REVIEW —Current Perspective—

Enkephalinergic Neurons in the Periaqueductal Gray and Morphine Withdrawal

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ABSTRACT—The effects of opioid (e.g., morphine) withdrawal on levels of endogenous opioid peptides and their mRNA in the various brain regions have been studied. However, the role of this opioidergic mechanism in the mediation of opioid withdrawal is not fully understood. Preproenkephalin (PPE) mRNA in the caudal periaqueductal gray (cPAG), an important brain region in opioid withdrawal, is increased by both opioid antagonist (naloxone)-precipitated and spontaneous morphine withdrawal, but not by various other stresses in rats, indicating a role of endogenous enkephalins in the cPAG in morphine withdrawal. In addition, PPE mRNA levels in the cPAG increase in the course of the dissipation of morphine withdrawal, and they are returned to the control levels after disappearance of morphine withdrawal signs. Local administration of an enkephalin analog or peptidase inhibitors into the cPAG suppresses morphine withdrawal signs. These facts suggest that enkephalinergic neurons in the PAG may have a critical role in the recovery phase of morphine withdrawal. Recently, an involvement of transcription factors in morphine withdrawal has been suggested. Thus, the possible role of transcription factors in the regulation of PPE gene expression in the cPAG during morphine withdrawal is also discussed.

Keywords: Enkephalin, Preproenkephalin mRNA, Morphine withdrawal, Periaqueductal gray, Transcription factor

Introduction

Chronic use of μ -opioid agonist (such as morphine) results in the development of physical dependence on the drug, which is defined as an occurrence of withdrawal signs after the cessation of the opioids or administration of opioid antagonist. In the central nervous system, there are three types of endogenous opioid peptides, that is, enkephalins, endorphins and dynorphins, which are processed from preproenkephalin (PPE), proopiomelanocortin and preprodynorphin, respectively. Changes in the levels of the endogenous opioid peptides and mRNA expression of their precursors have been reported in various brain regions during opioid withdrawal. However, the role of endogenous opioids in opioid withdrawal is not fully understood.

It was reported that escape behavior including jumping was induced by the systemic administration of an opioid antagonist in rats infused with an enkephalin analog or morphine chronically in the periaqueductal gray (PAG) (1, 2) or by the injection of opioid antagonist into the PAG in opioid-dependent rats (3). On the other hand, Maldonado et al. (4) showed that the ED₅₀ values of methylnaloxonium

for rearing and locomotor activity after PAG injection were lower than that after intracerebroventricular injection in morphine-dependent rats, although only a large dose (1000 ng per rat) of methylnaloxonium injected into the PAG could slightly induce jumping behavior. These facts indicate that PAG may play an important role in the occurrence of morphine withdrawal. Thus, we focus on the expression and the role of PPE mRNA in the PAG during morphine withdrawal and propose the relevance between enkephalinergic neurons in the PAG and the morphine withdrawal in this review. Recent studies have noted the participation of second messengers in neuronal gene expressions of endogenous opioids (5, 6). We also discuss the participation of transcription factors during morphine withdrawal, as these are thought to be key molecules involved in the changes in neuronal gene expression of PPE.

Modulation of enkephalinergic neurons in the PAG by morphine withdrawal

PPE mRNA expression in the caudal PAG (cPAG) (but not in the rostral PAG) is increased during morphine

Table 1. Comparison between opioid antagonist-precipitated withdrawal and spontaneous withdrawal on the changes in methionine-enkephalin content and prepro-enkephalin mRNA expression in brain regions in morphine-dependent rats

Tissue	met-Enk		PPE mRNA		
	precipitated withdrawal	spontaneous withdrawal	precipitated withdrawal	spontaneous withdrawal	
Striatum (CPu)	↔ (13) ↑ (14)	↓ (16)	↔ (15)	↔ (17)	↑ (18)
PVN	—	—	↑ (11, 12, 19, 20)	↔ (11, 12, 20)	
cPAG	—	—	↑ (11, 12)	↑ (11, 12)	

↑: increase, ↓: decrease, ↔: unchanged, —: not determined. met-Enk, methionine-enkephalin; PPE, preproenkephalin; CPu, caudate-putamen; PVN, paraventricular hypothalamic nucleus; cPAG, caudal periaqueductal gray. Numbers in parentheses indicate relevant references.

withdrawal (7, 8), although the PPE mRNA expression in the PAG is not significantly changed after chronic morphine (Y. Fukunaga et al., unpublished data) or after single naloxone in morphine-naïve rats (7). This increase in the PPE mRNA expression in the cPAG is elicited by both naloxone-precipitated and spontaneous withdrawal in morphine-dependent rats (Table 1) (8). In several other brain regions, different responses of enkephalinergic neurons occur during naloxone-precipitated withdrawal and spontaneous withdrawal (Table 1). For example, met-enkephalin content in the rat striatum or caudate-putamen was increased or unchanged during naloxone-precipitated withdrawal (9, 10), and the PPE mRNA expression was unchanged (11). However, met-enkephalin content was decreased (12), but the PPE mRNA expression was increased or unchanged during spontaneous morphine withdrawal (13, 14). In the paraventricular hypothalamic nucleus (PVN), opioid antagonist-precipitated withdrawal results in increases in PPE mRNA expression (7, 8, 15, 16), but this does not occur after spontaneous opioid withdrawal (7, 8, 16). These results suggest that the change of the PPE gene expression in the cPAG, rather than the striatum, caudate-putamen or PVN, is closely related with morphine withdrawal.

PPE mRNA expression in the PVN is increased by various kinds of stressors (8, 17–19). We have shown that restraint stress, hypertonic saline stress, isolation stress and naloxone-precipitated morphine withdrawal, but not spontaneous morphine withdrawal, increased the PPE mRNA expression in the PVN (8). These results suggest that the PPE gene expression in the PVN could respond to not only morphine withdrawal, but also other kinds of stressors. In contrast, PPE mRNA expression in the cPAG was not changed after these kinds of stresses, but was increased after both naloxone-precipitated and spontaneous morphine

withdrawal (8). These findings suggest that the increase in the PPE mRNA expression in the cPAG may be a specific response to morphine withdrawal.

A relationship between the time course of the morphine withdrawal and that of the increase in the PPE mRNA expression in the cPAG has been reported (8). Morphine withdrawal signs (body weight loss and plasma corticosterone increase) are observed at 1 h after naloxone in morphine-dependent rats, whereas the PPE mRNA expression in the cPAG is not changed. During recovery from morphine withdrawal (4 h to 2 days after naloxone injection), PPE mRNA expression in the cPAG is increased. The increased PPE mRNA expression in the cPAG returns to the control level after disappearance of the above withdrawal signs. During spontaneous withdrawal, PPE mRNA expression in the cPAG does not change, while body weight loss and plasma corticosterone increases are observed (12 h after the final morphine injection). The increment of the PPE mRNA expression in the cPAG is observed for 1–3 days after the cessation of morphine treatment, which could be the recovery phase of morphine withdrawal. Thus, PPE mRNA expression is restored to the control level after disappearance of the withdrawal signs. These results suggest that enkephalinergic neurons in the cPAG may play some role in the recovery phase of morphine withdrawal.

Role of enkephalinergic neurons in the PAG during morphine withdrawal

The effects of exogenously administered opioid peptides on morphine withdrawal have been studied. For example, intracerebroventricular (i.c.v.) injections of met-enkephalin and leu-enkephalin suppressed naloxone-precipitated withdrawal jumping in morphine-dependent mice (20, 21). Likewise, i.c.v. pretreatment with enkephalin degrading

enzyme inhibitors, e.g., thiorphan, an enkephalinase inhibitor (22–24); phelorphan, a dipeptidyl aminopeptidase inhibitor (23); kelatorphan ((*R*)-3-(*N*-hydroxycarboxamido-2-benzylpropanol)-L-alanine), a mixed inhibitor (24); and RB38A ((*R*)-3-(*N*-hydroxycarboxamido-2-benzylpropanol)-L-phenylalanine), a mixed inhibitor (24) suppress naloxone-precipitated morphine withdrawal. Because enkephalins are rapidly decreased by peptidases such as enkephalinase, dipeptidyl aminopeptidase, peptidyl dipeptidase A and aminopeptidase N, the inhibition of these peptidases is thought to potentiate actions of enkephalins and to subsequently suppress morphine withdrawal. Furthermore, i.c.v. injection of synthetic enkephalin analogs, [D-Ala², Met⁵]enkephalinamide and [D-Met², Pro⁵]enkephalinamide (which are resistant to enkephalin degrading enzymes) inhibited naloxone-precipitated withdrawal jumping and hypothermia in morphine-dependent mice (25). These results suggest that endogenous opioid peptides may play an important role in the suppression of morphine withdrawal.

The intra-PAG injection of thiorphan, kelatorphan and RB38A also appears to reduce the naloxone-precipitated withdrawal jumping in morphine-dependent rats (22, 26). In addition, the intra-cPAG injection of either a mixture of specific inhibitors of aminopeptidase N (amastatin), peptidyl dipeptidase A (captopril) and enkephalinase (phosphoramidon), which have been shown to protect met-enkephalin from degradation in vitro almost completely (27), or injection of [D-Ala², Met⁵]enkephalinamide also suppressed naloxone-precipitated withdrawal (especially jumping behavior) (28). These findings suggest that an increase in opioid receptor activation in the cPAG by exogenous or endogenous opioid peptides suppresses jumping during morphine withdrawal. The above findings, together with the increase in PPE mRNA expression in the cPAG during the recovery phase of morphine withdrawal, support the hypothesis that the increase in the PPE mRNA expression in the cPAG after morphine withdrawal may participate in the alleviation of morphine withdrawal through an increase in enkephalin release.

Possible mechanisms for the role of exogenous and endogenous opioid peptides in alleviation of withdrawal include the inhibition of binding to opioid receptors of opioid antagonist or compensation for the elimination of opioid peptide from the receptors. This may result in the attenuation of naloxone-precipitated and spontaneous morphine withdrawal, respectively. Alternatively, we could suggest that enkephalins released during morphine withdrawal affect the activity of other neural systems that modulate morphine withdrawal. Possible candidates are excitatory amino acid (EAA) and gamma-aminobutyric acid (GABA) systems in the PAG. For example, glutamate and aspartate immunoreactive neurons are located in the

PAG (29), and EAA antagonists have been reported to reduce morphine withdrawal (30). This indicates that EAA may participate in the occurrence of morphine withdrawal. An electrophysiological study has shown that the glutamatergic components of fast postsynaptic potentials in single PAG neurons in rat brain slices are inhibited by met-enkephalin (31). With regard to the GABA system, there are axo-somatic synapses from enkephalinergic axon terminals to GABAergic neurons in the PAG (32). In addition, chronic morphine treatment increases the efficacy of opioid agonists at μ -receptors on GABAergic nerve terminals in the PAG, and naloxone-precipitated withdrawal subsequently potentiates GABAergic synaptic currents in PAG neurons from morphine-dependent rats (33). Enhanced GABAergic synaptic transmission was also inhibited by the α_2 -adrenoceptor agonist clonidine (33), which is known to suppress morphine withdrawal signs (34). Thus, enhanced GABAergic inhibition during morphine withdrawal could excessively suppress activity of descending output neurons from the PAG and elicit withdrawal behaviors. An electrophysiological study has also shown that GABAergic components of fast postsynaptic potentials in single PAG neurons in rat brain slices are inhibited by met-enkephalin (31). These findings suggest that enkephalins may inhibit morphine withdrawal via inhibition of EAA or GABA neurotransmission in the PAG (Fig. 1).

Mechanisms underlying expression of PPE gene in the PAG during morphine withdrawal

Recently, much attention has been directed toward signal transduction pathways linking membrane receptors to gene expression, with regard to the mechanisms underlying the regulation of the PPE gene expression. Thus, PPE gene expression may be increased via a second messenger during morphine withdrawal (Fig. 1). μ -Opioid agonists inhibit adenylate cyclase activity in rat brain (35). Intracellular cAMP levels are increased by the addition of an opioid antagonist in neuroblastoma $\times$ glioma hybrid cells cultured with morphine (36). Morphine withdrawal facilitates phosphorylation of a transcription factor, cAMP response element binding protein (CREB), in the rat locus coeruleus (37). Evidence exists that the PPE gene expression is regulated by the cAMP pathway. For example, a putative cAMP-response element (CRE) is located upstream of the transcriptional start site of PPE gene, and its transcription can be activated by an increase in cAMP (38). These findings suggest that an increase in cAMP or an activation of protein kinase A during morphine withdrawal could influence the PPE gene expression.

Punch and coworkers (39) have shown that intra-PAG infusion of a protein kinase A inhibitor, Rp-cAMPS, attenuates withdrawal signs in morphine-dependent rats, while that of a protein kinase A activator, Sp-cAMPS, in-

Fig. 1. Scheme illustrating the possible role of enkephalinergic neurons, and mechanism underlying the increase in PPE mRNA in the caudal periaqueductal gray during morphine withdrawal. PKA, protein kinase A; CREB, cAMP response element binding protein; AP-1, activator protein-1; PPE, preproenkephalin; met-Enk, methionine-enkephalin; leu-Enk, leucine-enkephalin, GABA; gamma-aminobutyric acid.

duces quasi-withdrawal signs in morphine naive rats. These results contradict the concept that activation of the cAMP system induces an increase in the PPE mRNA expression in the PAG, which in turn may alleviate morphine withdrawal. There are two possibilities to explain this contradiction. First, protein kinase A may be involved in the occurrence of morphine withdrawal in the acute phase and thereafter in the alleviation of withdrawal in the recovery phase via a delayed and prolonged response such as PPE gene expression. Second, other signal transduction pathways besides the cAMP system may be critical for an increase in PPE mRNA in the PAG during morphine withdrawal. For example, CREB is phosphorylated by not only protein kinase A but also by Ca^{2+} /calmodulin-dependent protein kinase IV and mitogen-activated protein (MAP) kinase (40). Moreover, it has also been reported that naloxone-precipitated withdrawal activates MAP kinase in the rat locus coeruleus (41).

The 5' region of the PPE gene has a binding site for the activator protein-1 (AP-1), which is a dimer of Fos and Jun family proteins (42). The PPE gene is one of the possible target genes for Fos and Jun (43, 44). Pretreatment with an antisense oligonucleotide complementary to c-fos mRNA inhibits the induction of PPE mRNA in the rat hippocampus by kainic acid (43). This increase in Fos-like

immunoreactivity has been observed in the lateral and ventrolateral (particularly caudal ventrolateral) PAG neurons during naloxone-precipitated morphine withdrawal (45). These results suggest that Fos and Jun could facilitate the synthesis of PPE mRNA in the cPAG during morphine withdrawal.

Future directions

Recently, it has been reported that morphine withdrawal signs were significantly attenuated in CREB $\alpha\Delta$ mutant mice, which lack the α and Δ isoforms of CREB (46). Intra-locus coeruleus infusions of CREB antisense oligonucleotide reduce the development of physical dependence to morphine, based on attenuation of morphine withdrawal signs (47). The above findings, together with a report showing an increase in the CREB phosphorylation in rat locus coeruleus during opioid antagonist-precipitated morphine withdrawal (37), support the hypothesis that CREB-dependent gene transcription is a factor in the onset of behavioral signs of opioid withdrawal. In addition, evidence has accumulated that c-Fos is increased in the central nervous system after morphine withdrawal. These findings lead to the suggestion that transcription factor-dependent gene expression plays an important role in morphine dependence and withdrawal. However, the target genes of

these transcription factors, which may be activated during morphine dependence and withdrawal are presently unclear. In the future, the elucidation of these target genes and the role of their products will contribute to the understanding of the neuronal mechanisms of morphine dependence and withdrawal. Since the PPE gene is one of the candidate target genes of CREB and AP-1 (43, 44), it is especially valuable to clarify the role of these transcription factors in PPE gene expression during morphine dependence and withdrawal.

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