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The ambiguities of opioid tolerance mechanisms: barriers to pain therapeutics or new pain therapeutic possibilities

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Running title: Plasticity of tolerant mechanisms

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Non-standard abbreviations: μ-opioid receptor (MOR); Chinese Hamster Ovary cells stably expressing mu opioid receptor (MOR-CHO); adenylyl cyclase (AC); MOR-CHO overexpressing AC1 (AC1-MOR-CHO; MOR-CHO overexpressing AC2 (AC2-MOR-CHO); immunoprecipitate (IP); protein kinase C (PKC);

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Abstract

Identification of adaptations to chronic morphine that are causally associated with opioid tolerance formation has long been intensely pursued by the opioid research community. There is an impressive array of components of signaling pathways that are influenced by chronic opioid administration. This underscores the importance to tolerance mechanisms of the complex interplay of cellular adaptations that are downstream from the opioid receptor. A major impetus for this research remains the need to develop opioid agonists that are potent and efficacious activators of analgesic mechanisms without triggering opioid tolerance-producing adaptations. Implicit in most models of opioid tolerance is that their underlying mechanisms are invariant and independent of the system in which they have been observed. Reports that acute prior morphine treatment and pain could influence tolerance mechanisms were not understood on mechanistic levels and consequently not incorporated into commonly used models of opioid tolerance. The recent demonstration that adenylyl cyclase/cAMP-related cellular adaptations to chronic morphine depend on cell state demonstrates that ongoing cell physiology is a critical determinant of tolerance mechanisms. The plasticity and pliability of cellular adaptations that mediate tolerance formation indicates that mechanisms underlying opioid analgesic tolerance could be a moving target. While this might represent a daunting barrier to developing anti-tolerance pharmacotherapies, appreciation of this complexity could lead to the development of new pharmacotherapeutic approaches.

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Introduction

Of the armamentarium of pharmacological agents available to manage post-surgical and neuropathic pain, morphine and its congeners remain among the most widely employed. Nevertheless, the propensity of narcotics to induce analgesic tolerance (operationally defined as a reduction in responsiveness to an agent following repeated exposure) profoundly limits their therapeutic usefulness. Not surprisingly, an enormous research effort has been expended over the years toward elucidating the mechanistic underpinnings of opioid tolerance. A major impetus for this research remains the need to develop opioid agonists that are potent and efficacious activators of analgesic mechanisms without triggering opioid tolerance-producing adaptations. Central to this effort is the identification of tolerance substrates, i.e., adaptations causally associated with tolerance formation, on all organizational and functional levels. In this pursuit, conceptual formulations of tolerance become critical for they determine the contour map guiding the journey as well as the resting stops that are targeted along the way.

It is certainly humbling to recognize that the search for non-tolerance-forming potent narcotic analgesics, alone or in combination with adjunctive pharmacotherapy, has been ongoing for at least the past 50 years without notable success. Moreover, this failure has occurred in the face of huge advances in our molecular and cellular knowledge of opioid receptors and the cell signaling pathways that are activated by them. This could indicate that opioid analgesic and tolerance mechanisms are so inextricably intertwined that they cannot be differentially targeted. Alternatively, our conceptual models of tolerance might not be sufficiently inclusive to provide the perspectives needed to develop opioid-based medications with which to treat pain in the absence of tolerance.

This Perspective will advance the concept that models of tolerance need to embrace the influence of ongoing physiological state on opioid tolerance mechanisms that are utilized. Since this article is not intended to be a review, aspects of opioid tolerance have been selected that advance the idea that opioid tolerance mechanisms are pliable and context-dependent.

Cellular tolerance vs. adaptations involving neuronal networks

Adaptations to chronic morphine that occur on the level of individual neurons all occur within neuronal networks, which can amplify (or diminish) the functional consequences of those adaptations. A good exemplar of this is the ability of chronic opioids to increase activity of glutamatergic neurons and consequently augment *N*-methyl-D-aspartate (NMDA) receptor activity (Mao, 1999). This illustrates the important consideration that chronic morphine can induce tolerance adaptations in neurons that do not bear opioid receptors, which might amplify or compensate for the consequences of cellular adaptations that occur within individual neurons. This greatly complicates the generation of tolerance models of translational utility. This notwithstanding, delineation of cellular adaptations to chronic morphine enables the identification of putative pharmacologic cellular targets for the amelioration of tolerance development. It also can facilitate the development of organizing concepts and principals that would apply to chronic morphine-induced adaptations on cellular as well as network organizational levels.

Translational utility of tolerance models

It is important to keep in mind that opioid tolerance is not a unitary entity and that variable mechanisms might underlie the development of tolerance to each of the multiple effects of the same agonist in the same or different experimental systems. It is also essential to remember that there are multiple forms of tolerance, each of which could be mediated via a different subset of adaptations. These can often be differentiated by their specificity and temporal characteristics. For example, opioid receptor homologous desensitization resulting from G protein uncoupling has a very rapid (minutes) onset (Law and Loh, 1999), whereas adaptations

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involving upregulation of the adenylyl cyclase (AC) cascade requires hours for full manifestation (Nestler and Aghajanian, 1997; Nestler et al., 1994). Yet another proposed adaptation to chronic morphine that is heterologous and that has a much more delayed onset involves down-regulation of the sodium pump and a reduction in its electrogenic contribution to membrane potential (Fleming, 1999). The existence of multiple organizational levels on which opioid tolerance can occur and the multiplicity of functions that can be influenced by opioids require that mechanistic models that attempt to define tolerance be appropriately constrained and qualified in relationship to the system under study.

Impairment of opioid receptor functionality

Most models of opioid tolerance frequently employed revolve around the conceptual rubric that it is the direct result of the actual loss of specific opioid receptor-mediated signaling, i.e., opioid receptor desensitization. This desensitization is frequently envisioned to involve a reduction in spare opioid receptors, (Chavkin and Goldstein, 1984) increased opioid receptor internalization (Bohn et al., 2000), decreased opioid receptor density (Chakrabarti et al., 1995) and altered content of G proteins (Ammer and Schulz, 1995). A pivotal aspect of such models is the enhanced phosphorylation of the opioid receptor via G protein receptor kinase that accompanies its activation and is a prelude to its forming a complex with β-arrestin (Appleyard et al., 1999; Kovoor et al., 1997; Pei et al., 1995). This results in its targeting to clathrin-coated pits, G protein uncoupling and its subsequent internalization and intracellular trafficking to subcellular compartments, e.g., lysozomes where receptor degradation can occur.

These events directly parallel those that have been extensively described for the β_2-adrenergic receptor and they are shared by most, if not all G protein coupled receptors (GPCRs). The relevance of these events and models that revolve around them to *in vivo* pharmacological opioid tolerance is certainly suggested by the coincidence of the temporal characteristics of

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opioid receptor phosphorylation and G protein uncoupling with the onset of the acute loss of opioid receptor functionality, i.e., receptor desensitization or 'acute tolerance' (Appleyard et al., 1997; Zhang et al., 1996). Importantly, the above mechanistic formulations of opioid tolerance are invariably thought of as being invariant responses to chronic morphine, independent of ongoing physiological state.

Post opioid receptor adaptations to chronic morphine

Numerous post opioid receptor cellular adaptations to chronic morphine have been identified that challenge the centrality of the uncoupling theory of opioid tolerance by suggesting alternative mechanisms. Furthermore, these underscore the complex interplay of tolerance mechanisms with cell physiology and the need for more complex working models that reflect it. Many tolerance-related adaptations pertain to adenylyl cyclase (AC)-cAMP (Duman et al., 1988; Guitart and Nestler, 1989; Kim et al., 2006; Lane-Ladd et al., 1997; Nestler and Tallman, 1988) and protein kinase C (PKC) (Mao et al., 1995; Mayer et al., 1995; Narita et al., 1995; Wang et al., 1996; Wei and Roerig, 1998; Zeitz et al., 2001) signaling pathways. More recently, chronic morphine was shown to up-regulate specific AC isoforms (Chakrabarti et al., 1998a; Rivera and Gintzler, 1998), increase phosphorylation of AC (Chakrabarti et al., 1998b) and the G_β subunit of G proteins (Chakrabarti et al., 2005b), and increase association of the μ-opioid receptor (MOR) with G_s (Chakrabarti and Gintzler, 2007; Chakrabarti et al., 2005a). Notably, all of these adaptations not only occur concomitantly but also have convergent signaling consequences; in the aggregate they shift acute MOR-coupled signaling from AC inhibitory to stimulatory **(**Gintzler and Chakrabarti, 2006).

These cellular adaptations to long-term morphine underscores that at least a subset of tolerance mechanisms do not cause the loss of opioid receptor functionality but rather the alteration of the consequences of opioid receptor activation. This is very revealing because it indicates that the

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protective function served by opioid tolerance formation, i.e., the reinstatement of initial steady state conditions, does not result solely from unidirectional adaptations, e.g., restricted opioid receptor functionality, but also from the active assertion of compensatory opioid receptor-coupled cell signaling strategies.

Opioid receptor pleiotropy and duality of signaling

Identification of the interrelated cellular adaptations to long-term morphine treatment highlighted above is a poignant reminder that formation of tolerance utilizes the flexibility that is inherent in receptor G protein signaling. Opioid receptor pleiotropy (tolerance-associated enhanced coupling to G_s) (Chakrabarti et al., 2005a) and the duality of G protein signaling via the G_α and $G_{\beta\gamma}$ subunits (tolerance-associated enhanced AC $G_{\beta\gamma}$ stimulatory AC signaling) (Chakrabarti et al., 1998a) are both recruited in response to persistent opioid receptor activation. GPCR pleiotropy and duality of G protein subunit signaling are pillars of cell signaling plasticity and underlie much of the richness and diversity of signaling that is characteristic of GPCRs. Thus, it should not be surprising that they also underlie many of the cellular adaptations that enable cell survival in the face of prolonged opioid exposure. Chronic morphine-induced enhanced opioid receptor pleiotropy and duality of signaling enable opioid tolerance mechanisms to be pliable, as reflected by the shift in MOR-coupled signaling from $G_{i\alpha}/G_{o\alpha}$ inhibitory to $G_{s\alpha}$ /$G_{\beta\gamma}$ stimulatory AC signaling (Gintzler and Chakrabarti, 2006). This multi-dimensionality of cellular adaptations to long-term morphine treatment demands the development of mechanistic models of opioid tolerance that include a much broader spectrum of adaptational mechanisms than has thus far been the case if they are to be medicinally relevant.

Influence of prior treatment on spinal opioid tolerance and addiction

Although not concluded at the time of publication, some early behavioral studies do indicate that tolerance adaptations could depend on physiological state. For example, there is provocative data predating the biochemical demonstration of the opioid receptor, that the antinociceptive

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effect of morphine can be reduced by a single dose of systemic morphine administered months earlier (Cochin and Kornetsky, 1964). More recently (Lim et al., 2005), it was shown that the rate of onset and the magnitude of antinociceptive tolerance increases with serial intrathecal morphine injections. The authors suggest that repeated cycles of morphine exposure produce sustained changes in the spinal cord that modulate the development of opioid tolerance to subsequent morphine exposure. The demonstration that prior history of morphine-induced plasticity can influence the magnitude of subsequently observed tolerance adaptations can be construed to indicate that at least some of the adaptations to chronic morphine are not set in stone and in fact resonate with evolving physiological state. However, in the absence of any formal direct demonstration of this concept, these phenomena remained an enigma.

Behavioral studies conducted months following opioid withdrawal also support the notion that ongoing physiological state can be a major determinant of addiction predisposition. For example, morphine-dependent rats that had been successfully detoxified and showed no significant signs of morphine dependence consumed significantly larger volumes of morphine solution than opiate naïve controls and had recurrence of morphine tolerance and dependence (Dai et al., 1984). Subsequently, it was suggested (Bartoletti et al., 1987) that modification of the neuronal mechanism subserving the excitatory component of the action of opiates by chronic morphine treatment that had occurred months earlier could represent a neurobiological basis for recidivism in addicts. While the mechanisms responsible for the pliability of opioid responsiveness have remained unidentified, such observations further support the notion of the state dependence of the processes of tolerance and dependence.

Dependence of cellular opioid tolerance mechanisms on cell state

A review of the opioid tolerance literature reveals that while cellular biochemical parameters of morphine administration are often considered in studies of tolerance, the influence of ongoing physiology and cell state are not. The complexity and multi-dimensionality of cellular

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mechanisms underlying opioid tolerance is underscored by the recent report that a subset of interrelated cell signaling adaptations to chronic morphine exposure do not represent a fixed set of adaptations but are themselves cell state-dependent (Shy et al., 2007).

This notion was directly put to the test by comparing AC-cAMP-related adaptations to long-term morphine treatment among Chinese Hamster ovary cells (CHO) stably expressing MOR (MOR-CHO) and MOR-CHO overexpressing either AC2 (AC2–MOR-CHO) or AC1 (AC1-MOR-CHO). These cells manifest qualitatively opposite consequences of acute MOR activation as a result of differences in the relative abundance of specific AC isoforms (Federman et al., 1992; Tsu et al., 1995; Yoshimura et al., 1996) that are differentially regulated by $G_{\beta\gamma}$ (Tang and Gilman, 1991).

The qualitative difference in the consequences of acute MOR activation (AC inhibition vs. stimulation) has a profound effect on the manifestation of multiple, complementary AC-related adaptations to chronic morphine, many of which are diametrically opposite (Shy et al., 2007). Strikingly, none of the AC/cAMP-related adaptations to chronic morphine observed in MOR-CHO and AC1-MOR-CHO (increased AC and Gβ phosphorylation, membrane protein kinase Cγ translocation and MOR G_s association (Chakrabarti and Gintzler, 2003; Chakrabarti et al., 2005a; Chakrabarti et al., 2005b; Chakrabarti et al., 1998b) are observed in AC2-MOR-CHO. Instead, overexpression of AC2 negates the increment in Gβ phosphorylation and PKCγ translocation and reverses the increment in AC phosphorylation and MOR G_s association to a decrement (Shy et al., 2007).

These experiments formally tested the interrelatedness of tolerance adaptations and cell state. Results directly demonstrate that adaptational strategies in the AC/cAMP signaling pathway elicited by chronic morphine are not hard-wired but instead are conditional on cell state. In this

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particular case, the default acute responsiveness of cells to MOR activation is a determinant of the mechanisms harnessed by cells to cope with the persistent activation of MOR. In cells in which acute MOR activation inhibits AC activity (MOR-CHO, AC1-MOR-CHO, left panel of the schematic), chronic morphine elicits adaptations that augment a stimulatory arm of MOR-G protein coupling. Conversely, in cells manifesting acute stimulatory AC responsiveness to MOR (AC2-MOR-CHO, right panel of the schematic), the same treatment with morphine elicits adaptations that enhance AC inhibitory responsiveness. Notably, the substrates for tolerance formation remain the same but are differentially modulated. This underscores the plasticity of the cellular adaptations that mediate tolerance formation and provide a cellular basis for inferences to that effect drawn from much earlier behavioral studies.

Relevance to CNS

The cellular environment in which the plasticity of long-term morphine responsiveness was demonstrated contains differences in the abundance of AC isoforms that are not found in naturally occurring neuronal tissue. This notwithstanding, there is, undoubtedly, analogous variation in the distribution of AC isoforms (and other signaling molecules), albeit more subtle, across brain regions (Cali et al., 1994; Glatt and Snyder, 1993; Mons and Cooper, 1994; Mons et al., 1993; Xia et al., 1991). Consequently, nuanced differences in the state of cells present would likely occur, particularly following chronic morphine since many of the AC isoforms are differentially regulated by chronic opioid administration (Avidor-Reiss et al., 1997). Thus, the plasticity of adaptations related to AC/cAMP signaling in cells maintained in culture would seem to be applicable to the CNS.

Translational utility of pliability of tolerance mechanisms

The extent to which such plasticity generalizes to the multitude of other adaptations elicited by long-term morphine treatment, [e.g., opioid receptor downregulation/internalization, MOR G-protein uncoupling, increased activity of mitogen activated protein kinase (Bilecki et al., 2005), altered association/activity of RGS proteins], needs to be determined on all organizational and

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functional levels. This notwithstanding, a picture is emerging that suggests that opioid tolerance mechanisms represent a moving target. In the end, attempts to define a complete set of tolerance substrates may be successful. However, the nature of their modulation as well as altered interactions and functionality would seem to define a continuum of multidirectional changes rather than a rigid predetermined grid.

Future challenges and clinical implications

A poignant exemplar of the influence of physiological state on tolerance mechanisms that remains an enigma is the complex interrelationship that has been demonstrated between pain and opioid tolerance development. For example, tolerance did not compromise the efficacy of opioids, administered over three months, to significantly reduced back pain severity in a large cohort of patients with well-defined spinal diseases (Mahowald et al., 2005). Similarly, intrathecal opioid therapy was found to be effective in the management of chronic non-cancer pain, which was not inhibited by the development of tolerance (Roberts et al., 2001). Analogous results had been obtained in pre-clinical rat studies in which the analgesic action of the continuous systemic application of morphine on chronic thermal hyperalgesia due to sciatic constriction injury was unabated after seven days, which is considered long-term for animal models (Backonja et al., 1995). Notably, studies utilizing mice and a chronic inflammatory pain model demonstrated an opposite effect on tolerance development and that tolerance development could be modulated by an interaction between chronic inflammatory pain and genetics (Liang et al., 2006). At present, there is no mechanistic understanding of attenuated opioid tolerance development in some pain states. A complete understanding of the ways in which ongoing physiological state can influence opioid tolerance mechanisms could prove to be useful in identifying unique physiological parameters of painful states that are causally associated with diminished tolerance and a biochemical basis for this interaction.

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Validation of the generality of the perspective that opioid tolerance mechanisms are plastic would certainly represent a major paradigm shift that, on the surface, would make even more daunting attempts to develop pharmacological strategies that would eliminate or at least markedly attenuate opioid tolerance formation. However, realization of the pliability of opioid tolerance mechanisms could also open new possibilities. It could suggest the utility of developing anti-tolerance pharmacotherapies that target a very restricted CNS region, which is essential for opioid antinociception and contains cells utilizing a homogeneous set of tolerance adaptations. The unfolding increasing complexity of opioid tolerance represents a panoply of pharmacologic possibilities with which to play.

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References

Ammer H and Schulz R (1995) Chronic activation of inhibitory d-opioid receptors cross-regulates the stimulatory adenylate cyclase-coupled prostaglandin E1 receptor system in neuroblastoma X glioma (NG108-15) hybrid cells. *J Neurochem* **64**:2449-2457.

Appleyard SM, Celver J, Pineda V, Kovoor A, Wayman GA and Chavkin C (1999) Agonist-dependent desensitization of the kappa opioid receptor by G protein receptor kinase and beta-arrestin. *J Biol Chem* **274**:23802-23807.

Appleyard SM, Patterson TA, Jin W and Chavkin C (1997) Agonist-induced phosphorylation of the k-opioid receptor. *J Neurochem* **68**:2405-2412.

Avidor-Reiss T, Nevo I, Saya D, Bayewitch M and Vogel Z (1997) Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J Biol Chem* **272**:5040-5047.

Backonja MM, Miletic G and Miletic V (1995) The effect of continuous morphine analgesia on chronic thermal hyperalgesia due to sciatic constriction injury in rats. *Neuroscience Letters* **196**:61-64.

Bartoletti M, Gaiardi M, Gubellini C, Bacchi A and Babbini M (1987) Previous treatment with morphine and sensitization to the excitatory actions of opiates: dose-effect relationship. *Neuropharmacology* **26**:115-119.

Bilecki W, Zapart G, Ligeza A, Wawrzczak-Bargiela A, Urbanski MJ and Przewlocki R (2005) Regulation of the extracellular signal-regulated kinases following acute and chronic opioid treatment. *Cell Mol Life Sci* **62**:2369-2375.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ and Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408**:720-723.

JPET Fast Forward. Published on March 19, 2008 as DOI: 10.1124/jpet.107.135533
This article has not been copyedited and formatted. The final version may differ from this version.

Cali JJ, Zwaagstra JC, Mons N, Cooper DM and Krupinski J (1994) Type VIII adenylyl cyclase. A Ca2+/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. *J Biol Chem* **269**:12190-12195.

Chakrabarti S and Gintzler AR (2003) Phosphorylation of Gβ is augmented by chronic morphine and enhances Gβγ stimulation of adenylyl cyclase activity. Mol. Brain Res. **119**:144-151.

Chakrabarti S and Gintzler AR (2007) Phosphorylation of Gs{alpha} influences its association with the mu-opioid receptor and is modulated by chronic morphine. *Mol Pharmacol* **72**:753-760.

Chakrabarti S, Law P-Y and Loh HH (1995) Neuroblastoma neuro2A cells stably expressing a cloned μ-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. *Mol Brain Research* **30**:269-278.

Chakrabarti S, Regec A and Gintzler AR (2005a) Biochemical demonstration of mu-opioid receptor association with Gsalpha: enhancement following morphine exposure. *Mol Brain Res* **135**:217-224.

Chakrabarti S, Regec A and Gintzler AR (2005b) Chronic morphine acts via a protein kinase C©-Gβ-adenylyl cyclase complex to augment phosphorylation of Gβ and Gβ© stimulatory adenylyl cyclase signaling. Mol Brain Res **138**:94-103.

Chakrabarti S, Rivera M, Yan S-Z, Tang W-J and Gintzler AR (1998a) Chronic morphine augments Gβγ/Gsα stimulation of adenylyl cyclase: relevance to opioid tolerance. *Mol Pharmacol* **54**:655-662.

Chakrabarti S, Wang L, Tang W-J and Gintzler AR (1998b) Chronic morphine augments adenylyl cyclase phosphorylation: relevance to altered signaling during tolerance/dependence. *Mol Pharmacol* **54**:949-953.

Chavkin C and Goldstein A (1984) Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. *Proc Natl Acad Sci USA* **81**:7253-7257.

Cochin J and Kornetsky C (1964) Development and Loss of Tolerance to Morphine in the Rat after Single and Multiple Injections. *J Pharmacol Exp Ther* **145**:1-10.

Dai S, Hui SC and Ogle CW (1984) Morphine preference in rats previously morphine dependent. *Pharmacol Res Comm.* **16**:495-511.

Duman RS, Tallman JF and Nestler EJ (1988) Acute and chronic opiate-receptor regulation of adenylate cyclase in brain: specific effects in locus coerulus. *J Pharmacol Exp Ther* **246:** 1033-1039.

Federman AD, Conklin BR, Schrader KA, Reed RR and Bourne HR (1992) Hormonal stimulation of adenylyl cyclase through Gi-protein βγ subunits. *Nature* **356**:159-161.

Fleming WW (1999) Cellular adaptation: journey from smooth muscle cells to neurons. *The J Pharmacol Exp Ther* **291**:925-931.

Gintzler AR and Chakrabarti S (2006) Post-opioid receptor adaptations to chronic morphine; altered functionality and associations of signaling molecules. *Life sciences* **79**:717-722.

Glatt CE and Snyder SH (1993) Cloning and expression of an adenylyl cyclase localized to the corpus striatum. *Nature* **361**:536-538.

Guitart X and Nestler EJ (1989) Identification of morphine- and cyclic AMP-regulated phosphoproteins (MARPPs) in the locus coeruleus and other regions of rat brain: regulation by acute and chronic morphine. *J Neurosci* **9**:4371-4387.

Kim KS, Lee KW, Lee KW, Im JY, Yoo JY, Kim SW, Lee JK, Nestler EJ and Han PL (2006) Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. *Proc Natl Acad Sci U S A* **103**:3908-3913.

Kovoor A, Nappey V, Kieffer BL and Chavkin C (1997) Mu and delta opioid receptors are differentially desensitized by the coexpression of beta-adrenergic receptor kinase 2 and beta-arrestin 2 in xenopus oocytes. *J Biol Chem* **272**:27605-27611.

Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK and Nestler EJ (1997) CREB (cAMPresponse element-binding protein) in the locus coeruleus: biochemical,

JPET Fast Forward. Published on March 19, 2008 as DOI: 10.1124/jpet.107.135533
This article has not been copyedited and formatted. The final version may differ from this version.

physiological and behavioral evidence for a role in opiate dependence. *J Neurosci* **17**:7890-7901.

Law PY and Loh HH (1999) Regulation of opioid receptor activities. *J Pharmacol Exp Ther* **289**:607-624.

Liang DY, Guo T, Liao G, Kingery WS, Peltz G and Clark JD (2006) Chronic pain and genetic background interact and influence opioid analgesia, tolerance, and physical dependence. *Pain* **12**:232-240.

Lim G, Wang S, Zeng Q, Sung B and Mao J (2005) Evidence for a long-term influence on morphine tolerance after previous morphine exposure: role of neuronal glucocorticoid receptors. *Pain* **114**:81-92.

Mahowald ML, Singh JA and Majeski P (2005) Opioid use by patients in an orthopedics spine clinic. *Arthritis and Rheum* **52**:312-321.

Mao J (1999) NMDA and opioid receptors: their interactions in antinociception, tolerance and neuroplasticity. *Brain Res Brain Res Rev* **30**:289-304.

Mao J, Price DD, Phillips LL, Lu J and Mayer DJ (1995) Increases in protein kinase Cγ immunoreactivity in the spinal cord of rats associated with tolerance to the analgesic effects of morphine. *Brain Res* **677**:257-267.

Mayer DJ, Mao J and Price DD (1995) The development of tolerance and dependence is associated with translocation of protein kinase C. *Pain* **61**:365-374.

Mons N and Cooper DM (1994) Selective expression of one Ca(2+)-inhibitable adenylyl cyclase in dopaminergically innervated rat brain regions. *Mol Brain Res* **22**:236-244.

Mons N, Yoshimura M and Cooper DM (1993) Discrete expression of Ca2+/calmodulin-sensitive and Ca(2+)-insensitive adenylyl cyclases in the rat brain. *Synapse* **14**:51-59.

Narita M, Narita M, Mizoguchi H and Tseng LF (1995) Inhibition of protein kinase C, but not of protein kinase A, blocks the development of acute antinociceptive tolerance to an intrathecally administered mu-opioid receptor agonist in the mouse. *Eur J Pharmacol* **280**:R1-3.

JPET Fast Forward. Published on March 19, 2008 as DOI: 10.1124/jpet.107.135533
This article has not been copyedited and formatted. The final version may differ from this version.

Nestler EJ and Aghajanian GK (1997) Molecular and cellular basis of addiction. *Science* **278**:58-63.

Nestler EJ, Alreja M and Aghajanian GK (1994) Molecular and cellular mechanisms of opiate action: studies in the rat locus coeruleus. *Brain Re Bull* **35**:521-528.

Nestler EJ and Tallman JF (1988) Chronic morphine treatment increases cyclic AMP-dependent protein kinase activity in the rat locus coeruleus. *Mol Pharmacol* **33**:127-132.

Pei G, Keiffer BL, Lefkowitz RJ and Freedman NJ (1995) Agonist-dependent phosphorylation of the mouse δ-opioid receptor; involvement of G protein-coupled receptor kinase but not protein kinase C. *Mol Pharmacol* **48**:173-177.

Rivera M and Gintzler AR (1998) Differential effect of chronic morphine on mRNA encoding adenylyl cyclase isoforms: relevance to physiological sequela of tolerance/dependence. *Mol Brain Research* **54**:165-169.

Roberts LJ, Finch PM, Goucke CR and Price LM (2001) Outcome of intrathecal opioids in chronic non-cancer pain. *Eur J Pain (London, England)* **5**:353-361.

Shy M, Chakrabarti S and Gintzler AR (2007) Plasticity of adenylyl cyclase-related signaling sequelae following long-term morphine treatment. *Mol Pharmacol*. **73**: 868-879.

Tang W-J and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein βγ subunits. *Science* **254**:1500-1503.

Tsu RC, Chan JSC and Wong YH (1995) Regulation of multiple effectors by the cloned delta-opioid receptor: stimulation of phospholipase C and type II adenylyl cyclase. *J Neurochem* **64**:2700-2707.

Wang L, Medina VM, Rivera M and Gintzler AR (1996) Relevance of phosphorylation state to opioid responsiveness in opiate naive and tolerant/dependent tissue. *Brain Res* **723**:61-69.

Wei ZY and Roerig SC (1998) Spinal morphine/clonidine antinociceptive synergism is regulated by protein kinase C, but not protein kinase A activity. *J Pharmacol Exp Ther* **287**:937-943.

JPET Fast Forward. Published on March 19, 2008 as DOI: 10.1124/jpet.107.135533
This article has not been copyedited and formatted. The final version may differ from this version.

Xia ZG, Refsdal CD, Merchant KM, Dorsa DM and Storm DR (1991) Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain: expression in areas associated with learning and memory. *Neuron* **6**:431-443.

Yoshimura M, Ikeda H and Tabakoff B (1996) mu-Opioid receptors inhibit dopamine-stimulated activity of type V adenylyl cyclase but enhance dopamine-stimulated activity of type VII adenylyl cyclase. *Mol Pharmacol* **50**:43-51.

Zeitz KP, Malmberg AB, Gilbert H and Basbaum AI (2001) Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKCγ mutant mice. *Pain* **94**:245-253.

Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR and Wang JB (1996) Differential mu opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. *J Bio Chem* **271**:11449-11454.

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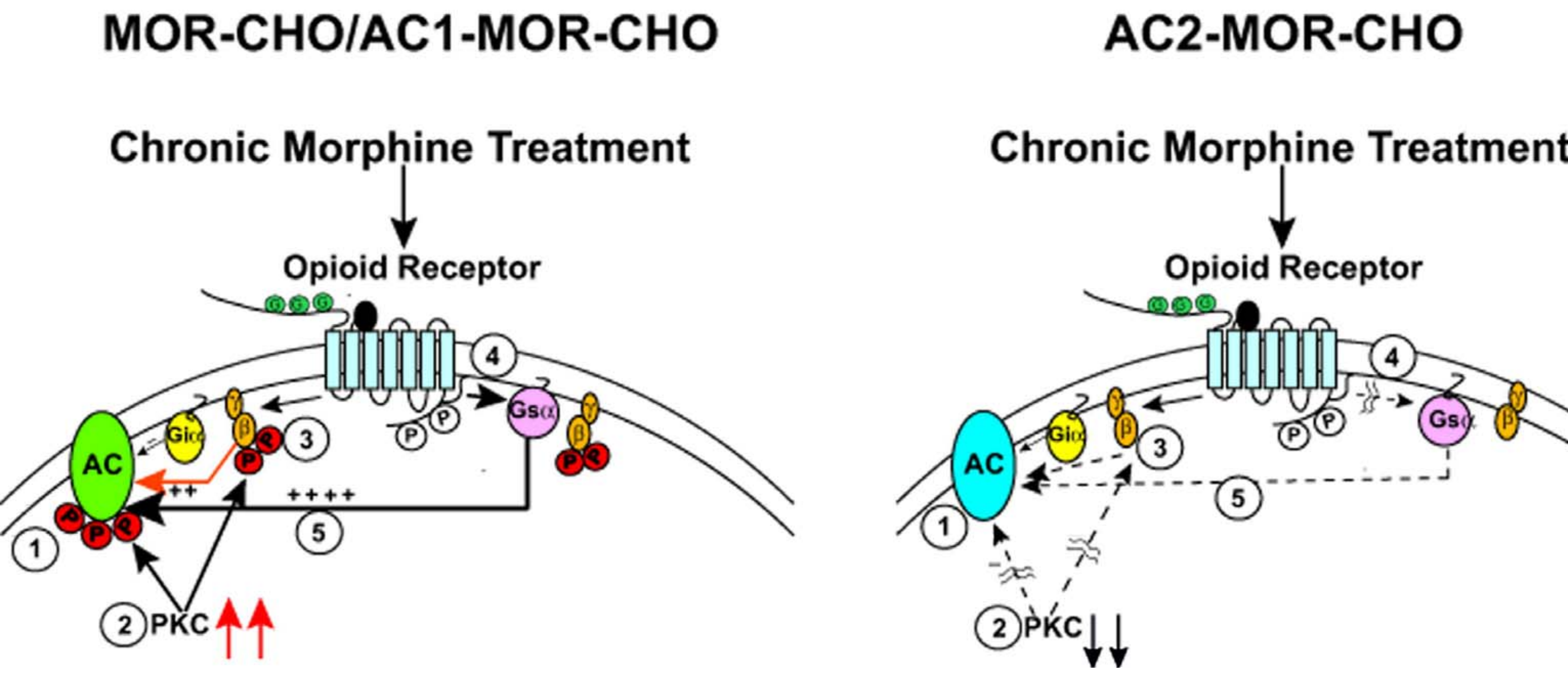
Legends for Figures

Comparison of AC/cAMP-related adaptations to chronic morphine when acute MOR activation inhibits or stimulates AC activity. In MOR-CHO and AC1-MOR-CHO cells, in which acute activation of MOR inhibits AC activity (left panel), chronic morphine elicits convergent adaptations that shift the consequences of MOR activation from $G_{i\alpha}/G_{o\alpha}$ inhibitory to $G_{s\alpha}/G_{\beta\gamma}$ AC stimulatory. These adaptations consist of (1) augmented AC phosphorylation, (2) increased membrane translocation of PKC, (3) augmented phosphorylation of the G_{β} subunit of $G_{\beta\gamma}$ and (4) increased association of MOR with G_s. Increased phosphorylation of some AC isoforms (e.g., AC2) increases their stimulatory responsiveness to $G_{s\alpha}$ (5) and $G_{\beta\gamma}$, which is further augmented by increased phosphorylation of G_{β}. In contrast, in cells in which acute activation of MOR results in stimulation of AC activity (AC2-MOR-CHO, right panel), none of these adaptations to chronic morphine occur. The increase in G_{β} phosphorylation and PKC translocation is negated and the increment in AC phosphorylation and MOR G_s association reverses to a decrement. These observations reflect that tolerance mechanisms are dynamic, pliable and interconnected with cell physiology. Dashed arrows in right panel (AC2-MOR-CHO) denotes a reduction in activity of signaling events 2,3,4,5 relative to the analogous events on the left (MOR-CHO/AC1-MOR-CHO), which is denoted by solid arrows. G=glycosylation sites on the N-terminus of MOR.

FIG 1


MOR-CHO/AC1-MOR-CHO
Chronic Morphine Treatment
Opioid Receptor
AC
Giα
Gsα
PKC
++
++++
1
2
3
4
5
AC2-MOR-CHO
Chronic Morphine Treatment
Opioid Receptor
AC
Giα
Gsα
PKC
1
2
3
4
5