.49	2697.20 ± 173.76	3763.60 ± 327.37	3269.60 ± 167.70	2732.00 ± 201.55
II	3602.48 ± 202.93	3940.24 ± 196.98	3107.20 ± 114.06	4110.00 ± 295.13	3353.60 ± 485.50	3308.00 ± 370.92
III	3914.00 ± 116.60	4186.24 ± 222.12	3110.40 ± 201.88	4219.60 ± 361.14	3800.80 ± 211.64	3349.60 ± 281.99
IV	3832.00 ± 165.15*	4107.68 ± 243.01	2938.80 ± 161.50*	3794.40 ± 240.30	3226.40 ± 305.52*	3425.60 ± 354.86
V	4047.34 ± 203.90	3612.88 ± 125.45	3146.00 ± 178.04	3681.20 ± 236.34	3325.60 ± 315.28	3329.20 ± 262.18
VI	3732.00 ± 103.80	3764.24 ± 114.44	3130.40 ± 120.76	3903.60 ± 344.01	3128.80 ± 438.73	3112.00 ± 290.76

MOR1-labeled neurons were examined in an average of eight fields per animal, yielding an absolute number of cells in forms of cells/mm^2. Morphine-abstinent (MA), morphine-dependent (MD) and 3 h after morphine-abstinent (3 h). The data were analyzed using analysis of two-way ANOVA. There was significant group and time differences in layer IV ($F[1, 24] = 4.55$, $P < 0.05$; $F[2, 24] = 4.01$, $P < 0.05$).

* Significantly different from the control group.

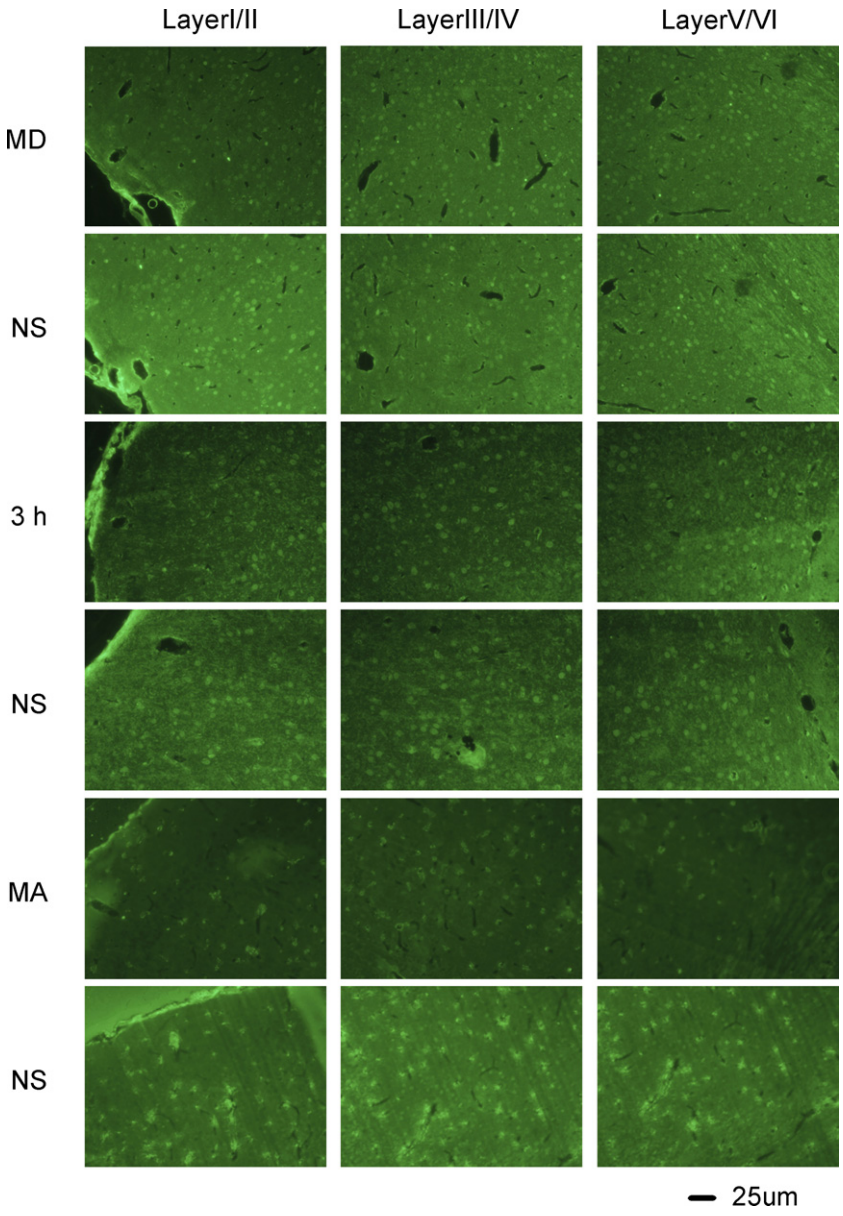

Fig. 2. GABA-LI immunoreactive neurons in layers I–VI in the visual cortex. Morphine-dependent visual cortical neurons (MD), morphine-abstinent neurons (MA) and 3 h after morphine-abstinent neurons (3 h) were stained with polyclonal rabbit antibody against GABA. NS represents normal control. Layer I/II (left), layer III/IV (center), layer V/VI (right) represent different layers of visual cortex I–VI. Scale bar = 25 μm.

Table 2
Numerical densities (of neurons/mm^2) of GABA-immunoreactive neurons in the visual cortex

Cortical layer	MA	Control of MA	MD	Control of MD	3 h	Control of 3 h
I	3008.80 ± 249.69*	3384.80 ± 162.75	3833.60 ± 200.21	3658.00 ± 291.49	3229.60 ± 305.50	2935.20 ± 144.15
II	3378.40 ± 230.19*	3556.00 ± 76.83	4027.20 ± 140.55	3658.00 ± 334.81	3393.60 ± 190.37**	3256.00 ± 163.96
III	3169.60 ± 234.11$^{\#}$	3971.20 ± 221.11	3445.60 ± 322.92	3645.00 ± 120.10	3504.00 ± 192.04	3341.60 ± 247.79
IV	3408.00 ± 149.18$^{\#}$	3871.20 ± 124.96	3764.80 ± 138.97	3465.00 ± 279.45	3309.60 ± 136.63**	3184.80 ± 57.32
V	3472.80 ± 432.53	3637.60 ± 116.64	3468.80 ± 346.76	3542.00 ± 302.15	3746.40 ± 338.81	3664.80 ± 199.34
VI	3344.80 ± 354.46	3496.00 ± 198.15	3558.40 ± 305.49	3607.00 ± 202.24	3416.00 ± 168.52	3696.80 ± 227.06

GABA-labeled neurons were examined in an average of eight fields per animal, yielding an absolute number of cells in forms of cells/mm^2. Morphine-abstinent (MA), morphine-dependent (MD) and 3 h after morphine-abstinent (3 h).

$^{\#}$ $P < 0.05$, compared with control of morphine-abstinent group.

* $P < 0.05$, compared with morphine-dependent group.

** $P < 0.05$, compared with morphine-dependent group.

distribution patterns varied. In morphine-dependent rats significantly fewer GABA-like immunoreactive neuronal cell bodies were found in the visual cortex. Three hours after the last morphine injection, GABA-like immunoreactive neurons were more than morphine-dependent group in layer II and IV of the visual cortex (Table 2). There was significant time differences in the density of GABA in layer I ($F[2, 24] = 5.13$, $P < 0.05$), layer II ($F[2, 24] = 4.11$, $P < 0.05$) and layer IV ($F[2, 24] = 4.60$, $P < 0.05$). In morphine-abstinent group, fewer GABA-like immunoreactive neurons were observed than morphine-dependent rats in layer I and II and there was a decrease than saline-control in layer III and IV. Above all, there is an interesting phenomenon that morphine-dependent rats show fewer GABA-like immunoreactive neurons than control, 3 h after the last morphine injection there is more GABA-like immunoreactive neurons in layer II and IV, and morphine-abstinent rats show fewer than morphine-dependent group.

MORs have been believed to play important roles in opiate addiction and analgesia [22]. Opioids interaction with cell surface receptors on neurons had exerted powerful influences upon a variety of physiological functions, such as neurotransmitter release and neuroendocrine secretion functions [2]. Pharmacological studies have shown that the μ-opioid receptor subtype appears to be particularly important for the reinforcing actions of heroin and morphine [14]. Our previous studies have shown that morphine affects the visual response properties [7], degrades response modulation of visual cortical cells of cat [8]. Moreover, short-term synaptic depression recovers to normal levels at several time points from 3 h to 6 h after morphine re-exposure [20]. We believed the degradation of GABAergic systems induced by chronic morphine exposure was involved. It is well known that activation of opioid receptors suppresses the presynaptic release of GABA, which may alter the neural network activity, and thus indirectly changes the function and morphology of dendrite spines [23]. Therefore, neuroanatomical experiments on visual cortex were carried and we found the changes of GABA level in visual cortex of rats following chronic morphine exposure. We have also found that GABA content and the activity of GAD (glutamic acid decarboxylase, GAD) in visual cortex of morphine-abstinent rats were significantly decreased compared with saline-control. Three hours after re-exposure to morphine, both GABA content and the activity of GAD recovered to normal level. These results suggest that GABAergic neurons are implicated in morphine-induced changes of visual cortical neurons' properties. In this paper, we provide morphological evidence that chronic morphine exposure influences the distribution pattern of μ-opioid receptor and GABA. These results indicate that chronic morphine exposure changes visual cortical neurons' response properties through modifying neurotransmitter content level.

Based on pharmacological studies, there are three major subtypes of opioid receptors: μ, δ, κ. Our evidence shows that μ-opioid receptor mediates opioid modulation of plasticity and neurotransmitter during chronic morphine treatment. The altered distribution of μ-opioid receptors may result from decrease of gene expression, or, receptor internalization [1,21]. However, the participation of other subtypes of opioid receptors cannot be excluded. The endogenous opioids, as well as the exogenous opioids, including morphine, may reduce the excitability of neurons through the postsynaptic μ-opioid receptors, and they may inhibit the release of neurotransmitters such as GABA or neuromodulators via the presynaptic μ-opioid receptors. It is possible that the decrease in the amount of detectable GABA between different groups of animals resulted indirectly from presynaptic accumulation of GABA molecules. When morphine is given systematically, it inhibits the presynaptic release of GABA. The accumulated GABA in the presynaptic termini may cause a feedback inhibition of GABA synthesis. Decreased synthesis of GABA may lead to a decrease in immunostaining of GABA. Additionally, potential candidates include adenosine and 5-HT, all of them have been shown to cause potent presynaptic inhibition of GABA release [24]. A number of mechanisms could be involved in these effects, such as the increase of adenylyl cyclase, a decline in cAMP-dependent phosphodiesterase activity reported in cell lines and various brain areas [15], or an upregulation of downstream cAMP-dependent processes. The evidence suggests that all three mechanisms can occur in response to chronic morphine treatment [15,26].

Besides, many other factors are also involved in GABAergic mediated opioid addiction. For example, different types of GABA receptors may also play an important role, for example, activation of $GABA_B$ receptor prevents self-administration of heroin and DA release of nucleus accumbens. $GABA_A$ receptor functions in modulating DA releasing and drug reinforcement [10,25]. Interestingly, in situ hybridization histochemistry and binding autoradiography studies in the central nervous system of the rat indicated that the distribution of μ-opioid receptor mRNAs mostly parallels to that of the receptor binding sites, but numbers of notable discrepancies exist between the distribution of the μ-binding sites and that of μ-opioid receptor mRNAs. This dissociation suggests that the receptor may be transported from the synthesis sites in the cell bodies to the dendrites and/or axon terminals [13]. So it is necessary to examine further the expression of μ-opioid receptor mRNA and μ-opioid receptor at the synthesis sites by in situ hybridization and histochemistry.

In fact, it has been found that the modulatory mechanism of GABAergic neurons involved in the opioid action also takes place in many other brain regions. In cerebral cortex, hippocampus and brainstem descending inhibitory system, the expression of μ-opioid receptor and GABA in neurons and opioid-evoked excitation of projection neurons that results from inhibiting the GABAergic interneurons have been found [3]. Opioid may change functions of neurons via Ì-opioid receptor and GABAergic system. In summary, the present study has provided new morphological evidence for the changes of density and distribution of μ-opioid receptor and GABAergic neurons at the visual cortex.

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