Mechanisms underlying morphine analgesic tolerance and dependence

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1. ABSTRACT

The mechanisms underlying opioid tolerance are not fully understood, but appear to be comprised of two types of plasticity or counter-adaptation, at the cellular level and through neuronal circuits. Current studies mostly emphasize the cellular adaptation mechanisms, which include altered gene expression and receptor desensitization due to phosphorylation and endocytosis. However, the mechanisms underlying opioid tolerance and dependence are not always explained by cellular adaptation mechanisms alone. This review focuses on the plasticity in neuronal circuits achieved through an enhancement of synaptic activities between glutamate and NMDA receptor due to up-regulation of receptor and racemase to produce D-serine, an allosteric NMDA receptor agonist, and down-regulation of gluatamate transporter, all which contribute to the counterbalance of opioid actions or anti-opioid mechanisms underlying opioid tolerance. This anti-opioid system is supposed to be also augmented by altered expression of key molecules regulating through neuron–glial networks. This review also introduces a new approach using *in vivo* electroporation to identify the brain loci responsible for morphine tolerance and dependence.

2. INTRODUCTION

The clinical utility of morphine and related opioids is often overshadowed by the development of tolerance and dependence following their chronic use. However, recent clinical evidence has revealed that opioid tolerance does not develop frequently, and that opioid treatments are not always problematic in terms of tolerance and dependence in patients with chronic pain owing to cancer or nonmalignant tissue injury, as long as the opioids are used appropriately in the clinic (1-3). As opioid dose escalation is still observed in a significant number of such patients, we have to consider the practical situations in which terminal cancer patients with severe pain should take higher doses of opioids during longer treatments. Higher doses of morphine are more likely to result in sub-sensitivity to the drug and worsened quality of life (QOL) by exerting other side effects; thus we need to study how to prevent adaptation to morphine, based on the molecular mechanisms. Analgesic tolerance that requires dose escalation regardless of the disease progression has often been correlated to sub-sensitivity to morphine. For many years, many investigators have been elucidating the molecular and cellular mechanisms underlying opioid

analgesic tolerance by different approaches. These studies have dealt with cellular opioid adaptation following long-term exposure through neural circuits, including anti-opioid systems. Since the mid-1970s, opioid tolerance and dependence have been discussed in terms of the so-called 'cyclic AMP (cAMP) hypothesis' or adapted loss of opioid-mediated inhibition of cAMP production (4-6). Studies using the neuroblastoma x glioma hybrid 108-15 (NG108-15) or related cells have long been the gold standard for investigations of opioid tolerance. In this hypothesis, opioids first decrease cAMP production; this is followed by a return to the basal level via a counterbalancing increase in cAMP level (tolerance). The removal of opioids or the addition of an opioid antagonist, on the other hand, manifests as an increase in cAMP levels (dependence). Physical dependence is manifested by withdrawal symptoms when morphine is withdrawn or an opioid antagonist is given. As withdrawal symptoms, such as hyperalgesia, hyperpnea and diarrhea, are quite opposite to the analgesia, respiratory depression and constipation observed following acute morphine treatment, the mechanisms underlying dependence also seem to be derived from cellular adaptation. Although the validity of this cAMP hypothesis remains elusive, there are reports that some key molecules that increase cAMP level are up-regulated following chronic opioid treatments (7). Maldonado *et al.* (8) reported that the development of chronic morphine-induced dependence is significantly inhibited in mutant mice with a genetic deletion of cAMP-response element binding protein/CREB. Although morphine-induced inhibition of adenylyl cyclase through G_i is unlikely to be related to CREB activation, the activation of the RAS-MAP kinase pathway through G_i beta-gamma subunits might be an alternative mechanism leading to CREB activation (9). Furthermore, it should be noted that CREB activation promotes the gene expression of adenylyl cyclase (10). In the last decade, the cellular and molecular mechanisms underlying opioid tolerance have been discussed in relation to opioid receptor signaling and trafficking.

In addition to these cellular mechanisms, plasticity in neuronal counterbalancing mechanisms has also been proposed as a possible mechanism underlying opioid tolerance and dependence. A pioneering study showed that MK-801, an NMDA receptor antagonist, blocks opioid tolerance and dependence (11). This finding suggests that the anti-opioid NMDA receptor system is enhanced during chronic opioid treatments, thereby counteracting the actions of opioids. The peptidergic systems of nociceptin/OFQ, neuropeptide FF and cholecystokinin, which also show anti-opioid actions, were found to be similarly enhanced following chronic opioid treatments and to mediate opioid tolerance (12). The plasticity of these anti-opioid systems has now been shown by use of specific antagonists or transgenic mice with genetic deletions, as described later.

3. OPIOID RECEPTOR SIGNALING

The molecular events underlying the reduction of opioid receptor function following morphine pretreatments have been correlated with receptor trafficking, including 1) phosphorylation, 2) internalization/endocytosis and 3) sequestration/recycling or 4) down-regulation/breakdown of these receptors (13-17). Phosphorylation of opioid

receptors is the most important step for desensitization. We firstly reported that partially purified mu-opioid receptors (MOPs) undergo functional coupling with purified G_i or G_o (18), and that cAMP-dependent protein kinase (PKA)-treated MOPs lose this functional coupling ability (19, 20). It is accepted that longer exposure to opioids leads to phosphorylation of the C-terminal region of opioid receptors, followed by desensitization (21, 22). However, there are reports that opioid receptors are phosphorylated by many different kinases, such as cAMP-dependent protein kinase (PKA) (20), protein kinase C (PKC) (23, 24), Ca^{2+}/calmodulin-dependent protein kinase (25), G protein-coupled receptor (GPCR) kinases (GRKs) (26, 27), and mitogen-activated protein kinase (28, 29). As seen with many other GPCRs, the internalization of opioid receptors is closely related to GRK-mediated phosphorylation (Figure 1). According to current understanding, the desensitization process is largely mediated by receptor phosphorylation in the membrane, but the internalization and subsequent sequestration processes are presumed to play roles in resensitization. As details of the proposed opioid receptor phosphorylation and trafficking machineries underlying opioid tolerance and desensitization have been described elsewhere (15-17, 30, 31), the present section describes the mechanisms mediated by protein kinase C (PKC), namely, receptor phosphorylation, endocytosis and tolerance/desensitization.

3.1. Receptor phosphorylation by protein kinase C

We first demonstrated that that phospholipase C (PLC) activation is mediated by G_i-coupled receptors in brain membranes (32). This finding suggested the possibility that opioid receptors showing G_i-coupling may also activate PKC. In Xenopus oocytes expressing mu, delta or kappa-opioid receptors (MOP, DOP or KOP), repeated applications of opioid agonists caused rapid desensitization through a PKC-dependent mechanism in the measurement of evoked Ca^{2+}-dependent chloride currents (24, 33, 34). In this study we successfully developed the functional reconstitution of different receptors - G_{i1} coupling by exogenous expressions of DOP, muscarinic M2 receptors and the alpha-subunit of G_{i1} (24). Opioid receptor desensitization was characterized to be quasi-homologous through activation of PKC because it was caused by repeated challenges with a DOP agonist, but not by a M2 agonist. As DOP desensitization rapidly recovers after the application of a PKC inhibitor, the desensitization is unlikely to be due to receptor down-regulation, but rather to the temporary inhibition of receptor function by PKC. The view that opioid receptors are inactivated by PKC has recently been supported by an electrophysiological study using cells expressing opioid receptors with a point mutation at the PKC binding site (35, 36). In this study, a PKC inhibitor selectively blocked morphine-stimulated ^{32}P-phosphorylation of MOP1 as well as G protein-coupled inwardly rectifying K^+ channel activity.

3.2. Modulation of receptor internalization by protein kinase C

As shown in Figure 1, the internalization or endocytosis of seven transmembrane GPCRs, including opioid receptors, is now believed to contribute to
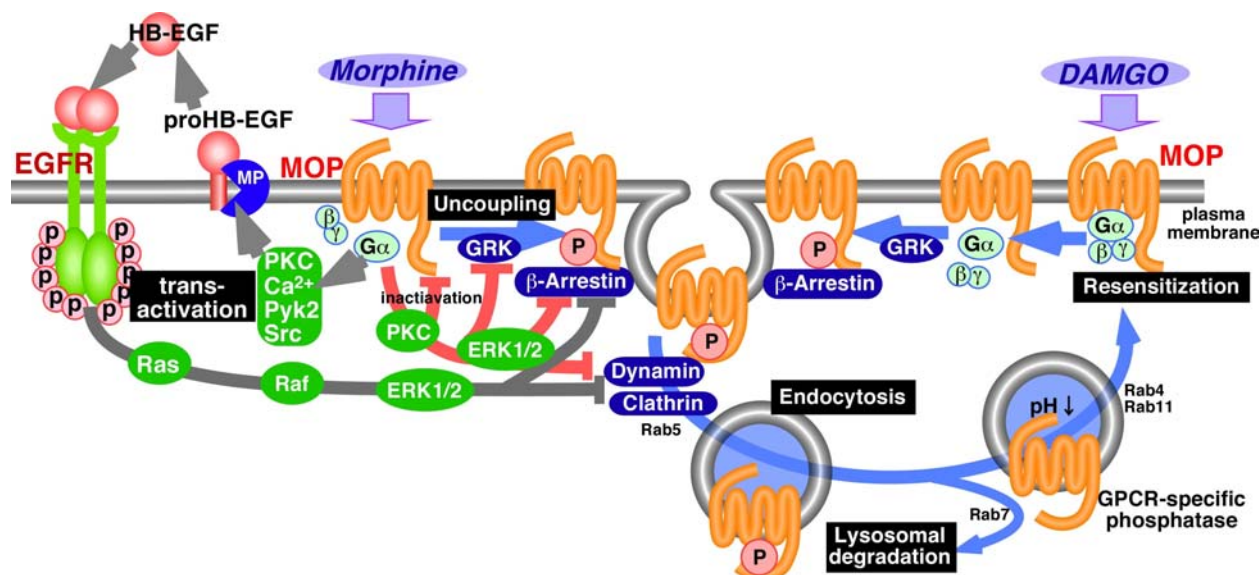
Figure 1. Differential mu-Opioid receptor trafficking upon the stimulation by different agonists. When mu-Opioid receptor (MOP) is stimulated by DAMGO, the signal is coupled to an activation of G protein (G_i or G_o), followed by G protein-coupled receptor kinase (GRK)-catalyzed phosphorylation of MOP and association with beta-arrestin. Clathrin and dynamin are then associated to the membrane containing beta-arrestin-associated MOP to form receptor-containing endosome. Thus endocytosed MOP in the endosome has two fates, lysosomal degradation and resensitization via dephosphorylation. When MOP is stimulated by morphine, on the other hand, the MOP endocytosis does not occur. However, morphine stimulation causes more potent desensitization (acute tolerance) of MOP than DAMGO stimulation, as seen in so-called RAVE hypothesis (see details in the text). As morphine-induced MOP endocytosis is facilitated in the presence of protein kinase C (PKC) inhibitor, morphine-induced PKC phosphorylation of MOP may precede GRK-mediated one and prevent the endocytosis. PKC-phosphorylated MOP seems to be a major mechanism underlying desensitization of MOP in the membrane. The DAMGO-induced PKC activation seems to be simply slower than GRK activation, since similar PKC-mediated desensitization (acute tolerance) occurs when the endocytosis is prevented in the presence of dominant negative mutant of dynamin. MOP : mu-opioid receptor, GRK : G protein coupled receptor kinase, PKC : protein kinase C, ERK1/2 : extracellular signal-regulated kinase1/2, GPCR : G-protein coupled receptor, MP : Metalloprotease, EGF : epidermal growth factor, EGFR : epidermal growth factor receptor, Pyk2 : proline-rich tyrosine kinase 2.

resensitization through dephosphorylation or lysosomal degradation upon normal or excess stimulation of the receptor (15-17, 30, 31). Receptor phosphorylation following stimulation by agonist is performed by GRKs. Agonist-occupied and GRK-phosphorylated receptors are supposed to recruit beta-arrestin with high affinity. According to current understanding, beta-arrestin turns off receptor signaling by sterically inhibiting G protein coupling or stimulating phosphodiesterase and receptor internalization (37). Mechanistically, beta-arrestins serve as adaptors linking the receptors to elements of the endocytotic machinery, such as clathrin and the clathrin adaptor AP2 (38). In this sense, receptor phosphorylation by various second messenger-activated kinases other than GRKs is presumed to modulate the desensitization status. It is well known that MOPs are phosphorylated by GRKs and PKC. Although little is known of the specific sites in MOP phosphorylated by these kinases, there is a report that serine 375 of the mu-opioid receptor is a plausible candidate for the PKC phosphorylation and prolonged desensitization (39). Interestingly, the GRK and PKC sites are independent, and phosphorylation by these two different kinases appears to have opposing effects on endocytosis (35). This assumption was evidenced by the treatment of MOP-expressing CHO cells with a PKC

inhibitor (40). In this study we used two MOP agonists, DAMGO and morphine, which have different effects on MOP endocytosis. As seen in many studies (14, 15), DAMGO at 1 μM causes MOP endocytosis, while morphine at 10 μM does not. However, morphine-induced MOP endocytosis was observed selectively in the presence of an inhibitor of conventional PKC isoforms (alpha, beta and gamma). The modes of DAMGO-stimulated and morphine plus PKC inhibitor-stimulated MOP endocytosis seem to be similar because they were both abolished in cells transfected with adenovirus expressing gene of dominant-negative dynamin (K44A/dynamin), which has a defect in pinching off clathrin-coated raft membranes. Thus, it is evident that there are agonist-specific modes of opioid receptor trafficking. However, Haberstock-Debic et.al revealed that acute morphine causes an opioid receptor membrane trafficking in dendrites (41). This study may suggest the possibility that there are subcellular compartment-selective trafficking machineries as well as agonist-specificity.

3.3. PKC hypothesis for morphine tolerance/desensitization

Several lines of evidence suggest that there is a close relationship between the tolerance/desensitization

ability of opioid agonists and endocytosis of MOP. In the central nervous system, opioid receptors in various brain loci and spinal cord may have different states of endocytosis, depending on the differential levels of GRK or arrestins. The involvement of glial cells and anti-opioid neurons may increase the complexity of the degree and nature of opioid actions. Based on this background, we adopted a peripheral nociception test called the algogenic-induced paw flexion (APF) test (40), which clarifies the relationship between opioid receptor endocytosis and acute tolerance/desensitization. In this approach, we evaluated the inhibitory action of intraplantarly (i.pl.) administered opioids against algogenic bradykinin (BK, pain-producing substance, i.pl.)-induced nociception. In our previous study, BK was characterized to stimulate B2 receptors on unmyelinated C-fibers, which use substance P as a pain transmitter (42). Furthermore, the sites of action of BK and opioids were specified to be on nociceptor endings because the antisense oligodeoxynucleotide (AS-ODN, i.t.) treatments abolished both peripheral actions. Thus it was proved that the peripheral morphine (i.pl.) analgesia against BK (i.pl.)-induced nociception is the event without involvement of central neuronal circuits. In this model, the local application of morphine dose-dependently inhibited BK (i.pl.)-induced nociception in the range of 0.1 to 1 nmol (i.pl.) with an ED_{50} of approximately 0.3 nmol. Four hours after the administration of morphine at a supramaximal dose (3 nmol, i.pl.), the morphine analgesic activity in terms of BK nociception level completely disappeared. However, the analgesic potency of a second challenge with morphine was reduced (ED50: approximately 10 nmol, i.pl.). This acute morphine analgesic tolerance was completely abolished by pretreatment (i.pl.) with conventional PKC inhibitors (43), consistent with the finding that morphine-induced opioid receptor endocytosis was induced by the conventional type of PKC inhibitor (40). Interestingly, DAMGO (i.pl.) pretreatment did not produce acute tolerance. The above-findings that there was an analgesic tolerance with morphine, which does not cause agonist-specific opioid receptor endocytosis, while no tolerance was observed with DAMGO, which cause endocytosis are consistent to so-called relative activity versus endocytosis (RAVE) hypothesis proposed by Von Zastrow, Whistler and their colleagues (15, 44). When MOP endocytosis was blocked by the intrathecal pretreatment with adenovirus expressing dominant-negative dynamin (K44A/dynamin) gene, DAMGO-induced analgesic tolerance was observed. Furthermore, pretreatment with a PKC inhibitor also abolished analgesic tolerance. Thus it is speculated that MOPs stimulated by morphine are first phosphorylated by PKC, and show resistance to phosphorylation by GRKs. PKC-phosphorylated MOPs show a loss of function and no longer activate $G_{i/o}$ (desensitization). On the other hand, DAMGO does not activate PKC to phosphorylate MOPs prior to GRK phosphorylation. GRK-phosphorylated MOPs are then endocytosed and resensitized via the actions of a phosphatase. Thus it is hypothesized that acute tolerance to morphine is mediated by desensitization and down-regulation of the MOP receptor by PKC mechanisms.

3.4. Heterologous regulation of opioid receptor endocytosis

Repeated morphine exposure (10-20 μM) for 6 days increased the number of neurons expressing phosphorylated mitogen-activated protein (MAP) kinases, including p38 MAPK propose the view that p38 MAPK plays a key role in the MOP endocytosis, from the findings that the DAMGO stimulation of GFP-tagged MOP in HEK293 cells only transiently activates p38 MAPKs signaling, and the MOP endocytosis was abolished in cell deficient of p38alpha, which regulates endocytosis through a phosphorylation of Rab5 effectors (45, 46). These findings suggest that the cross-talk through other receptor signaling may affect the status of MOP endocytosis. This heterologous regulation of MOP endocytosis mechanism seems to be more important *in vivo*.

4. PLASTICITY IN NEURONAL NETWORKS

When mice were chronically treated for 5 days with systemic morphine at a dose of 10 mg/kg (s.c.), morphine (10 mg/kg s.c.)-induced analgesia, as assessed by the tail pinch test, which involves higher central nervous mechanisms, was markedly attenuated. However, the above-mentioned peripheral morphine analgesia was not affected (47) in such morphine-tolerant mice. 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. It should be noted that acute tolerance to peripheral morphine analgesia was observed 4 h after the initial morphine administration (i.pl.), but not 24 h later (40, 47). Therefore, we must separately consider the molecular mechanisms underlying acute and chronic morphine analgesic tolerance in terms of synaptic plasticity. Compared with morphine tolerance, it appears to be difficult to clarify the plasticity of neuronal circuits involved in opioid dependence. One of major reasons for this, which make the understanding of this plasticity more complicated, seems to be related to the fact that opioid systems are present at multiple locations in addiction pathways, as well as the involvement of other non-opioid systems (48). Although accumulated findings demonstrate that several brain loci are closely related to the cause of morphine tolerance and dependence, it remains unclear whether the neuronal plasticity involved in morphine analgesic tolerance and dependence is caused by locus-specific activation of opioid receptors. Therefore, we attempted to obtain evidence for a locus-specific mechanism of opioid tolerance and/or dependence in order to further study such complicated plasticity of neuronal networks at a fixed brain locus.

4.1. Anti-opioid glutamate-NMDA receptor system

NMDA receptors have long been supposed to play important roles in the development of morphine tolerance and dependence (11, 49, 50). As many known competitive or non-competitive NMDA antagonists potentiate morphine-induced catalepsy, lethality (51) and hypothermia (52), or retard learning behaviors (53), the development of NMDA receptor antagonists that specifically block morphine tolerance has been explored.
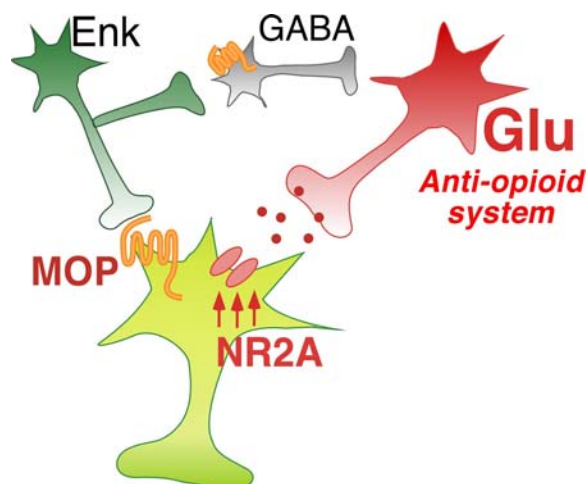
Enk GABA Glu

Anti-opioid system

MOP

NR2A

Figure 2. Anti-opioid NMDA receptor hypothesis underlying morphine tolerance. As morphine action (analgesia) is enhanced to some extent in NR2A$^{-/-}$ mice, compared with wild-type (WT) mice, without changes in basal nociceptive threshold, morphine stimulation may also cause a glutamate-NMDA (NR2A) receptor activation (possibly mediated by a dis-inhibition of GABA neuronal activity), which in turn limits the morphine inhibitory action. Following chronic morphine treatments, NR2A proteins are up-regulated and cancel the morphine analgesic activity (tolerance).

One approach to find specific antagonists began with the identification of the subunit of NMDA receptor involved in morphine tolerance and addiction. In our experiments (54), GluRepsilon1 (NR2A) knockout mice showed an enhancement of acute morphine analgesia in the tail pinch test. 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 inhibit endogenous and exogenous opioid actions (Figure 2). Chronic pretreatments with morphine (10 mg/kg s.c.) for 5 days produced a tolerance to morphine analgesia on the 6th day in wild-type mice. However, such analgesic tolerance was not observed in NR2A knockout mice. As the level of NR2A was significantly increased by 100 – 200% of the control level only in the periaqueductal grey matter (PAG), ventral tegmental area (VTA) and nucleus accumbens (NAcc), we speculated that enhanced activation of the anti-opioid NMDA receptor system abolishes morphine analgesia during chronic treatments. The restoration of this gene through a novel electroporation technique into the PAG or VTA, but not the NAcc successfully restored morphine analgesic tolerance (Figure 3). The rescued protein level in the PAG was almost the same as that in wild-type mice, and remained at this level until 9 days after electroporation. This electroporation technique also has the advantage that it caused no morphological damage to the PAG. 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 3 days showed withdrawal behaviors when 1 mg/kg i.p. naloxone was administered 2 h after the last morphine (100 mg/kg s.c.)

injection on the 4th day. The withdrawal behaviors, such as jumping, withdrawal locomotion, sniffing and defecation, which were observed in wild-type mice, were markedly inhibited in NR2A knock-out mice. The significant increase (by 100% of the control level) in protein expression of NR2A following chronic morphine administration was observed in the NAcc of wild-type mice. Locus-specific recovery of withdrawal behaviors was observed when NR2A gene expression was restored in the NAcc of knock-out mice. 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. The most interesting conclusion is that chronic morphine-induced plasticity in neuronal networks is also mediated through such anti-opioid systems through NR2A, and it occurs, at least to some extent, in locus-specific brain regions. As all these studies have been performed using pharmacological tools, however, we have to wait for the study using specific receptor gene deletion to clarify which types of glutamate receptors as well as NR2A contribute best to the mechanisms underlying morphine analgesic tolerance.

In addition to NR2A-mediated mechanisms, NR2B has 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 has a role to reduce the expression of analgesic tolerance to morphine (55). Since NMDA NR2B receptors in the anterior cingulated cortex (ACC) play roles in the establishment of 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 not to alter morphine analgesia, possibly due to the use of different doses of antagonist and morphine, as well as experimental animals and tests for nociception (56). 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 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 (57).

4.2. BDNF system that supports anti-opioid NMDA receptor systems

In the hypothesized mechanisms based on the anti-opioid NMDA receptor system, NR2A receptor up-regulation following chronic morphine treatments seems to play a key role. We speculate that BDNF might support this anti-opioid system. This speculation is primarily based on following findings: 1) the addition of BDNF to cultured rat cortical neurons up-regulates NR2A gene expression (58); 2) morphine up-regulates BDNF expression in cultured microglia through an autocrine machinery (59); 3) chronic morphine treatments up-regulate BDNF expression in brain
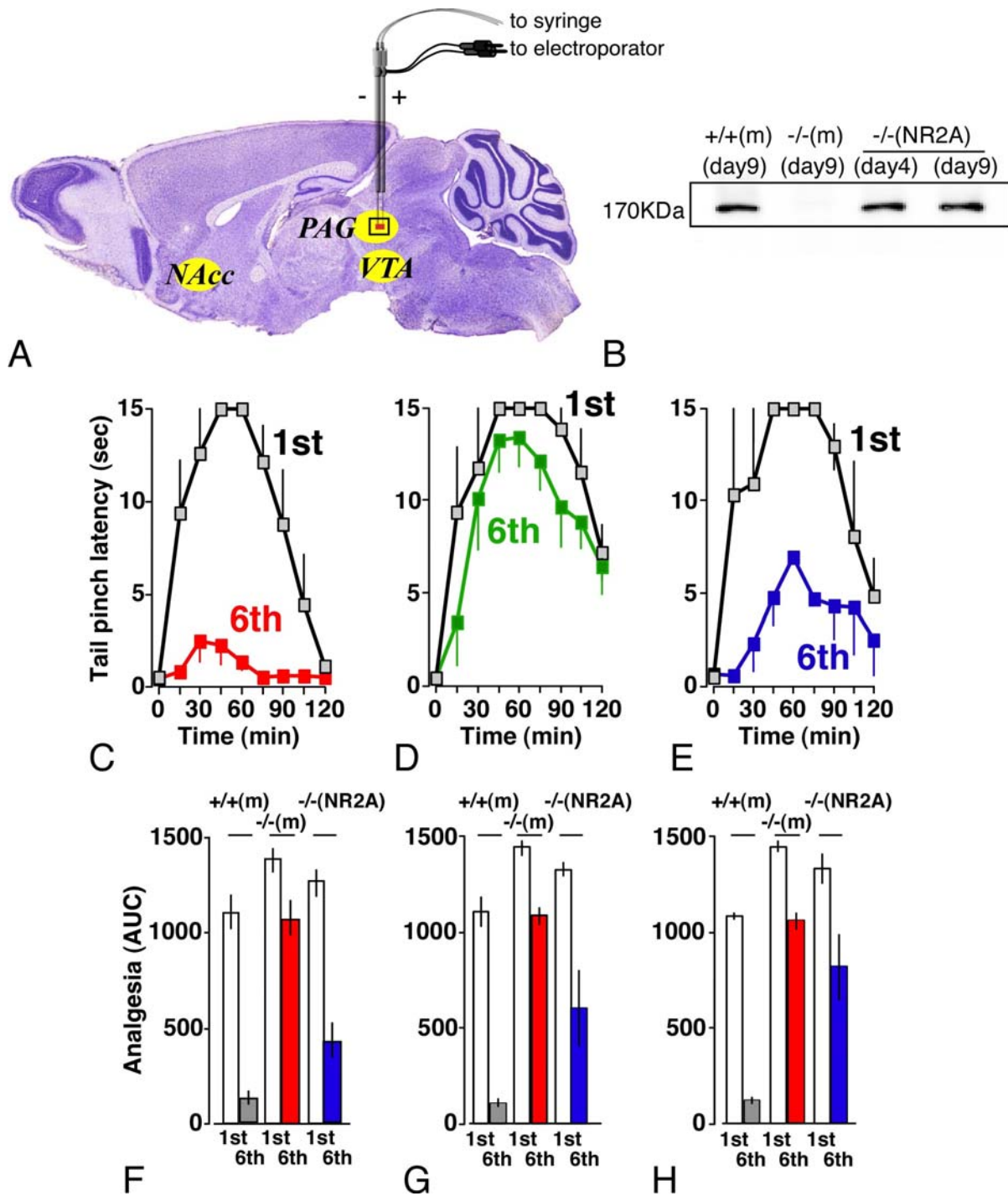
Figure 3. Recovery of morphine analgesia and tolerance by locus-specific rescue of the NR2A gene in the NR2A$^{-/-}$ mouse brain. Rescue of NR2A gene by *in vivo* electroporation (A). Western blot of NR2A in the PAG from mock-transferred NR2A$^{+/+}$ (+/+(m)) or NR2A$^{-/-}$ (-/-(m)) and recombinant NR2A-transferred NR2A$^{-/-}$ (-/-(NR2A)) mice, respectively. Comparable levels of NR2A protein are expressed in the PAG of NR2A$^{-/-}$ mouse, following NR2A gene transfer by *in vivo* electroporation at day 4 and 9 (B). Acute morphine analgesia (day 1, 1st) and reduced one following chronic treatments (day 6, 6th) are shown in WT mice (NR2A$^{+/+}$) with mock gene (C), NR2A$^{-/-}$ mice with mock gene (D) and NR2A$^{-/-}$ mice with NR2A gene (E), respectively. Morphine analgesic tolerance is observed by *in vivo* electroporation of NR2A gene. Similar recovery of analgesic tolerance is observed when the gene transfer is performed into PAG (F) or ventral tegmental area (VTA) (G), but not into nucleus accumbens (Nacc) (H).Reproduced with permission from (54).

Figure 4. Glial involvement in the anti-opioid glutamate/NMDA receptor hypothesis. Following chronic opioid treatments, glutamate neurotransmission and NMDA receptor signaling are up-regulated through neuron-glia interactions. Down-regulation of glutamate transporter (GLAST) levels in astrocytes is expected to increase the glutamate level in synaptic cleft, and up-regulation of D-serine levels to facilitate NMDA receptor signaling through an allosteric mechanism. On the other hand, microglial activation may up-regulate the levels of BDNF, which in turn up-regulates NMDA receptor NR2A subunit, a key molecule for anti-opioid system. Epigenetic control via CBP activation is also involved in the morphine tolerance through BDNF expression.

neurons *in vivo* through a neuron-microglia interaction (60); 4) central injection of anti-BDNF antibody abolished morphine tolerance (61); and 5) morphine physical dependence was lost in forebrain-specific BDNF knock-out mice(62). Although little is known about the molecular mechanisms underlying BDNF-induced NR2A gene expression, three functional GC-boxes in the NR2A promoter were found to interact with Sp1 and Sp4 transcription factors, and to be important for the activity of the core promoter. It is interesting to speculate that BDNF-TrkB activation increases the transactivating capacity of Sp1 through phosphorylation (63, 64). On the other hand, it is a very interesting subject how chronic morphine treatments up-regulate BDNF expression. When mice were pretreated with morphine at a dose of 10 mg/kg s.c. (a maximal dose for analgesia) for 5 days, the substantial analgesic activity of morphine (10 mg/kg, s.c.) was lost on

the 6th day. At this time point, there was a significant up-regulation of BDNF levels in the PAG (61), which is the major brain region involved in morphine analgesia. The intense 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 up-regulates BDNF expression in cultured microglia (59), but not in cultured neurons. From this point of view, we are attempting to find microglia-derived bioactive molecules, which in turn up-regulate BDNF in neurons. Thus the neuron-microglia interaction would play a key role in morphine analgesic tolerance through an action of BDNF (Figure 4). However, these results seem to conflict with a report that forebrain-specific BDNF knock-out mice lose morphine physical dependence, but not morphine analgesic tolerance. 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 (Ueda *et al.*, unpublished data). We recently found that curcumin, an inhibitor of histone acetyltransferase activity of CREB-binding protein inhibitor, blocked the chronic morphine-induced expression of exon I and IV BDNF transcripts, and morphine analgesic tolerance (61). This 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.

4.3. Other systems that support the anti-opioid NMDA receptor system

In addition to the role of microglia, astrocytes also play important roles in the anti-opioid glutamate-NMDA receptor system. Following chronic morphine treatments, the expression levels of glutamate transporters in astrocytes and neurons are down-regulated (50, 65). This phenomenon seems to enhance glutamate signals in the synaptic cleft, which in turn give more opportunity for the stimulation of NMDA receptors (Figure 4). In this sense, D-serine, a key molecule that activates NMDA receptors as an allosteric agonist (66), seems to also be involved in this anti-opioid system because chronic morphine induced up-regulation of glial racemase to increase D-serine levels (67). The knock-down of spinal cord postsynaptic density protein-95 (PSD-95), a scaffold protein for NMDA receptors, prevented the development of morphine tolerance in rats (68) . 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 (69). As ania-3 interferes with the function of constitutively active long forms of Homer proteins, which build bridges between NMDA and metabotropic glutamate receptors (70), this change in Ania-3 expression may contribute to the development of morphine dependence. These findings are consistent with the recent studies by Befort *et al.* (71), in which chronic morphine up-regulates 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 (72). These findings seem to be very interesting because there are reports that Cdk5 phosphorylates the NR2A subunit and activates NMDA receptor function (73), 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 (74). However, there is a conflicting report that no significant change in Cdk5 expression was observed in similar brain regions of morphine-sensitized rats (71).

4.4. Anti-opioid neuropeptide systems

Nociceptin/orphanin FQ (75, 76), cholecystokinin (77, 78) or neuropeptide FF (79) are also key molecules in terms of the development of opioid tolerance and dependence. As the details are well described elsewhere, here we introduce the case with nociceptin. The first observation comes from a study in which centrally administered nociceptin/orphanin FQ inhibited morphine analgesia (80). Based on the counter-balance hypothesis, we successfully demonstrated that morphine analgesic tolerance is attenuated in nociceptin receptor (NOP) knock-out mice (76). Following this demonstration, we have elucidated more details of the NOP-mediated mechanisms underlying morphine analgesic tolerance(81), as follows. In this study, the loss of morphine analgesic tolerance in NOP knock out mice was more evident in the nociception test based on the spinal reflex (tail flick test) rather than in tests involving systemic biting behavior (tail pinch test). This was supported by experiments using a NOP antagonist. The intrathecal injection of NOP antagonist abolished morphine analgesic tolerance in the tail flick test, but intracerebroventricular injection did not. Naloxone-precipitated morphine withdrawal behaviors were also markedly attenuated in NOP knockout mice and following systemic injection of this antagonist. NOP gene expression measured by RT-PCR was enhanced specifically in the spinal cord following daily morphine administration, according to the tolerance paradigm. On day 5, the NOP level in the spinal cord increased by 50% of the control level following chronic morphine treatments. A similar increase in the NOP level (by 60% of the control level) was observed in the spinal cords of mice pretreated with morphine, according to dependence paradigm. Thus enhanced NOP expression could contribute to the plasticity underlying morphine tolerance and dependence, and this mechanism seems to be more specific in the spinal cord. As NOP knockout mice did not show any changes in acute morphine analgesia, the nociceptin/orphanin FQ system is unlikely to be located downstream of opioid neurons, in contrast to the anti-opioid glutamate-NMDA receptor system.

4.5. Opioid-induced hyperalgesia by neuropeptides

Opioid-induced hyperalgesia often occurs in some patients receiving chronic opioid therapy. This hyperalgesia arises at different places and of a different quality than the original pain problem (82). Substance P (SP) and the NK-1 receptor play an important role in mediating morphine-induced hyperalgesia (83). Although blocking SP activity does not alter responses to acute nociceptive stimuli (84), SP is known to contribute to chronic pain and participate in central sensitization and associated hyperalgesia (85, 86). SP preferentially binds to NK-1 receptors located in the dorsal horn of spinal cord. Once activated by SP, NK-1 receptors internalize rapidly in cells located in the superficial regions of the spinal dorsal horn and recycle to the plasma membrane (87). Sustained exposure to morphine has previously been shown to reliably produce increased sensitivity to noxious thermal stimuli (82). Long-term spinal administration of NK-1 receptor antagonist, L-732,138, following morphine treatment rat reversed the morphine-induced thermal hyperalgesia. The possible role of the NK-1 receptor in the mediation of chronic morphine-induced thermal hypersensitivity was also explored using mice lacking the

NK-1 receptor (NK-1$^{-/-}$) and wild type controls (NK-1$^{+/+}$). Chronic morphine-treatment induced a time-dependent reduction in heat-induced paw-withdrawal latency in NK-1$^{+/+}$ mice, but not in NK-1$^{-/-}$ mice, showing that morphine-induced hyperalgesia is mediated by SP activation of NK-1 receptor (83).

4.6. Glial cell involvements in morphine tolerance

Glial cell responses to chronic morphine treatment were examined by immunohistochemistry of glial fibrillary acidic protein (GFAP), a specific marker for astroglial cells (88). Systemic administration of morphine (50 mg/kg, i.p.) once daily for 9 consecutive days led to significant increase in GFAP immunostaining density 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, i.t.) was delivered in combination with fluorocitrate (1 nmol/1 μl, i.t.), 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*. Chronic morphine treatments down-regulate the neuronal and astrocyte glutamate transporters (50, 65), which lead to an enhancement of synaptic glutamate or an increase in the anti-opioid level. These treatments also activate microglia (88). On the other hand, accumulating findings demonstrate that microglia also play key roles in morphine actions or analgesic tolerance. Takayama and Ueda (2005) firstly reported that morphine up-regulates microglial gene expression of BDNF, which is known to up-regulate NR2A (58, 59). This study was succeeded by the *in vivo* studies, in which the treatments with minocycline to inhibit microglial activation or liposome-encapsulated chrodronate to kill activated microglia abolished morphine analgesic tolerance (60). Of interest is the recent study by Matsushita and Ueda (2009) that morphine analgesic tolerance and BDNF up-regulation was both inhibited by curcumin, which is an inhibitor of CREB-binding protein (CBP) (61). As CBP is known to cause a chromatin remodeling through histone acetyltransferase (HAT) activity and stimulate BDNF gene expression, these finding may be the first evidence that morphine analgesia could be suppressed by epigenetic regulation using curcumin. Similar results were also reported by several investigators. Hutchinson reported that minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia (89). Cui *et al.* reported that morphine analgesic tolerance is attenuated by the inhibition of p38 MAPK in the activated spinal microglia (90).

5. CONCLUSION

It is accepted that chronic or long-term pain is a disease by itself, and that such pain should be appropriately suppressed. Opioids are becoming more widely used, not only as palliative medicines for terminal cancer patients, but also as successful analgesics for neuropathic pain patients. Although appropriate medical use of opioids in the clinic is said to be safe, unlike experiments using animals without chronic pain, the long-term use of opioids has a risk of inducing analgesic tolerance and dependence. Therefore, we have to determine how opioid tolerance and dependence develop, and how these side effects could be avoided. The present review proposes several target analgesic adjuvants for use in palliative care. For instance, the suppression of anti-opioid systems can be used together with established opioid treatments. NR2A-specific antagonists and compounds that suppress BDNF transcription, such as curcumin, seem to be good candidates. Chemical modification of opioid analgesics toward more efficient endocytosis may also represent a viable approach. Alternatively, hybrid analgesic adjuvants that apply both mechanisms might offer a better approach.

6. ACKNOWLEDGMENTS

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Abbreviations: cAMP, cyclic AMP; CREB, cAMP response element-binding protein; glutamate-NMDA receptor, glutamate-N-methyl-D-aspartate receptor; MOPs, mu-opioid receptors; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; GPCR, G protein-coupled receptor; GRKs, G protein-coupled receptor (GPCR) kinases; PLC, phospholipase C; MOP, DOP and KOP, mu, delta and kappa-opioid receptors; CHO cells, Chinese Hamster Ovary cells; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; RAVE, relative activity versus endocytosis; i.pl., intraplantarly; APF, algogenic-induced paw flexion; BK, bradykinin; AS-ODN, antisense oligodeoxynucleotide; i.t., intrathecal injection; s.c., subcutaneous injection; PAG, periaqueductal grey matter; VTA, ventral tegmental area; NAcc, nucleus accumbens; BDNF, brain-derived neurotrophic factor; TrkB, tyrosine kinase B; PSD-95, postsynaptic density protein-95; Cdk5, cyclin-dependent kinase 5; NOP, nociceptin receptor

Key Words: NMDA receptor, PSD95, Homer1, Racemase, Glutamate transporter, Astrocyte, Microglia, *In vivo* electroporation, Epigenetic, BDNF, Rescue, Knock-out, Review

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