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Publication Date

2016

DOI

10.1016/bs.pmbts.2015.10.005

Peer reviewed

Molecular Changes in Opioid Addiction: The Role of Adenylyl Cyclase and cAMP/PKA System

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Abstract

For centuries, opiate analgesics have had a considerable presence in the treatment of moderate to severe pain. While effective in providing analgesia, opiates are notorious in exerting many undesirable adverse reactions. The receptor targets and the intracellular effectors of opioids have largely been identified. Furthermore, much of the mechanisms underlying the development of tolerance, dependence, and withdrawal have been delineated. Thus, there is a focus on developing novel compounds or strategies in mitigating or avoiding the development of tolerance, dependence, and withdrawal. This review focuses on the adenylyl cyclase and cyclic adenosine 3,5-monophosphate (cAMP)/protein kinase A (AC/cAMP/PKA) system as the central player in mediating the acute and chronic effects of opioids. This chapter also reviews

the neuronal adaptive changes in the locus coeruleus, amygdala, periaqueductal gray, and ventral tegmental area induced by acute and chronic actions of opioid because these neuronal adaptive changes in these regions may underlie the behavioral changes observed in opiate users and abusers.

1. INTRODUCTION

Opioid use and abuse has increased significantly over the past several decades. Using the Drug Abuse Warning Network (DAWN) and Automation of Reports and Consolidated Orders System (ARCOS), a study published in 2014 showed higher rates of use for seven of the eight most commonly used opioid analgesics (buprenorphine, fentanyl, hydrocodone, hydromorphone, methadone, morphine, and oxycodone) between 1996 and 2011.¹ The use of these drugs had increased over 40-fold during the same time period. As a result of the increased use and misuse of opioid analgesics, substance abuse rehabilitation increased nearly 200%, with the largest increases observed in buprenorphine, hydromorphone, and oxycodone. In 2012, it was estimated that 2.1 million people were dependent on or abused opioid analgesics, representing a 50% increase from 2004 to 2012. It is second only to marijuana use.² Among illicit users of opioids, addiction rates may be as high as 30%. Notably, the clinical use of opioids has been on the rise. In 2012, 259 million prescriptions for painkillers were written for patients in the United States.³

Opioid analgesics have tremendous utility in the relief of moderate to severe pain. However, chronic opioid treatment has a high propensity to induce tolerance and dependence, and perhaps addiction, which is a serious and prevalent medical condition in the United States, resulting in a tremendous economic burden. The phenomenon of tolerance is defined as a rightward shift in the analgesic and other actions of morphine and related analgesics. Therefore, it is not unusual to see patients taking doses substantially larger than their original dose over a period of time.⁴ For patients suffering from opioid dependency, there is both a physiological and psychological need to continue taking the drug. Opioid withdrawal occurs with abrupt cessation of chronic use. In the absence of the drug, withdrawal syndrome emerges that produces negative-affective states and physical symptoms, which can be exaggerated by stress. This often leads patients to relapse to seeking opioid consumption. Not only stress, but environmental cues associated with drug administration can elicit cravings and reinstate drug-seeking and drug-taking behaviors in abstinence individuals. In animal models of withdrawal, symptoms include hyperalgesia, ptosis, wet-dog shakes, escape

attempts, tachypnea, and diarrhea. The various symptoms can be correlated with changes in neuronal functions.⁵ Furthermore, an “incubation” phenomenon in which cravings for drug use is increased and may linger for weeks to months following exposure to cues associated with drug administration in subjects undergoing withdrawal from opiates.⁶ Overall, all of these neuronal adaptive changes contribute to the development and maintenance of drug addiction. Much research has focused on the neurobiological processes involved in opioid tolerance, dependence, withdrawal, and addiction. The adenylyl cyclase (AC) pathway has been implicated in these processes and is the focus of this review.

2. THE ADENYLYL CYCLASE PATHWAY

There are at least three types of opioid receptors—mu (μ ; MOP), kappa (κ ; KOP), and delta (δ ; DOP), which are also referred to as traditional or classical opioid receptors. In addition, there is a related receptor, nociceptin receptor (NOP), also known as the opioid receptor-like (ORL1) receptor. Sigma (σ) receptors were previously categorized as opioid receptors but they are no longer classified as opioid receptors. Opioid receptors are G-protein-coupled receptors containing seven transmembrane domains with an extracellular N-terminus with various glycosylation sites and an intracellular C-terminus.⁷ The MOP, KOP, and DOP receptors are coupled with heterotrimeric, pertussis toxin (PTX)-sensitive, inhibitory G (G_i/G_o) proteins, with all three receptors having the ability to activate both G_i and G_o with similar potencies and subsequently, regulate AC, Ca^{2+} channels, phospholipase C, and mitogen-activated protein kinases.^{8–10} The involvement of each component of AC/cAMP/PKA pathway in analgesia, tolerance, and dependence induced by opioids.

2.1 Adenylyl Cyclase

AC is an enzyme that is activated following stimulation of receptors that are coupled to the AC via G_s , such as beta-adrenergic receptors. On the other hand, the activity of the enzyme is inhibited by activation of receptors that are coupled to $G_{i/o}$, such as opioid receptors. Following the stimulation of opioid receptors, the G_α subunit inhibits AC.^{11,12} Inhibition of AC results in reduced production of intracellular cAMP.¹³ The decrease in intracellular cAMP levels leads to a reduction in protein kinase A (PKA) activity. The activity of AC is altered differentially with acute and chronic exposure to opioids.

Multiple studies have demonstrated the involvement of AC and cAMP in pain and opioid-induced analgesia. Increased nociception is correlated with elevated levels of cAMP.¹⁴ Administration of inhibitors (rolipram and RP-73401) of phosphodiesterase (PDE), which break down cAMP, and activators of AC (forskolin) caused hyperalgesia in rat models of pain.¹⁵ Mammalian genes encode for ten AC isoforms denoted 1–10. AC isoforms I, III, V, VI, and IX are involved in spinal pain transmission. AC V is involved in the mediation of morphine analgesia.¹⁶ High densities of AC V are expressed in the striatum along with MOP, KOP, and DOP receptors.¹⁷ It was further established that AC V mediates the effects of MOP and DOP but not KOP agonists.¹⁶ In wild-type mice, the administration of MOP agonist, DAMGO (D-Ala², NME-Phe⁴, Gly-ol⁵]-enkephalin) or DOP agonist, DPDPE ([D-Pen^{2,5}]Enkephalin), both reduced forskolin-stimulated AC activity in striatal tissues. Following acute administration of opioids, the activity of AC isoforms I, V, VI, and VIII were reduced due to MOP activation. DOP activation by endogenous opioid peptide enkephalin reduced cAMP levels via inhibition of ACII.¹⁸ In AC knockout (isoforms 1, 8, and 1 and 8) mice, forskolin-induced nociception was reduced.^{19,20} Morphine-induced antinociception was not different between wild-type and AC knockout (isoforms 1, 8, and 1 and 8) mice. However, as expected, tolerance to morphine was blunted in the knockout compared to wild-type mice.²¹

While the acute effects of opioid administration involve inhibitory effects on AC mediated by G α_i and G α_o subunits, chronic administration reveals superactivation of AC (also termed supersensitization or cAMP overshoot) and a compensatory increase in cAMP production. Sharma and coworkers first demonstrated that chronic morphine exposure increases AC activity, as measured by increased cAMP levels, following the initial decrease in AC activity.²² At the time, the group proposed that a yet unidentified mechanism underlying the adaptive changes to positively regulate AC activity is related to the development of tolerance and withdrawal observed clinically in patients. Similarly, Collier and Francis hypothesized that elevated cAMP level following chronic morphine exposure is related to opioid dependence.²³ The superactivation of AC was observed in a recombinant CHO (Chinese hamster ovary) cell line expressing DOP.²⁴ Treatment with a DOP agonist reduced DOP density, and moreover, inhibited forskolin-stimulated cAMP accumulation. The inhibition and subsequent superactivation of AC was also isoform specific. The various isoforms of AC have different intracellular signaling mechanisms in response to chronic opioid activation.²⁵

With chronic morphine administration, several steps of the AC/cAMP/PKA pathway are amplified, notably the activity of AC.^{26,27} Withdrawal symptoms associated with morphine dependence are reduced in AC V knockout mice.¹⁶ The locus coeruleus (LC) plays a central role in opiate dependence and withdrawal and its activity is regulated by opioids.²⁸ While neuronal firing in the LC is reduced with acute opioid administration, AC activity and cAMP levels return toward basal levels during chronic opiate exposure.²⁹ Overshoots of cAMP occur following naloxone-precipitated withdrawal along with increased firing rates of LC neurons.²⁸ Furthermore, chronic morphine stimulates G_i and G_o subunits in the LC.³⁰ Chronic administration of opioids causes an uncoupling of the G_α and $G_{\beta\gamma}$ proteins.³¹

PTX-induced inhibition of $G\alpha_i$ and $G\alpha_o$ proteins prevented sensitization of AC as demonstrated with the lack of cAMP accumulation, suggesting that $G\alpha_i$ and $G\alpha_o$ proteins are required for this process.³² Additional insight into the response of $G\alpha_i$ and $G\alpha_o$ subunits under constant stimulation were studied in NS20Y-2_{2L} cells expressing dopamine (D_{2L}) receptors.³³ $G\alpha_i$ and $G\alpha_o$ subunits were genetically altered to be PTX-insensitive, allowing for the examination of which G subunit was involved in the sensitization process. $G\alpha_o$ but not $G\alpha_i$ proteins produced sensitization of forskolin-induced accumulation of cAMP. Under these conditions, $G\alpha_s$ may be stimulated, leading to $G_{\beta\gamma}$ -subunit-enhanced AC activity.³⁴ Furthermore, chronic morphine has been shown to produce a superactive state of AC³⁴ via $G_{\beta\gamma}$ stimulation of plasma-membrane-bound AC 1 and 8.³⁵ Under continuous agonist exposure, receptors appear to be desensitized or down-regulated. However, removal of the agonist unveils the overshoot of cAMP due to $G\alpha_s$ activation. A reduction in PDE activity has also been observed with chronic exposure.³⁶ This process may contribute to the withdrawal symptoms observed upon removal of the agonist.

The sensitization process of AC also involves $G\alpha_s$.³⁷ Following chronic morphine administration, $G\alpha_s$ subunits coupling to PGE₁ (prostaglandin) receptors are increased.³⁷ This resulted in PGE-stimulated AC activity and increased cAMP levels. While most work with MOP signaling involves $G\alpha_i$ and $G\alpha_o$, other studies have shown that the stimulation of MOP with chronic morphine reduces $G\alpha_s$ phosphorylation.³⁸

2.2 Protein Kinase A

PKA is a cAMP-dependent kinase that is a major downstream effector of opioid receptor activation. The phosphorylation of PKA leads to the activation of the enzyme and has numerous intracellular roles, including

the upregulation of gene expression through cAMP response element-binding protein (CREB), upregulation of tyrosine kinase, and increasing Na^+ -dependent inward current.

Acute exposure to opioids inhibits AC activity and reduces the production of cAMP, and therefore, decreases the activity of PKA. With chronic exposure of opioids, AC activity returns toward basal levels, resulting in increased cAMP levels and PKA activity. Chronic morphine exposure decreases phosphorylation of G_s , and enhances its coupling with MOP, increasing AC activity.³⁹ Several studies indicate that PKA phosphorylates and interferes with MOP coupling to G_i subunits.^{40,41} Other studies have shown a reduction in PKA-mediated phosphorylation of MOP.⁴² Phosphorylation of GM_1 ganglioside by PKA converts MOP coupling to G_s from $G_{i/o}$. This conversion from inhibitory $G_{i/o}$ to stimulatory G_s via GM_1 ganglioside phosphorylation may be one of the underlying mechanisms for the development of opioid dependence.⁴³ Furthermore, we have previously shown that this shift may also enhance the toxic effects of morphine. Morphine-induced antinociception was reduced but seizure-like activity increased in mice treated intracerebroventricularly with PTX (ADP-ribosylates the inhibitory G proteins).⁴⁴ The role of GM_1 was further substantiated by the work of Crain and Shen who showed that low-dose naloxone produced hyperalgesia in the tail-flick assay in naive mice treated with GM_1 ganglioside, a response that is seen in subjects chronically treated with morphine and exposed to the same dose of naloxone.⁴⁵ This response was shown to be mediated via the KOP, as administration of high doses of naltrexone or KOP antagonist nor BNI blocked hyperalgesia induced by naloxone in these mice.

The role of PKA in the development of tolerance and withdrawal is not completely clear. PKA activators directly infused into the NAc increased drug-seeking behaviors observed in animals while PKA inhibitors mitigated these behaviors.⁴⁶ Some studies demonstrate that inhibition of PKA has minimal or no effect on the development of acute tolerance. Injections of MOP agonist DAMGO in mice produced acute tolerance.⁴⁷ Administration of PKC inhibitors prevented the development of acute tolerance produced by intrathecal DAMGO. Injection of PKA inhibitors, however, did not attenuate the development of acute tolerance to DAMGO. Similar findings regarding the role of PKA and PKC in acute tolerance to the antinociceptive effect of peripherally administered morphine were reported in the bradykinin-induced flinching test.⁴⁸ Calphostin C, an inhibitor of PKC, prevented morphine-induced tolerance, whereas KT-5720, an inhibitor of PKA, did

not. Bilsky and coworkers treated mice with morphine (100 mg/kg) to induce acute tolerance to its analgesic effect and 4 h post morphine treatment injected them with naloxone to precipitate withdrawal. They determined that the effect of a neutral antagonist, such as, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), and naloxone, a nonselective opioid antagonist, which acts as an inverse agonist in opioid-dependent subject, in the presence and absence of the protein kinase C inhibitors, H7 and its related analog, H8. Central administration of naloxone elicited withdrawal jumping and this response was blocked by H7 but not H8. While CTAP induced limited jumping, it suggested that mu-constitutive activity required PKC in this response.⁴⁹

The role of PKA in opioid withdrawal was assessed with the application of Rp-cAMPS, a PKA inhibitor.⁵⁰ Infusion of Rp-cAMPS directly into the LC and PAG attenuated naloxone-induced withdrawal symptoms whereas infusion into the amygdala did not. Furthermore, the infusion of PKA activator Sp-cAMPS into the LC or PAG in opioid-naïve animals produced withdrawal-like symptoms. Rp-cAMPS significantly increased phosphorylation of tyrosine hydroxylase in the LC.

3. OPIOID EFFECT ON cAMP-RESPONSIVE ELEMENT-BINDING PROTEIN

A variety of upstream signals regulate gene expression through phosphorylation of CREB. Phosphorylation of CREB by PKA promotes a high-affinity binding site with transcriptional coactivators CREB-binding protein and p300.^{51,52} The secondary structure of CREB is unaltered. The serine residue of the kinase-inducible domain is the site for phosphorylation by Ca²⁺ and PKA.⁵³ CREB plays a functional role in the regulation of gene expression.⁵⁴ Acute morphine administration reduces AC activity, which ultimately leads to reduced PKA-induced phosphorylation of CREB. However, the level of phosphorylated CREB is altered differentially by acute and chronic morphine and this alteration is brain region-dependent.

Chronic morphine administration reduced CREB immunoreactivity in the NAc⁵⁵ but increased it in the LC.⁵⁶ Acute opioid administration inhibits AC activity, therefore reducing cAMP-dependent phosphorylation of CREB.⁵⁷ Maldonado and coworkers created CREB-mutant mice to further examine the role of CREB in opioid dependence.⁵⁸ Wild-type animals treated with chronic morphine displayed expected signs of withdrawal upon

precipitation by naloxone. However, in CREB-mutant mice, all signs of withdrawal were reduced. Seven-day treatment with morphine via pellets implantation showed elevated levels of phosphorylated CREB in the lateral hypothalamus, ventral tegmental area (VTA), and the hypothalamus.⁵⁹ Alterations in CREB levels were not observed in the NAc and amygdala.

Mapping of CREB response to opioid administration was obtained using transgenic mice encoding β -galactosidase (β -gal).⁶⁰ β -Gal levels were increased in the LC and amygdala but decreased in the VTA or dorsal raphe nucleus (DRN). The reduction in CREB activity in the VTA during withdrawal may be due to inhibition of dopaminergic activity by γ -aminobutyric acid (GABA) mechanisms. The reduction in serotonergic firing in the DRN is also mediated by GABAergic interneurons.

Prohormone convertases, PC1/3 and PC2, are enzymes that cleave prohormones to hormones. The expression of PCs is dependent upon CREB activation.^{61,62} These enzymes are found in high concentrations in the pituitary gland and some brain regions.⁶³ Most of the endogenous peptides are secreted as inactive precursors and converted into an active form by PC1/3 and PC2. PC1/3 and PC2 response to opioid exposure appears to be biphasic. We have previously shown that short-term morphine exposure downregulated the enzymes while chronic (7 days) morphine administration upregulated PC1/3 and PC2 levels in the midbrain.^{64,65} The level of phosphorylated CREB (P-CREB) is increased with chronic morphine exposure in the hypothalamus.⁶⁴ Short-term exposure of morphine (6 h) reduced P-CREB levels, indicating that P-CREB is involved in the downregulation of PC1/3 and PC2 in the pituitary.⁶⁴ Lastly, pituitary proopiomelanocortin (POMC) and β -endorphin levels were reduced by morphine administration. This may be due to feedback mechanisms responding to exogenous opioid exposure by reducing endogenous opioid peptide levels via the CREB system. Naltrexone, a nonselective opioid antagonist, increased POMC, PC1/3, PC2, and β -endorphin levels. Thus, CREB may be a target for the action of exogenous opioid to regulate the level of endogenous opioids although alterations in the POMC levels could lead to changes in the expression of nonopioid peptides, such as adrenocorticotrophic hormones and α -melanocyte-stimulating hormone. During extinction of morphine-induced conditioned place preference (CPP), CREB levels in the hippocampus, NAc, and PFC return toward baseline levels.⁶⁶ However, extracellular signal-regulated protein kinase (ERK)-CREB activity is elevated during extinction of conditioned place aversion following morphine withdrawal.⁶⁷

Downstream effectors of $G\alpha_i$ and $G\alpha_o$ proteins regulate AC through phosphorylation, thereby, providing additional mechanisms for sensitization. Following activation of $G\alpha_i$ and $G\alpha_o$, the $G_{\beta\gamma}$ subunit interacts with various protein kinases. Protein kinase Raf was shown to phosphorylate AC VI and sensitize the enzyme.⁶⁸ Varga *et al.* proposed that Raf-1 is the site of convergence for the effects of Ras and PKC.⁶⁹ $G_{\beta\gamma}$ activates receptor tyrosine kinase (RTK), β -arrestin, and PKC. RTK and β -arrestin both increase the activity of nonreceptor tyrosine kinase, leading to RAS activation. RAS and PKC increase the activity of Raf-1 during the chronic opioid exposure. Given that ERK is downstream of Raf-1 and RAS, the alteration in RAS and Raf-1 may play a role in the incubation of opiate craving. Indeed, a low dose of morphine (3 mg/kg) induced a long-lasting CPP in rats. Along with this enduring CPP response, there was a significant increase in the level of phosphorylated ERK as well as CREB in the central amygdala. Interestingly, injection of ERK and CREB inhibitors in this brain region prevented the incubation of opiate craving.⁷⁰ Thus, ERK could be a potential target for the development of medications to treat opiate addiction and particularly opiate craving. However, ERK is also implicated in neuronal plasticity and thus the task to develop small-molecule ERK inhibitors that selectively reduce opiate craving needs further investigations.

4. MOLECULAR CHANGES IN BRAIN REGIONS THAT MAY UNDERLIE OPIATE DEPENDENCE

4.1 Molecular Changes in the Locus Coeruleus

The LC has been studied extensively as a site of mediating opiate withdrawal.^{71–74} The LC is densely populated with adrenergic neurons^{75,76} and has a substantial role in manifestation of behavioral signs of withdrawal.⁷⁷ The LC primarily receives neuronal input from the hippocampus, prefrontal cortex (PFC), and the amygdala. The LC projects to the limbic system, cerebral and cerebellar cortices as well as various nuclei throughout the CNS such as the hypothalamus, spinal cord, and brainstem (paragigantocellularis) nuclei.⁷⁸ All three opioid receptors are expressed in this brain area. However, MOP is the predominate opioid receptor in the region.⁷⁹ Chronic administration of opioids induces substantial changes in intracellular second messenger systems in the LC. Behavioral studies in morphine-dependent rats showed that the LC is the most sensitive structure in the CNS during the withdrawal phase induced by opioid antagonist methylnaloxonium.⁸⁰

Acute administration of levorphanol, an opioid receptor agonist, produced rapid suppression of LC firing.⁸¹ Naloxone prevented and reversed the inhibitory effects of levorphanol, showing that opioid receptors are involved in this response. Morphine and clonidine, an α_2 -adrenoreceptor receptor agonist, each decreases LC firing in brain slices.⁸² Chronic morphine (but not clonidine) treatment induces tolerance, that is, LC neurons show reduced sensitivity to morphine.

Increases in LC neuronal firing have been demonstrated following opioid withdrawal.⁷¹ Direct administration of kynurenate, an antagonist of excitatory amino acids, 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX), a non-NMDA receptor antagonist, or AP5, a selective NMDA-receptor antagonist, into the LC attenuated withdrawal induced neuronal firing.⁸³ Microdialysis studies following administration of naltrexone showed increases in excitatory amino acids (EAA) glutamate and aspartate release, which correlated with increased neuronal firing in the LC.⁸⁴ The changes in LC firing during withdrawal are accounted primarily by EAA mechanisms within the LC.⁸⁵

The α_2 -adrenoreceptors are present in the LC as both presynaptic and postsynaptic receptors and are negatively coupled to AC.⁸⁶ The effects of chronic opioids on α_2 -adrenoreceptor density and binding have produced mixed results. Chronic morphine showed a reduction in receptor binding⁸⁷ and density.⁸⁸ In contrast, no change in α_2 -adrenoreceptor density was observed in a neurotumor cell line⁸⁹ while another study showed an increase in density.⁹⁰ In hippocampal slices, chronic administration of morphine reduced α_2 -adrenoreceptor binding.⁸⁸

Clonidine has been used successfully in the treatment of acute opiate withdrawal.⁹¹ Both objective signs and subjective symptoms were reduced in a human trial using clonidine, which suggests that α_2 -adrenoreceptors were involved in mediating opioid dependence and withdrawal.⁹² However, the LC was not investigated as the CNS site for mediating the signs of physical dependence on opiates as the site of action for clonidine until a decade later. Naloxone-induced withdrawal in subjects exposed to chronic morphine was reversed by local infusions of clonidine into the LC.⁹³ As stated earlier, clonidine is an agonist at presynaptic α_2 -adrenergic receptors in the LC, decreasing adrenergic outflow responsible for the observed withdrawal symptoms (wet-dog shakes, weight loss, diarrhea, and ptosis). Naloxone, a nonselective opioid receptor antagonist, directly injected into the LC induced withdrawal symptoms in rats chronically administered with morphine for 11 days.⁹⁴ The application of

methylnaloxonium intracerebroventricularly precipitated withdrawal symptoms in subjects with chronic opioid exposure.⁹⁵ Withdrawal symptoms were attenuated following intraperitoneal injection of clonidine, indicating that the symptoms were mediated through the adrenergic neurons of the LC.⁹⁶

Rasmussen and Aghajanian provided insight to the role of LC by examining behavioral, biochemical, and electrophysiological changes.⁹⁷ A behavioral assessment, composed of 14 behaviors characteristic of opioid withdrawal, showed a rapid reduction in withdrawal symptoms during the first 4 h after naloxone administration in opioid-dependent rats. However, withdrawal symptoms gradually declined and continued to last for 72 h. Within several minutes of naltrexone exposure, neuronal firing in the LC increased to a sixfold peak within 15–30 min in comparison to prenaltrexone exposure in opioid-dependent rats. In congruence with the withdrawal symptoms, the elevation in LC firing rats remained elevated for 72 h compared to controls. Biochemical studies of AC and PKA showed elevated activity 20 min after naltrexone administration, but no differences in activity were observed at this time point compared to morphine-dependent subjects exposed to abstinence withdrawal. The behavioral and electrophysiological parameters showed changes after the induction of opioid withdrawal. In contrast, the biochemical results were already elevated prior to the induction of withdrawal. The rendering of a nonfunctional LC by electrolytic lesion mitigated the withdrawal symptoms.⁹⁵ Lesions of the nucleus paragigantocellularis (PGi) reduced withdrawal induced by LC activation.⁹⁷

Neuronal firing in the LC of anesthetized rats was gradually suppressed with implantation of morphine pellets, with complete suppression of firing achieved 1–2 h.⁹⁸ Within 48–72 h, firing rates returned toward basal level. The administration of MOP antagonist induced neuronal firing substantially above baseline levels. Nestler and Tallman also investigated the effects of chronic opioid administration on LC neuronal firing.³⁰ Rats treated with chronic but not acute morphine showed elevated PKA activity. This paralleled the time course of the development of tolerance and dependence on morphine as demonstrated by electrophysiologic studies. Chronic morphine exposure increases $G\alpha_{i/o}$, AC, and tyrosine hydroxylase in the LC, all of which may mediate the development of tolerance and withdrawal.⁹⁹ Proto-oncogene c-FOS levels are increased within several hours during withdrawal from opioid use.¹⁰⁰

PKA plays a pivotal role in mediating opiate withdrawal in the LC. Administration of a PKA antagonist directly into the LC reduced withdrawal

behaviors induced by naloxone in morphine-treated animals.¹⁰¹ Injection into the PAG produced a lesser effect to attenuate withdrawal behaviors compared to injections into the LC. The use of a tyrosine kinase inhibitor did not attenuate the withdrawal symptoms.

Changes in CREB response in LC have been observed.¹⁰² Acute morphine exposure reduced CREB phosphorylation. However, CREB phosphorylation returned toward basal levels with chronic morphine administration. Furthermore, increased phosphorylation of CREB was demonstrated following induction of withdrawal with a MOP antagonist. Changes in CREB phosphorylation in the LC may be linked to gene expression involved in addiction. AC levels and gene expression did not increase during withdrawal in mice with CREB gene disruption.⁵⁸ Thus, to examine the role of CREB, antisense oligonucleotides were infused directly into the LC of rats under anesthesia.¹⁰³ The antisense sequence was directed at the start site of the CREB mRNA translation. Morphine-induced upregulation of AC 8 and tyrosine kinase, but not PKA or G_i, were attenuated in animals receiving CREB antisense oligonucleotides. Electrophysiologic studies showed a reduction in LC firing in CREB antisense-treated animals. Results from behavioral studies showed decreased withdrawal symptoms induced by naltrexone in CREB antisense-treated animals.

Long-term glutamate receptor desensitization was observed during opioid withdrawal due to elevated glutamate levels.^{84,104} Desensitization of glutamate receptors occurred within 20 min of continuous glutamate administration, with resensitization occurring within several hours. Phosphorylation of glutamate receptors by PKA¹⁰⁵ or PKC¹⁰⁶ may regulate the desensitization process.

While the LC has been shown to play an important role in dependence and withdrawal, it does not appear to mediate the development of opioid tolerance.¹⁰⁷ Pretreatment with *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4), a noradrenergic neurotoxin, produced lesions specific to LC projections. Behavioral studies using the tail-flick test showed no changes in the development of tolerance to chronic morphine administration in DSP-4-lesioned animals. However, DSP-4-induced lesions of the LC attenuated opioid withdrawal.

4.2 Molecular Changes in the Amygdala

The amygdala plays a functional role in integrating stress and arousal and producing negative reinforcement related to addictive disorders.¹⁰⁸ Furthermore, the amygdala mediates signs of withdrawal in subjects with

either acute or chronic opioid exposure.¹⁰⁹ Acute administration of morphine stimulates MOP-activated K^+ channels in the amygdala.¹¹⁰ The resultant hyperpolarization inhibits GABAergic neurotransmission. DAMGO application decreased inhibitory postsynaptic currents (IPSCs) in neurons projecting to the PAG, an effect that was blocked with the administration of bicuculline, a GABA_A receptor antagonist.¹¹¹ The modulation of GABAergic neurotransmission by acute morphine appears to be mediated by a PTX-insensitive process.¹¹² The administration of RP-cAMPS triethylammonium, a cAMP inhibitor, reduced IPSC and attenuated morphine-induced GABAergic neurotransmission. The authors proposed that a switch to $G_{\alpha s}$ along with $G_{\beta\gamma}$ -stimulated AC mediated the superactivation of cAMP observed in the amygdala.

There is evidence of a differential response or switch by dopamine (DA) in the pyramidal neurons of the amygdala under acute versus chronic opioid exposure.^{113,114} In particular, the pyramidal neurons of the basolateral amygdala receive input from the VTA. In saline-treated amygdala slices, DA reduced the amplitude of excitatory postsynaptic currents (EPSCs) by inhibiting α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors.¹¹³ However, in slices exposed to chronic morphine, DA increased the amplitude of EPSCs by inducing presynaptic glutamate release. This effect was abolished by the application of SCH23390, a D1 (dopamine) receptor antagonist. The enhanced glutamate release is mediated via increased PKA activity in the presynaptic neuron. The switch from an inhibitory to excitatory modulation of DA receptors is dependent on cAMP. Behavioral studies showed that administration of SCH23990, directly into the amygdala prevented naloxone-induced aversion.

CREB levels following chronic morphine exposure remain unchanged or elevated. Phosphorylated CREB levels were not increased in the amygdala with chronic morphine exposure.⁵⁹ The role of the central amygdala during the “incubation” phenomenon was examined after chronic morphine administration. Interestingly, animals treated with low but not high doses of morphine exhibited increased increases in CPP in a three-chambered apparatus after 14 days of withdrawal.⁷⁰ Prior studies using the two-chambered apparatus showed decreased CPP after withdrawal.^{115,116} ERK expression is increased by glutamate release during withdrawal, enhancing ERK phosphorylation.⁷⁰ The authors postulated that ERK-inhibited K^+ channels cause depolarization, which perhaps exposed the neurons to be more sensitive to drug cues as observed with increased CPP. UO126, a MEK inhibitor, reduced ERK and CREB activity and reversed CPP, thus substantiating a role for ERK and

CREB in the mediation of drug-seeking behaviors following withdrawal. As stated earlier, ERK then could be a potential target for the development of novel pharmacotherapy of opiate addiction.

4.3 Molecular Changes in the Periaqueductal Gray

The periaqueductal gray (PAG) plays a vital role in mediating pain transmission as injections of morphine or partial MOP-agonist enkephalin produced analgesia.^{117,118} The analgesic effects are mediated by serotonergic fibers arising from the PAG. Serotonergic neurotransmission in the PAG is inhibited by local GABAergic neurons.^{119,120} Exogenous opioids and endogenous peptide endomorphin-activating MOP in the PAG, which inhibits GABAergic neurons, resulting in disinhibition of serotonergic neurotransmission.^{117,121}

The electrical stimulation¹²² of neurons or direct injection of morphine¹²³ into the PAG produces analgesia. The PAG receives input from the amygdala and PFC. DAMGO suppressed AC activity, an effect that was reversed by the application of naloxone. Direct injection of PKA inhibitors, H7 or Rp-cAMPS, into the PAG reduced precipitated withdrawal symptoms.^{50,101} Electrophysiologic data support the development of tolerance by PAG neurons to chronic morphine exposure.¹²⁴ PKC but not PKA modulates Ca^{2+} channels in the PAG following MOP activation.¹²⁵ Administration of a protein kinase C activator, phorbol-12-myristate-13-acetate (PMA), prevented DAMGO-induced inhibition of Ca^{2+} currents.

Acute treatment with morphine increased MOP coupling to $G_{i/o}$ in the PAG.¹²⁶ However, chronic exposure reduced MOP coupling to $G_{i/o}$ and increased coupling to G_s . $G_{\beta\gamma}$ interaction with AC II and IV also occurred during chronic treatment. Concurrent administration of low-dose naloxone (10 ng/kg, subcutaneously or 0.05 ng, intrathecally) inhibited morphine-induced G_s coupling and $G_{\beta\gamma}$ activation of AC. Low-dose naloxone coadministered with morphine enhanced acute analgesic effect of morphine and attenuated its dependence and tolerance liability.

GABA transporter-1 (GAT) activity in the PAG is increased during withdrawal from chronic opioid use. GABA_B receptors are expressed extensively in the PAG¹²⁴ and are coupled to $G_{i/o}$ subunits,¹²⁷ AC is a downstream effector of GABA_B receptors.¹²⁸ Elevated PKA activity increased GABA currents, causing depolarization and stimulation of GABAergic neurons in the PAG.¹²⁹ Thus, PAG neuronal activity in the hypothalamus and medulla is reduced by GABAergic activation in the PAG.¹³⁰ However, it does not appear that the activation of GABA_B receptors affect GAT activity.¹³¹

Baclofen, a GABA_B receptor agonist, mitigates some of the behavioral symptoms of withdrawal. However, Bagley *et al.* suggested that the GABA_B receptors display differential coupling to AC during chronic opioid treatment.¹³¹

5. MOLECULAR CHANGES IN THE VENTRAL TEGMENTAL AREA

The VTA plays a prominent role in the reward-related behaviors. Therefore, the effects of drugs of abuse have been studied extensively in the VTA. The VTA contains a high density of dopaminergic neurons projecting to the NAc, which is likewise involved in drug reward. The VTA also projects to the PFC and amygdala, which are thought to mediate thought process, decision-making, and the emotional aspects of reward. The VTA primarily receives excitatory neuronal input from the PFC while receiving inhibitory input from the amygdala.¹³²

Acute administration of morphine directly into the VTA induces release of DA in the NAc as measured by microdialysis in rats.¹³³ This coincides with increased firing of dopaminergic neurons in the VTA with acute IV morphine.¹³⁴ Naloxone reversed this effect. The putative mechanism by which morphine increases dopaminergic firing is via coupling of MOP to G α_i and G α_o proteins and causing hyperpolarization of local GABAergic interneurons in the VTA. Morphine administration disinhibits GABAergic influence on dopaminergic neurons, allowing DA release. Furthermore, intracellular recordings demonstrate that morphine does not directly affect dopaminergic neurons in the VTA, but primarily through disinhibition of GABAergic neurons. KOP, on the other hand, produces inhibition of dopaminergic neurons in the VTA.¹³⁵ Intracellular recordings of the VTA neurons show that KOP agonists decrease neuronal firing of dopaminergic neurons mediated by the opening of inward rectifying K⁺ channels. Thus, acute administration of opioids can stimulate and inhibit DA activity in the VTA.

Chronic administration of morphine produces sustained dopaminergic activity, with a return toward baseline levels during withdrawal. Intracellular recordings from midbrain slices containing the VTA showed increased IPSCs in GABA neurons following withdrawal from morphine.¹³⁶ The increase in GABA activity may be due to the sensitization of AC and the buildup of cAMP levels. The application of forskolin increases GABA IPSCs in morphine-treated brain slices while dideoxyforskolin, which does not have an effect on AC, did not increase GABA IPSC.^{136,137}

6. MOLECULAR CHANGES IN OTHER CNS REGIONS

Within the amygdala, the bed nucleus of the stria terminalis (BNST) serves as the output pathway. The BNST receives considerable noradrenergic input and has been shown to mediate the neurocircuitry and behaviors of reward and stress.¹³⁸ Immunoreactivity of tyrosine hydroxylase increased considerably in morphine-dependent animals in this brain region following the induction of withdrawal by naltrexone.¹³⁹ Withdrawal-induced aversion assessed by CPP was also significantly higher in morphine-treated animals. Lesion of axons from the LC to BNST did not affect place aversion during withdrawal. However, lesion of axons from A1 and A2 noradrenergic neurons reduced withdrawal-induced aversion, implicating the influence of the ventral noradrenergic bundle in stimulating behavioral symptoms mediated by the BNST. Local application of ST-91 into BNST, a clonidine analog, reduced aversion. Taken together, the BNST may be a site of action for the therapeutic effects of clonidine. The BNST projects inhibitory fibers to the VTA, GABAergic, and GABAergic/enkephalinergic, to modulate response to MOP activation.¹⁴⁰ While behavioral changes are associated with BNST modulation upon chronic morphine administration, AC activity is not affected.¹⁴¹

While opioid antagonists have been employed experimentally to induce withdrawal in animals treated chronically with opioids, several studies have examined the chronic use of low-dose opioid antagonists to alleviate withdrawal symptoms.^{142–144} Withdrawal jumping was reduced significantly in both acutely and chronically treated animals with morphine and low-dose naltrexone.¹⁴⁴ Moreover, tail-flick tests showed that low-dose naltrexone appeared to potentiate the analgesic effects of morphine compared to animals treated with morphine alone. The hypothesis was that blocking the Gs-coupled MOP facilitates morphine analgesia and dependence liability, which is in line with the theory of opiate tolerance and involves superactivation of AC. Low-dose naltrexone (5 mg/L) was dissolved in drinking water for rats treated for eight days with morphine.¹⁴² On the eighth day, animals were injected with naltrexone to induce withdrawal. In the naltrexone pretreated animals, withdrawal symptoms were significantly reduced. Furthermore, c-FOS labeling, PKA protein levels, and phosphorylated CREB levels were lower in the LC and the nucleus of the solitary tract (NTS) compared to nontreated animals. The reduction in c-FOS, PKA, and P-CREB is correlated with the reduction in withdrawal symptoms. Further examination

under similar conditions showed increased MOP expression in the NTS but not in the LC in animals pretreated with naltrexone.¹⁴³ However, DOP expression remained unchanged in both the NTS and LC. No changes in PKA and P-CREB were observed in the frontal cortex, striatum, amygdala, and VTA.

7. CONCLUSIONS

The need for the use of opioids in the treatment of pain associated with various disease-states will continue in healthcare. This increases the propensity for the development of tolerance, dependence, withdrawal, and addiction in patients who are prescribed to use opioid chronically. The molecular targets and changes due to acute and chronic opioid administration have been delineated. Earlier studies showed that chronic opioid administration induces desensitization and internalization. However, changes in the number of opioid receptors following chronic opioid administration have yielded mixed results. Targeting second messenger systems may be proven useful to treat opioid withdrawal. In particular, the AC/cAMP/PKA pathway may be a potential target to reduce pain and prevent symptoms of opioid withdrawal. Indeed, the use of α_2 -adrenergic receptor agonists, such as clonidine, have been proven useful in this regard. Thus, small-molecule compounds that target the AC/cAMP/PKA pathway can be useful as adjunct therapeutic agents to reduce pain and the undesirable side effect of the opioid that may develop following their chronic use.

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