Cellular Mechanisms Underlying Morphine Analgesic Tolerance and Hyperalgesia

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INTRODUCTION

Opioid dose escalation or analgesic tolerance is observed during longer treatments in a significant number of patients with chronic pain owing to cancer or nonmalignant tissue injury. Higher doses of morphine are more likely to result in subsensitivity to the drug and worsened quality of life (QOL) by exerting other side effects. Many investigators have been studying the molecular and cellular mechanisms underlying opioid analgesic tolerance by different approaches. They studied the underlying mechanisms in terms of cellular opioid adaptation following long-term exposure. In so-called cyclic AMP hypothesis in mid-1970s, adapted loss of opioid-mediated inhibition of cyclic AMP production and abrupt increase in this level following opioid withdrawal were proposed as mechanistic models for opioid tolerance and dependence, respectively (1–3). In the current cellular models, it is well documented that the molecular events underlying the reduction of opioid receptor function following morphine pretreatments are closely correlated with receptor trafficking, including (i) phosphorylation, (ii) internalization/endocytosis, (iii) sequestration/recycling,
or (iv) downregulation/breakdown of these receptors (4–8). Among these steps, the phosphorylation of opioid receptors is the most important step for desensitization. The direct evidence that opioid receptor function is lost by phosphorylation has been first reported in the studies using partially purified μ-opioid receptors (MOPs) and purified cAMP-dependent protein kinase (PKA) (9–11). It is accepted that longer exposure to opioids leads to phosphorylation of the C-terminal region of opioid receptors, followed by desensitization (12,13). However, there are reports that opioid receptors are phosphorylated by many different kinases, and details of the proposed opioid receptor phosphorylation and trafficking machineries underlying opioid tolerance and desensitization have been described elsewhere (9,14–21).

In addition to these cellular mechanisms, the plasticity in neuronal counterbalancing mechanisms including different neurons has also been proposed as a possible mechanism underlying opioid tolerance. The rationality of this plasticity is found in the fact that the degree of tolerance differs in different opioid actions. The morphine-induced respiratory inhibition and analgesia is known to develop tolerance, while the constipation is not (22–25). Trujillo and Akil (1991) first reported that MK-801, an N-methyl-D-aspartic acid (NMDA) receptor antagonist, blocks opioid tolerance and dependence (26). This finding suggests that the anti-opioid NMDA receptor system is enhanced during chronic opioid treatments, thereby counteracting the actions of opioids. This type of plasticity through anti-opioid NMDA receptor system seems to be augmented by various supporting mechanisms, as described later.

**PLASTICITY IN NEURONAL NETWORKS**

The plasticity in central neuronal networks underlying the mechanism of opioid tolerance following chronic treatments may be evidenced by the following study (27,28). Although the morphine analgesia assessed by the tail pinch test, which involves higher central nervous mechanisms, was markedly attenuated on the sixth day following daily systemic injection of morphine at a relatively high dose of 10 mg/kg (SC), the peripheral morphine analgesia was not observed in such morphine-tolerant mice. In this study, the peripheral morphine analgesia by local (intraplantar, IPI) injection was evaluated to measure the inhibition of nociceptive flexor responses by bradykinin (IPI). Thus the discrepancy in susceptibility to chronic morphine analgesic tolerance seems to be attributed to the absence (peripheral) or presence of neuronal circuits (systemic or central) that affect morphine analgesia. Most importantly, the tolerance to peripheral morphine analgesia was not observed as late as 24 hours later. Therefore, we must separately consider the molecular mechanisms underlying acute and chronic morphine analgesic tolerance in terms of synaptic plasticity. Although accumulated findings demonstrate that several brain loci are closely related to the cause of morphine analgesia (29), it remains unclear whether the neuronal
plasticity involved in morphine analgesic tolerance is caused by locus-specific or translocus systems.

**Anti-Opioid Glutamate-NMDA Receptor System**

Since the report by Trujillo and Akil (1991), NMDA receptors have long been supposed to play important roles in the development of morphine tolerance and dependence (30,31). As many known competitive or noncompetitive NMDA antagonists potentiate morphine-induced catalepsy, lethality (32), and hypothermia (33), or retard learning behaviors (34), the development of NMDA receptor antagonists that specifically block morphine tolerance has been explored. One approach to find specific antagonists began with the identification of the subunit of NMDA receptor involved in morphine tolerance and addiction. We first revealed that GluRepsilon1 (NR2A) knockout mice showed an enhancement of acute morphine analgesia in the tail pinch test (35). As NR2A knockout mice did not show any change in the basal nociceptive threshold, glutamatergic neurons that stimulate NR2A subunit are located downstream of opioid neurons, and attenuate endogenous and exogenous opioid actions. Chronic daily pretreatments with morphine (10 mg/kg SC) produced a tolerance to morphine analgesia on the 6th day in wild-type mice, but not in NR2A knockout mice. As the level of NR2A was significantly increased by 100 to 200% of the control level only in the periaqueductal gray matter (PAG), ventral tegmental area (VTA), and nucleus accumbens (NAcc), we speculated that enhanced activation of the anti-opioid NMDA receptor system counterbalances or cancels morphine analgesia during chronic treatments. The restoration of this gene through a novel in vivo electroporation technique into the PAG or VTA, but not the NAcc of NR2A knockout mice, successfully restored the morphine analgesic tolerance, without significant changes in the basal nociceptive threshold (Fig. 1). The rescued NR2A protein level by in vivo electroporation (1 µg DNA, 10 mA and 10 Hz) in the PAG was almost the same as that in wild-type mice, and remained at this level until nine days after electroporation, although the nonspecific CMV promoter is used for NR2A gene transfer. It probably suggests that extra-NR2A proteins not used for the right NMDA receptor complex may be removed by protein quality-check systems, such as ubiquitin-proteasome system (36).

Our developed technique also has the advantage that it caused no morphological damage to the PAG under the condition with 10 to 40 mA at 10 Hz, or with 10 and 20 Hz at 10 mA at day 4 after in vivo electroporation, although slightly fragmented nuclei were observed with 40 Hz at 10 mA. Similar approaches were carried out to study the mechanisms underlying morphine dependence. Mice given increasing doses of morphine from 20 to 100 mg/kg for three days showed withdrawal behaviors when 1 mg/kg IP naloxone was administered two hours after the last morphine (100 mg/kg SC) injection on the fourth day. The withdrawal behaviors such as jumping, withdrawal locomotion,
sniffing, and defecation were similarly observed by both paradigms of morphine treatments. These withdrawal behaviors were markedly inhibited in NR2A knockout mice and there was a significant increase in protein expression of NR2A of wild-type mice. Locus-specific recovery of some, but not all withdrawal behaviors was observed when NR2A gene expression was restored in the NAcc of knockout mice, suggesting that both locus-specific and translocus NR2A systems are involved in the development of opioid dependence. Thus, we propose the view that enhanced anti-opioid systems may attenuate the actions of morphine following chronic morphine treatments, and deprivation from morphine may lead to withdrawal symptoms through anti-opioid NR2A systems.

On the other hand, NR2B has also been proposed to contribute to the mechanisms of morphine tolerance or the plasticity in opioid actions. Ro 256981, an antagonist of the NMDA receptor subunit NR2B, reduces the expression of analgesic tolerance to morphine (37). Since NMDA NR2B receptors in the anterior cingulated cortex (ACC) play roles in the establishment of long-term potentiation (LTP) and fear memory, both systemic and intra-ACC inhibition of NR2B in morphine-tolerant animals inhibited the expression of analgesic tolerance. Although there is an abundance of evidence from animal studies that NMDA receptor inhibition using antagonists during opioid exposure attenuates chronic opioid tolerance, there are also some reports that NMDA receptor antagonists potentiate, inhibit, or do not alter morphine analgesia, possibly due to

![Figure 1 Method for in vivo electroporation. A pair of stainless steel electrodes, 0.5 mm in length and 0.3 mm in outer diameter, was stereotaxically inserted into specific brain regions according to the stereotaxic atlas in anesthetized mice. Electric pulses were generated with a square electroporator (CUY21, Nepagene, Tokyo, Japan) at 10 pulses/s (10 Hz). The shape of the pulse was a square wave; that is, the voltage remained constant during the pulse duration. For the best gene transfer, cDNA at a dose of 1 μg was diluted in Tris-EDTA buffer (1 μL) immediately before use and electroporated into brain regions by the electric pulses (2 ms) to get 10 mA current.](image-url)
the use of different doses of antagonist and morphine, as well as experimental animals and tests for nociception (38). In addition, there are reports that different types of glutamate receptors are also involved in the development of opioid analgesic tolerance. Kozela et al. (2003) have pointed out the role of metabotropic glutamate receptor 5 (mGluR5) by showing that chronic administration of 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a specific antagonist of group 1 mGluRs, markedly attenuated morphine tolerance (39).

**Intracellular Systems That Support The Anti-Opioid NMDA Receptor System**

The knockdown of spinal cord postsynaptic density protein-95 (PSD-95), a scaffold protein for NMDA receptors, prevented the development of morphine tolerance in rats (40). On the other hand, microarray studies revealed increased expression of Ania-3, a short variant of Homer 1 protein, in the frontal cortices of rats showing naloxone-precipitated morphine withdrawal (41). As Ania-3 interferes with the function of constitutively active long forms of Homer proteins, which build bridges between NMDA and metabotropic glutamate receptors (42), this change in Ania-3 expression may contribute to the development of morphine dependence, or tolerance. These findings are consistent with the recent studies (43), in which chronic morphine upregulates the gene expression of PSD-95 and Homer-1 in the central extended amygdala, a key site for the drug craving and seeking behaviors. On the other hand, there is a report that cyclin-dependent kinase 5 (Cdk5) levels were markedly reduced in the prefrontal cortices of opioid addicts and the cerebral cortices of morphine-sensitized rats (44). These findings seem to be very interesting, since Cdk5 phosphorylates the NR2A subunit and activates NMDA receptor function (45), while the inhibition of Cdk5 increases Src-mediated phosphorylation of NR2B and blocks the binding to AP-2, resulting in the promotion of cell surface expression of NMDA receptors (46). However, there is a conflicting report that no significant change in Cdk5 expression was observed in similar brain regions of morphine-sensitized rats (43).

**BDNF System That Supports Anti-Opioid NMDA Receptor Systems**

Although NR2A receptor upregulation following chronic morphine treatments seems to play a key role in the above-mentioned hypothesized mechanisms, details of mechanisms underlying NR2A upregulation remain to be determined. We speculate from the recent findings that brain-derived neurotrophic factor (BDNF) may support this anti-opioid system as follows: (i) the addition of BDNF to cultured rat cortical neurons upregulates NR2A gene expression (47), (ii) morphine upregulates BDNF expression in cultured microglia through an autocrine machinery (48), (iii) chronic morphine treatments upregulate BDNF
expression in brain neurons in vivo through a neuron-microglia interaction (49), (iv) central injection of anti-BDNF antibody abolished morphine tolerance (50), and (v) morphine physical dependence was lost in forebrain-specific BDNF knockout mice (51). Although little is known about the molecular mechanisms underlying BDNF-induced NR2A gene expression, three functional GC-boxes in the NR2A core promoter are reported to interact with Sp1 and Sp4 transcription factors. It is interesting to speculate that the phosphorylation signaling through BDNF-TrkB couples with the transcriptional activity of Sp1 (52,53).

On the other hand, the machinery underlying chronic morphine-induced upregulation of BDNF expression is also an interesting subject. When mice were pretreated with morphine at a dose of 10 mg/kg SC (a maximal dose for analgesia) for five days, the substantial analgesic activity of morphine (10 mg/kg, SC) was lost on the 6th day. At this time point, there was a significant upregulation of BDNF levels in the PAG (50), which is the major brain region involved in morphine analgesia. However, these results seem to conflict with a report that forebrain-specific BDNF knockout mice lose morphine physical dependence, but not morphine analgesic tolerance (51). This contradictory observation is unlikely to be important because it is well known that lower brain stem regions, but not forebrain regions, are important for morphine analgesia. Indeed, the injection of adenovirus expressing Cre recombinase gene into the PAG of floxed BDNF-transgenic mice markedly reduced morphine analgesic tolerance (Matsushita and Ueda, unpublished data). We recently found that curcumin, an inhibitor of histone acetyltransferase (HAT) activity of CREB-binding protein inhibitor, blocked the chronic morphine-induced expression of exon I and IV BDNF transcripts, and morphine analgesic tolerance (50). As CBP is known to cause a chromatin remodeling through HAT activity and stimulate BDNF gene expression, these findings may be the first evidence that morphine analgesia could be suppressed by epigenetic regulation. This also indicates that a health food product, curcumin, might reduce morphine analgesic tolerance, and that underlying epigenetic control could be a new strategy useful for the control of this problem.

**Glial Systems That Support Anti-Opioid NMDA Receptor Systems**

Astrocytes play important roles in the anti-opioid glutamate-NMDA receptor system. As the expression levels of glutamate transporters in astrocytes and neurons are downregulated by chronic morphine treatments (30,54), glutamate signals in the synaptic cleft are expected to increase and give more opportunity for the stimulation of NMDA receptors. On the other hand, d-serine, a key molecule that activates NMDA receptors as an allosteric agonist (55), is also a candidate of supporting machineries involved in this anti-opioid system, since chronic morphine induced the upregulation of glial racemase, which is expected to increase d-serine levels (56). Glial cell responses to chronic morphine
treatment were examined by immunohistochemistry of glial fibrillary acidic protein (GFAP), a specific marker for astroglial cells (57). Chronic administration of morphine (50 mg/kg, IP, once daily for nine consecutive days) increased the immunoreactive level of GFAP, a specific marker for astroglial cells, in the spinal cord, posterior cingulate cortex, and hippocampus, but not in the thalamus. This increase was attributed primarily to hypertrophy of astroglial cells rather than their proliferation or migration. When chronic morphine (20 µg/2 µL, IT) was delivered in combination with fluorocitrate (1 nmol/L µL, IT), a specific and reversible inhibitor of glial cells, spinal tolerance to morphine analgesia was partly but significantly attenuated as measured by behavioral tests and the increase in spinal GFAP immunostaining was also greatly blocked. This report may be the first evidence for the role of glial cells in the development of morphine tolerance in vivo.

On the other hand, accumulating findings demonstrate that microglia also play key roles in morphine actions or analgesic tolerance. Takayama and Ueda (2005) reported that morphine upregulates microglial gene expression of BDNF, which is known to upregulate NR2A. However, the BDNF-like immunoreactivity in the brains of chronic morphine-treated mice was mostly observed in neurons, but slightly in microglia (Ueda et al., unpublished data). This selective expression is very intriguing because morphine upregulates BDNF expression in cultured microglia, but not in cultured neurons. From this point of view, we are attempting to find microglia-derived bioactive molecules, which in turn upregulate BDNF in neurons, followed by the development of tolerance via an upregulation of NR2A, as mentioned earlier. Liu et al. have recently reported that a selective nNOS inhibitor, 7-nitroindazole (7-NINA, sodium salt), attenuated morphine analgesic tolerance and p38 MAPK in the activated spinal microglia (58). Taking into consideration that NMDA receptor mediates an activation of nNOS, it is interesting to speculate that microglia activation downstream of NMDA receptor activation may exert an amplifying mechanism for the anti-opioid NMDA receptor systems.

**CONCLUSION**

Opioids are becoming more widely used, not only as palliative medicines for terminal cancer patients, but also as successful analgesics for neuropathic pain patients. When the side effects such as tolerance and dependence are appropriately avoided by the use of adjuvant analgesics, which will be developed through the studies of underlying mechanisms, the pain control by opioids will be more idealistic. The present review proposes several target analgesic adjuvants for use in palliative care (Fig. 2). They are NR2A-specific antagonists and compounds that suppress BDNF transcription, such as curcumin. Various types of compounds to inhibit the activation of microglia, astrocytes, racemase, and nNOS would be also added to the lineup of adjuvants to inhibit opioid analgesic tolerance.
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Figure 2  Candidate molecules to inhibit opioid tolerance in the anti-opioid glutamate/NMDA receptor hypothesis. In this hypothesis, glutamate neurotransmission and NMDA receptor signaling are upregulated, following chronic opioid treatments. Some parts of this hypothesized mechanism are mediated by neuron-glia interactions, as stated in the text. Candidate molecules to inhibit opioid tolerance are indicated by the number in the figure, as follows: (i) NMDA receptor (NR2A) antagonists; (ii) anti-BDNF antibody or TrkB antagonists; (iii) CBP inhibitors, such as curcumin; (iv) unknown compounds to inhibit the GLT-1 downregulation, racemase inhibitors; (v) astrocyte inactivators, such as fluoroacetate; (vi) microglia inactivators, such as minocycline; and (vii) nNOS inhibitors.


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