Plasticity of Opioid Adaptations to Chronic Morphine

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By

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Table of Contents

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Key Abbreviations

AC, adenylyl cyclase

cAMP, cyclic adenosine monophosphate

CHO, Chinese Hamster Ovary cells

CTX, cholera toxin

$G_{i\alpha}$, α-subunit of inhibitory G-protein

$G_{s\alpha}$, (α_s), α-subunit of stimulatory G-protein

G_{β}, β-subunit of G-protein

$G_{\beta\gamma}$, ($\beta\gamma$), $\beta\gamma$-subunit of G protein

HEK, Human Embryonic Kidney cells

IP, immunoprecipitate

LMMP, longitudinal muscle/myenteric plexus

MOR, μ-opioid receptor

MOR-CHO, Chinese Hamster Ovary cells stably transfected with μ-opioid receptor

PKA, protein kinase A

PKC, protein kinase C

PTX, pertussis toxin

QEHA, blocking peptide of $\beta\gamma$-subunit of G protein

List of Tables, Figures and Legends

Abstract

Prolonged administration of opioids in the clinical setting leads to eventual resistance to its effects. Many mechanisms have been proposed to explain the phenomenon of opioid tolerance with the common theme that tolerance reestablishes initial steady state conditions and neutralizes the perturbing consequences of continued opioid presence. The Gintzler laboratory has previously shown that tolerance results, in part, from two parallel pathways that intersect to negate acute inhibitory opioid signaling: (1) change in G-protein signaling, from $G_{i\alpha}$ inhibitory to G_i-derived $G_{\beta\gamma}$ stimulatory, through multiple changes in signaling molecules which converge to the shift to $G_{\beta\gamma}$ stimulation of adenylyl cyclase (AC) and (2) increased association between the μ-opioid receptor (MOR) and the stimulatory G protein (G_s). In the current study, we demonstrate that these adaptations to chronic morphine are not hard-wired, but can exhibit plasticity based on the internal cellular milieu. In Chinese Hamster Ovary (CHO) cells stably transfected with the rat μ-opioid receptor (MOR-CHO) and overexpressing ACII (ACII MOR-CHO), a $G_{\beta\gamma}$ stimulatory isoform, we have engineered a cell that both exhibits default opioid stimulation and manifests chronic opioid tolerance. In ACII MOR-CHO, the tolerance adaptations that previously caused a shift to $G_{\beta\gamma}$ stimulation of AC are either negated or reversed, decreasing the stimulatory interactions between $G_{\beta\gamma}$ and AC, manifest by the following observations: chronic morphine fails to augment G_β phosphorylation, decreases AC phosphorylation, membrane translocation of PKCγ and MOR-G_s coupling. The importance of isoform-specific $G_{\beta\gamma}$ regulation of AC in the plasticity exhibited by chronic morphine is underscored by its ability to elicit adaptations in cells overexpressing ACI, a $G_{\beta\gamma}$-inhibitory isoform, which are identical to those in OR-CHO. These observations suggest pre-existing levels of isoform-specific AC is a critical

determinant of adaptations to the persistent presence of morphine. This could explain the lack of ubiquity and uniformity of the degree and mechanistic underpinnings of opioid tolerance throughout the nervous system.

1) Background

The opioid receptor

The opioid receptor is one of many receptors that belong to the superfamily of guanine nucleotide-binding protein (G-protein) coupled receptors. All receptors in this class contain seven transmembrane spanning domains, an extracellular N-terminus and an intracellular C-terminus. Three major classes of opioid receptors (μ, δ, and κ) exist. These can be distinguished by their binding affinities for agonists. The μ-opioid receptor (MOR) is the most prevalent of the opioid receptors and morphine, the most common opiate alkaloid, is a strong MOR agonist. Analogous to opioid receptors, there are multiple (four) main classes of endogenous opioid peptides (enkephalins, dynorphins, β-endorphin, and endomorphins). The enkephalins exhibit δ-opioid receptor (DOR) preference, dynorphins have highest affinity for the κ -opioid receptor (KOR) β-endorphins have a slight preference for DOR, and endomorphins are highly selective for (MOR) (see review, ref. 1).

Mechanisms and sites of opioid action

Most opioid agonists exert their effects through μ-opioid receptors. These drugs affect a wide range of physiological systems. They produce analgesia, affect mood and rewarding behavior, and alter respiratory, cardiovascular, gastrointestinal, and neuroendocrine function (see review, ref. 2). In order to further examine the effects of opioids at a cellular and molecular level, it is important to view opioid action in a systemic context.

In modulating pain transmission, the most extensively studied area of the CNS is the spinal cord dorsal horn. The analgesic effects of opioids arise from their ability to directly inhibit the ascending transmission of nociceptive information from the spinal cord dorsal horn and to activate pain control circuits that descend from the midbrain via the rostral ventromedial medulla to the spinal cord dorsal horn [3]. In addition to opioids, there is a multitude of neurotransmitters involved in modulation of signaling in these pathways: serotonin (5-HT), GABA, norepinephrine, neurotensin, and excitatory amino acids [3].

Opioid peptides and their receptors are found throughout these descending pain control circuits. μ-opioid receptor mRNA and/or ligand binding can be observed in the periaqueductal gray (PAG), pontine reticular formation, median raphe, nucleus raphe magnus, and adjacent gigantocellular reticular nucleus in the rostral ventromedial medulla (RVM) and spinal cord [4,5]. There is presence of significant μ-opioid receptor ligand binding in the superficial dorsal horn but scarce mRNA expression [4], which suggest that the majority of these spinal μ-receptor ligand-binding sites are located presynaptically on the terminals of primary afferent nociceptors, consistent with the high levels of μ-opioid receptor mRNA observed in dorsal root ganglia. A similar situation is seen in the dorsolateral PAG [5].

As for the other opioid receptors, δ-opioid receptor mRNA and ligand binding have been demonstrated in the ventral and ventrolateral quadrants of the PAG, the pontine reticular formation, and the gigantocellular reticular nucleus, but only low levels are seen in the median raphe and nucleus raphe magnus [4]. κ-opioid receptor mRNA and ligand binding are widespread throughout the PAG, pontine reticular formation,

median raphe, nucleus raphe magnus, and adjacent gigantocellular reticular nucleus [4]. Although all three receptor mRNAs are found in the DRG, they are localized on different types of primary afferent cells: μ-Opioid receptor mRNA is present in medium- and large-diameter DRG cells, δ-opioid receptor mRNA in large-diameter cells, and κ-opioid receptor mRNA in small- and medium-diameter cells [4]. This differential localization may be linked to functional differences in pain modulation.

Opioid tolerance: an overview

A major problem that confounds the use of opioid analgesic drugs is the development of tolerance; prolonged and repeated use of morphine in a clinical setting frequently results in the loss of its analgesic efficacy. Accordingly, progressively higher dosages are required to achieve a similar analgesic effect. This profoundly limits the medicinal usefulness of narcotics for the relief of pain. Tolerance also develops to the other drugs effects of opioids, albeit at varying rates. While the development of tolerance to the euphorigenic effects of opioids, such as those exhibited by heroin abusers, may be rapid, the drug effects on the vital functions such as cardiovascular function and respiration are much slower in development of tolerance. Hence, the extreme danger of accidental overdoses exists in the population of opioid abusers.

Physiologic tolerance can exist in many forms [2]: tolerance that is *innate* refers to a level of drug sensitivity that is determined genetically; its effects (or lack thereof) can be seen the first time that the drug is administered. The mechanisms underlying observed physiologic tolerance is usually the basis for *acquired* tolerance which can exist as *pharmacokinetic, pharmacodynamic, or learned* tolerance (*acute, reverse, and*

cross-tolerance). *Pharmacokinetic* tolerance refers to metabolic or distributional changes in a drug after prolonged use such that the same dose produces a lower blood concentration than previously (e.g. increase in the rate of metabolism of the drug by hepatic enzymes). The basis for the working model of opioid tolerance in our laboratory deals with cellular and molecular adaptations of *pharmacodynamic* tolerance: adaptive changes within systems affected by the drug so that response to a given concentration of the drug is reduced, such as changes in receptor density or efficiency of receptor coupling to signal-transduction pathways. Tolerance can also be *learned*, where the drug effect diminishes due to compensatory mechanisms shaped by past experiences, including behavioral tolerance, where skills are developed to overcome mild impairment, and conditioned tolerance, where sights, sounds, smells and other environmental cues are paired with the administration of a drug.

Acute tolerance can occur rapidly during a singular "binge" occasion. After repeated doses over several hours, there may be a decrease in response to subsequent doses during the binge. This is contrasted with *reverse* tolerance, or *sensitization*, where there is an increase in response with repetition of the same dose of the drug such that repeated daily administration of cocaine can produce increased motor activity. *Cross-tolerance* occurs when repeated use of a drug in a given category confers tolerance other drugs in the same category. The clinical implications of understanding cross-tolerance are serious; for example, when undergoing detoxification for heroin users who are tolerant to other opioids, detoxification can be accomplished with any medication that activates opioid receptors.

Tolerance and dependence: opioid withdrawal

Another phenomenon that is often described in conjunction with tolerance is the state of physical dependence. Physical dependence develops as a result of the adaptations (tolerance) produced in order to reset equilibrium in response to repeated drug use [2]. Drugs can affect numerous systems that previously were in equilibrium followed by the development of tolerance to balance the continued presence of inhibition or stimulation. A person in this adapted state requires continued administration of the drug to maintain normal function. If the drug is suddenly withdrawn, there is another imbalance, and resetting equilibrium must happen again. This observed withdrawal syndrome is the only actual evidence of physical dependence and can be either due to the removal of the drug of dependence and/or CNS hyperstimulation following a reset to the absence of the drug [2]. As expected, withdrawal symptoms are usually opposite those of the original effects produced by the drug. Thus, abrupt termination of an opioid that normally produces miosis and bradycardia will cause mydriasis and tachycardia as the withdrawal syndrome.

Tolerance, physical dependence, and withdrawal are all biological phenomena and can be reproduced in experimental animals and in any human being. Although these adaptations can be complex and have yet to be fully elucidated, they stand in stark contrast to the behavioral complexities of abuse and addiction (see discussion). However, as will be discussed later, these symptoms do not imply abuse or addiction. Appropriate prescription and dosing may still lead to tolerance, physical dependence, and withdrawal symptoms if the drug is stopped abruptly rather than gradually. They are not signs of abuse and treatment must be continued.

Molecular basis of opioid action

Initial opioid binding to its receptor leads to its association with a GDP-bearing G-protein. This leads to a GTP-GDP exchange on the α-subunit of the G-protein, resulting in its disassociation from the $G_{\beta\gamma}$ subunit. The α-subunit can now exert its effects on some effector molecule (e.g. adenylyl cyclase), which in turn either changes channel permeability directly or utilizes a second messenger (e.g. cyclic adenosine monophosphate, cAMP). The predominant consequence of opioid binding to any of the three types of receptors is inhibition of some physiological function, i.e., inhibition of adenylyl cyclase (AC), opening potassium channels, [6,7] or closing voltage-gated calcium channels [8,9] (both of which result in a hyperpolarized state).

The identity of the G-protein with which opioid receptors interact can affect its interaction with the downstream effector molecules, thus altering the functional consequences of opioid receptor activation. Much of the early data indicating the opioid receptor coupled predominantly to the inhibitory G proteins G_i/G_o was consistent with observations of predominantly inhibitory opioid actions [10]. However, more recently there has since been mounting evidence that opioid receptors can couple simultaneously to multiple G-proteins (e.g., G_s and G_q) [11-14]. Moreover, there has been evidence of concentration-dependent opioid receptor-coupled stimulation; nanomolar and micromolar concentrations of opioid receptor type selective agonists have excitatory and inhibitory effects, respectively [15]. This observed bimodality is thought to be due to differential coupling of the opioid receptor to stimulatory G_s- and inhibitory G_i-like proteins [16].

Opioid tolerance – early mechanisms

On a pharmacodynamic basis, the phenomenon of opioid tolerance has been extensively investigated on systemic cellular and molecular levels. There have been many proposed mechanisms and models of opioid tolerance. Regardless of the tolerance mechanism thought to be central to its formation, perspective recently developed in the Gintzler laboratory is that tolerance is adaptive and protective. Its development helps to reestablish initial steady state conditions and neutralizes the perturbing consequences of the continued presence of opioids.

A phenomenon that was originally thought to be seminal to the development of tolerance was desensitization. On the level of the opioid receptor itself, i.e. loss of opioid receptor function, desensitization was modeled after mechanisms shown to mediate the loss of activity of β-adrenergic receptor agonists. Desensitization was shown to occur through several mechanisms. These include downregulation and internalization of opioid receptors [17], altered G-protein content [18], and decreased receptor-G-protein coupling [19]. Notably, these tolerance mechanisms imply that opioid tolerance is essentially no different than those shown to mediate β-adrenergic receptor desensitization [20]. Other modifications were also observed that suggest receptor desensitization is not the sole contributor to opioid tolerance. Some of these findings include: a lack of receptor downregulation accompanying physiologic tolerance, i.e., receptor downregulation of mostly observed in cultured cell and not the CNS [21,22], the relative inability of chronic morphine to induce receptor internalization [23-27] and increasing $G_{i\alpha}$ content in guinea pig longitudinal muscle/myenteric plexus (LMMP) [28] and locus coeruleus [29]. Even with these observations, the strongest evidence for additional tolerance mechanisms remains the acute withdrawal that is precipitated by exposing opioid treated animals, or cultured cells, to opioid receptor antagonists such as

naloxone. This suggests that opioid receptors must maintain their functionality despite the loss of analgesic responsiveness.

Concurrent work in opioid tolerance all studied cellular adaptations under opioid withdrawal. It was shown that when chronic morphine was suddenly withdrawn, there was a profound increase in adenylyl cyclase (AC) activity [30-33]. This upregulation is often called the "overshoot" phenomenon or AC superactivation. The presence of increased AC activity suggests that it is a cellular adaptation of the cell that balances the inhibitory effects of morphine on AC activity and that it is this adaptation, not the loss of opioid receptor functionality that results in "normal" levels of cAMP despite the continued presence of an opioid. In other words, the loss of the ability of morphine to inhibit AC activity is apparent, not real. It results from the masking of inhibitory actions, once again leading to the conclusion that the morphine still maintains its inhibitory influence. This interpretation is validated by the observation that the AC overshoot produced by exposure of chronic morphine-treated cells to the opioid-receptor antagonist naloxone can be re-reversed by increasing the concentration of opioids. This demonstrates that loss of opioid receptor functionality cannot be the sole underpinning of opioid tolerance [30,31,33].

Opioid tolerance involve altered consequences of receptor-G_i-AC coupling

Our working model of opioid tolerance evolved from two basic observations: there is a return to "opioid-naïve" levels of cAMP in chronic morphine-treated tissue [34], and that chronic morphine increases coupling of the opioid receptor to G_i [35]. The reestablishment of "normal" levels of cAMP in chronic morphine-treated tissue [34] underscores the idea that opioid tolerance involves a reversal of opioid inhibition to

opioid enhancement of cAMP production, despite the continued presence of inhibitory concentrations of morphine and the maintenance of fully functional receptors. The second observation of increased receptor-G_i coupling, while on its face, seemingly contradictory to the notion of a reversal to opioid enhancement, does suggest the possibility that tolerance formation is in part due to altered consequences of coupling to inhibitory G proteins [36]. While the receptor remains coupled to G_i, changes occur downstream of that coupling that enable restoration of steady state conditions in effect prior to morphine exposure equilibria.

Opioid tolerance: role of the βγ-subunit

In the conventional model of G protein activation, ligand binding to a GPCR leads to activation of the α-subunit of the G protein and its consequent dissociation from $G_{\beta\gamma}$. Thus, generation of an activated α-subunit is always accompanied by the generation of $G_{\beta\gamma}$. Generation of the βγ-subunit was originally thought to play minimal importance in second messenger systems. However, the Gilman laboratory showed [37] that the regulation of effectors such as AC can also be a critical function of the $G_{\beta\gamma}$ -subunits and that the directionality of this regulation is in large part determined by the nature of the effectors. Tang et al. [37] showed that ACII, and subsequently, type II AC (ACII, IV, and VII), has a positive regulatory effect on βγ [38-42]. However, the opposite was shown to be true for βγ regulation of ACI (inhibitory) [37,42]. The isoform-specific regulation of AC by $G_{\beta\gamma}$ is summarized in Table 1.

Table 1: Isoform-specific regulation of AC by the $G_{\beta\gamma}$ subunit

G-protein Subunit	Adenylyl Cyclase Isoform								
	AC1	AC2	AC3	AC4	AC5	AC6	AC7	AC8	AC9
Giα	↓	---	↓	---	↓	↓	---	---	---
G$\beta\gamma$	↓	↑	---	↑	↓	↓	↑	↓	---

(table compiled from ref. 42 and 43)

Thus for a given inhibitory G-protein, its regulatory fate is not so much determined by it being G_i, but by its associated-AC isoforms. This role of isoform-specific $G_{\beta\gamma}$ regulation of AC has been demonstrated in several cell lines. Federman et al. showed in human embryonic kidney (HEK293) cells that the default response to D1 receptor binding can be altered by changing the starting conditions of AC isoforms [39]. Like the MOR, the D1 receptor is linked to G_i, but following ACII overexpression, the default acute response shifts from inhibition to stimulation of AC via $G_{\beta\gamma}$.

One critical component of the working model of opioid tolerance formulated by the Gintzler laboratory is the hypothesis that adaptations to chronic morphine involve the plasticity of signaling conferred by AC isoform-specific signaling. Specifically, the Gintzler laboratory has demonstrated that the formation of tolerance involves the shift in MOR-coupled signaling from a $G_{i\alpha}$-inhibitory to a (G_i-derived) $G_{\beta\gamma}$-stimulatory AC state. This enables the restoration of steady state conditions existing prior to chronic morphine exposure. This is achieved by taking advantage of AC isoform-specific signaling [38,44] and modulation thereof. Keeping in mind that the isoforms in the type II family (ACII, IV,

and VII) are stimulated by $G_{\beta\gamma}$ [37-42], a brief summary of the modulations follows (Figure 1, modified from ref. 6):

A. After chronic morphine, AC protein of isoforms ACII, ACIV, and ACVII are upregulated [45,46], while mRNA encoding ACIV [47] and ACVII [45] are upregulated.

B. Chronic morphine increases PKC-mediated phosphorylation of ACII and ACVII [45,47-50] (#2, Figure 1). Both of these effects (A, B) converge to make AC more responsive to $G_{\beta\gamma}$ stimulation and contribute to the shift from $G_{i\alpha}$ inhibition to $G_{\beta\gamma}$ stimulation.

C. In addition to AC modification, chronic morphine also increases the potency of $G_{\beta\gamma}$ to stimulate AC by the covalent modification of $G_{\beta\gamma}$ itself. $G_{\beta\gamma}$ can exist as a phosphorylated protein and its phosphorylation state has been shown to increase in guinea pig LMMP and spinal cord tissue treated with chronic morphine [51]. Notably, the G_{β} subunit of $G_{\beta\gamma}$ can be phosphorylated by PKA and PKC [51]. This has two main consequences: (1) phosphorylation of G_{β} increases its potency to stimulate Type II AC (#3, Figure 1) and (2) phosphorylation of G_{β} decreases its interaction with G protein receptor kinase (#4, Figure 1), making it more available for interaction with AC [51].

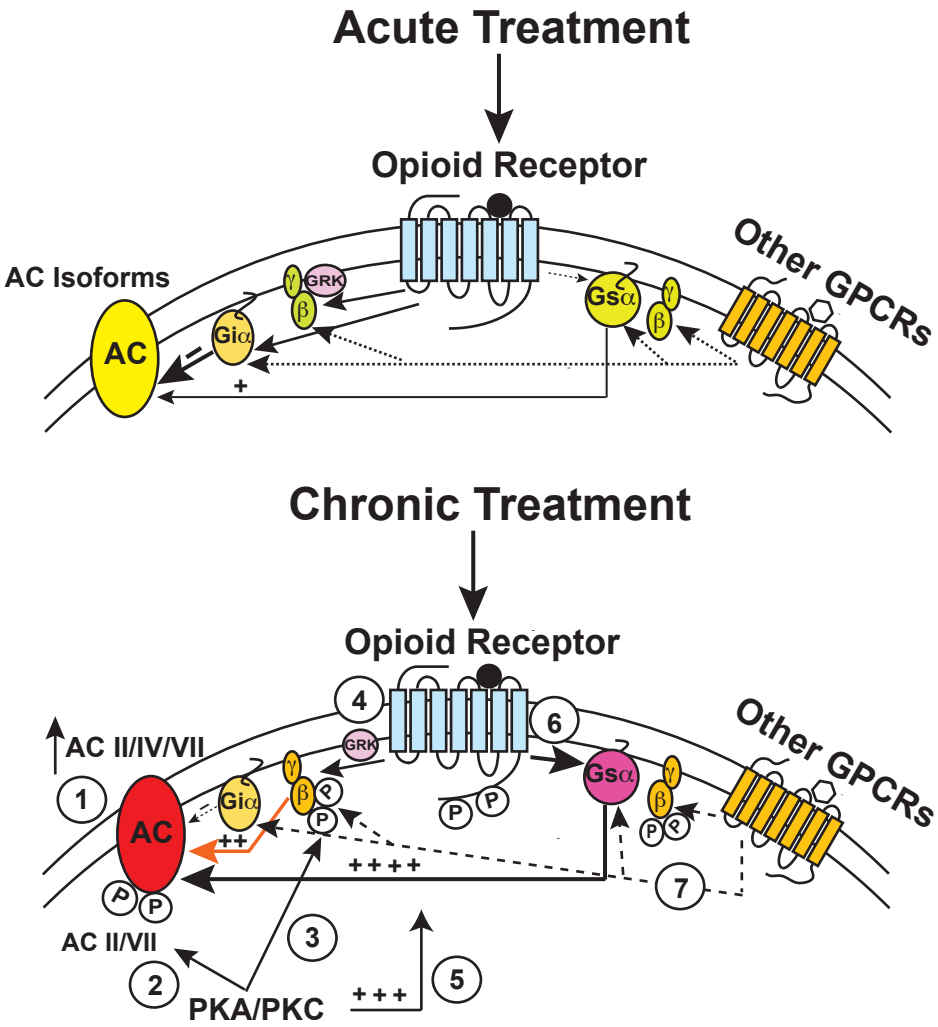

Figure 1: Post-opioid receptor adaptations to chronic morphine. Models of **(A)** Opioid receptor signaling during acute opioid treatment followed by **(B)** Altered opioid receptor signaling after chronic opioid treatment. These pathways make use of G protein signaling via Gα and Gβγ subunits as well as opioid receptor pleiotropy. In acute opioid signaling **(A)**, there is a predominant state of Giα signaling with AC inhibition and decreased cAMP production and subsequent inhibition of cell signaling. The key adaptations to chronic morphine **(B)** identified to date are: 1: Protein up-regulation of AC isoforms II, IV and VII, which are Gβγ stimulated; 2: Augmented PKC-mediated phosphorylation of ACII/VII, increasing stimulation by Gβγ; 3: Phosphorylation of Gβ by PKC (PKCγ) increases the potency of Gβγ to stimulate AC; 4: Increased PKA and PKC phosphorylation of GRK/2,3 and Gβ, decreasing the association of Gβγ with GRK, increasing the availability of Gβγ to interact with AC; 5: Augmented membrane translocation of PKCγ to effect above adaptations; 6: Increased MOR-coupled AC stimulation, mediated via the MOR-Gs association, augmented following chronic morphine; 7: The above noted changes in effector enzymes and signaling molecules elicited by chronic morphine would also be expected to alter responsiveness to other (non-opioid) neurotransmitters that signal via Gi/Go, Gs and AC, enhancing the stimulatory effects of generated Gsα and Gβγ. (Taken and adapted with permission from Gintzler and Chakrabarti, 2006)

Role of PKC in tolerance formation

PKC, and in particular PKCγ, has also been shown to be critical to the formation of opioid tolerance. Pretreatment with the PKC-selective inhibitor chelerythrine can block the shift from opioid receptor-coupled inhibition to stimulation observed following chronic morphine [48] and the increased presence of PKCγ in the neuronal membrane is critical to opioid tolerance. Mayer et al. showed that by blocking translocation of PKC from the cytosol to the membrane, there is decreased development of morphine tolerance [52]. Furthermore, Mao et al. showed that with respect to analgesic tolerance, there is specifically an increase in spinal cord PKCγ immunoreactivity [53]. This strongly suggests that PKCγ could be the isoform that is involved in such translocation during the development of morphine tolerance. Indeed, chronic morphine induces up-regulation of PKCγ in membranes obtained from Chinese hamster ovary cells (CHO) stably transfected with MOR (MOR-CHO) [54] (#5, Figure 1).

Notably, as mentioned above, G_β can be phosphorylated by PKC. Furthermore, the stoichiometry of G_β phosphorylation achieved by PKCγ is comparable to that achieved by the catalytic subunits of PKA and PKC [55]. In contrast, PKCα, PKCβ, and PKCζ produces negligible phosphorylation of G_β[55,56]. Importantly, both G_β and PKCγ have been shown to exist in a stable protein complex [57] and both phosphorylated G_β and PKCγ have been shown to co-immunoprecipate with AC [57], indicating that AC is a target for phosphorylated G_β ($G_{\beta\gamma}$) (#3, Figure 1). These observations, taken with the finding of increased G_β phosphorylation in chronic morphine treated tissue, underscore the physiologic relevance of PKCγ and phosphorylated G_β in augmenting the AC stimulatory potential.

MOR and $G_{s\alpha}$: role in opioid tolerance

So far, our model of opioid tolerant mechanisms revolves around the shift in opioid receptor-coupled signaling from $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory AC stimulatory. This is brought about by multiple post receptor adaptations involving the upregulation of $G_{\beta\gamma}$-stimulated AC isoforms and their phosphorylation as well as augmented phosphorylation of the G_{β} subunit of $G_{\beta\gamma}$. In the aggregate, these adaptations have convergent multiplicative effects to counteract MOR-coupled inhibitory modulation of AC by amplifying the AC stimulatory consequences of the $G_{\beta\gamma}$ that is generated in parallel with its corresponding G_{α} subunit. Notably, however, although involving a shift in signaling from predominantly inhibitory to stimulatory, these adaptations do not require any change in opioid receptor-G protein coupling.

An additional dimension of the model of tolerance recently developed by the Gintzler laboratory does involve altered opioid receptor coupling to G proteins. Early association of opioid receptors with $G_{s\alpha}$ was posited based on the observation that opioid G_i-mediated inhibition of AC activity could be blocked with pertussis toxin (PTX), resulting in a stimulation of AC activity by the same inhibitory opioid [35]. Importantly, the unmasked facilitation of AC activity was sensitive to cholera toxin (CTX), which is very selective for G_s [35]. Heretofore, there had been insufficient biochemical evidence of MOR coupling to G_s [58,59], e.g., demonstration of the co-immunoprecipitation of $G_{s\alpha}$ with MOR. Recently, Chakrabarti et al. was able to demonstrate the presence of a subset of MORs in in immunoprecipitate obtained using anti-$G_{s\alpha}$ antibodies [60]. Moreover, the content of MOR in $G_{s\alpha}$ immunoprecipitate (MOR-$G_{s\alpha}$ association) is augmented following chronic

morphine [60] (#6, Figure 1). Concurrently, there is a decrease in association between MOR and $G_{i\alpha}$ [60]. This demonstrates a parallel mechanism by which chronic morphine induces a shift from predominantly inhibitory to stimulatory signaling, i.e., opioid receptor coupled stimulation of all AC isoforms via $G_{s\alpha}$ [60]. Thus, chronic morphine induces the emergence of dual stimulatory pathways that would negate inhibitory MOR signaling (produce tolerance). One involves the altered consequences of signaling via the same inhibitory G protein (increased importance of the $G_{\beta\gamma}$ AC stimulatory arm of G protein signaling) and the other, enhanced MOR coupling to the stimulatory G_s G protein [10,36].

Significance of Study

The multitude of adaptations to chronic morphine undertaken by a cell in an opioid- tolerant state leads to the question of whether these adaptations are themselves hard-wired. It would be important to investigate the nature to which the starting conditions of a cell prior to opioid treatment influence the observed adaptations to chronic morphine.

One way to alter the starting conditions of a cell would be to engineer a cell where the default response to chronic morphine is different from the normally observed opioid inhibition. This paradigm had been previously utilized in other cell systems with the understanding that the relative preponderance of specific AC isoforms can determine the nature of responsiveness to drugs acting via G protein-coupled receptors. In HEK-293 cells, Federman et al. demonstrated the importance of isoform distribution, using ACII as a conditional activator of cAMP synthesis through G_i-coupled receptors via the generation of $G_{\beta\gamma}$ [39,44,61,62]. Similarly, Yoshimura et al. showed that in HEK-293 cells

stably transfected with ACV, dopamine inhibited AC activity [38]. However in the same cells overexpressing ACVII (ACII family), responses to the same dopamine agonist shifted to a stimulation of AC activity, despite the fact that the complement of G proteins had not been altered [38].

Thus, in order to engineer a cell where the default response to MOR activation is excitatory, we attempted to do this through ACII overexpression. Utilization of such a cell culture system allowed us to test the hypothesis that observed tolerant mechanisms are not hard wired but depend on initial starting conditions. By altering initial starting conditions, we observed the default opioid response could be reversed and that the adaptations that had counteracted the inhibitory signaling could be negated, or even reversed. Such adaptations to chronic morphine, mediating the shift from MOR-coupled inhibition to stimulation, were no longer observed in MOR-CHO overexpressing ACII (chronic morphine-induced augmented phosphorylation of ACII/ACVII and G_{β}, increased association of G_{β} with PKCγ, increased membrane translocation of PKCγ, increased association of MOR with G_s). To strengthen the notion that opioid tolerance is adaptive and designed to mitigate the continuance of any perturbations produced by the constant presence of opioids we engineered a cell exhibiting a $G_{\beta\gamma}$ inhibitory isoform, ACI. Subsequent observations revealed adaptations to counteract the $G_{\beta\gamma}$-mediated AC inhibition with stimulation. This suggests cellular adaptations to chronic morphine are not invariant and exhibit plasticity, which could explain the lack of ubiquity and uniformity of the degree and mechanistic underpinnings of opioid tolerance throughout the nervous system.

2) Methods and Materials

Cell culture

Chinese Hamster Ovary (CHO) cells stably transfected with rat MOR (MOR-CHO) were maintained in DMEM high glucose with L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin (P/S), and 100 μg/ml geneticin and plated at 3.8 x 10^6 cells/150 mm^2 dishes. At 65-70% confluency, half of the plates were transiently transfected with either the ACII isoform or the ACI isoform (and other half with empty vector, untransfected). ACII cDNA had been cloned into a mammalian expression vector, pRC/CMV. ACI cDNA had been cloned into a mammalian expression vector, (pcDNA 3.1). Cells were incubated with serum-free media, Lipofectamine transfection reagent (Invitrogen) and plasmid for 6 hours prior to supplementation with serum. At 90-95% confluency, cells were further divided into groups treated with or without morphine (1 μM) for 48 hours. Fresh media was provided after 24 hours as well as the morphine for the chronic morphine treatments.

Preparation of MOR-CHO cell membranes with and without $^{32}P_i$ labeling

For MOR-CHO cells undergoing $^{32}P_i$ labeling, cells were thoroughly washed with serum and phosphate free media and incubated in such media for 2 hours. Thereafter, cells were incubated in the same media containing $^{32}P_i$ (100 μCi /10 mL) for another 2 hours prior to harvest. All cells, with and without $^{32}P_i$ labeling, were then washed thoroughly with cold phosphate buffered saline (pH 7.3) and harvested directly into 20 mM HEPES, pH 7.4, containing 10% sucrose, 5 mM EDTA, 2 mM Dithiothreitol [DTT],

10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.2 mM sodium orthovanadate; protease inhibitors: 1 mM benzamidine, 0.2 mg/ml bacitracin, 2 mg/L Aprotinin, 3.2 mg/L each of soybean trypsin inhibitor and Leupeptin, 20 mg/L each of N-tosyl-L-phenylalanine choloromethyl ketone, N^{α}-p-tosyl-L-lysine choloromethyl ketone and phenylmethylsufonyl fluoride, complete cocktail inhibitor tablet/50 ml and phosphatase inhibitor 25 nM calyculin A. Cells were homogenized and centrifuged at 1000g, 4°C for 10 minutes. Supernatants obtained from the low-speed spin were subjected to a high-speed spin at 30,000g for 40 minutes at 4°C. Membranes were resuspended in HEPES buffer (pH 7.4) containing 1 mM each of EDTA and DTT, 10 mM sodium pyrophosphate and the same protease and phosphates inhibitors as mentioned above.

Acute opioid agonist treatment and determination of AC activity in membrane preparations

AC activity was determined by measuring the synthesis of [^{32}P]cAMP from [α-^{32}P]ATP (MP Biomedicals, Irvine, CA). Membranes (MOR-CHO, naïve ACII MOR-CHO, and chronic morphine-treated ACII MOR-CHO) were treated with the strong μ-opioid agonist sufentanil at varying dosages. The role of $G_{\beta\gamma}$ in the responsiveness of AC was investigated by pretreatment of the membranes with the $G_{\beta\gamma}$ blocking peptide QEHA [63] (50 μM; 20 minutes on ice).

Assays were initiated by the addition of the reaction mixture (50 mM HEPES buffer, pH 8.0, containing 10 mM $MgCl_2$, 20 mM creatine phosphate, 10 units/sample creatine phosphokinase, 0.1 mM ATP, 10 μM GTP, 20 mM NaCl, 1 mM DTT, 50 mM EGTA, 0.125 mM rolipram, 0.1% BSA, and [α-^{32}P]ATP; 1 μCi/sample) to cell membranes

(50 μg untransfected, 5 μg transfected). Reactions (30°C for 15 minutes) were terminated by the addition of 10 μl of 2.2 N HCl (4°C). Then, [^{32}P]cAMP generated was separated by neutral alumina column chromatography [64]. [^{3}H]cAMP (0.005 μCi), added to each sample before fractionation, was used as an internal standard to correct for column recovery of the [^{32}P]cAMP. Radioisotopes were quantified using liquid scintillation spectroscopy.

$G_{s\alpha}$ stimulation of AC and determination of AC activity in membrane preparations (as described in ref. 46)

We used recombinant $G_{s\alpha}$ ($rG_{s\alpha}$) that was expressed in and purified from E. coli [65]. $rG_{s\alpha}$ (5 μg) was activated by incubation for 60 minutes at 30°C with 100 μM GTPγS in 50mM Na-HEPES buffer, pH 8.0, containing 1 mM EDTA, 1 mM DTT, 5 mM $MgSO_4$, and BSA (1.25 mg/ml) [37]. Separation from free GTPγS was achieved through gel filtration (Microspin G-25 columns, Amersham Biosciences). The role of $G_{\beta\gamma}$ in the stimulatory responsiveness of AC to $G_{s\alpha}$ was investigated by pretreatment of the membranes with the $G_{\beta\gamma}$ blocking peptide QEHA [63] (50 μM; 20 minutes on ice).

AC activity was determined by measuring the synthesis of [^{32}P]cAMP from [α-^{32}P]ATP (MP Biomedicals, Irvine, CA). Assays were initiated by the addition of the reaction mixture (50 mM HEPES buffer, pH 8.0, containing 10 mM $MgCl_2$, 20 mM creatine phosphate, 10 units/sample creatine phosphokinase, 0.1 mM ATP, 10 μM GTP, 20 mM NaCl, 1 mM DTT, 50 mM EGTA, 0.125 mM rolipram, 0.1% BSA, and [α-^{32}P]ATP; 1 μCi/sample) to cell membranes (50 μg untransfected, 5 μg transfected) with or without activated $rG_{s\alpha}$. Reactions (30°C for 15 minutes) were terminated by the addition of 10 μl

of 2.2 N HCl (4°C). Then, [^{32}P]cAMP generated was separated by neutral alumina column chromatography [64]. [^{3}H]cAMP (0.005 μCi), added to each sample before fractionation, was used as an internal standard to correct for column recovery of the [^{32}P]cAMP. Radioisotopes were quantified using liquid scintillation spectroscopy.

Immunoprecipitation

Membranes were prepared for immunoprecipitation by solubilizing in the same buffer containing 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 10% glycerol, agitated 60 minutes at 4°C and centrifuged 16,000g for 20 minutes at 4°C. Clear supernatants were used for immunoprecipitation.

AC was immunoprecipitated using antibodies generated against the carboxyl terminus common to all AC isoforms (5 μl/500 μg protein; BBC-4, provided by Dr. S. Mollner, Heinrich Heine University, Dusseldorf, Germany) [49,66]. PKCγ was immunoprecipitated using a mouse monoclonal antibody (5 μl/500 μg protein, Sigma Chemical Co.) generated against rat PKCγ amino acid residues 684-697. $G_{s\alpha}$ was immunoprecipitated from 50 μg of solubilized membrane using a rabbit polyclonal antibody generated against the C-terminus of the $G_{s\alpha}$ subunit (aa 385-394; BD Biosciences Pharmingen, San Diego, SA; 1 μl/100 μg protein.) Pre-washed Protein G slurry was used for AC and PKCγ immunoprecipitation (Protein A for $G_{s\alpha}$) overnight at 4°C. The beads were washed in 20 mM HEPES buffer (pH 7.4) containing 1 mM each of DTT and EDTA, 150 mM NaCl, 0.05% NP-40 and the same protease inhibitors as mentioned above. The immunoprecipitates were eluted via heating in 30 μl of sample

buffer (15 minutes at 85^{o}C). All samples were separated on 4-12% gradient Bis-Tris gels (Invitrogen) and then electro-transferred onto nitrocellulose membranes, which were exposed to Phosphorimager screens and scanned in Phosphorimager Storm 860 (Molecular Dynamics, Sunnyvale, CA). $^{32}P_i$ incorporated into phosphorylated samples was determined using densitometric analysis (Imagequant, Molecular Dynamics).

Western analysis

Nitrocellulose membranes previously scanned for phosphorylation analyses were sequentially processed for Western blots (as described in refs. 46, 57). This was done to validate chemical identity of autoradiographic bands through coincidence with Western bands. Membranes were incubated with blocking solution (5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 hour). Selected lanes were then incubated overnight at 4^{o}C with the appropriate primary antibody. AC protein (in transfected and untransfected membranes) was visualized using the monoclonal antibody, BBC-4. PKCγ protein was visualized using a rabbit polyclonal antibody generated against the carboxyl terminus of PKCγ (Santa Cruz Biotech, Santa Cruz, CA). G_β proteins were visualized using a polyclonal antibody against the carboxyl terminus of G_β [67] (BC-1, generously supplied by Dr. John Hildebrandt, Medical University of South Carolina). $G_{s\alpha}$ protein was visualized using a 1:15000 dilution of a rabbit polyclonal anti-$G_{s\alpha}$ antibody generated against the C-terminus of $G_{s\alpha}$. MOR protein was visualized using a 1:10,000 dilution of anti-MOR antibodies generated against the C-terminal 50 aa of MOR [59] (generously provided by Dr. Thomas Cote, Uniformed Services University of the Health Sciences, Bethesda, Maryland). The secondary antibody used was either peroxidase labeled anti-mouse (for

monoclonal BBC-4) or anti-rabbit (for polyclonal PKCγ, BC-1, $G_{s\alpha}$, and MOR) (Amersham Biosciences). The antibody/substrate complex was visualized using Supersignal West Dura Chemiluminescence Detection Kit (Pierce) and a CCD camera (GeneGnome, Syngene Corp.). The signal intensity was quantified using Syngene imaging software.

Statistical analyses

Either one-way or mixed model analysis of variance (ANOVA) was used to locate statistically significant interactions between the main effects of opioid treatment, adenylyl cyclase (AC) overexpression, and the $G_{\beta\gamma}$ blocking peptide QEHA. Analysis of co-variance was employed to assess AC phosphorylation while adjusting for AC protein changes. Subsequently, post-hoc analyses using Tukey Honestly Significantly Different (HSD) or Least Significant Difference (LSD) tests were performed to detect the sources of the significant interactions. To assess the effect of opioid treatments within an AC overexpression group, we used one-sample t-tests. Finally, linear regression analysis was utilized to assess AC overexpression as a predictor of changes in AC phosphorylation.

3) Results

3.1a) Acute activation of MOR in ACII MOR-CHO vs. MOR-CHO results in qualitatively divergent modulation of AC activity

It is very well established that acute activation of MOR in MOR-CHO results in inhibition of AC activity and a consequent lowering of cAMP production. In order to validate that overexpression of a $G_{\beta\gamma}$–stimulated isoform of AC, e.g., ACII, would reverses acute opioid inhibition of ACII to a stimulation, as was described in Federman et al. [39] for acute activation of dopamine receptors in HEK293 cells, we determined the effect of acute MOR activation in ACII MOR-CHO. Given the receptor specificity of the cell system (exclusively μ-opioid receptors), we utilized the potent μ-selective agonist, sufentanil, in order to maximize any differences between effects of acute MOR activation in MOR-CHO vs. ACII MOR-CHO.

As predicted, in MOR-CHO, acute activation of MOR inhibited AC activity (Figure 2, solid line). A decrement in cAMP production was observed at all sufentanil concentrations tested (3, 10, 100, 1000 nM). In contrast, in ACII MOR-CHO, a reverse response to MOR activation was observed; acute MOR activation stimulated AC activity (Figure 2, dotted line), producing an increment in cAMP at all sufentanil dosages employed.

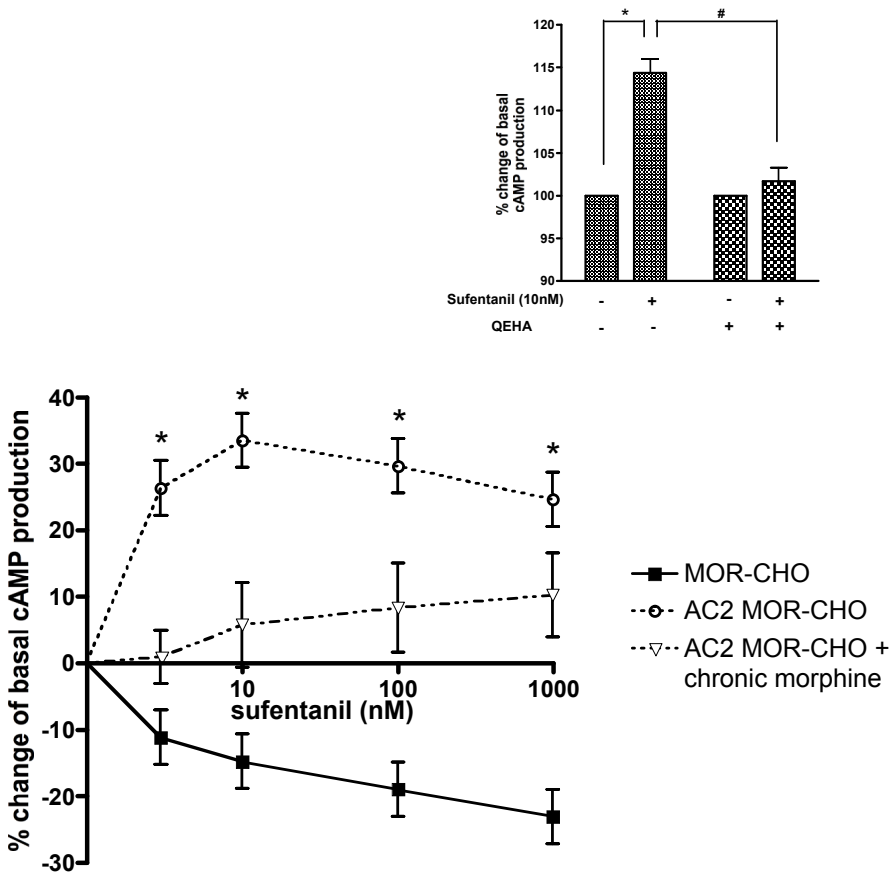

Figure 2: Activation of MOR produces opposite modulation of AC activity in MOR-CHO vs. ACII MOR-CHO. The ability of MOR activation to modulate AC activity was reflected by cAMP production in membranes obtained from MOR-CHO (●), ACII MOR-CHO (○), and ACII MOR-CHO treated with chronic morphine (▼), all subjected to sufentanil (3 min). *Inset*, effect of QEHA (50 mM) on the stimulatory responsiveness of ACII-MOR-CHO membranes to sufentanil (10 nM) *, $p < 0.01$ for effect of MOR activation on AC activity in membranes of naïve ACII MOR-CHO vs. chronic morphine-treated ACII MOR-CHO (n = 4) and MOR-CHO (n = 8). #, $p < 0.001$ for the effect of sufentanil (10 mM) on AC activity in the presence vs. absence of QEHA in membranes of ACII-MOR-CHO) (n = 6). However, MOR activation inhibits AC activity in MOR-CHO yet enhances AC activity in ACII-MOR-CHO, which is attenuated following chronic morphine. The increment of AC activity that results from MOR activation is abolished by QEHA indicating mediation by $G_{\beta\gamma}$.

As was expected based on Federman et al. [39], the MOR-coupled activation of AC in ACII MOR-CHO is $G_{\beta\gamma}$-dependent. This was assessed by determining the effect of acute MOR activation on AC activity in the presence vs. absence of the $G_{\beta\gamma}$ blocking peptide, QEHA (*inset*, Figure 2). ANOVA revealed effects of QEHA that were dose-dependent ($F_{1,10} = 31.41$, $p < 0.001$). QEHA (50 μM) abolished the previously observed increment in AC stimulation that was observed in response to acute MOR activation by 10 nM sufentanil (*inset* to Figure 2; without QEHA: $t_5 = 14.11$, $p < 0.001$ versus no effect in the presence of QEHA, $t_5 = 0.870$, $p = 0.424$). This indicates that the observed AC stimulation in ACII MOR-CHO is dependent upon $G_{\beta\gamma}$ regulation.

3.1b) Acute activation of MOR in chronic morphine-treated ACII MOR-CHO fails to stimulate AC: manifestation of tolerance

In untransfected cells, chronic morphine elicits adaptations that shift $G_{i\alpha}$-inhibition to $G_{\beta\gamma}$-stimulation of AC to counteract the continued presence of opioid inhibition. Thus there is the reestablishment of "normal" levels of cAMP in chronic morphine-treated tissue [34]. Tolerance exhibited by untranfected cells has been extensively investigated [68]. We demonstrate here that ACII overexpressing MOR-CHO, in addition to exhibiting default opioid AC stimulation, also exhibit tolerance when exposed to chronic morphine; MOR activation has minimal effects on cAMP production. In ACII MOR-CHO acute activation of MOR failed to alter AC activity (Figure 2, mixed line). At all sufentanil concentrations tested (3, 10, 100, 1000 nM), there was no significant change in cAMP production ($n = 4$, $p > 0.05$), suggesting that chronic morphine was able to abolish the opioid stimulation of AC activity upon MOR activation.

To formally assess the relationship between sufentanil responsiveness, ACII overexpression, and effect of chronic morphine, a mixed model ANOVA was used to compare sufentanil dose responsiveness in membranes obtained from MOR-CHO, naïve ACII MOR-CHO, and tolerant ACII MOR-CHO. There were significant interactions between effects of MOR activation, AC overexpression, and effect of chronic morphine ($F_{8,68} = 19.48$, $p < 0.001$). In other words, the consequence of acute MOR activation on AC activity is dependent upon the presence vs. absence of AC overexpression and upon the presence vs. absence of chronic morphine. To examine this effect more closely, we performed post-hoc analysis (LSD), which demonstrated that the effect of ACII overexpression was significant at all sufentanil concentrations tested ($n = 8$, $p < 0.001$) and the effect of chronic morphine in ACII MOR-CHO on the increment of AC stimulation was significant at all concentrations ($n = 4$-8, $p < 0.01$).

These observations confirm that overexpression of ACII in MOR-CHO shifts the default acute response to MOR activation from an inhibition to a stimulation of AC activity. Furthermore, these cells manifest opioid tolerance when treated with chronic morphine such that cAMP production remains unchanged despite the continued presence of opioid stimulation. In contrast to MOR-CHO, this suggests in ACII MOR-CHO adaptations that shift the cell to a state of AC inhibition, validating the use of ACII MOR-CHO to test whether or not initial starting conditions are relevant to adaptations elicited by chronic morphine.

3.2a) Chronic morphine augments $G_{s\alpha}$-dependent $G_{\beta\gamma}$ stimulation of AC activity in MOR-CHO

Chronic activation of opioid receptors leads to increased $G_{s\alpha}$-dependent $G_{\beta\gamma}$ stimulation of AC [46]. This was first demonstrated in mammalian tissue by Chakrabarti et al. in guinea pig longitudinal muscle myenteric plexus (LMMP) tissue [46]. These observations have been confirmed in MOR-CHO. As was observed in LMMP [46], $rG_{s\alpha}$ dose dependently stimulates AC activity in membranes obtained from both opioid naïve and chronic morphine-treated MOR-CHO (Figure 3A). In addition, as was also observed in LMMP tissue [46], the magnitude of the increment in AC activity produced by all $rG_{s\alpha}$ concentrations tested (2.5, 5, 10, 20 nM) in membranes obtained from chronic morphine-treated cells is significantly greater than that observed in opioid naive membranes. ANOVA revealed significant main effects for morphine ($F_{1,44} = 78.52$, $p < 0.001$) and for $rG_{s\alpha}$ concentration ($F_{4,47} = 160.22$, $p < 0.001$). Importantly, significant interactions between the effect of chronic morphine and $rG_{s\alpha}$ concentrations were also observed ($F_{4,44} = 9.4$, $p < 0.001$). As the concentration of $rG_{s\alpha}$ increased, so did the chronic morphine-induced increment in AC activity, which achieved significance at all $rG_{s\alpha}$ concentrations ($p < 0.005$; $n = 4$-9).

To determine whether this increase in $G_{s\alpha}$ stimulation of AC after chronic morphine treatment is $G_{\beta\gamma}$-dependent, as has been reported for LMMP tissue [46], the effect of QEHA on responsiveness of AC to $rG_{s\alpha}$-stimulated AC activity was determined in membranes obtained from opioid naïve and chronic morphine-treated cells. A significant interaction was observed between the presence vs. the absence chronic morphine and the ability of QEHA to alter $rG_{s\alpha}$ responsiveness ($F_{1,9} = 24.33$, $p = 0.001$). In membranes from opioid naïve cells, the increment in AC activity above basal produced by $rG_{s\alpha}$ (5 mM) was not altered by QEHA (50 uM), indicating that the

increased activity of AC resulted from its direct stimulation by $rG_{s\alpha}$. In contrast, QEHA (50 mM)

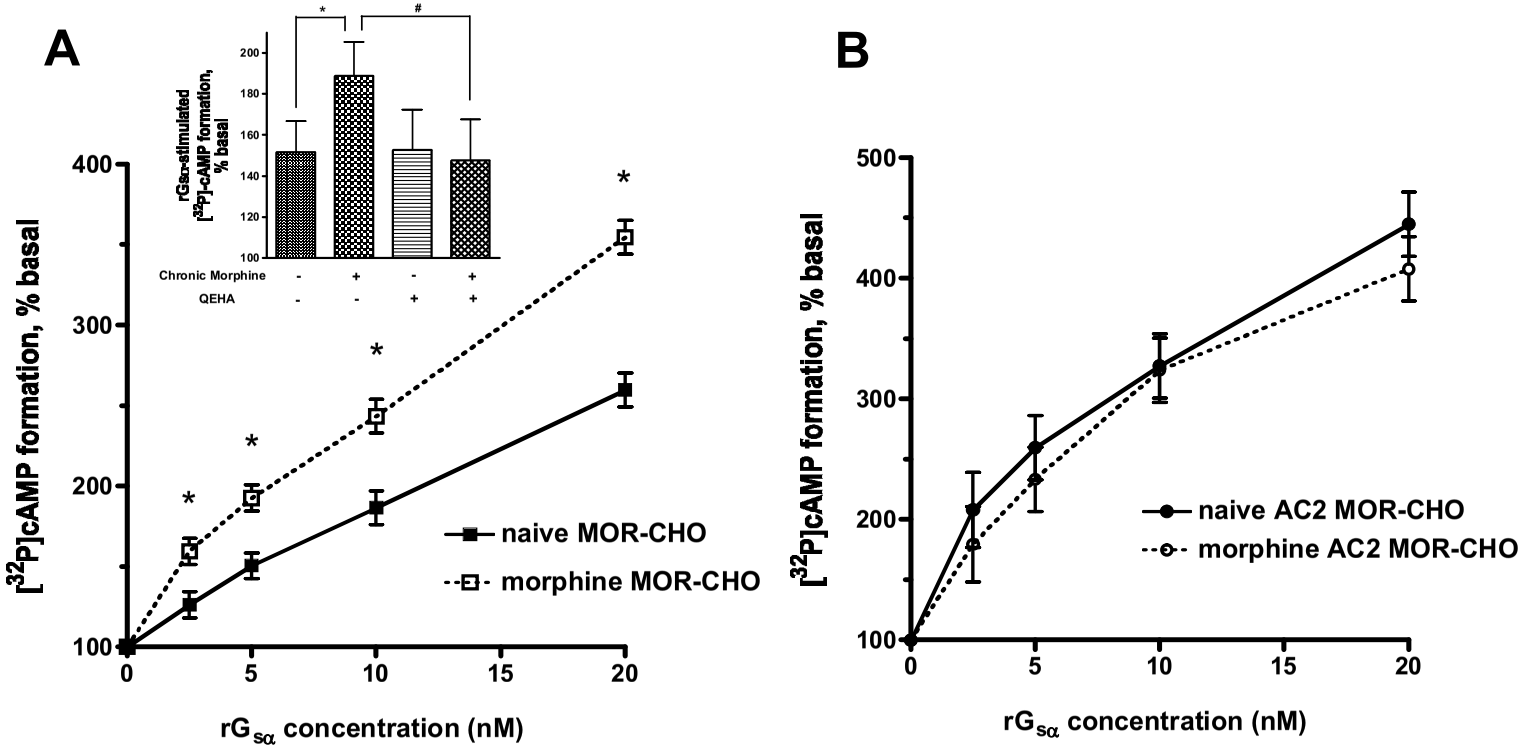

Figure 3: Chronic morphine augments Gsα-dependent Gβγ stimulation of AC activity in MOR-CHO but not in ACII MOR-CHO. **A**, rGsα stimulatory responsiveness of AC in membranes obtained from opioid naive (■) and chronic morphine treated (□) MOR-CHO tissue. *Inset*, effect of QEHA (50 mM) on the rGsα (5 nM) stimulatory responsiveness of membranes obtained from opioid naive versus chronic morphine-treated MOR-CHO. **B**, rGsα stimulatory responsiveness of AC in membranes obtained from opioid naive (●) and chronic morphine-treated ACII MOR-CHO (○). The level of ACII overexpression was assessed by comparing the basal activity of AC in MOR-CHO versus that of ACII MOR-CHO (cAMP production: 1.3-5.2 pmol/mg/min in MOR-CHO; 61-173 pmol/mg/min in ACII MOR-CHO). *, $p < 0.002$ for the magnitude of rGsα stimulation in chronic morphine-treated versus naive membranes (n = 4-9). #, $p = 0.001$ for effect of QEHA on rGsα stimulatory responsiveness in chronic morphine-treated MOR-CHO membranes (*inset*) (n = 4). Chronic morphine treatment produces augmented stimulatory responsiveness to rGsα, which can be abolished by QEHA. In contrast, chronic morphine treatment does not increase rGsα stimulatory responsiveness in ACII MOR-CHO.

abolished the chronic morphine-induced increment in $rG_{s\alpha}$ AC stimulation in membranes obtained from chronic morphine-treated MOR-CHO; in the presence of QEHA, the magnitude of the increment in AC activity produced in membranes of chronic morphine-treated and opioid naïve MOR-CHO was indistinguishable ($p = 0.421$) (*inset*, Figure 3A). Thus, in MOR-CHO as in LMMP, chronic morphine treatment augments $G_{s\alpha}$-dependent $G_{\beta\gamma}$ stimulation of AC.

3.2b) Chronic morphine fails to augment $G_{\beta\gamma}$-AC signaling in ACII-transfected MOR-CHO

AC assays using ACII MOR-CHO demonstrated that in both naïve and chronic morphine-treated preparations, $rG_{s\alpha}$ was still able to stimulate AC in a dose-dependent manner (Figure 3B). The level of ACII overexpression was assessed by comparing the basal activity of AC in MOR-CHO versus that of ACII MOR-CHO (cAMP production: 1.3-5.2 pmol/mg/min in MOR-CHO; 61-173 pmol/mg/min in ACII MOR-CHO). However, unlike untransfected MOR-CHO, in ACII MOR-CHO chronic morphine failed to augment the magnitude of $G_{s\alpha}$ stimulation of AC. Mixed model ANOVA revealed a significant main effect for $rG_{s\alpha}$ concentration ($F_{4,32} = 84.04$, $p < 0.001$), but not for presence or absence of chronic morphine ($F_{1,32} = 2.23$, $p = 0.143$). Additionally, there were no significant interactions between treatment condition (presence or absence of chronic morphine) and $rG_{s\alpha}$ concentration ($F_{4,32} = 0.36$, $p = 0.834$). The absence of any effect of chronic morphine on $rG_{s\alpha}$ stimulation of AC was observed for all $rG_{s\alpha}$ concentrations ($p > 0.17$; $n = 5$). Thus, overexpression of ACII in MOR-CHO resulted in the inability to manifest increased $G_{s\alpha}$-dependent $G_{\beta\gamma}$ stimulation of AC in response to chronic

morphine. This suggested the utility of investigating the influence of ACII overexpression on those adaptations that underlie augmented $G_{\beta\gamma}$-AC stimulation following chronic morphine (increased phosphorylation of AC, G_{β} subunit of $G_{\beta\gamma}$, and membrane translocation of PKCγ).

3.3a) ACII overexpression abolishes the ability of chronic morphine to augment AC phosphorylation

Here we show that the ability of chronic morphine to augment AC phosphorylation, observed in untransfected MOR-CHO [50] (in which AC activity is inhibited by acute MOR activation) is no longer manifest in ACII-overexpressing MOR-CHO (in which acute MOR activation stimulates AC activity).

We quantified metabolic phosphorylation of AC in ACII MOR-CHO and MOR-CHO via incubation with $^{32}P_i$ added during the last 2 hours of a 48-hour pretreatment with or without morphine (1 mM). Autoradiograms of immunoprecipitate obtained using anti-AC antibodies (AC IP) from membranes of untransfected MOR-CHO contained two radiolabeled bands of ≈155 kDa and ≈130 kDa (Figure 4A, lanes 1-2). Analogous bands were observed in AC IP from membranes of ACII MOR-CHO cells. As seen in previous studies with ACII [49], in contrast to untransfected MOR-CHO, the molecular mass of the larger, predominant band in ACII MOR-CHO encompassed ≈130 kDa to ≈110 kDa (Figure 4A, lanes 3-4), reflecting the greater abundance of phosphorylation of the overexpressed ACII.

To determine the effect of ACII overexpression on the influence of chronic morphine on AC phosphorylation, densitometric analyses were performed on the $^{32}P_i$-

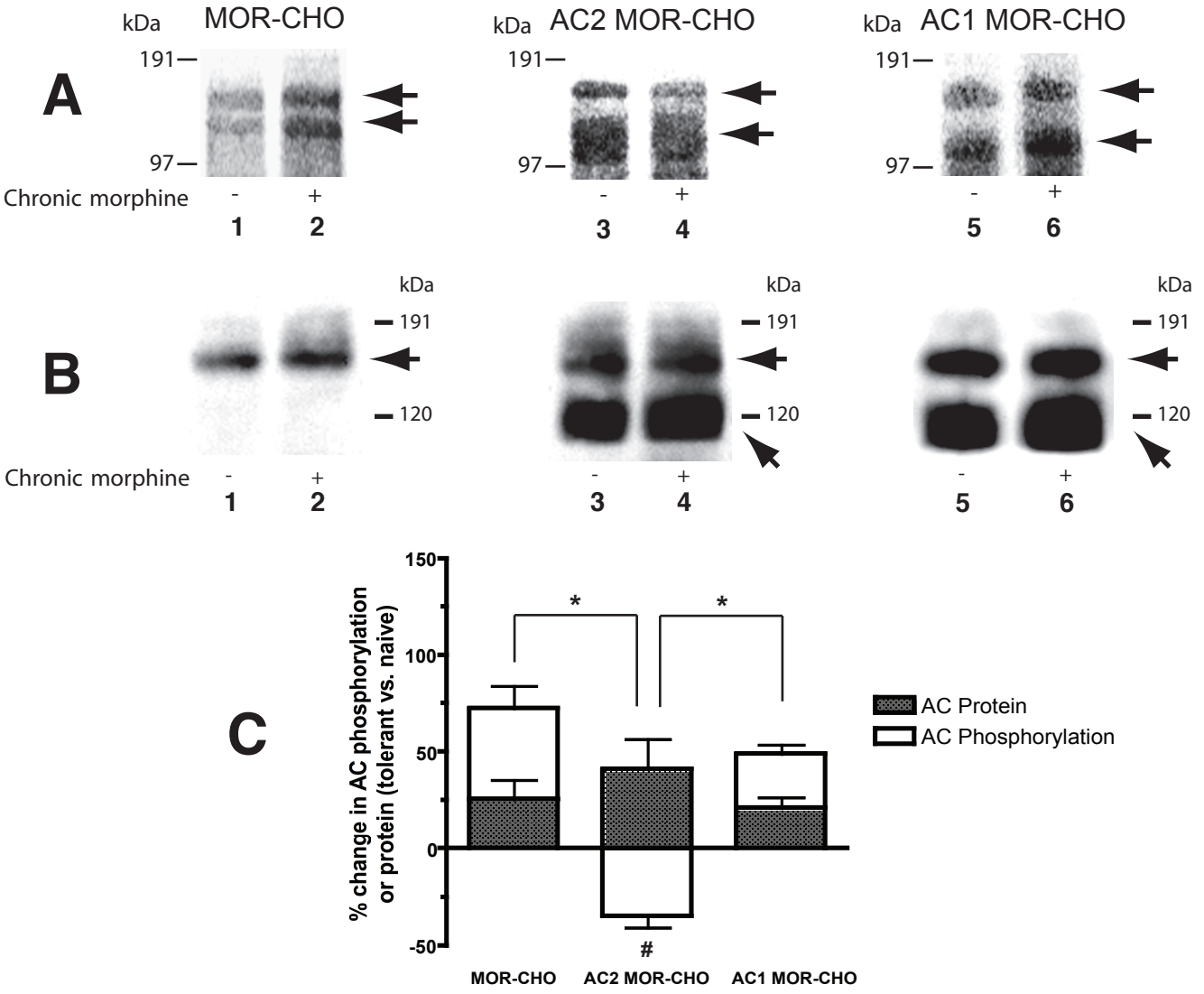

Figure 4A-C: Chronic morphine augments AC phosphorylation in in AC IP obtained from membranes of MOR-CHO and ACI MOR-CHO but not ACII MOR-CHO. (A) Autoradiograms of ^{32}P incorporation into AC IP obtained from membranes of opioid naive (lane 1) and chronic-morphine treated (lane 2) MOR-CHO (A), membranes of opioid naive (lane 3) and chronic morphine treated (lane 4) ACII MOR-CHO and membranes of opioid naive (lane 5) and chronic morphine-treated (lane 6) ACI MOR-CHO. Left arrowheads represent phosphorylated AC protein: MOR-CHO (~155 kDa, ~130 kDa), ACII MOR-CHO (~155 kDa, ~120 kDa), and ACI MOR-CHO (~155 kDa, ~115 kDa). As seen in previous studies with ACII [49], in contrast to untransfected MOR-CHO, the molecular mass of the larger, predominant band in ACII MOR-CHO encompassed ≈130 kDa to ≈110 kDa (Figure 4A, lanes 3-4), reflecting the greater abundance of phosphorylation of the overexpressed ACII. **(B)** Sequential AC Western analyses of corresponding autoradiograms above. AC chemical identity of autoradiographic signals above (indicated by arrowheads) is validated by coincidence of molecular mass with Western signals. **(C)** Quantification of the influence of AC overexpression state on the ability of chronic morphine to modulate phosphorylation of AC (open bars) and AC protein (solid bars) present in the AC IP. *, $p < 0.001$ for effect of chronic morphine on AC phosphorylation in ACII MOR-CHO vs. MOR-CHO or ACI MOR-CHO (n = 4-6 for MOR-CHO and ACI MOR-CHO; n = 11 for ACII MOR-CHO). #, $p < 0.001$, for effect of chronic morphine on AC phosphorylation in ACII MOR-CHO.

radiolabeled AC bands present in AC IP obtained from membranes of MOR-CHO and ACII MOR-CHO that had been maintained with and without chronic morphine. In the untransfected cells, chronic morphine increased AC phosphorylation (≈72%; $t_5 = 6.19$, $p = 0.002$, Figure 4C), as was initially reported for LMMP tissue [46]. In contrast, in ACII MOR-CHO (Figure 4A, lanes 3-4), the significant increase in AC phosphorylation was no longer observed; on the contrary, in ACII MOR-CHO, chronic morphine decreased AC phosphorylation (≈34%; $t_{10} = -6.024$, $p < 0.001$, Figure 4C). Chronic morphine is no longer able to enhance AC phosphorylation in ACII–overexpressing cells. This suggests that the effect of chronic morphine on AC phosphorylation is dependent upon the consequences of acute activation of MOR (inhibitory vs. stimulatory).

3.3b) Chronic morphine augments the phosphorylation state of AC in ACI-overexpressing MOR-CHO

The $G_{\beta\gamma}$-subunit differentially regulates ACI and ACII [37]. Whereas $G_{\beta\gamma}$ stimulates ACII, it inhibits ACI. Thus, overexpression of ACI would not be expected to alter consequences of acute MOR activation. In order to differentiate between effects of ACII that result from its stimulation by $G_{\beta\gamma}$ (i.e., the reversal of acute activation of MOR from inhibition to stimulation) versus an effect of AC overexpression per se, the effect of ACI overexpression on modulation of AC phosphorylation by chronic morphine was determined. Autoradiograms of AC IP were obtained from ACI MOR-CHO metabolically labeled with ^{32}P during the last 2 hours of a 48-hour treatment with morphine or vehicle. Two radiolabeled bands of ≈155 kDa and ≈115-120 kDa were prominent (Figure 4A, lanes 5-6). Semi-quantitative autoradiographic analyses revealed that chronic exposure

to morphine resulted in a significant increment in AC phosphorylation (≈49%; $t_3 = 9.98$, $p = 0.002$; Figure 4C), analogous to observations made in untransfected MOR-CHO.

In order to formally compare the changes in levels of AC phosphorylation in untransfected, ACI, and ACII MOR-CHO resulting from chronic morphine exposure (Figure 4C), we used one-way ANOVA and Tukey HSD post-hoc analysis to assess and locate a statistical difference between the groups (untransfected MOR-CHO, ACI MOR-CHO, ACII MOR-CHO, each with and without chronic morphine). There was a significant interaction between the presence and absence of chronic morphine, AC overexpression state and the change in AC phosphorylation ($F_{2, 20} = 57.424$, $p < 0.001$). There was no significant difference between the effects of chronic morphine on AC phosphorylation in untransfected versus ACI MOR-CHO ($p = 0.221$). There was, however, a significant difference between the effects of chronic morphine on AC phosphorylation in untransfected versus ACII MOR-CHO ($p < 0.001$), and in ACI MOR-CHO versus ACII MOR-CHO ($p < 0.001$).

3.3c) Chronic morphine increases AC protein content of AC immunoprecipitate in untransfected, ACII-, and ACI-transfected MOR-CHO

We determined the AC protein content of the AC IP used for phosphorylation analysis. Sequential AC Western analysis of the AC IP used for phosphorylation studies was performed to validate chemical identity of autoradiographic bands through coincidence with Western bands. Although autoradiographic analysis revealed two radiolabeled AC bands, sequential Western blot analysis of untransfected MOR-CHO membranes revealed only a single ≈155 kDa band (Figure 4B, lanes 1-2). In contrast,

both radiolabeled bands that were present in each of the autoradiograms of ACII MOR-CHO and ACI MOR-CHO samples have corresponding Western signals (Figure 4B, lanes 3-6). This suggests that the smaller species of metabolically phosphorylated ACII (≈130 kDa) exists in quantities sufficient for the autoradiographic detection, but insufficient for chemiluminescence (Western) detection. This difference between MOR-CHO and ACII/ACI MOR-CHO is most likely explained by the greater amount of AC protein that results from AC overexpression.

The amount of AC immunoprecipitated from membranes of chronic morphine-treated MOR-CHO was ≈25% higher than from opioid naïve cells (t_5 = 2.55, p = 0.05; Figure 4C). Importantly, this increment in AC protein (≈25%) was less than the observed increase in AC phosphorylation (≈72%). This indicates that chronic morphine results in a net increase in AC phosphorylation [49]. There was an analogous increment in the magnitude of AC immunoprecipitated from chronic morphine-treated ACII-MOR-CHO vs. opioid naïve ACII-MOR-CHO (Figure 4B, lanes 3-4), (≈41%; t_{10} = 2.683, p = 0.023; Figure 4C). In contrast to untransfected cells, however, this increment in AC protein occurred concomitant with a diminution in AC phosphorylation. Thus, the decrement in AC phosphorylation produced by chronic morphine in these cells (≈36%) is an underestimate of its true magnitude. These data indicate that overexpression of ACII not only obliterates the chronic morphine-induced increment in AC phosphorylation but it reverses the previously observed increment to a net decrease.

In ACI MOR-CHO (Figure 4B, lanes 5-6), chronic morphine augments the amount of immunoprecipitated AC protein (≈21%; t_3 = 3.89, p = 0.030; Figure 4C), which like the untransfected MOR-CHO, was smaller than the chronic morphine-induced

increment in AC phosphorylation (≈49%). This indicates that in ACI MOR-CHO, as in untransfected MOR-CHO, chronic morphine results in a net increase in AC phosphorylation. One-way ANOVA was used to formally compare the chronic morphine-induced increase in levels of AC protein in untransfected, ACI, and ACII MOR-CHO. There were no differences between the groups ($F_{2,20} = 0.501$, $p = 0.614$).

In order to formally eliminate the chronic morphine-induced increment in immunoprecipitated AC as a variable confounding interpretation of effects of AC overexpression on AC phosphorylation, we performed an analysis of co-variance. This revealed that the chronic morphine-induced increment in AC protein levels was not a significant covariant ($F_{1,20} = 2.072$, $p = 0.168$); the presence or absence of ACII overexpression remained a significant determinant of the effect of chronic morphine on AC phosphorylation after adjusting for AC protein changes ($F_{2,20} = 54.689$, $p < 0.001$).

3.3d) Change in AC phosphorylation is dependent upon the level of ACII overexpression in ACII MOR-CHO

In ACII MOR-CHO, chronic morphine elicited varying decrements in AC phosphorylation. Analysis of variance revealed a significant interaction between levels of ACII overexpression and magnitude of change in AC phosphorylation ($F_{1,10} = 6.515$, $p = 0.031$). A scatterplot showing the relationship between the level of ACII overexpression and the level of chronic morphine-induced decrement in AC phosphorylation reveals a trend (Figure 5, $r = -0.648$, $p= 0.031$) such that as ACII overexpression levels went up, the decrement in AC phosphorylation due to chronic morphine was greater.

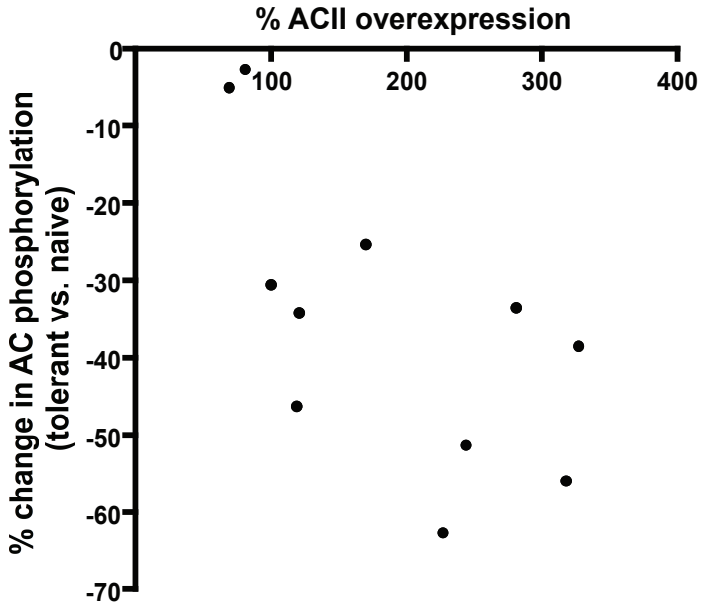

Figure 5: Modulation of AC phosphorylation by chronic morphine in ACII MOR-CHO is dependent upon ACII overexpression levels. Quantification of the interaction between level of ACII overexpression and changes in AC phosphorylation (chronic morphine-treated versus naïve). Analysis of covariance revealed a significant interaction between ACII overexpression level and change in AC phosphorylation ($F_{1,10} = 6.515$, $p = 0.031$).

3.4a) Chronic morphine fails to augment G_β phosphorylation in ACII MOR-CHO, as was previously reported for MOR-CHO

The phosphorylation state of the G_β subunit of $G_{\beta\gamma}$ is another important determinant of $G_{\beta\gamma}$-stimulatory AC signaling [51]. PKCγ endogenously phosphorylates G_β, which increases in chronic morphine-treated MOR-CHO [57]. This augments both the availability and potency of $G_{\beta\gamma}$ to stimulate ACII [57] and is thus a component of the chronic morphine-induced shift from opioid receptor-coupled $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory AC signaling. As such, we investigated the effect of ACII overexpression on G_β phosphorylation following chronic treatment with morphine. Since a causal association between PKCγ and chronic morphine-induced G_β phosphorylation has been established [57], we assessed the phosphorylation state of G_β that was present in immunoprecipitate obtained, using anti-PKCγ antibodies (PKCγ IP), from ACII MOR-CHO vs. MOR-CHO, grown in the presence or absence of chronic morphine.

Autoradiographic analysis of the PKCγ IP of $^{32}P_i$-metabolically radiolabeled MOR-CHO and ACII MOR-CHO, each maintained with and without chronic morphine, revealed two radiolabeled bands of ≈37 kDa and ≈33 kDa. These correspond to molecular masses of G_β previously observed [51,57] (Figures 6A, lanes 1-6). In the untransfected cells (Figure 6A, lanes 1-2), chronic morphine treatment increased phosphorylation of the ≈37 kDa and ≈33 kDa bands by ≈73% ($t_3 = 4.13$, $p = 0.026$) and ≈56% ($t_3 = 6.19$, $p = 0.009$) respectively (Figure 6C). This confirms previously reported observations in MOR-CHO and rat spinal cord [57]. In contrast, the augmentation of G_β phosphorylation

following chronic morphine treatment of ACII MOR-CHO, similarly metabolically radiolabeled, was not discernable (Figure 6A, lanes 3-4); autoradiographic analyses

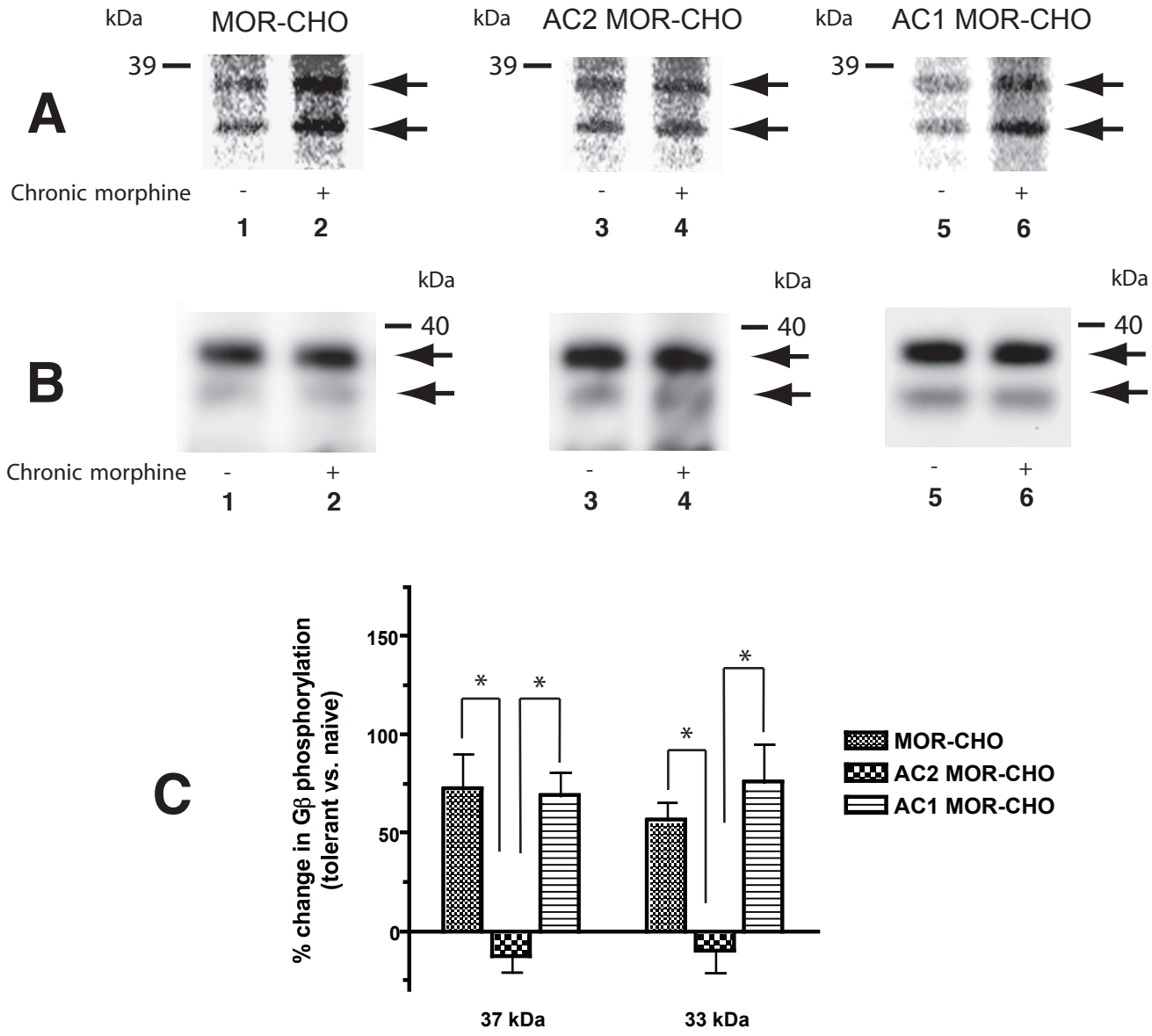

Figure 6A-C: Chronic morphine augments Gβ phosphorylation in PKCγ IP obtained from membranes of MOR-CHO and ACI MOR-CHO, but not ACII MOR-CHO. (A) Autoradiograms of ^{32}P incorporation into PKCγ IP obtained from membranes of opioid naive (lane 1) and chronic morphine-treated (lane 2) MOR-CHO, membranes of opioid naive (lane 3) and chronic morphine-treated (lane 4) ACII MOR-CHO and membranes of opioid naive (lane 5) and chronic morphine-treated (lane 6) ACI MOR-CHO. Left arrowheads represent phosphorylated Gβ protein: MOR-CHO (~37kDa, ~33kDa), ACII MOR-CHO (~37kDa, ~33kDa), and ACI MOR-CHO (~37kDa, ~33kDa). **(B)** Sequential Gβ Western analyses of corresponding auto-radiograms above. Gβ chemical identity of autoradiographic signals above (indicated by arrowheads) is validated by coincidence of molecular mass with Western signals. **(C)** Quantification of the influence of AC overexpression state on the ability of chronic morphine to modulate phosphorylation of Gβ present in the PKCγ IP in MOR-CHO (stippled bars), ACI MOR-CHO (lined bars), and ACII MOR-CHO (checkered bars). *, $p < 0.001$ for effect of chronic morphine on Gβ phosphorylation in ACII MOR-CHO vs. MOR-CHO or ACI MOR-CHO (n = 4-5 for MOR-CHO and ACI MOR-CHO; n = 9 for ACII MOR-CHO).

revealed no significant effect of chronic morphine on G_β phosphorylation (37kDa: t_8 = -1.54, p = 0.161; 33kDa: t_8 = -0.92, p = 0.383) (Figure 6C).

Negation of the ability of chronic morphine to increase G_β phosphorylation in ACII MOR-CHO would contribute to the loss of a chronic morphine shift in MOR signaling from $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory AC signaling. This suggests that like AC phosphorylation, the ability of chronic morphine to increase G_β phosphorylation is dependent upon the direction of acute responsiveness to MOR activation (inhibitory vs. stimulatory).

Sequential G_β Western analysis of the PKCγ IP used for phosphorylation studies was performed to validate chemical identity of autoradiographic bands and to assess if altered G_β membrane protein content could confound interpretation of the effect of ACII overexpression on the increment in G_β phosphorylation that occurs following chronic morphine. Chronic morphine did not produce any discernible change (<10%) in the protein content of either the ≈37 kDa or ≈33 kDa G_β species that co-immunoprecipitates with PKCγ from either MOR-CHO [57] (Figure 6B, lanes 1-2) or ACII MOR-CHO (Figure 6B, lanes 3-4).

3.4b) Chronic morphine augments the phosphorylation state of G_β in ACI-overexpressing MOR-CHO

In order to eliminate any potential confound of AC overexpression per se on G_β phosphorylation and effects thereon of chronic morphine, we determined whether or not

overexpression of ACI had effects on G_β phosphorylation similar to those of ACII. The ability of chronic morphine to augment G_β phosphorylation was once again manifest in membranes of ACI-overexpressing MOR-CHO treated chronically with morphine (Figure 6A, lanes 5-6). Autoradiographic analyses revealed that chronic morphine increased $^{32}P_i$ incorporation into both the ≈37 kDa (≈69%; $t_4 = 5.72$, $p = 0.005$), and ≈33 kDa (≈76%; $t_4 = 3.89$, $p = 0.018$) molecular mass species (Figure 6C). This occurred in the absence of any detectable change in G_β membrane protein content (Figure 6B, lanes 5-6).

We used one-way ANOVA to formally compare the changes in levels of G_β phosphorylation in untransfected MOR-CHO, ACI, and ACII MOR-CHO subject to chronic morphine (≈37kDa, $F_{2, 17} = 20.666$, $p < 0.001$; ≈33kDa, $F_{2, 17} = 12.308$, $p = 0.001$). Post-hoc analysis (Tukey HSD) revealed there was no significant difference in the magnitude of chronic morphine-induced G_β phosphorylation in untransfected MOR-CHO versus ACI MOR-CHO (≈37kDa, $p = 0.979$; ≈33kDa, $p = 0.666$). But chronic morphine did significantly enhance G_β phosphorylation in untransfected versus ACII MOR-CHO (≈37kDa, $p < 0.001$; ≈33kDa, $p = 0.013$), and in ACI versus ACII MOR-CHO (≈37kDa, $p < 0.001$; ≈33kDa, $p = 0.001$).

3.5) Chronic morphine fails to increase membrane translocation of PKCγ in ACII overexpressing MOR-CHO

It is well established in spinal cord and cell lines that chronic morphine increases membrane translocation of PKCγ [52]. In MOR-CHO, chronic morphine elicits an ≈80% increase in membrane content of PKCγ [54]. Increased membrane content of PKCγ and its association with $G_{\beta\gamma}$ following chronic morphine treatment of MOR-CHO could be a

major contributor to augmented phosphorylation of G_β under this condition. Conversely, the inability of chronic morphine to increase G_β phosphorylation and PKCγ membrane translocation in ACII MOR-CHO could suggest a relative decrease in PKCγ activity and/or in translocation to the membrane. This possibility was investigated by performing semi-quantitative PKCγ Western analyses of membranes of untransfected, ACI, and ACII MOR-CHO. Lack of the chronic morphine-induced increase in PKCγ translocation in ACII MOR-CHO would also explain the previously observed decrease in AC phosphorylation in the same preparations.

Western analyses of membranes of MOR-CHO revealed a signal of ≈82-85 kDa (Figure 7A), consistent with previous western analyses of membrane PKCγ [56]. In untransfected MOR-CHO (Figure 7A, lanes 1-2), there was a ≈120% increase in membrane PKCγ in the chronic morphine-treated cells compared with naïve cells (t_2 = 25.06, p = 0.002; Figure 7B). However, in ACII MOR-CHO, chronic morphine failed to elicit a significant increase in membrane PKCγ (Figure 7A, lanes 3-4) (t_6 = -1.52, p = 0.180; Figure 7B). In contrast, in ACI MOR-CHO, chronic morphine once again produced a significant increment in the membrane content of PKCγ (Figure 7A, lanes 5-6) (≈70%; t_2 = 38.63, p = 0.001; Figure 7B).

We used one-way ANOVA to formally compare the changes in levels of membrane PKCγ in untransfected MOR-CHO, ACI, and ACII MOR-CHO subject to chronic morphine. This analysis revealed a significant interaction between the ability of chronic morphine to augment membrane PKCγ content and AC overexpression state ($F_{2, 12}$ = 39.432, p < 0.001). Post hoc analysis (Tukey HSD) revealed a significant difference in the effect of chronic morphine on membrane PKCγ content in untransfected MOR-

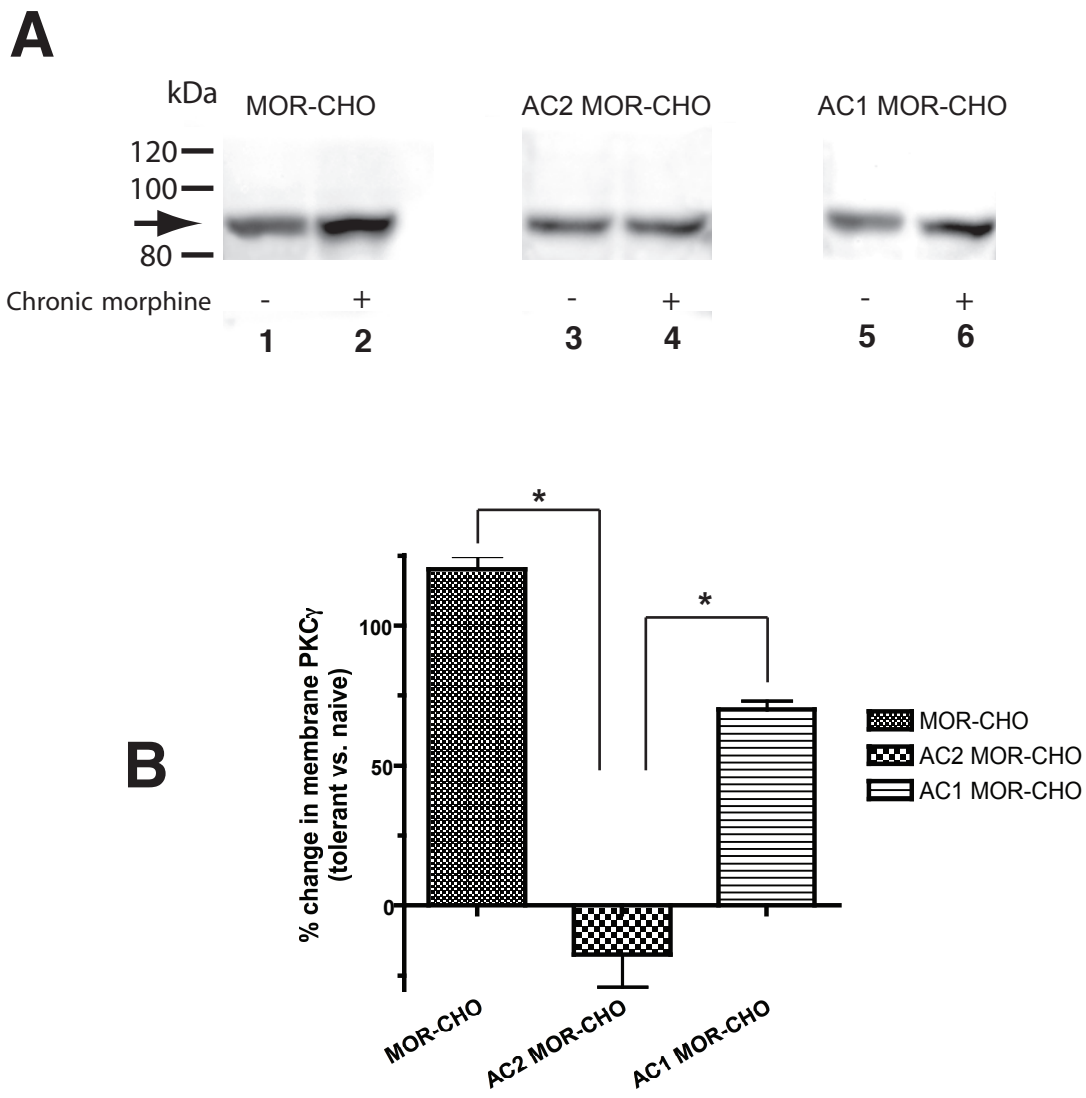

Figure 7A-B: Chronic morphine augments membrane translocation of PKCγ in MOR-CHO and ACI MOR-CHO, but not ACII MOR-CHO. (A) PKCγ Western analyses of membranes obtained from opioid naive (-) and chronic-morphine treated (+) MOR-CHO (lanes 1 and 2), ACII MOR-CHO (lanes 3 and 4), and ACI MOR-CHO (lanes 5 and 6). Arrowheads indicate membrane PKCγ protein (~85 kDa). **(B)** Quantification of the influence of AC overexpression state on the ability of chronic morphine to modulate membrane PKCγ levels in MOR-CHO (stippled bars), ACI MOR-CHO (lined bars), and ACII MOR-CHO (checkered bars). *, $p < 0.001$, for effect of chronic morphine on membrane PKCγ in ACII MOR-CHO versus MOR-CHO or ACI MOR-CHO (n = 3 for MOR-CHO and ACI MOR-CHO; n = 7 for ACII MOR-CHO).

CHO versus ACII MOR-CHO ($p < 0.001$), as well as that in ACI versus ACII MOR-CHO ($p = 0.001$).

3.6) Reversal of chronic morphine-induced augmentation to diminution of MOR-$G_{s\alpha}$ signaling in ACII-transfected MOR-CHO

The increased association of MOR and G_s following chronic morphine treatment, first biochemically demonstrated by Chakrabarti et al. [60], represents a parallel mechanism for shifting MOR-coupled signaling from inhibition to stimulation of AC. The adaptation of augmented MOR-G_s coupling, resulting in the increased generation of activated $G_{s\alpha}$ and its direct activation of AC, would act in concert with those that mediate increased MOR-coupled $G_{\beta\gamma}$ stimulation of AC to mask any ongoing G_i/G_o-mediated inhibition of AC and thus contribute to the manifestation of opioid tolerance [10,36]. If tolerance adaptations are protective and thus depend on initial cellular starting conditions, the enhanced coupling of MOR to G_s following chronic morphine should not be observed in ACII MOR-CHO. To test this prediction, we assessed the ability of chronic morphine to modulate MOR-$G_{s\alpha}$ association in untransfected, ACI, and ACII MOR-CHO. MOR-$G_{s\alpha}$ association was determined by quantifying the content of MOR in immunoprecipitate obtained, using anti-$G_{s\alpha}$ antibodies ($G_{s\alpha}$ IP), from membranes of the above cells, as was done in the initial biochemical demonstration of MOR-$G_{s\alpha}$ association and its augmentation by chronic morphine [60].

A Western signal of ≈85 kDa was observed in $G_{s\alpha}$ IP in all groups (Figure 8A), corresponding to presence of a MOR species [60]. The content of MOR in the IP obtained from membranes of chronic morphine treated MOR-CHO was significantly greater than

that obtained from membranes of naïve MOR-CHO (Figure 8A, lanes 1-2) (≈93%; t_5 = 3.77, p = 0.013; Figure 8B). Importantly, chronic morphine did not increase the content of $G_{s\alpha}$ present in the $G_{s\alpha}$ IP validating previous findings that chronic morphine produced a net increase in MOR-$G_{s\alpha}$ association [60].

Strikingly, chronic morphine failed to increase the co-immunoprecipitation of MOR with $G_{s\alpha}$ in ACII-MOR-CHO (Figure 8A, lanes 3-4). In fact, as was observed for AC phosphorylation, in ACII MOR-CHO, chronic morphine results in a diminution (≈15%) of MOR-$G_{s\alpha}$ co-immunoprecipitation. This diminution was modest in magnitude but did reach statistical significance (t_5 = -2.81, p = 0.037; Figure 8B). Notably, in ACI MOR-CHO, the ability of chronic morphine to augment MOR $G_{s\alpha}$ co-immunoprecipitation was again manifest. In ACI overexpressing MOR-CHO, chronic morphine augmented the MOR co-immunoprecipitate by ≈121% (t_4 = 11.77, p < 0.001; Figure 8B), eliminating the potential confound of increased AC overexpression per se on this adaptation (Figure 8A, lanes 5-6).

We used one-way ANOVA to formally compare the effect of chronic morphine on MOR $G_{s\alpha}$ co-immunoprecipitation from membranes of untransfected, ACI, and ACII MOR-CHO. There was a significant interaction between the ability of chronic morphine to increase MOR-$G_{s\alpha}$ co-immunoprecipitation and the state of AC overexpression ($F_{2, 16}$ = 26.694, p < 0.001). Post hoc analysis (Tukey HSD) revealed a significant difference in the ability of chronic morphine to alter MOR-$G_{s\alpha}$ co-immunoprecipitation in membranes of untransfected MOR-CHO versus ACII MOR-CHO (p = 0.001), ACI versus ACII MOR-CHO (p < 0.001), and in untransfected MOR-CHO versus ACI MOR-CHO (p = 0.039).

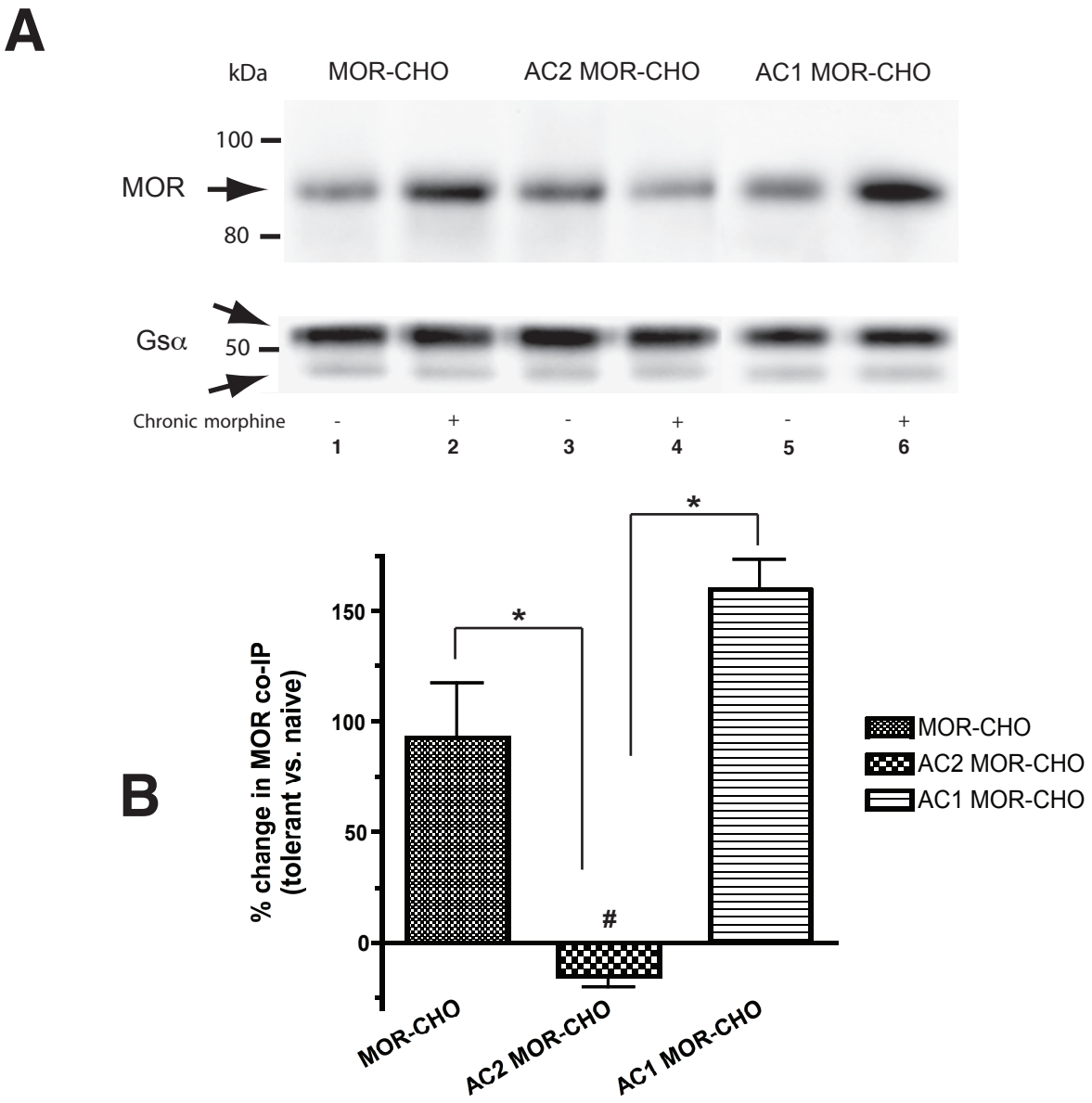

Figure 8A-B: Chronic morphine augments the content of MOR in Gsα IP obtained from membranes of MOR-CHO and ACI MOR-CHO, but not ACII MOR-CHO. (A) MOR Western analyses of Gsα IP obtained from membranes of opioid naive (-) and chronic-morphine treated (+) MOR-CHO (lanes 1 and 2), ACII MOR-CHO(lanes 3 and 4), and ACI-CHO (lanes 5 and 6). The upper and lower portions of nitrocellulose membranes were immunoblotted with anti-MOR and anti-Gsα antibodies, respectively. Upper arrowhead indicates MOR (~85kDa) that co-immunoprecipitates with Gsα (lower arrowheads). Since the content of Gsα in the Gsα IP was not influenced by chronic morphine, its ability to augment MOR-Gsα coimmunoprecipitation reflects their increased association in membranes of MOR-CHO and ACI MOR-CHO, which is prevented by overexpression of ACII. **(B)** Quantification of the influence of AC overexpression state on the ability of chronic morphine to modulate MOR-Gsα association in MOR-CHO(stippled bars), ACI MOR-CHO (lined bars), and ACII MOR-CHO (checkered bars). *, $p < 0.001$, for the chronic morphine-induced increment in MOR-Gsα association in ACII MOR-CHO vs. MOR-CHO or ACI MOR-CHO (n = 5-6 for MOR-CHO and ACI MOR-CHO; n = 5 for ACII MOR-CHO). #, $p < 0.001$, for the chronic morphine-induced decrement in MOR-Gsα association in ACII MOR-CHO.

4) Discussion

Plasticity of opioid tolerance: effect of AC overexpression

Experiments were designed to test the hypothesis that adaptations to chronic morphine are not hard-wired and invariant but instead depend on initial starting dynamic interactions among signaling molecules. This hypothesis rests on the formulation that opioid tolerance is protective and is designed to counteract consequences of the sustained opioid receptor activation. It also rests on the formulation that the loss of action of opioids following their sustained presence does not result from loss of opioid receptor functionality but instead from the alteration of interactions among signaling molecules that initially resulted from opioid receptor activation.

To test this hypothesis, we compared several chemical sequelae of chronic morphine in three cell lines: untransfected MOR-CHO, ACII MOR-CHO and ACI MOR-CHO. This approach was predicated on the demonstration by Henry Bourne that overexpression of a $G_{\beta\gamma}$ stimulated AC isoform would shift the consequences of activating a G_i-coupled receptor, e.g., dopamine, from inhibition to stimulation of AC [39]. Accordingly, we decided to compare responsiveness to chronic morphine of ACII MOR-CHO and MOR-CHO based on the prediction that they would manifest opposite responsiveness to acute MOR activation; whereas acute activation of MOR in MOR-CHO inhibits AC activity and cAMP formation, the opposite should be observed in ACII MOR-CHO. This prediction was validated in initial experiments that re-affirmed that acute MOR activation inhibited AC activity in MOR-CHO but stimulated AC activity in MOR-CHO overexpressing ACII. Furthermore, in these cells exhibiting opioid stimulation, opioid tolerance was demonstrated through chronic morphine treatment

such that AC activity remained unchanged despite continued AC stimulation. The demonstrations of default opioid stimulation and tolerance in ACII MOR-CHO validated our investigation of tolerance adaptations in these cells.

Use of ACI MOR-CHO: importance of isoform-specific $G_{\beta\gamma}$ regulation of AC

If opioid tolerance subserves a protective function by minimizing the continued cellular perturbations caused by the persistent activation of MOR, chronic morphine-induced adaptations present in MOR-CHO should not be observed, or should be reversed, in ACII MOR-CHO. Chronic morphine responsiveness of ACI MOR-CHO was also selected for comparison because in contrast to ACII MOR-CHO, ACI MOR-CHO is overexpressing an AC isoform that is negatively regulated by the $G_{\beta\gamma}$ subunit [37]. As such, manifestation of the same adaptations to chronic morphine in MOR-CHO and ACI MOR-CHO would eliminate AC protein overexpression per se as a confounding variable.

Opioid tolerance in MOR-CHO

Chemical sequelae of chronic morphine exposure selected for comparison among the three cell lines are those discovered in the Gintzler laboratory, all of which converge to shift acute responsiveness to MOR activation from AC inhibitory to AC stimulatory [10,36]. This reversal occurs from the chronic morphine-induced modulation of two parallel signaling pathways. It involves a shift in MOR-coupled signaling from predominantly $G_{i\alpha}$-inhibition to $G_{\beta\gamma}$-stimulation of AC [10,36], as well as through enhanced coupling of MOR to G_s [60]. Both alterations would result in a convergence of MOR-coupled signaling from inhibition to activation of AC.

In MOR-CHO as well as LMMP tissue and spinal cord, the shift from MOR-coupled signaling from $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory is multi-factorial. Chronic morphine up-regulates $G_{\beta\gamma}$ stimulated AC isoforms [47], their increased phosphorylation [45,47-50] as well as the phosphorylation of the G_{β} subunit of $G_{\beta\gamma}$ [51]. Phosphorylation of G_{β} and AC occurs as a result of the activity of protein kinase Cγ (PKCγ), whose membrane concentration is significantly augmented by chronic morphine [52-54]. Notably, PKCγ exists, at least in part, in a multi-molecular signaling complex containing AC and $G_{\beta\gamma}$ [57].

The consequences of the above sequelae of chronic morphine are not only convergent but are also multiplicative. For example, phosphorylation of G_{β} not only increases the potency of $G_{\beta\gamma}$ to stimulate AC but also increases its availability to do so [51]. Similarly, an increase in $G_{\beta\gamma}$-stimulated AC isoforms would augment the AC stimulatory consequences of generating $G_{\beta\gamma}$ subunits from heterotrimeric G proteins that are coupled to MOR [46]. This is further augmented by their enhanced phosphorylation [50], which increases their stimulatory responsiveness to $G_{\beta\gamma}$ [69,70]. Because of the interconnectedness and synergistic consequences of the above adaptations, their offset would have reciprocal consequences of comparable magnitude. Loss of any, alone or in combination, would impede any shift in signaling from a MOR-coupled inhibitory to a MOR-coupled excitatory state. Moreover, any net reduction in phosphorylation of AC and G_{β} and membrane translocation of PKCγ would produce a qualitatively opposite shift in MOR responsiveness, i.e., from excitatory to inhibitory.

Chronic morphine-induced augmented MOR-G_s coupling occurs concomitantly with enhanced MOR-coupled $G_{\beta\gamma}$ stimulation of AC. Increased MOR signaling via G_s is

a parallel adaptation that would act in concert with the emergence/augmentation of the $G_{\beta\gamma}$ stimulatory arm of G_i/G_o coupling. As a result, activation of MOR would not only stimulate AC via the generation of $G_{\beta\gamma}$ (from both G_i and G_s) but would also stimulate AC via the generation of $G_{s\alpha}$ and its direct activation of AC. Notably, phosphorylation of AC (a sequelae of chronic morphine) increases its stimulatory responsiveness to $G_{s\alpha}$ [41,70,71]. As is the case for adaptations that subserve MOR-coupled $G_{\beta\gamma}$ activation of AC, attenuation of the increment in MOR-G_s coupling or a net decrement thereof would further block or reverse, respectively, the chronic morphine-induced shift from MOR-coupled inhibition to AC activation.

Opioid tolerance in ACII MOR-CHO: reversal of MOR-CHO findings

Of critical importance, all of the sequelae of chronic morphine delineated above (augmented AC/G_β phosphorylation, increased membrane translocation of PKCγ, increased MOR G_s coupling), all of which converge to shift the acute consequences of MOR activation from inhibition to stimulation of AC, continued to be manifest in ACI MOR-CHO. These findings demonstrate that overexpression of AC protein per se do not alter previously demonstrated adaptations to chronic morphine. This was a key finding because it indicated that effects of overexpressing ACII on adaptations elicited by chronic morphine could be attributed to specific attributes of the ACII isoform and that mechanistic inferences and interpretations would not be confounded by non-specific consequences of a per se increase in AC protein.

In contrast to findings in MOR-CHO and ACI MOR-CHO, chronic morphine neither augmented AC and G_β phosphorylation, increased membrane translocation of

PKCγ nor increased MOR-$G_{s\alpha}$ coupling in chronic morphine-treated ACII-MOR-CHO. Thus, in a cell line overexpressing ACII, in which acute activation of MOR, in the absence of prior exposure to chronic morphine, produces an activation of AC, adaptations resulting in MOR-coupled stimulation of AC are no longer manifest.

Strikingly, not only was the previously observed increment in AC phosphorylation in ACII MOR-CHO obliterated, there was a net decrease in AC phosphorylation, PKCγ membrane translocation and MOR-G_s coupling observed following chronic morphine. The decrement in AC phosphorylation and MOR-G_s coupling reached statistical significance. The decrement in membrane PKCγ translocation just missed significance; an increase in the number of determinations would have enabled significance to be reached. This could explain the observation that the increment in phosphorylation of the G_β subunit following chronic morphine is no longer manifest in MOR-CHO overexpressing ACII. The inability of chronic morphine to increase G_β phosphorylation in ACII MOR-CHO means these cells have less phosphorylated G_β with which to stimulate type II AC. The decrement in the chronic morphine-induced increase in PKCγ translocation in ACII MOR-CHO may also explain the decrement in AC phosphorylation in the same preparations. These observations reveal that chronic morphine elicits adaptations that shift the consequences of MOR-coupled signaling from stimulation to inhibition of AC in ACII MOR-CHO, opposite the consequences of those adaptations to chronic morphine demonstrated in MOR-CHO and ACI MOR-CHO.

Opioid tolerance is not hard-wired but a graded phenomenon

Thus to underscore the main conclusion, our observations in ACII MOR-CHO indicate tolerant mechanisms are not hard-wired but instead are conditional on cell state; the default acute responsiveness of cells to MOR activation is a determinant of the direction of the mechanisms established in response to chronic morphine. Our findings in ACII MOR-CHO are graphically summarized in Figure 9. In many, if not all, cell systems, opioids have an initial inhibitory effect on cell signaling. In such cells, this elicits adaptations that augment a stimulatory arm of MOR-G protein coupling that is present in opioid naïve cells but masked by the more predominant inhibitory MOR-coupled signaling cascades. By augmenting this $G_{\beta\gamma}$-stimulatory arm, which does not require any change in the identity of the G proteins coupled to MOR, as well as the parallel association of MOR with the stimulatory G protein G_s, persistent inhibitory effects of MOR activation in MOR-CHO and ACI MOR-CHO are offset or neutralized. This gives the appearance of the loss of MOR-coupled signaling, and such loss of signaling is often (erroneously) inferred to be a predominant, if not exclusive underpinning of opioid tolerance. Inversely, in ACII MOR-CHO, the sustained stimulatory effects of MOR activation are offset by decreasing those parameters that are responsible for the shift to stimulation in MOR-CHO and ACI MOR-CHO (AC and G_β phosphorylation, PKCγ translocation, MOR-G_s coupling) (Figure 9). The current demonstration that adaptations utilized to counteract the continued activation/signaling of MOR are dependent on cell state underscore the paradox that seems to arise regarding mechanisms of tolerance formation. Tolerance mechanisms take advantage of the inherent plasticity of receptor-G protein-coupled signaling such that MOR activation produces functional consequences that oppose those that are triggered in the opioid naïve condition.

In cells that manifest acute stimulatory responsiveness to MOR activation prior to chronic morphine exposure, adaptations that augment that response would only exacerbate the initial perturbing effects. If tolerance were to be protective and counteract the persistence of initial perturbing effects of MOR activation, a qualitative

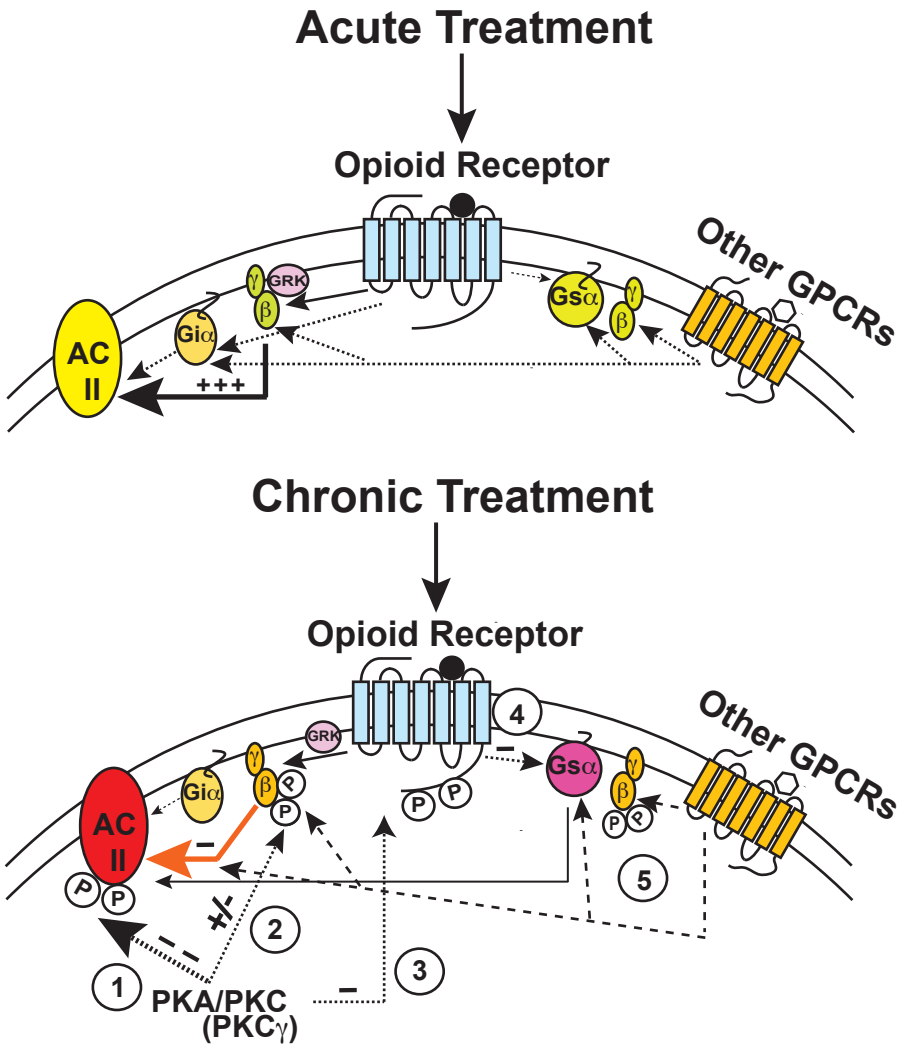

Figure 9: Post-opioid receptor adaptations to chronic morphine are reversed in a cell over-expressing ACII. Models of **(A)** Opioid receptor signaling during acute opioid treatment followed by **(B)** Altered opioid receptor signaling after chronic opioid treatment in a cell overexpressing ACII, a Gβγ-stimulatory AC isoform. In acute opioid signaling **(A)**, the preponderance of G protein signaling is via Gβγ subunits with AC stimulation, increased cAMP production and subsequent enhanced cellular signaling. The key adaptations to chronic morphine observed in the current study **(B)** seek to counterbalance the continued AC-stimulatory opioid presence by shifting the cell into a Gβγ-inhibitory state: 1: Down-regulation of AC phosphorylation, which decreases stimulation by Gβγ; 2: Failure of chronic morphine to augment phosphorylation of Gβ by PKC (PKCγ) and thereby the potency of Gβγ to stimulate AC; 3: Decreased membrane translocation of PKCγ to effect above adaptations; 4: Decreased MOR-coupled AC stimulation, mediated via the MOR-Gs association, which is weakened following chronic morphine; 5: Upon opioid withdrawal, the above noted changes in effector enzymes and signaling molecules elicited by chronic morphine would also be expected to alter responsiveness to other (non-opioid) neurotransmitters that signal via Gi/Go, Gs and AC, maintaining their AC-inhibitory effects. (Taken and adapted with permission from Gintzler and Chakrabarti, 2006)

change in acute MOR responsiveness should change the tolerant mechanisms required for adaptation to chronic morphine. Thus it is not surprising that in ACII MOR-CHO, those adaptations that were documented in MOR-CHO and ACI MOR-CHO were no longer observed. Indeed, tolerant-producing mechanisms are dynamic, are interconnected intricately with cell physiology, and exhibit substantial plasticity.

Adaptations to chronic morphine in ACII MOR-CHO: AC phosphorylation

Interpretation of the offset of some of the previously identified adaptations to chronic morphine in ACII MOR-CHO could be confounded by the augmentation of AC protein. For example, the observed decrease in AC phosphorylation in chronic morphine-treated ACII MOR-CHO could be due to a dilutional effect (i.e. saturation of kinase, etc). Several factors, however, mitigate this possibility. (1) AC phosphorylation in ACII-MOR-CHO was always determined in the presence and absence of chronic morphine, in parallel. Thus, any effect of overexpression, per se would be common to both. (2) As has been previously reported, chronic morphine increases AC protein [46]. However, the magnitude of this increment varied among experiments. Importantly, there was no correlation between the varied ability of chronic morphine to produce an additional increment in AC membrane content of ACII MOR-CHO and a decrement in AC phosphorylation. In fact, in some experiments, chronic morphine-induced decreases in AC phosphorylation occurred without any corresponding change in AC protein. In such cases, the chronic morphine-induced decrement in AC phosphorylation in ACII

MOR-CHO cannot be due to a dilutional effect. (3) The chronic morphine-induced increase in AC phosphorylation was manifest in MOR-CHO overexpressing ACI at levels comparable to those of ACII MOR-CHO, which did not manifest chronic morphine-induced increased AC phosphorylation.

Interestingly, there is an association between the level of ACII overexpression and the subsequent decrement in AC phosphorylation. The significant interaction between ACII overexpression levels and the magnitude of chronic morphine-induced decrement in AC phosphorylation shows that the plasticity exhibited by tolerance mechanisms is not an all-or-none phenomenon; graded responses can occur according to the starting conditions of the cell. This suggests the occurrence of a gradient of mechanisms and their magnitude, across regions of the brain and spinal cord, which are averaged in biochemical studies that utilize homogenates of these regions. It could also explain, at least in part, observations that many electrophysiological manifestations of tolerance are observed in some percentage of cells examined, never all.

In MOR-CHO, the inability of QEHA, a $G_{\beta\gamma}$ blocker [63], to fully abolish $rG_{s\alpha}$ stimulation of AC demonstrates a $G_{\beta\gamma}$-independent component of $G_{s\alpha}$-AC stimulation. This component may be attributed to the increased stimulatory responsiveness manifested by phosphorylated ACII and ACVII to $G_{s\alpha}$ [41,70,71]. The central role of AC phosphorylation by PKCγ in opioid tolerance is supported by taking into consideration the finding that the magnitude of the chronic morphine-induced increment in AC phosphorylation is substantially attenuated by chelerythrine, a PKC-selective inhibitor [48], and our observed chronic morphine-induced decrements in AC phosphorylation and membrane PKCγ content in ACII MOR-CHO. In targeted analysis of the AC isoform-

specific mRNA present in MOR-CHO revealed the presence of mRNA encoding ACs of the type II family, predominantly ACVII [57]. Similarly, in ACII MOR-CHO, the observed chronic morphine induced-decrement in AC phosphorylation most likely reflects not only decreased stimulatory responsiveness to $G_{\beta\gamma}$, but also to $G_{s\alpha}$.

Adaptations to chronic morphine in ACII MOR-CHO: relevance to dependence and withdrawal

The role of AC phosphorylation in tolerance formation in is underscored when we consider it in the context of dependence and withdrawal. The adaptations to chronic morphine described in ACII MOR-CHO may also explain the observation of a decrease in AC activity in ACII overexpressing cells upon opioid withdrawal [44]. As demonstrated in our study, in a cell exhibiting default opioid stimulation, tolerance mechanisms would counteract such stimulation with inhibition. When the opioid is subsequently removed, the inhibitory adaptations remain, and subject to interactions within other remaining receptor-G protein signaling pathways (#7, Figure 1; #5, Figure 9). Thus in ACII overexpressing cells treated with chronic morphine, opioid withdrawal would leave in place our observed adaptations (decreased stimulatory interactions between phosphorylated AC and G_{β}, decreased membrane translocation of PKCγ, and decreased MOR-$G_{s\alpha}$ coupling), converging to inhibit AC activity (#5, Figure 9). Indeed, Schallmach et al., in COS-7 cells overexpressing ACII, demonstrated that there was default opioid stimulation, but that upon chronic opioid withdrawal, there was a decrease in ACII activity [72]. In addition, this decrease in AC activity was mediated by phosphorylation in part by PKC [73]. This suggest that our observed decrements in AC phosphorylation and

membrane PKCγ in ACII MOR-CHO may be responsible for the decreased AC activity observed in opioid withdrawal.

Opioid tolerance vs. opioid addiction

In any discussion of opioid tolerance, the topic of abuse and addiction will invariably be raised. Whereas tolerance is often a phenomenon that is observable via concrete cellular and molecular adaptations, it is the drug-seeking behavior exhibited by abusers and addicts that are more readily apparent in the clinic. Furthermore, many physicians are concerned about contributing to an addicted patient, since even proper use of medications for pain, anxiety, and even hypertension produces tolerance and dependence. These patients who benefit from therapy may be denied treatment due to the manifestation of symptoms of tolerance and dependence, which are often mistaken for abuse. Thus, it is important to distinguish between physiologic tolerance and addiction.

Abuse and addiction can be considered behavioral syndromes that exist along a continuum from minimal use to abuse to addictive use [74]. In the best case, the definition for abuse and addiction can be described as flexible, and arbitrary in the worst. The most influential system of diagnosis for mental disorders is that published by the American Psychiatric Association. The APA defines addiction (or substance dependence) as a cluster of symptoms indicating that the individual continues use of the substance despite significant substance-related problems [75]. Tolerance and dependence may be part of the symptoms, but are neither necessary nor sufficient for the diagnosis. Addiction requires three or more of the symptoms, whereas abuse needs only one or two.

In discussing these events in a neurobiological context, abuse and addiction occur as drugs vary in their capacity to produce immediate good feelings, or euphoria in the user and those that have more intense euphoria are more likely to be taken repeatedly. The abuse liability of a drug is also enhanced by rapidity of onset because effects that occur sooner are more likely to initiate the chain of events that leads to loss of self-control. These reinforcing effects can establish the foundation for addiction. Reinforcing properties of a drug can be measured reliably in animals, and as such, medications can be screened for their potential for abuse in humans.

Reinforcing properties of drugs are associated with their capacity to increase neuronal activity in critical brain areas. Cocaine, amphetamine, ethanol, opioids, cannabinoids, and nicotine all reliably increase extracellular fluid dopamine levels in the ventral striatum, specifically the nucleus accumbens region [76-82]. Despite strong correlative findings, a causal relationship between dopamine and euphoria/dysphoria has not been established, and other neurotransmitters may be implicated as having a contributory role.

On a biochemical level, the mechanisms of tolerance and addiction do share some components; the dopaminergic reward pathway also acts via G-protein coupled receptors. The observations in our study suggest that the graded nature of opioid tolerance can extend to other neurotransmitter systems, including that of dopamine. The action of dopamine has already been shown to be dependent upon the initial starting condition (i.e. AC isoform distribution) [39], and indeed, physiologically, effects of drugs vary among individuals and the great variance, both qualitative and quantitative, in the enzymes involved in the receptor-mediated signaling may contribute to the different

degrees of reinforcement or euphoria observed. Future isoform-specific targeting of this pathway could have great implications on the diagnosis and treatment of abuse and addiction.

AC isoform distribution in the CNS

Our findings naturally lead to the question of the relevance of current findings to in vivo opioid tolerance mechanisms and variations thereof. To what extent does the relative abundance of AC isoforms among cells in the central nervous system, under physiological conditions, influence tolerant mechanisms that are utilized? Within the brain, there exist a multitudinous variety of cell types, which virtually assures a wide range of heterogeneity amongst even those cells within the same anatomical structure.

Studies which have attempted to map out the distribution patterns of AC isoforms have had mixed results. Most isoforms are expressed in varying quantities throughout the brain, and yet a few tend to be localized. To further complicate matters, within specialized areas such as the hippocampus and cerebellum, the distribution may change by individual layer. Among the type II family of AC isoforms (ACII, IV, and VII), ACII mRNA is found in several rat brain areas; strong mRNA hybridization signals are observed in cerebellum, hippocampus, and piriform cortex, and moderate signals in neocortex and substantia nigra [83]. ACVII mRNA is localized primarily to the cerebellar granule cell layer. Among isoforms that are not $G_{\beta\gamma}$ stimulatory, such as ACV and ACVIII, ACV is mainly restricted to the striatum and may be important for dopamine functions [84,85]. ACVIII is expressed in regions associated with learning and memory. The strongest signals are in the pyramidal cell layers of the CA2 and CA3 fields of the hippocampal formation and the granular cell layers of the dentate gyrus [86].

Future studies

Despite the breadth of distribution of all AC isoforms in the brain, virtually all of the opioid studies have shown that the default opioid action is inhibition. There are a couple of interesting isolated exceptions. First, work with the canine enteric nervous system demonstrated that the endogenous release of opioid peptides in the canine circular muscle layer would suppress or inhibit release of an inhibitory neurotransmitter, thereby increasing contractile activity [87,88]. The second instance is in the central nervous system and involves the $G_{\beta\gamma}$-mediated opioid stimulation pathway. The work of Olianas and Onali has investigated opioid action in the rat olfactory bulb, another location where type II AC isoforms are known to be preferentially expressed [89]. They demonstrated opioid receptor agonists stimulate AC activity in rat olfactory bulb [90] via the δ- or μ-opioid receptor [91]. Furthermore, through work with QEHA binding to ACII, it was demonstrated that this AC stimulation was mediated by $G_{\beta\gamma}$ subunits, the first example that opioid receptor-mediated AC stimulation can occur in native brain membrane tissue where the receptors, G proteins, and AC isoforms (mainly type II AC) are naturally expressed [92]. These examples notwithstanding, there remains little evidence that there are other brain regions or nervous systems (i.e. myenteric plexus) that exhibit opioid stimulation exclusively upon MOR activation; this explains why most work with opioid stimulation has mainly been conducted utilizing AC overexpression systems.

Furthermore, a current limitation to AC isoform analysis is the lack of a reliable commercial antibody to a singular AC isoform. Those used in our past and current studies either recognized all AC isoforms or type II AC (ACII, IV, and VII). Certainly,

distinguishing each and the role of each in the $G_{\beta\gamma}$-stimulatory shift seen in opioid tolerance would be desired in conjunction with a better understanding of their distribution in the CNS.

Finally, to frame our findings in a more global context, we remain far from fully elucidating the mechanisms of opioid tolerance and mapping out precise distributions of AC isoforms in the CNS, but we have underscored the graded nature of signaling between G-proteins and AC. Ideally, reaching such goals would resolve the problem of why there is a lack of uniformity in the presence of mechanisms of opioid tolerance. With any G protein-coupled receptor, as techniques to isolate local neuronal populations become more refined, future attempts to define AC isoform distribution will be more successful. Since the stoichiometry of cyclase within a receptor-G protein-AC signaling complex has proven to be crucial to neurotransmitter action, the identification of regional neuronal populations that share common cyclase regulation would help in understanding the local neurotransmitter response. With many disease states targeting localized regions of the CNS, such an understanding is pivotal in taking the first steps to more targeted and successful drug design.

Conclusion

The engineered cell overexpressing ACII and ACI utilized in these studies represent an extreme example of isoform distribution, as the AC mapping studies and the lack of available native cell systems indicate. *In vivo* circumstances will most likely represent a gradient of relative preponderance of one AC isoform versus another. It is unlikely that even within the olfactory bulb, there exists a cell containing a single isoform. Thus the implications of our findings go beyond simply the AC overexpression cell

systems. Extrapolation to complex integrated *in vivo* systems from our cell culture models suggests a parallel multiplicity in tolerance mechanisms harnessed that will vary in their individual contribution to tolerance depending on the functional state of each cell.

As exemplified by our finding of an association between the level of ACII overexpression and AC phosphorylation, the cell type and the starting conditions of AC isoforms are variables that have not always been accounted for, so when one evaluates the findings in a set of experiments for the purposes of defining a general tolerance mechanism, one must do so with extreme caution. As our studies have indicated, merely inserting opioid receptors or overexpressing a certain protein into a cell system without regard for the internal cellular milieu can have distorting effects on the observed sequelae. What we have shown in this study is that opioid tolerance is by no means hard-wired; within a unique internal cellular environment, there is a plasticity and pliability to the tolerance-producing mechanisms that allow the cell to deal with any external perturbations in search of equilibrium.

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