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Cellular and Synaptic Adaptations Mediating Opioid Dependence

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Williams, John T., MacDonald J. Christie, and Olivier Manzoni. Cellular and Synaptic Adaptations Mediating Opioid Dependence. *Physiol Rev* 81: 299–343, 2001. —Although opioids are highly effective for the treatment of pain, they are also known to be intensely addictive. There has been a massive research investment in the development of opioid analgesics, resulting in a plethora of compounds with varying affinity and efficacy at all the known opioid receptor subtypes. Although compounds of extremely high potency have been produced, the problem of tolerance to and dependence on these agonists persists. This review centers on the adaptive changes in cellular and synaptic function induced by chronic morphine treatment. The initial steps of opioid action are mediated through the activation of G protein-linked receptors. As is true for all G protein-linked receptors, opioid receptors activate and regulate multiple second messenger pathways associated with effector coupling, receptor trafficking, and nuclear signaling. These events are critical for understanding the early events leading to nonassociative tolerance and dependence. Equally important are associative and network changes that affect neurons that do not have opioid receptors but that are indirectly altered by opioid-sensitive cells. Finally, opioids and other drugs of abuse have some common cellular and anatomical pathways. The characterization of common pathways affected by different drugs, particularly after repeated treatment, is important in the understanding of drug abuse.

I. INTRODUCTION

The original notion that perturbations in central nervous system (CNS) functions produced by opioid drugs initiate homeostatic processes leading to the development of opioid dependence (191) stimulated attempts to explain the nature of the relevant adaptations in neurons responsible for opioid addiction. These studies have focused on identifying the biological basis of the core features of addiction to opioid drugs, particularly tolerance, the withdrawal syndrome, and compulsive use of the drug in the face of known harm. With repeated administration of opioid drugs, adaptive mechanisms are initiated that result in short-term as well as protracted changes in the functioning of opioid-sensitive neurons and neural networks. One such mechanism is the development of tolerance to opioid drugs, such that higher doses are required to gain the desired effect. Although associative or conditioned tolerance, where morphine treatment is always paired with a distinctive environment, plays an important

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role and is mediated by specific neural systems in behaving animals (e.g., Ref. 325), nonassociative or cellular tolerance is a process that has received considerable attention. There are two very general forms of nonassociative tolerance that develop in the CNS, isolated tissues, and cells: one is at the level of the opioid receptor, where effector coupling is reduced, and the second is at the cellular, synaptic, and network levels, where counteradaptive changes occur to bring about normal function despite the continued activity of the drug.

The mechanisms involved in the initiation of compulsive self-administration of many drugs seem to be seated in common central pathways, particularly those thought to mediate endogenous reward. With repeated administration of these drugs, adaptive mechanisms are initiated. One such mechanism is the development of tolerance. Another results from development of counteradaptations such that once the drug is removed a sequence of rebound signs and symptoms are manifested. This withdrawal syndrome has short-, long-, and very-long-term features that may include craving and relapse to drug use long after acute withdrawal has ended. Thus long-term adaptations induced by chronic opioid treatment are expressed in the absence of the triggering drug and indicate long-lasting change in the functioning of specific neural systems. It is intriguing that the mechanisms that seem to be responsible for these adaptive processes in neurons and synapses are reminiscent of mechanisms involved in "normal" plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), which are thought to form the cellular basis of memory. Indeed, recent work suggests that these adaptive processes at the cellular, synaptic, and network levels downstream from the receptor may hold the keys to understanding of addiction.

Perhaps the most important adaptations that develop as a result of chronic opioid administration occur in neural systems responsible for the transition from casual to compulsive drug use. Although tolerance and withdrawal surely contribute to this process, mechanisms involved in the initiation of compulsive self-administration of opioids as well as other major drugs of abuse seem to be seated in common central neural systems. The mesolimbic dopaminergic system, thought to have a crucial role in the rewarding actions of drugs of abuse, is a prime candidate for mediating this process. There is growing evidence (reviewed in Refs. 398, 449) that mesolimbic dopaminergic neurons are involved in strengthening formation of associations between salient contextual stimuli and internal rewarding or aversive events. The common long-term adaptations produced by opioids and other drugs of abuse in this system could enhance these processes and thereby play a major role in initiation and maintenance of compulsive drug use.

This review focuses on the adaptive changes in cellular and synaptic function induced by chronic morphine treatment. Opioids are known to be intensely addictive and share some general actions with other addictive drugs including psychostimulants and nicotine. One advantage that studies of the opioid system have over other addictive drugs has resulted from the massive research effort to find an opioid that is effective for the treatment of pain but lacks addictive properties. A plethora of compounds are available with varying affinity and efficacy at all the known opioid receptor subtypes. Although compounds of extremely high potency have been produced, the problem of tolerance to and dependence on these agonists persists.

The initial steps of opioid action are mediated through the activation of G protein-linked receptors. As is true for all G protein receptors, opioid receptors activate and regulate multiple second messenger pathways associated with effector coupling, receptor trafficking, and nuclear signaling. These initial effects are critical for understanding the early events leading to tolerance and dependence in cells that have opioid receptors. Equally important are network changes that occur as a result of the altered synaptic regulation that may affect downstream neurons that may not have opioid receptors. Finally, opioids and other drugs of abuse have some common cellular and anatomical pathways. The characterization of common pathways particularly after chronic drug treatment is an important extension in the understanding of drug abuse.

II. INITIAL STEPS OF OPIOID ACTION

A. Receptors/Ligands

Multiple opioid receptors were initially predicted on the basis of the actions of various alkaloid agonists and antagonists in whole animal preparations (155, 315). Soon after the discovery of endogenous opioid peptides, multiple opioid receptors were confirmed functionally using isolated pharmacological preparations (286). From these studies three major receptor subtypes were identified: μ, δ, and κ (161). Highly selective and potent ligands have been developed for each of the three general receptor subtypes (Fig. 1). At each of these receptors both agonists and antagonists exist that are >1,000-fold selective. As is always the case, however, the selectivity window of any ligand can be exceeded such that results obtained even with a highly selective agent may be misinterpreted. The issue of binding selectivity of ligands at the opioid receptor has been critically examined and reviewed by Goldstein (160).

Pharmacological studies have attempted to further divide opioid receptors in each of the three major subgroups. Although suggestive, pharmacologically defined subclasses of μ, δ, and κ receptors are not well estab-

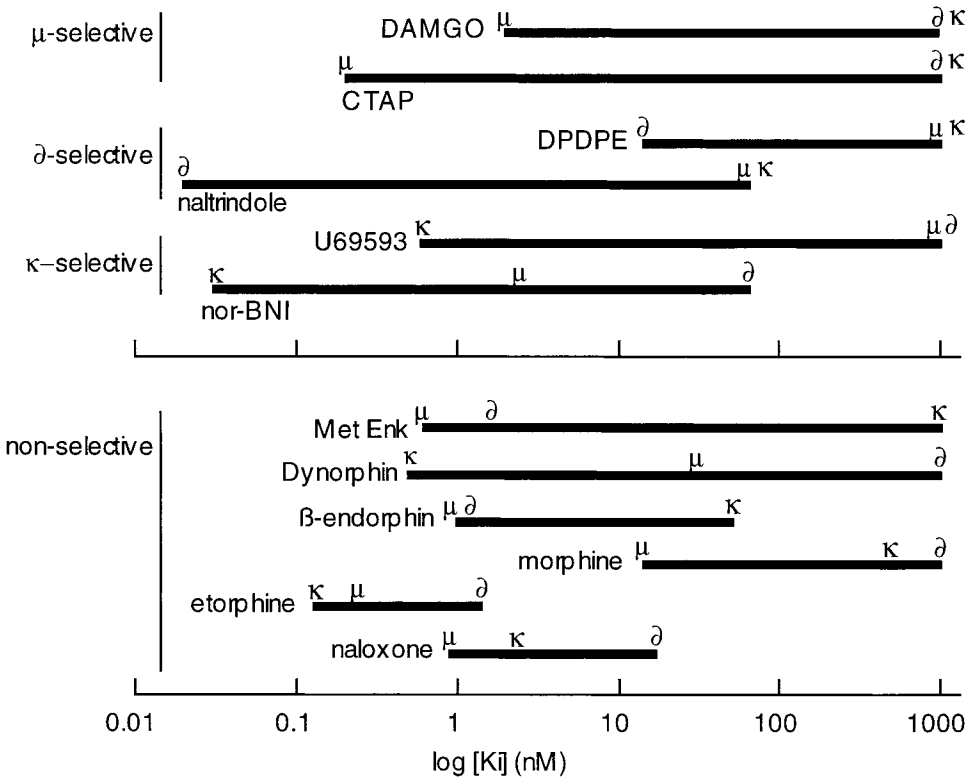

FIG. 1. An illustration of the selectivity windows of some commonly used opioid agonists and antagonists, determined in an expression system (392). *Top*: compounds tht are selective for each of the opioid receptors. Note that although nor-BNI is highly selective, the inhibition constant (K_i) at μ-receptors is ~3 nM. *Bottom*: the selectivity of the endogenous opioids and other commonly used opioids. Again note that none of the endogenous opioids show a high degree of selectivity. DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; nor-BNI, norbinaltorphimine; CTAP, H-D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2; DPDPE, [D-Pen(2),(5)]-enkephalin.

lished. The cloning of each of the three major opioid receptors has done little to support further expansion of opioid receptor classification (392, 393). There is ~60% sequence homology between the μ, δ, and κ receptors. Unless undiscovered opioid receptors are significantly different from receptors described to date, it appears that the opioid receptor family has been defined. There are reports of alternate splice variants, although it is not clear at what level they are expressed or if they can be distinguished pharmacologically (153, 256, 368, 421). The lack of molecular evidence for more than three opioid receptor subtypes indicates that further subclassification of receptors may result from mechanisms that may include post-translational regulation, receptor dimerization, or even interactions with accessory proteins.

The use of ligands with differing efficacy in tissues having varying receptor reserve is one potential confounding problem in the pharmacological classification of multiple opioid receptors. The description of the epsilon opioid receptor in the rat vas deferens is one such example. In this preparation β-endorphin decreased the muscle contraction evoked by electrically stimulating transmitter release from the nerves. Morphine was ineffective in this preparation. From this observation the β-endorphin selective epsilon receptor was characterized (151, 419). Subsequent work showed that the receptor reserve of μ-receptors in this preparation was low enough that a partial agonist, such as morphine, acted as a pure antagonist (442, 427). The characterization of multiple receptors based on results obtained in more complex tissues using indirect assays are subject to the same difficulties in interpretation.

It now appears that many G protein-linked receptors exist as dimers (108). The most dramatic demonstration of dimerization of G protein-linked receptors is with the $GABA_B$ receptor, where heterodimerization with two subtypes of the receptor are required for functional expression (228, 242, 271, 519). Both κ- and δ-opioid receptors have been reported to form homodimers. Recently, heterodimers of κ- and δ-opioid receptors were expressed in Chinese hamster ovary (CHO), HEK 293, and COS cells (229). The pharmacological profile of heterodimers was not completely characterized but differed from the homodimers of both δ- and κ-receptors. Heterodimerization of receptors in vivo could account for complex pharmacology even if there is only a single gene for each receptor.

The cellular and anatomical distribution of opioid receptors is important for the identification of neuronal systems and local networks involved in the initiation of drug action and the subsequent development of adaptations resulting in repeated drug use. Distinct distributions and developmental patterns of receptor and mRNA subtypes have been identified throughout the neuroaxis as well as in paracrine and exocrine tissues (14, 15, 307, 308, 547, 548) . The widespread occurrence of these receptors indicates that opioids have the potential for affecting multiple systems, both nervous and hormonal. The cellular distribution of μ- and κ-receptors seems to be largely

along the plasma membrane, both at somata as well as dendrites and nerve terminals. Receptors are generally found in perisynaptic areas, rather than in subsynaptic sites (336). The δ-receptor differs in that it is most often found within cells associated with vesicles (547). The activity-dependent redistribution of both κ- (432) and δ-receptors (R. Elde, personal communication) from vesicles to the plasma membrane suggests that the localization of receptors is not static and may vary considerably with activity. The identity and distribution of receptor subtypes in local circuits will be discussed in specific sections on neuronal systems.

B. Second Messengers/Effectors

Activation of any of the three opioid receptor subtypes produces common cellular actions. Each receptor is coupled to pertussis toxin-sensitive G proteins, although some coupling to the pertussis toxin-insensitive G protein G_z has also been recognized (see Ref. 99 for review). The profile of coupling of the three opioid receptors to the spectrum of G proteins is similar, although subtle differences have been identified (99). The most commonly reported actions include inhibition of adenylyl cyclase, activation of a potassium conductance, inhibition of calcium conductance, and an inhibition of transmitter release (Fig. 2). More recent observations have extended the actions of opioids to include the activation of protein kinase C (PKC), the release of calcium from extracellular stores, the activation of the mitogen-activated protein kinase (MAPK) cascade, and the realization that receptor trafficking plays an important role in receptor function.

1. Inhibition of adenylyl cyclase

Until recently, nothing was known of the physiological consequences of the acute inhibition of adenylyl cyclase by opioids. Two effects have now been identified: one is mediated by the modulation of a voltage-dependent current (I_h), which is also termed the pacemaker current (205, 471). This cation nonselective current is activated at hyperpolarized potentials to cause an inward current that depolarizes the membrane potential toward threshold. The voltage dependence of this current is regulated by cAMP, being activated at less negative potentials when cAMP levels are elevated (204). Opioids shift the voltage dependence to more negative potentials by decreasing intracellular cAMP. This inhibition was most easily observed after the activation of adenylyl cyclase with forskolin or PGE_2 (205) but has also been observed without prior activation (471). The consequence of this action of opioids was to decrease the amplitude of the inward current that drives spontaneous activity and thus decreases excitability. This action of opioids could have been predicted based on work done on the pacemaker current in sinoatrial nodal cells of the heart where the activation of M_2 muscarinic receptors shifted the voltage dependence of I_h through an inhibition of adenylyl cyclase (121). A family of these cation channels has now been cloned, some of which show the same cAMP-dependent changes in voltage dependence (152, 289, 412). A similar effect of opioids has also been observed on a tetrodotoxin-insensitive, cAMP-sensitive sodium current in cultured sensory neurons (158). Activation of adenylyl cyclase with prostaglandin E increased a sodium current that was depressed by [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-en-

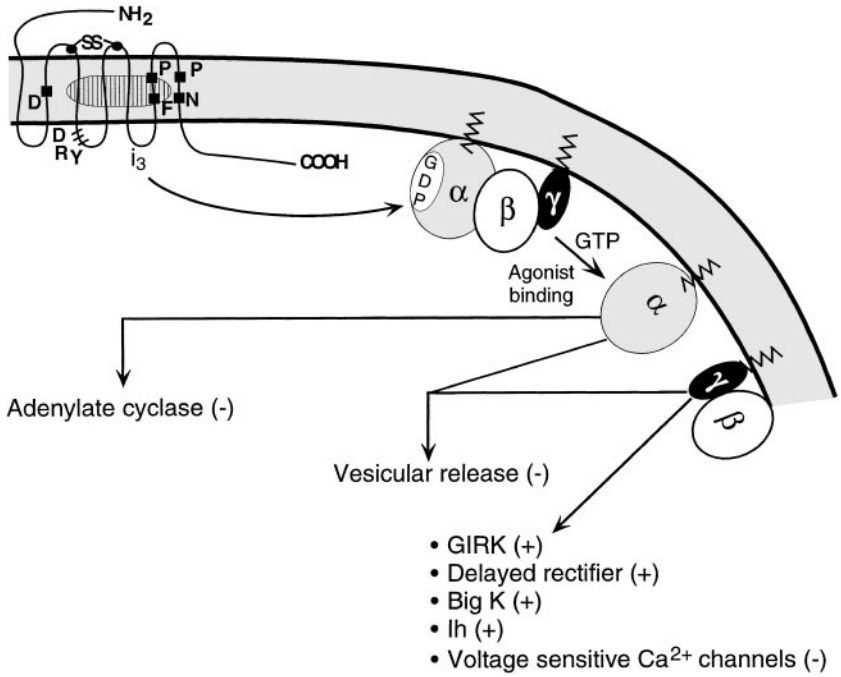

FIG. 2. An illustration of the best-characterized pathway of effector activation of opioids. Three primary classes of effectors include the inhibition of adenylyl cyclase, inhibition of vesicular release, and interactions with a number of ion channels. These effectors are affected by both the GTP-bound form of the α-subunit as well as free β/γ-subunits of pertussis toxin-sensitive G proteins. GIRK, G protein inwardly rectifying conductance.

kephalin (DAMGO). This effect, similar to the decrease in I_h, would be expected to reduce excitation caused by agents that are thought to mediate hyperalgesia.

The second consequence of the inhibition of adenylyl cyclase was an inhibition of transmitter release that was dependent on the activation of adenylyl cyclase (86, 203, 430). Previously there was no indication that the inhibition of adenylyl cyclase affected transmitter release. Under conditions where adenylyl cyclase was activated and caused an increase in transmitter release through activation of cAMP-dependent protein kinase (PKA), opioids decreased transmitter release via a PKA-dependent mechanism. This action of opioids was not observed at all opioid-sensitive synapses, which may suggest differential distribution of adenylyl cyclase isoforms at individual synapses (see sect. III*B4*).

An activation of adenylyl cyclase by opioids has been reported in both primary afferent neurons (105) and the olfactory bulb (362). Studies in the olfactory bulb indicate that the pA_2 for naloxone was ~8 [dissociation constant (K_d) = 10 nM], suggesting that this response was mediated through activation of δ-opioid receptors (362). The increase in adenylyl cyclase activity was not affected by pretreatment with cholera toxin and was blocked with pertussis toxin. More recently, the same group has found that the increase in adenylyl cyclase activity was mediated by the release of β/γ-subunits from pertussis toxin-sensitive G proteins (360). A similar mechanism for the opioid activation of adenylyl cyclase was proposed in a study using a membrane preparation of longitudinal muscle-myenteric plexus from guinea pigs chronically treated with morphine (72). Thus it appears that the opioid regulation of adenylyl cyclase is dependent on the isoform under study and the absence or presence of coactivated $G_s\alpha$. In the olfactory bulb it appears that the conditions are such that acute administration of opioids can activate the cyclase, whereas in other tissues, this response is observed only after adaptations induced by chronic morphine treatment.

2. Activation of potassium conductance

Opioids have been shown to activate at least three separate potassium conductances. The most commonly observed is the G protein-activated inwardly rectifying conductance (GIRK; Fig. 3, Table 1). All three opioid receptors have been shown to activate this conductance. The second messenger pathway is membrane delimited, mediated by a pertussis toxin-sensitive G protein (7), and it is presumed that the potassium conductance is activated by the β/γ-subunits (212). Rapid application and washout of opioids allowed the determination of the kinetics of opioid action using acutely dissociated cells (202). The activation of the potassium conductance had a latency to onset of 50–100 ms and a time constant of activation of ~700 ms, which is similar to that observed for other receptors coupled to GIRK channels (202, 444, 445). The termination of the GIRK current was dependent on the agonist applied. The rate of recovery was slower when higher affinity agonists were used, suggesting that receptor unbinding may be the rate-limiting step for deactivation.

The coupling of receptor to this potassium conductance was quite dependent on the experimental conditions. Whereas in brain slice experiments morphine produced an increase in potassium conductance that was equivalent to that induced by the peptide agonists DAMGO and [Met^5]enkephalin, it was an antagonist when tested in isolated cells. Under the same conditions, however, morphine had an agonist action on the inhibition of calcium currents. Thus it appears that the coupling of opioid receptors to GIRKs may be less efficient than to other effectors (202).

Opioids have also been shown to activate a voltage-dependent potassium conductance in acutely dissociated cells from hippocampus (526) and in brain slices (295). The activation of a voltage-dependent potassium conductance was also suggested based on the blockade of opioid inhibition of transmitter release by 4-aminopyridine and dendrotoxin (see sect. II *B4*). In addition, opioids have been reported to activate the BK calcium-sensitive potassium conductance (490). This effect, coupled with recent reports of opioid-induced calcium release from internal stores (100, 217, 219, 475, 476), indicates the diversity of opioid action, which has been recognized to occur with other G protein-coupled receptors.

3. Inhibition of calcium conductance

There are many examples of the inhibition of calcium currents by activation of all opioid receptor subtypes (Table 1). The inhibition of high-threshold calcium currents by opioids, in common with other receptors linked to pertussis toxin-sensitive G proteins, *1*) is membrane delimited, *2*) is mediated by the β/γ-subunits of G proteins, *3*) decreased the rate of current activation such that the inhibition was greater immediately after the voltage step, and *4*) showed relief of inhibition following a depolarization to positive potentials (521). The kinetics of activation of this effect of opioids were similar to that reported for the activation of potassium conductance, having a latency of onset of ~150 ms and peaking in ~5 s (Fig. 3, Ref. 521).

4. Inhibition of transmitter release

The opioid inhibition of acetylcholine release in the guinea pig ileum and ATP release in the vas deferens has been used as pharmacological assays for many decades (187, 265, 371). In various peripheral preparations from

different species, the activation of all three receptor subtypes has been found to cause inhibition of transmitter release (264, 318, 507). The activation of potassium conductance and/or the inhibition of calcium conductance and not the inhibition of adenylyl cyclase have been argued to account for this action (35, 355, 415), although recent work suggests that under some conditions the inhibition of adenylyl cyclase can also account for some of the decrease in transmitter release (see sect. III *B*). Direct inhibition of the release machinery, independent of potassium and calcium conductances, has also been reported (65).

Depending on the site, opioids inhibit release of excitatory and/or inhibitory transmitters (Table 1). Opioid inhibition of GABA release in local circuits, first observed in the hippocampus, has become a common observation that accounts for indirect excitatory, or disinhibitory, effects of opioids (351, 549). Opioids caused direct hyperpolarization of interneurons, thus decreasing excitability of these cells (296). In addition, spontaneous quantal release of GABA from terminals was decreased by opioids, suggesting that opioids also acted directly on axon terminals to decrease the probability of GABA release (92). A similar disinhibitory mechanism mediated by opioids acting on local circuits has now been described in brain regions where the local circuitry is not as well defined, such as the raphe magnus (370), ventral tegmental area (225), periaqueductal gray (PAG) (498, 499), and dorsal raphe (226, 227). This indirect action of opioids on output neurons from nuclei such as the VTA and PAG may be critical in the understanding of circuit adaptations in response to chronic morphine treatment.

Of equal significance is the fact that transmitter release is the result of a complex series of events with numerous protein-protein interactions such that there are multiple sites of potential regulation. Opioid receptors are one of a vast number of G protein-linked receptors that modify transmitter release. Given the potential interactions between these receptors and the effects of prior activity in any given terminal, the effects of opioids may vary considerably. Some of the consequences of the receptor interactions have been identified in the form of

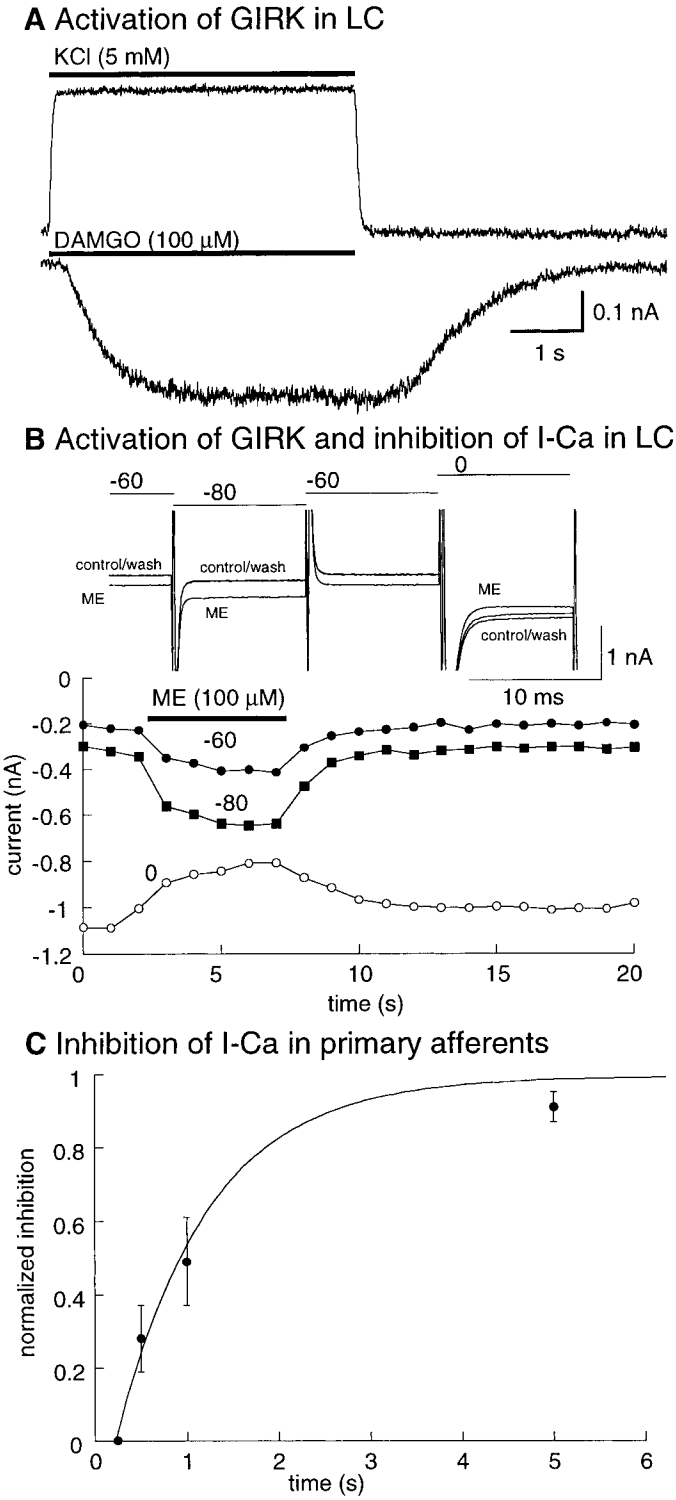

FIG. 3. The kinetics of opioid action on potassium and calcium conductances are similar. *A*: activation of opioid receptors on actuely isolated locus coeruleus (LC) neurons increases a potassium conductance with a time constant between 0.6 and 0.8 s (*bottom trace*; Ref. 202). The opioid activation of the potassium currents is much slower than the time course of solution exchange as illustrated in the *top trace*. *B*: time course of opioid action on potassium and calcium currents is comparable. *Top traces* show the voltage-clamp protocol. The traces below are superimposed current traces carried out in the absence and presence of [Met]enkephalin (ME) and showing an inward current at −60 and −80 mV and a decrease in the inward (calcium) current measured at 0 mV. The sharp inward current seen at the start of the step to 0 mV is a sodium current (no tetrodotoxin). The plot below shows the time course of these actions. The increase in potassium conductance and the inhibition of calcium current are mirror images. *C*: the onset of opioid inhibition of calcium current (I_{Ca}) measured in outside-out membrane patches from primary afferent neurons (521). With the rapid application of DAMGO (1 μM), there was a delay of 150 ms before any inhibition, and the time course of inhibition was fit by an exponential having a time constant of 1.3 s. [From Wilding et al. (521). Copyright 1995 by the Society for Neuroscience.]

TABLE 1. *Opioid receptors and effectors*

Effector	Receptor	Area/Cell	Reference No.
$G_{K(GIRK)}$	μ	Locus coeruleus	11, 202, 326, 374, 485, 522, 523
		Substantia gelatinosa	170, 414, 540
		PAG, medulla	84, 124, 178, 184, 365, 370
		Hippocampus	296, 470, 471, 526
		Nigra/VTA	225, 273
		Hypothalamus	76, 241, 248, 285, 339
		Striatum	214
		Thalamus	55
		Amygdala	462
		Parabrachial	88
		Myenteric	146
		Xenopus oocytes	267
	δ	Striatum	214
		Submucous plexus	324, 356, 478
		Cingulate cortex	474
		Hippocampus	470
		Vestibular	463
		Xenopus oocytes	199
	κ	Substantia gelatinosa	169
		Raphe magnus	369
		Xenopus oocytes	188, 199, 294
$G_{K(Ca)}$		Adrenal chromaffin	490
$G_{K(voltage)}$		Hippocampus	295, 331
		Supraoptic	339
G_{Ca}	μ	Primary afferent	177, 228, 327, 354, 407, 416, 417, 446, 472, 521
		Parasympathetic	4
		Locus coeruleus	97, 202
		PAG	98, 251
		Supraoptic	447
		Striatum	451
		NG108-15	190, 217, 218, 332
		GH_3	381
		SH-SY5Y	424
	κ	Primary afferent	167, 461
		Purkinje	238
		Neuroendocrine	406
		Xenopus oocytes	237
		GH_3	380
		PC12	473
	δ	Primary afferent	2
		Parasympathetic	335
		Striatum	451
		NG108-15	332
		SH-SY5Y	483
G_{Na}	μ	Primary afferent	158
G_{cation}	μ	Primary afferent	205
		LC	11
		PAG	E. E. Bagley and M. J. Christie, unpublished data
		Hippocampal	471
		Synaptic inhibition	
Glutamate	μ	LC	359
		Spinal cord	57, 157, 168, 193, 213, 239
		PAG	83, 394, 498
		Hippocampus	65, 66, 103, 131, 410
		Hypothalamus	131
		Striatum	214
		Accumbens	54, 312, 542
		VTA	311
	κ	LC	317, 379
		Hippocampus	123, 147, 209, 215, 410, 437–439, 503, 504, 513
		Hypothalamus	131
		Substantia gelatinosa	389
		Striatum	391
	δ	Spinal cord	157
		Striatum	214
		Accumbens	542
		NTS	394
		Cingulate cortex	474
GABA	μ	Amygdala	462
		Raphe magnus	370
		Accumbens	85, 450, 542; J. M. Brundege and J. T. Williams, unpublished data
		PAG	83, 87, 394, 499
		Hippocampus	70, 92, 145, 216, 275, 290–292, 526, 527, 536
		Substantia gelatinosa	168, 258
		VTA	225, 430
	κ	VTA	430
	δ	Amygdala	462
		Hippocampus	92, 145, 216, 290–292, 536
		Substantia gelatinosa	168, 258
		Accumbens/GP	450, 542
		NTS	394
Glycine		Substantia gelatinosa	168
Acetylcholine		Hypogastric	399

$G_{K(GIRK)}$, G protein inwardly rectifying potassium conductance; $G_{K(Ca)}$, calcium-activated potassium conductance; $G_{K(voltage)}$, voltage-activated potassium conductance; $G_{calcium}$, calcium conductance; G_{Na}, sodium conductance; G_{cation}, cation conductance; PAG, periaqueductal gray; VTA, ventral tegmental area; LC, locus coeruleus; NTS, nucleus tractus solitarius.

modulation of activity-dependent plasticity such as post-tetanic potentiation, LTP, and LTD.

5. *Activation of protein kinase C*

A long-term, selective augmentation of *N*-methyl-D-aspartate (NMDA)-mediated glutamate currents by activation of μ-opioid receptors was observed in brain slices of trigeminal nucleus (80). This augmentation was mimicked by phorbol esters and blocked by the peptide inhibitor of protein kinase C (PKC). It was concluded that opioids activated PKC, which then increased the conductance activated by NMDA receptor agonists. This was the first and remains the strongest evidence that opioids augment postsynaptic glutamate currents by a mechanism involving the activation of PKC. There have, however, been more recent studies showing augmented

NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) by μ-opioid agonists in both the nucleus accumbens and hippocampus (312–314, 384). This augmentation was not observed in the locus coeruleus (LC) (359), suggesting that it may be dependent on the makeup of NMDA receptor subunits and/or the isoforms of PKC present on any given cell type.

The activation of PKC by opioids appears to result from the activation of phospholipase C and/or phospholipase A_2, which is thought to result from an interaction of β/γ-subunits of pertussis toxin-sensitive G protein and may require coactivation with the α-subunits of pertussis toxin-insensitive G proteins (144, 342, 358, 441). The results suggest that in order for opioids to have a robust effect, coactivation with $G_q\alpha$ subtype G proteins is required. A similar pathway is also thought to mediate the release calcium from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (see sect. II*B6*).

6. Release of calcium from internal stores

Initial studies demonstrating a transient increase in intracellular calcium in NG108–15 cells were unexpected (217). Most of the rise in calcium found in both NG108–15 and ND8–47 cells was blocked with dihydropyridine calcium channel blockers or removal of extracellular calcium, suggesting that calcium entry across the plasma membrane was the primary source (217, 475). Further investigation in both NG108–15 and SH-SY5Y cells indicated that a component of the increase in calcium resulted from release from intracellular stores (100, 219). This effect of opioids was sensitive to pertussis toxin, depletion of stores by thapsigargin, and an inhibitor of phospholipase C, U73122 (219). Microinjection of a peptide that binds to β/γ-subunits (QEHA) blocked the opioid-induced increase in calcium in NG108–15 cells (539). Injection of a peptide that blocked bradykinin-induced activation of $G_q\alpha$ did not block the opioid-induced increase in calcium (539). Thus the interaction of β/γ-subunits with phospholipase was not dependent on coactivation of $G_q\alpha$, although other potential α-subunits were not excluded. In experiments on SH-SY5Y cells, the increase in intracellular calcium was dependent on coapplication of agonists (muscarinic) that activated receptors coupled to phospholipase C (100). Taken together, it appears that the opioid activation of phospholipase results from an interaction with β/γ-subunits of pertussis toxin-sensitive G proteins and subsequent production of IP_3 and diacylglycerol (DAG), which release stores of calcium or activate PKC, respectively. This action of opioids is either significantly enhanced or completely dependent on the coactivation of receptors that are directly coupled to phospholipase. Although opioids have been shown to increase intracellular calcium in primary afferent neurons (476), the signaling pathway for this effect has not been identified.

7. Receptor trafficking

With the cloning of opioid receptors has come a better understanding of the mechanisms that regulate the life cycle of receptors. It is clear that opioid receptors, as is the case with many G protein-linked receptors, are not static and cycle to and from the plasma membrane. Most opioid binding studies were primarily directed toward sites on the plasma membrane in both neuronal and nonneuronal tissues; however, opioid binding sites in intracellular compartments have been recognized for some time (32). These binding sites were considered to be newly synthesized or recycled receptors. On the basis of studies using antibodies directed at each of the opioid receptors, it was realized that significant immunoreactivity for both the δ- and κ-opioid receptors was associated with intracellular compartments, particularly in axonal projections (432, 547). From this observation, it was hypothesized that the intracellular receptors were associated with a regulated secretory pathway in terminals. κ-Opioid receptors were found on vesicles in nerve terminals of vasopressin-containing neurons and were translocated to the plasma membrane after a physiological stimulus (432). Interestingly, 1 h after the stimulus, the receptors disappeared from the plasma membrane and reappeared in the vesicular compartment. Thus receptors found in vesicular membranes could be both newly synthesized and/or recycled. Although it has not been determined if the receptors freshly inserted into the plasma membrane were functional, this observation suggests an interesting form of feedback inhibition that would be dependent on prior activity in any given terminal.

Receptor trafficking initiated by agonist binding and internalization through the endosomal pathway may be involved in desensitization and/or the initiation of nuclear signaling (see below). The COOH-terminal tail of opioid receptors, as other G protein-linked receptors, regulates the extent and efficiency of internalization. The events leading to internalization are based primarily on the β-receptor model (Fig. 4). Upon agonist occupation, a receptor kinase (BARK2) phosphorylates the receptor, uncoupling the G protein and increasing the affinity of the receptor for arrestin (269). This triggers a series of events that carry the receptor complex to clathrin-coated pits and into an endosomal compartment.

Internalization of the δ-opioid receptor was suggested to depend on phosphorylation of the COOH terminal as indicated by experiments using either point mutations, at Ser-344 and Ser-363 or COOH-terminal truncations (484). Similar experiments were carried out using the two alternatively spliced isoforms of the μ-receptor, MOR1 and MOR1B (256). The MOR1B isoform is the shorter of

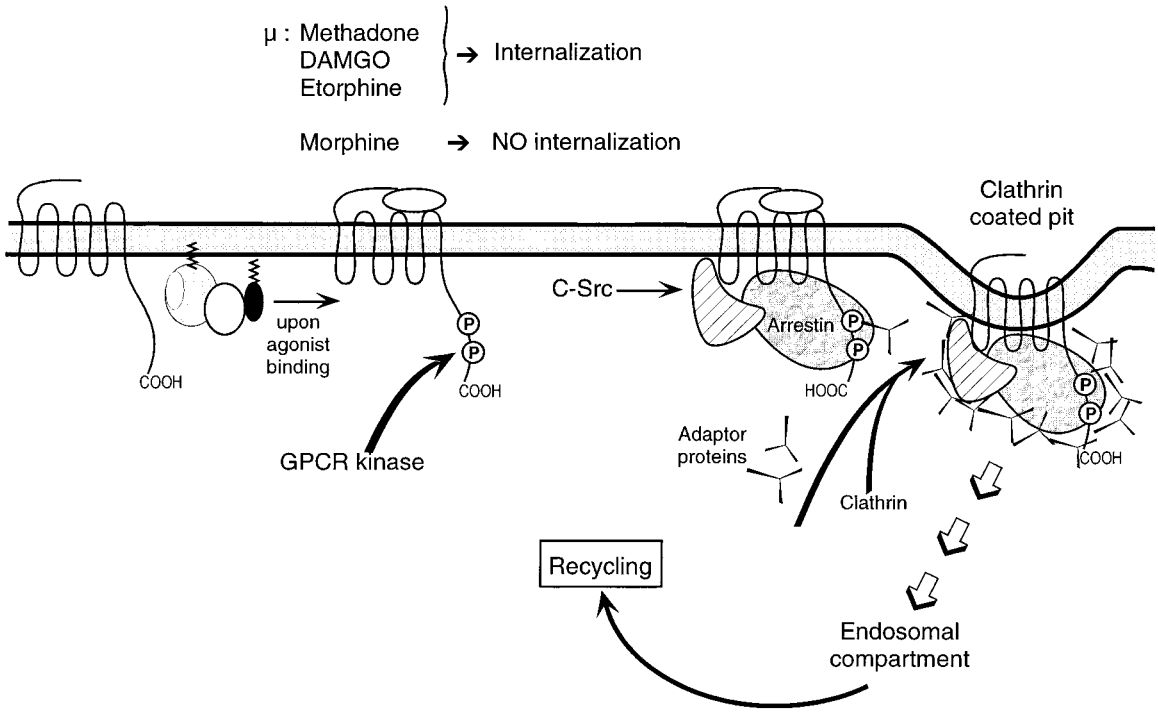

FIG. 4. An illustration of the sequence of events leading to receptor internalization. Some but not all (notably morphine) opioid agonists can activate the pathway. The activated opioid receptor is phosphorylated by a G protein receptor kinase. The affinity of interaction between this complex and arrestin is increased. The arrestin-bound complex recruits c-Src adaptor proteins (AP-2 complex) that link arrestin and clathrin to promote endocytosis (reveiwed in Ref. 252).

the two and lacks one phosphorlyation site, Thr-394. The MOR1B receptor was more resistant to desensitization, more rapidly internalized, and recovered from desensitization more rapidly than MOR1. It was also suggested that MOR1B was internalized constitutively. Thus it appears that regulation of internalization is highly dependent on the COOH-terminal tail.

Internalization is also agonist dependent (245, 516). The amount of internalization induced by a series of agonists was compared with efficacy in standard signal transduction assays using the μ-opioid receptor transfected into HEK 293 cells (516). Morphine was among the agonists that did not cause internalization. Morphine is well known as a partial agonist so the lack of activity was not surprising (266). Methadone, another partial agonist like morphine, resulted in efficient internalization (516). In addition, morphine was capable of causing internalization under conditions where the coupling efficiency was increased by overexpression of GRK2 (547). Finally, with the use of a chimera of the μ-receptor combined with the COOH terminus of the δ-opioid receptor, morphine mediated internalization in both a cell line and in primary cultures of hippocampal cells, suggesting that this portion of the receptor was responsible for lack of morphine-induced internalization (516). Given this result, it would be interesting to examine the effect of morphine on the MOR1 and MOR1B splice variants. Internalization of δ-receptors in NG108–15 cells was facilitated by overexpressing arrestin, such that morphine was capable of causing internalization. It therefore appears that morphine is a differentially weak partial agonist at producing internalization. The quantification of relative efficacies of morphine and other opioids was incomplete in these experiments, relying only on maximal responses. It is therefore not yet clear how much the relative efficacies of morphine, methadone, and other opioids differed for different signal transduction processes. Similarly, differential rank orders of efficacy of DAMGO, methadone, L-α-acetyl methadol, morphine, and buprenorphine were previously reported for μ-receptor phosphorylation, potassium channel activation, and desensitization (541). The results of these studies suggest that the efficiency of agonists to couple with different effectors varies and that coupling to mechanisms responsible for phosphorylation and internalization is rather inefficient. It appears that all of the endogenous agonists and a number of alkaloid agonists (but not others) are potent activators of internalization regardless of their ability to induce G protein activation. Similar interpretations have been made for phosphorylation of μ-receptors by PKA (71) and efficiency of activation of different G protein α-subunits (150). If this conclusion proves correct, then it implies that distinct

μ-opioid receptor conformational states exist for coupling to different effectors (96), with the implication that agonists with selective conformational state profiles can be developed deliberately.

The inability for morphine to cause internalization under most circumstances may also be an important issue in the eventual understanding of the cellular, synaptic, and network effects of chronic morphine treatment. Given the idea that receptor internalization may be a mechanism that mediates receptor turnover and resensitization, the inability of morphine to mediate this basic response could result in a chronic receptor activation. The continuous stimulation of transduction pathways may recruit signaling pathways to cause downstream adaptations (counteradaptations) with repercussions unrelated to direct actions of opioids.

8. Nuclear signaling

The regulation of cellular events through altered expression of proteins signaled by the chronic activation of opioid receptors is critical for understanding tolerance and dependence to opioids. The signaling pathways that lead to altered genetic expression are simply another effector system. Unlike membrane delimited effectors such as potassium and calcium channels, the extracellular signal-regulated kinase (ERK)/MAPK pathway that mediates nuclear signaling involves multiple protein-protein interactions, translocations, and phosphorylation events (Fig. 5). The activation of this pathway by opioids, like other opioid effectors, is sensitive to pertussis toxin. The kinetics of activation are longer than that for other effectors but occur over a period of several minutes to 1–2 h.

It appears that there are at least three general pathways following the activation of G_i/G_o-linked receptors that eventually converge on the activation of MAPK. One pathway involved ßγ-subunit activation of phosphatidylinositol 3-kinase, which activates MAPK activity through a series of phosphorylation steps including the activation of c-Src (139, 382, 383). The second pathway involves phosphorylation of the receptor with a receptor kinase; the translocation and binding of arrestin to the receptor is followed by translocation of c-Src to the membrane before internalization of this receptor complex through clathrin-coated pits (198, 293). Once internalized, the complex activates ERK, which is translocated into the nucleus to affect gene regulation by any of a number of transcription factors (250). In the CNS, ERK/MAPK can be activated by PKA (200). The PKA-dependent activation of ERK/MAPK by opioids may not be important during acute opioid administration but may be facilitated by an upregulation of adenylyl cyclase activity with chronic treatment. Activated ERK/MAPK can phosphorylate multiple targets in the cytoplasm and in the nucleus (i.e., transcription factors such as CREB).

This second pathway is significant in that internalization is a necessary step. Morphine, an agonist that does not activate internalization, would therefore be ineffective at activating the MAPK pathway, at least through this mechanism. It is interesting to note that, with one exception (279), all reports on the activation of the MAPK pathway have used agonists, such as etorphine, DAMGO, [D-Pen(2),(5)]-enkephalin (DPDPE) and U69593, that effectively cause receptor internalization (30). In the study where morphine did activate MAPK activity, the activation was transient relative to that caused by etorphine, DAMGO, and PLO17 (279). In an in vivo study where phosphorylated MAPK was examined immunohistochemically after acute and chronic treatment of animals with morphine, the distribution of immunoreactivity was unchanged by acute morphine treatment (420). Withdrawal from chronic morphine treatment precipitated by injection of naloxone, however, produced a robust increase in phospho-MAPK immunoreactivity in specific brain regions, among them the LC. Taken together, these observations suggest that in many systems morphine alone may be ineffective at activating the MAPK pathway. After chronic morphine treatment, however, adaptive mechanisms may facilitate the activation of MAPK. For example, in many brain areas, MAPK can be increased by excitatory transmission (17). The increased release of glutamate in the LC during withdrawal could be a trigger that activates the MAPK pathway.

III. CELLULAR ADAPTATIONS INDUCED BY CHRONIC MORPHINE TREATMENT

A spectrum of cellular adaptations resulting from chronic exposure to opioids is responsible for nonassociative tolerance and physical dependence. Humans and experimental animals can develop profound tolerance to opioids over periods of several weeks of escalating chronic treatment. Thus hundreds of times the normal analgesic dose of morphine have been reported to produce only mild physiological effects in some addicts (211) and chronic pain patients (166). Tolerance development involves a number of distinct cellular and neural processes (see below). The desensitization/downregulation mechanisms involved in tolerance are necessarily passive and do not engage the rebound mechanisms that could underlie maintenance of drug dependence and the opioid withdrawal syndrome. The latter require development of counteradaptations as outlined as follows: *1*) acute desensitization of opioid receptor to effector coupling and internalization that develops during and abates shortly (minutes to hours) after exposure to agonists; *2*) long-term desensitization of receptor to effector coupling and downregulation of receptors that slowly develop and then persist for many hours to days after removal of agonists;

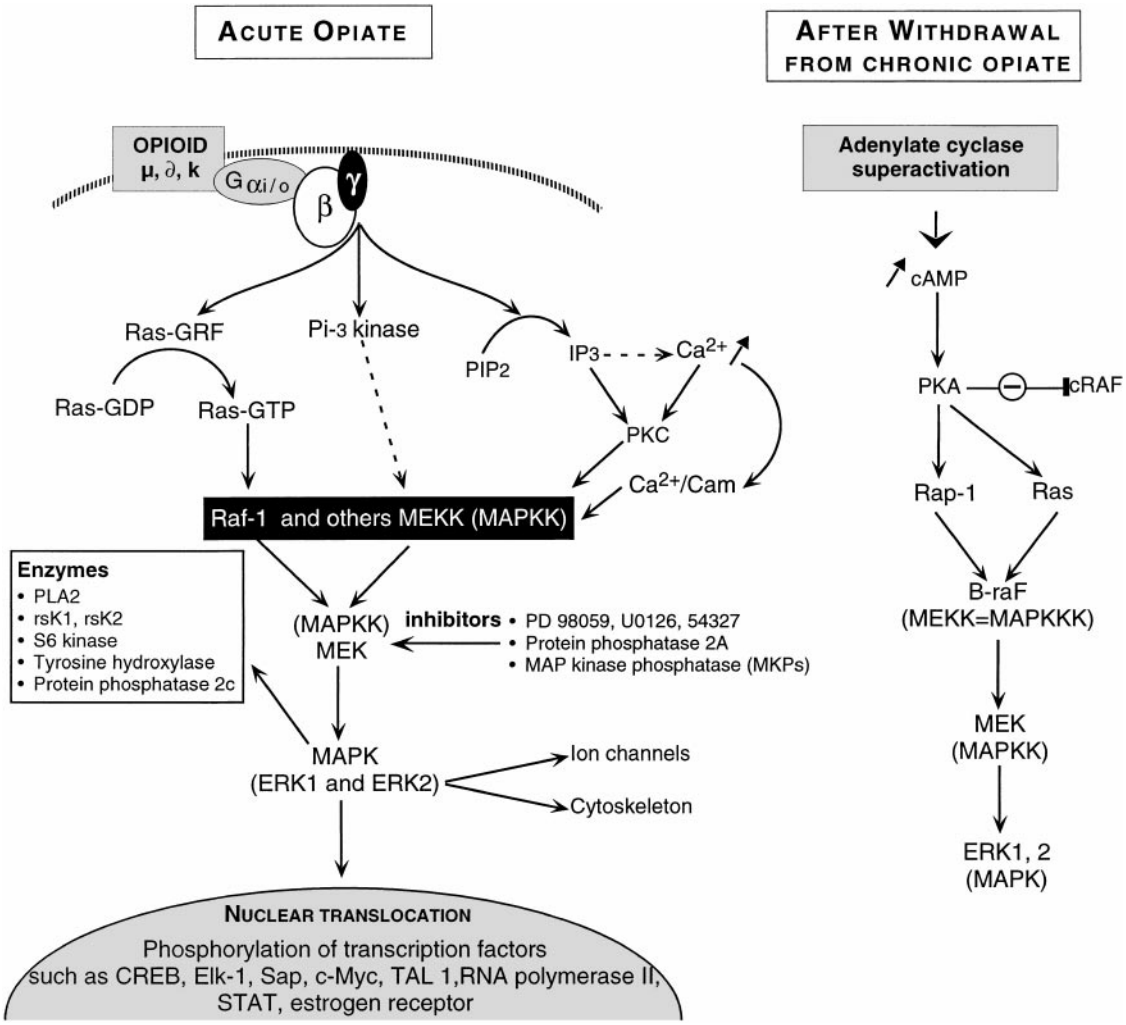

FIG. 5. Multiple pathways can lead to the activation of the extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) transduction cascade by opioids applied acutely or after chronic treatment. Three main transduction pathways can cause the activation of ERK/MAPK during acute application of opioid agonists. β/γ-Subunit release could *1*) stimulate phospholipase C and cause the release of calcium from internal stores and the production of diacylglycerol, which will in turn activate protein kinase C; *2*) recruit to the membrane proteins such as Ras-GRF; and *3*) activate the phosphatidylinositol 3-kinase (PI 3-kinase). The ERK/MAPK cascade can be blocked at the level of the MEK by pharmacological agents such as PD-98059 or by phosphatases. Activated ERK/MAPK has multiple targets, including nuclear transcription factors (such as CREB), cytoplasmic enzymes (including tyrosine hydroxylase), cytoskeletal proteins, and ion channels. Because morphine causes no or little internalization of μ-receptor, it is possible that the MAPK pathway is under permanent opioid stimulation during chronic drug treatment. After withdrawal, the adenylyl cyclase superactivation could now lead to the activation of the ERK/MAPK cascade through intracellular elevation of cAMP and activation of the protein kinase A (PKA). PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C.

3) counteradaptations of intracellular signaling mechanisms in opioid sensitive neurons; and *4*) counteradaptations in neuronal circuitry.

Two phases of development of and recovery from tolerance can be distinguished in humans and experimental animals. The onset of the rapid phase of tolerance occurs within minutes. It is difficult to quantify in whole animals but dissipates with a time course approximating the elimination of opioids (104). The slowly developing phase dissipates over several weeks regardless of the opioid used for induction (104). The rapid phase seems to predominantly involve acute desensitization, but counteradaptations can also play a role (see sect. III). The slow, persistent phase of tolerance involves the latter three mechanisms that are less fully understood than acute desensitization. The relative contributions of each of the above mechanisms to net tolerance development depends on the physiological system in question. However, the mechanisms involved have important implications in determining the extent of tolerance development, which varies greatly between different organ systems. The following will focus primarily on mechanisms identified for

μ-receptor desensitization and downregulation because actions at μ-receptors are of major relevance for tolerance development. The involvement of κ- and δ-receptor desensitization and downregulation in tolerance development is unclear and has not been extensively studied.

A. Desensitization, Internalization, and Downregulation of Receptor to Effector Coupling

1. Acute desensitization

Acute desensitization may be homologous, heterologous, or can involve both mechanisms. Homologous desensitization is by definition restricted to the opioid receptor occupied by the agonist and its specific interactions with signal transduction cascades. In contrast, heterologous desensitization generalizes to other receptors present in the same cells, and/or other elements of the signal transduction cascade such as G protein and ion channel activity. As discussed below, homologous desensitization can potentially involve phosphorylation of occupied receptors, occupancy-dependent protein-protein interactions, and/or occupancy-dependent compartmentalization and internalization. Homologous desensitization of μ-receptors has been described in a variety of cellular models using different end points. Although a general consensus on the mechanisms involved is emerging, both qualitative and quantitative differences have been reported in various experimental systems. These differences may arise from expression of diverse signaling elements, e.g., G protein receptor kinases, the stoichiometry of signaling elements, and the end points measured.

Several mechanisms of homologous desensitization of μ-receptors have been recognized. The most thoroughly studied for μ-receptor desensitization and internalization involve G protein-coupled receptor kinase (GRK)-mediated receptor phosphorylation that promotes the binding of β-arrestin proteins. This process not only uncouples opioid receptors from their cognate heterotrimeric G proteins, but also targets them for endocytosis. The processes by which this is thought to occur have been reviewed elsewhere (58, 277) and are outlined in Figure 4. Agonist binding to the receptor promotes a conformational change that results in G protein activation and dissociation from the receptor. Free G protein β/γ-subunits facilitate translocation of GRKs to the membrane where they phosphorylate serine and threonine residues in the COOH-terminal region. The phosphorylated receptor binds with high affinity to the cytoplasmic protein arrestin, which prevents association of inactive G proteins with the receptor and initiates internalization. Many of the details of this scheme have been confirmed for the μ-receptor, but a number of contentious issues remain.

Phosphorylation of the μ-receptor by GRKs does not appear to be necessary in all cases for desensitization to occur in neurons and some test systems, implying that other mechanisms mediating desensitization exist. Truncation of the COOH-terminal tail of the μ-receptor, the likely region for interaction with and phosphorylation by GRKs, greatly attenuates desensitization. When reconstituted in *Xenopus* oocytes, homologous desensitization of μ-receptor-mediated coupling to potassium currents was dependent on GRK2 (268). However, the desensitization kinetics were much slower (tens of minutes vs. minutes) than observed in CNS neurons (364), suggesting that components of the signaling cascade or stoichiometry of signaling elements were inappropriate. Other studies in reconstituted systems have also suggested that GRK phosphorylation and activation of MAPK pathways are essential for μ-receptor desensitization to occur (382, 383). Inhibitors of GRK-mediated phosphorylation such as Zn and heparin should be able to address the issue of GRK requirement, but unfortunately, they disrupt many aspects of cell function. Nonetheless, these inhibitors have generally not been shown to reduce desensitization (27, 364). In LC neurons, rapid (τ = 120 s), homologous desensitization of coupling between μ-receptors and inwardly rectifying potassium channels was not affected by heparin or staurosporine (180, 364), suggesting that GRK-dependent processes are not involved. Dominant negative GRK mutants (kinase activity deficient) should provide more conclusive results but have not yet been used to examine μ-receptor desensitization. Desensitization of some other GPCRs, e.g., adenosine A_{2a} and A_{2b} receptors, were greatly inhibited by dominant negative GRK2, but others such as prostanoid and somatostatin receptors were completely unaffected (27). GRK-β/γ inhibitory peptides also failed to inhibit somatostatin receptor-mediated desensitization (27). The possibility that subtype specific interactions of different G protein β/γ-subunits with the MAPK signaling cascade (109) cannot adequately resolve these difficulties because receptor coupling through the same elements in single cells showed different results. Desensitization of m_2 muscarinic receptors required the presence of either authentic GRK2 or the dominant negative form (431), suggesting that binding of GRK2 to the receptor is sufficient to cause desensitization in the absence of phosphorylation and initiation of internalization processes. However, it was not established whether or not desensitization was homologous in those studies, so the importance of the mechanism is still unclear. Enhanced morphine antinociception in mice lacking β-arrestin-2 (37) is suggestive of an involvement of GRK-dependent mechanisms in μ-receptor desensitization, but it has not been established that rapid desensitization is disrupted in these knockouts.

Other phosphorylation-dependent mechanisms of μ-receptor desensitization have also been examined. Although PKA-mediated desensitization occurs in some G

protein-coupled receptor systems, this has not been established for the μ-receptor. Inhibitors of PKA signaling have generally had no effect on desensitization (180, 183, 333, 344, 364) and activators of PKA signaling have an inhibitory effect on desensitization (81, 180), but it is not clear whether this inhibition was homologous or heterologous. PKC-mediated phosphorylation of μ-receptors has also been reported to reduce sensitivity to opioids (494, 546), but the process is independent of occupancy by agonists and therefore is heterologous. Phosphorylation by calmodulin kinase II of Ser-261/Ser-266 in the third intracellular loop of the μ-receptor has also been implicated in increasing the rate of desensitization (255). The third intracellular loop of the μ-opioid receptor has also been implicated as a binding site for calmodulin, which when associated with the receptor reduces coupling to G proteins (506). However, the functional significance of the latter observations for signaling and desensitization has not been characterized. Various other kinases such as cGMP-dependent protein kinase (323) and casein kinase (481) have been shown to modulate the activity of other G protein-coupled receptors, but these have not been examined for the μ-receptor.

It is not yet clear if phosphorylation-independent mechanisms are involved in initial events of homologous μ-receptor desensitization. The temporal correlation between desensitization, measured by inhibition of adenylyl cyclase activity, and at least some types of receptor phosphorylation events is poor (129). As indicated above, it is possible that phosphorylation-independent interactions of GRKs with μ-receptors functionally uncouple G protein interactions. In LC neurons using coupling to activation of potassium currents as an end point, μ-receptor desensitization was largely homologous (135, 180, 363) but was not affected by GRK inhibitors such as heparin or serine/threonine kinase inhibitors including staurosporine (Fig. 6). The phosphatase inhibitors okadaic acid and microcystin had no effect on onset of desensitization but markedly slowed recovery. The time course of desensitization was rapid under these conditions with onset and offset

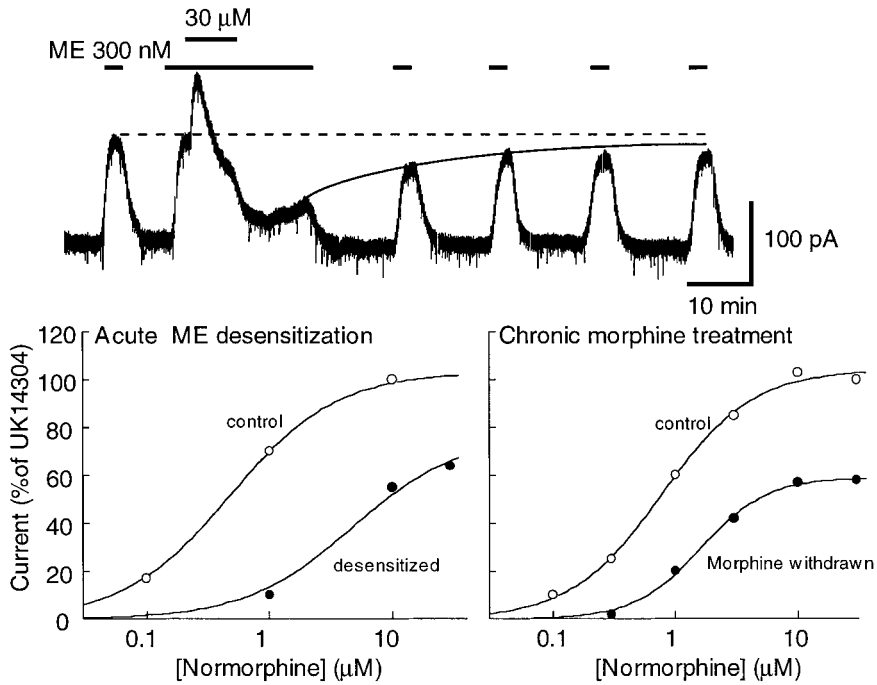

FIG. 6. μ-Opioid receptor desensitization and tolerance in locus coeruleus neurons. *Top trace* is an example recording of the protocol used to measure acute opioid desensitization. This is a current record of a cell voltage-clamped at −60 mV. Superfusion of [Met]enkephalin (ME; 300 nM) caused an outward current. Superfusion of ME (30 μM) for 5 min resulted in a peak response followed by a decline to ~50% of the peak. Immediately after washing the high concentration of ME, the outward current caused by ME (300 nM) increased over a period of 15–25 min to a value close to that at the beginning of the experiment. *Bottom left*: a concentration-response curve to normorphine in control and immediately after a desensitizing treatment with ME (30 μM, 5 min). The dose-response curve is shifted to the right, and the peak response is reduced. [From Osborne and Williams (363).] *Bottom right*: a similar experiment done in brain slices taken from control animals (control) and animals that were treated chronically with morphine. As was observed with the acute desensitization, chronic morphine treatment shifted the concentration-response curve to the right and depressed the maximum response. [From Christie et al. (89).] In each experiment, the response to normorphine was normalized to the outward current induced by a maximal concentration of the α_2-adrenoceptor agonist UK-14304. The conclusion of these experiments was that acute desensitization as well as more long-term tolerance results in a dramatic decline in μ-opioid receptor reserve. The acute desensitization recovers to a large degree within 30 min; however, in morphine-treated animals, there was little evidence for recovery over a period of 2–6 h.

time constants of ~3 min. This is comparable to desensitization rates measured by others using electrophysiological methods in neurons (e.g., Refs. 27, 333) but are markedly faster than time courses usually measured (when they have been) in reconstituted systems using electrophysiological techniques or biochemical measures such as GTPase activity and inhibition of adenylyl cyclase. The latter time courses often proceed over periods of several hours (e.g., Refs. 256, 266, 382, 383). Whether or not these differences reflect fundamentally different mechanisms or differences in stoichiometry of elements in native versus reconstituted systems has not been resolved.

Heterologous desensitization has been described in both model systems (267) and neurons (135, 353). By definition, heterologous desensitization generalizes to receptors and transduction mechanisms distinct from the one activated by the primary ligand and as such can represent at the cellular level the compensatory and incidental adaptive mechanisms discussed below. For example, heterologous desensitization that involves changes in the activity of kinases such as PKA and PKC can affect multiple elements of signal transduction cascades including diverse receptors, G proteins, and effectors such as ion channels.

2. Internalization

It has long been recognized that internalization is not required for desensitization to occur, but when it does, the process necessarily affects sensitivity to agonists by removing surface receptors. Pak et al. (366) demonstrated that μ-receptor desensitization was associated with a loss of binding sites on the plasma membrane. As discussed above, agonist-evoked internalization has been observed for μ-receptors in model systems (246, 517) as well as neurons (246, 455). Internalization occurs predominantly via clathrin-coated pits. Receptor phosphorylation by GRKs appears to be a critical event in internalization (64), although others have suggested that MAPK activation is critical (382).

A number of groups have examined residues in the COOH-terminal region of the μ-receptor, known to be essential for internalization via the endosomal pathway. Pak et al. (367) found that Thr-394 was the primary recognition site for G protein-coupled receptor kinases, but Thr-383 was also required for complete agonist-induced desensitization. The specificity of Thr-394 as the primary initiation site appears to be dependent on the preceding acidic amino acid stretch, because a mutant in which glutamic acid residues at 388, 391, and 393 were replaced by glutamines the receptor was not internalized. Truncated μ-receptors suggested that Ser-356 and Ser-363 were important for agonist-induced internalization of the receptor but not phosphorylation (59).

Although GRKs and endosomal mechanisms have been established to play a major role, other internalization processes have not been ruled out (337). The importance of GRK-mediated processes for acute tolerance, usually studied using morphine (or heroin), is doubtful because morphine does not induce internalization in vivo. Speculations that differences in the abilities of different opioid agonists to induce internalization are related to their addiction liability or therapeutic value in opioid management of opioid dependence are intriguing but have yet to be fully established (516, 541). It is possible that agonists employed therapeutically in dependency management, such as methadone, *l*-α-acetyl methadol (LAAM), and buprenorphine, have beneficial effects on adaptive processes because they more readily induce internalization than morphine (and heroin). As discussed above, endosomal internalization mechanisms involve dephosphorylation, resensitization, and MAPK activation which initiates nuclear signal transduction cascades that can influence downstream adaptive processes and may regulate μ-receptor function. Any such mechanisms and potential therapeutic benefits must be purely fortuitous because methadone, LAAM, and buprenorphine were developed for opioid dependency management solely on the basis of their agonist efficacy, bioavailability, safety, and plasma half-lives.

3. Long-term desensitization and downregulation

Long-term adaptive processes play a major role in the capacity of animals and humans to tolerate profoundly escalating doses of opioids over periods of weeks to months, but the mechanisms involved have not been fully resolved. It is not yet clear whether or not the early events of desensitization and internalization are necessary antecedents or perhaps contribute directly to these longer term adaptive processes. Where they have been studied, however, evidence for a direct role is lacking (see below). Such long-term adaptations presumably involve chronic functional uncoupling of μ-receptors from signaling pathways, perhaps as a consequence of counteradaptations (see below) and/or downregulation of surface receptors. Early studies of μ-receptor density in whole brain or brain regions following chronic morphine treatment almost invariably found no reduction in total number of receptor binding sites (e.g., Ref. 514). Although some studies of surface receptors in cultured cells observed downregulation following chronic morphine and other agonists (386, 537, 544), the findings in brain appeared to indicate that adaptive processes must be targeted at intracellular domains of the receptor involved in coupling with G proteins and not the density of receptors at the plasma membrane surface. Radiolabeled opioid ligands used in early studies could not readily distinguish surface from intracellular receptors and ligand binding studies are also confounded by continued occupancy of receptors, during chronic

treatment. Subsequent to the cloning of μ-receptors and the development of highly selective antibodies, more direct assessment of surface receptor densities has indicated that densities of μ-receptors are indeed downregulated in some brain regions after chronic treatment with morphine (34, 277, 477). In model systems expressing truncated μ-receptors, serine residues were identified in the COOH-terminal region (Ser-355 and Ser-363) that were necessary for opioid-induced (etorphine) downregulation (5, 59).

The mechanisms of μ-receptor downregulation do not appear to substantially involve GRK-dependent mechanisms. Studies in SH-SY5Y cells have suggested that μ-receptor downregulation is blocked by nonspecific serine-threonine kinase inhibitors but that a putative GRK2 inhibitor, suramin, had no effect (130). The absence of a role for GRK phosphorylation in downregulation is consistent with similar findings in GRK2 phosphorylation-deficient β_2-adrenoceptor mutants (172). The failure of morphine to engage endosomal internalization mechanisms is also consistent with a lack of involvement of GRK because morphine produces similar downregulation as other agonists (537). Although phosphorylation has been implicated in the process of downregulation, the nature of its role remains uncertain. It is possible that receptor cycling is affected in a GRK-independent fashion or that kinase activity affects synthesis or degradation rates.

Studies of μ-receptor coupling to proximal cellular effectors, such as GTPase activity, adenylyl cyclase inhibition, inwardly rectifying potassium channels, or calcium currents have also found functional uncoupling of receptors from effectors. ^{35}S-labeled guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) binding reflects GTPase activity and is decreased following chronic morphine treatment in brain, including LC (423, 435) and in cultured cells (130). Similar results have been reported for coupling of μ-receptors to inwardly rectifying potassium channel currents (89) and calcium channel currents (97) in LC neurons, and calcium channel currents in SH-SY5Y cells (249). Where studied, the uncoupling processes were found to be homologous. Reduced coupling efficacy could have arisen in these studies from functional uncoupling of receptors from G proteins, or a loss of surface receptors. Although the former was generally assumed on the basis of negative results from ligand binding studies in brain (see above), a reduction in surface μ-receptor density actually was found in the only study that directly addressed the issue (130). It therefore remains uncertain whether or not the functional uncoupling of μ-receptors from proximal effectors widely observed in neurons and model systems arises solely from a reduction in the density of μ-receptors in the plasma membrane.

In summary, acute desensitization, internalization, and downregulation of μ-receptors all play roles in opioid tolerance measured at the cellular and synaptic level. Although these mechanisms can explain the development of nonassociative tolerance at the cellular level, adaptive mechanisms that occur with repeated and/or continuous morphine treatment to mediate associative tolerance are probably mediated by separate mechanisms (e.g., Ref. 325). The counteradaptive mechanisms not only mediate forms of tolerance to morphine but are also involved in opioid withdrawal and dependence. The relative contributions of each process to the extent and persistence of tolerance in different physiological systems in the behaving organism have not been elucidated. The extent of tolerance is usually rather small when examined in single cells (e.g., Ref. 89) compared with tolerance in whole animals (e.g., Ref. 104). Tolerance at systems levels must involve interaction of mechanisms of tolerance at molecular, cellular, and neural network levels throughout each system, but the details of such interactions are completely unknown.

B. Counteradaptations

Mechanisms subsequent to receptor activation that adapt to restore function in the presence of drug mediate a second form of tolerance (222). Tolerance produced by compensation, by definition, requires the presence of opioid agonists to maintain normal function. The adaptive responses observed at the cellular, synaptic, and network levels are therefore the core of acute aspects of opioid withdrawal. As with tolerance, different processes may mediate short- and long-term and protracted compensatory changes associated with chronic opioid treatment. Very-short-term counteradaptations can be observed after only several minutes of opioid application and abate just as rapidly (e.g., Refs. 221, 143). As discussed below, long-term compensatory changes have been most thoroughly studied.

1. Adenylyl cyclase

The first and best-studied example of tolerance resulting from compensation used the inhibition of adenylyl cyclase as an assay (46, 425, 426). Acutely, opioids acting on δ-receptors inhibited adenylyl cyclase, but in the continued presence of morphine, there was an increase (upregulation) of adenylyl cyclase activity (46, 425, 426). When agonist was removed, the compensatory increase in adenylyl cyclase activity remained. The increased adenylyl cyclase activity was taken as an example of withdrawal at the cellular level.

Since these early studies, several isoforms of adenylyl cyclase have been identified and classified into three primary groups based on sequence similarities (102, 329, 330). All these enzymes are differentially regulated by a number of messenger pathways including calcium, $G_i\alpha$,

$G_s\alpha$, $G\beta/\gamma$, and PKC (102, 329, 330). In addition, each isoform has a distinct anatomical distribution. Three isoforms are primarily neuronal, ACI, ACII, and ACV. The distribution of cells expressing high levels of mRNA for each subtype has a distinct pattern. Type I is found in the dentate granule cells of hippocampus, cerebellar granule cells, and cortex; type V is found almost exclusively in striatum and nucleus accumbens, and type II is more diffusely located in cortex, hippocampus, cerebellar granule cells, and substantia nigra. At the subcellular level, the distribution seems to be highly localized to synapses (330). Subunit-selective antibodies have not been used extensively, limiting the interpretations of exact composition at many synapses, although suggestions have been made based on results from in situ hybridization experiments. The high density of immunoreactive substrates found both pre- and postsynaptically place this highly regulated molecule that is sensitive to a number of second messengers in an ideal position to mediate synaptic plasticity.

The sensitivity of various isoforms of adenylyl cyclase to opioids was examined in a series of studies done in COS-7 and CHO cells (18–20). Isoforms that were acutely inhibited by opioids (I, V, VI, and VIII) were upregulated or supersensitized by chronic treatment. Adenylyl cyclase I and V are expressed at high levels in the CNS, thus encouraging speculation of a similar upregulation in vivo (see below). The results of this series of experiments were similar in ways to the early experiments in NG108–15 cells with native opioid receptors and adenylyl cyclase. The upregulation varied between two- and fivefold, required 6–10 h of treatment, recovered within 2–3 h, and was sensitive to pertussis toxin and agents that scavenged free $G\beta/\gamma$ subunits. There were two significant differences. First the kinetics of the upregulation were faster in the CHO and COS-7 cells than the NG108–15 cells. This may have resulted from different expression levels of receptor and/or adenylyl cyclase. The recovery after morphine treatment in the NG108–15 cells was complete after 24 h (426). The second potentially more significant difference was that the supersensitization in CHO cells was insensitive to cycloheximide and to a dominant negative ras mutant that blocked the activation of MAPK (18, 19). In NG108–15 cells, however, a large portion of the increased adenylyl cyclase activity was blocked by cycloheximide (426). The interpretation of these two observations is completely different. The result in CHO cells indicates that activity of adenylyl cyclase is increased, by an as yet uncharacterized mechanism, whereas the work in NG108–15 cells suggests that the upregulation is dependent on new protein, which may be but is not necessarily adenylyl cyclase itself. It is important to revisit the NG108–15 cell model, particularly the effects that inhibition of the MAPK pathway may have on the upregulation of adenylyl cyclase.

Studies examining the effects of chronic morphine on adenylyl cyclase in the brain and peripheral tissues have produced mixed results (71, 125, 479, 497). Acutely, opioids produced only a small inhibition in most areas (125, 479, 497) and increased activity in some areas (72, 105, 362). The upregulation of adenylyl cyclase activity induced by chronic morphine treatment, where it has been observed, was generally <0.5-fold. Preparation of brain and peripheral tissues has problems of heterogeneous cell types, multiple receptors, and adenylyl cyclase isoforms. Expression levels of each component can also reduce the signal-to-noise ratio and thus cloud interpretation of effects. Such difficulties are inherent in biochemical assays from complex tissues. Despite the fact that the upregulation of adenylyl cyclase in cell lines and expression systems is a robust and reliable measure, a similar approach measuring the bulk production of cAMP in tissues from animals treated with morphine has not brought new insights to this adaptive mechanism. That is not to say that it does not happen and is not important. In fact, there is building evidence that it may be critically important in the local area surrounding specific synapses. There may be additional adaptations resulting from the increased adenylyl cyclase activity mediated by changes in gene expression under the regulation of CREB (346).

2. Counteradaptations on potassium and calcium channels

One disappointing and recurring observation has been the lack of any change in the regulation of potassium or calcium conductances during acute morphine withdrawal. Probably the most thoroughly studied example of the lack of an adaptive process in response to chronic treatment is the potassium conductance in the LC. Aghajanian (6) was the first to record the firing rate of LC neurons in vivo from chronically morphine-treated rats. Morphine applied systemically caused an acute inhibition of firing. After 5 days of continuous morphine treatment, the spontaneous firing had returned to control values, indicating that LC neurons were tolerant to the levels of circulating morphine. An increase in firing rate above control levels upon application of naloxone by iontophoresis was taken as a cellular sign of withdrawal. More recent experiments in brain slices showed that opioids acutely activated an inwardly rectifying potassium conductance that caused a hyperpolarization to decrease the firing rate (374, 523). In addition, LC cells have a resting potassium conductance that is inwardly rectifying, suggesting that during withdrawal a reduction of this conductance could depolarize the cell and increase excitability (523). A decrease in inwardly rectifying potassium conductance in these and other cells by other G protein-linked receptors has been demonstrated, suggesting that opioid withdrawal could increase excitability by this

mechanism (343, 500). When experiments were done with slices taken from morphine-treated animals, there was no evidence of a depression in the resting inwardly rectifying potassium conductance by naloxone (89). Subsequent experiments have indicated that the increase in firing observed in vivo was largely the result of an increase in glutamate release from the excitatory inputs to the LC (8).

Calcium currents have not been extensively characterized during withdrawal. Although biochemical studies suggest an increased calcium channel density after chronic morphine treatment, these results have not been confirmed in electrophysiological studies in isolated neurons. There was no change in the calcium channel current density or relative proportions of pharmacologically isolated calcium channel subtypes in acutely isolated LC neurons taken from morphine-treated animals (97). There was a modest reduction in efficacy of coupling between μ-opioid receptors and inhibition of calcium channel currents, but no rebound during naloxone precipitated withdrawal. Thus withdrawal from morphine treatment produced no compensatory action on either potassium or calcium conductances in LC neurons.

3. Cation channel

The acute inhibition of a cation conductance by opioids has been reported in several preparations (Table 1). In addition, an increase in a cation conductance has been reported to account for the withdrawal-induced increase in excitability in the PAG (84; E. E. Bagley and M. J. Christie, unpublished data) and proposed but not directly demonstrated in LC (10, 257), and indirectly implicated on GABAergic neurons in the vicinity of the dorsal raphe (227). This cation conductance is thought to be regulated by the cAMP cascade and is more pronounced during acute withdrawal.

In morphine-dependent rats, naloxone cause a pronounced depolarization in a subset of neurons in the PAG (84; Bagley and Christie, unpublished data). This depolarization was not simply the reversal of the hyperpolarization induced by morphine because it was associated with an increase in conductance. A reversal of the acute action of morphine would decrease the opioid-sensitive potassium conductance only. The underlying current has been more fully characterized using perforated patch recordings from PAG neurons in brain slices from mouse (84). The reversal potential of this current was near -30 mV and was not mediated by a chloride conductance, suggesting that it is mediated by a nonselective cation conductance. It was sensitive to inhibitors of PKA, was mimicked by forskolin, and was kinetically distinct from I_h. Thus it appears that unlike opioid actions on I_h that are dependent on cAMP but not PKA, this current is kinase dependent.

The activation of an inward current has been proposed but not established in LC during acute withdrawal. In a series of papers (reviewed in Ref. 347), Aghajanian and co-workers (9, 10, 257) showed that cAMP analogs caused a small glutamate-independent increase in the firing rate of LC neurons. The increase in activity was blocked by PKI (a blocker of PKA), as was the spontaneous activity. It was concluded that a cAMP-dependent mechanism regulated an inward current that was responsible for spontaneous firing of LC neurons, and this current was increased during acute opioid withdrawal. It has not been possible to study this current in isolation (10).

The LC has been used as a model system at the single-cell level for both the acute and chronic opioid actions. Recent experiments have indicated, however, that the electrotonic coupling between neurons as well as glia within the LC limit the interpretation of results obtained with voltage-clamp studies (12, 208, 359, 485). The effects of electrotonic coupling were dependent on the experimental circumstances (364). For example, the outward current caused by opioids in the LC was significantly increased by forskolin (364). The increase in the opioid current was reduced or abolished with manipulations that decreased electrotonic coupling (364, 485, 486). This result was taken to indicate that forskolin, through the activation of adenylyl cyclase, increased electrotonic coupling within the LC, thus adding an unexpected layer of complexity to experiments done with morphine-treated animals.

4. Adenylyl cyclase and synaptic transmission

Activation of the cAMP cascade facilitates synaptic transmission by both presynaptic (42, 47, 63, 77–79, 86, 165, 203, 402, 409) and postsynaptic mechanisms (388, 505). Increased release by this mechanism has been accomplished with the use of agonists that activate G_s-coupled receptors, forskolin, and/or cAMP analogs. In most cases the increase in cAMP production results in the activation of PKA that results in the facilitation of transmitter release. The dependence on PKA is generally determined with enzyme inhibitors, such as staurosporine, H-89, or blocking analogs of cAMP, such as the Rp-isomer of adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS).

Several studies have used recordings of synaptic potentials to examine the cAMP-dependent regulation of transmitter release in control and morphine-withdrawn tissues. The results of these studies indicate that during acute withdrawal, transmitter release from many, but not all, opioid-sensitive synapses is increased through a cAMP-dependent mechanism (Fig. 7). Although the experimental protocols varied somewhat, there were common features found during acute morphine withdrawal at four GABA-mediated synapses. First, both basal (spontaneous) and evoked release was greater in acutely withdrawn

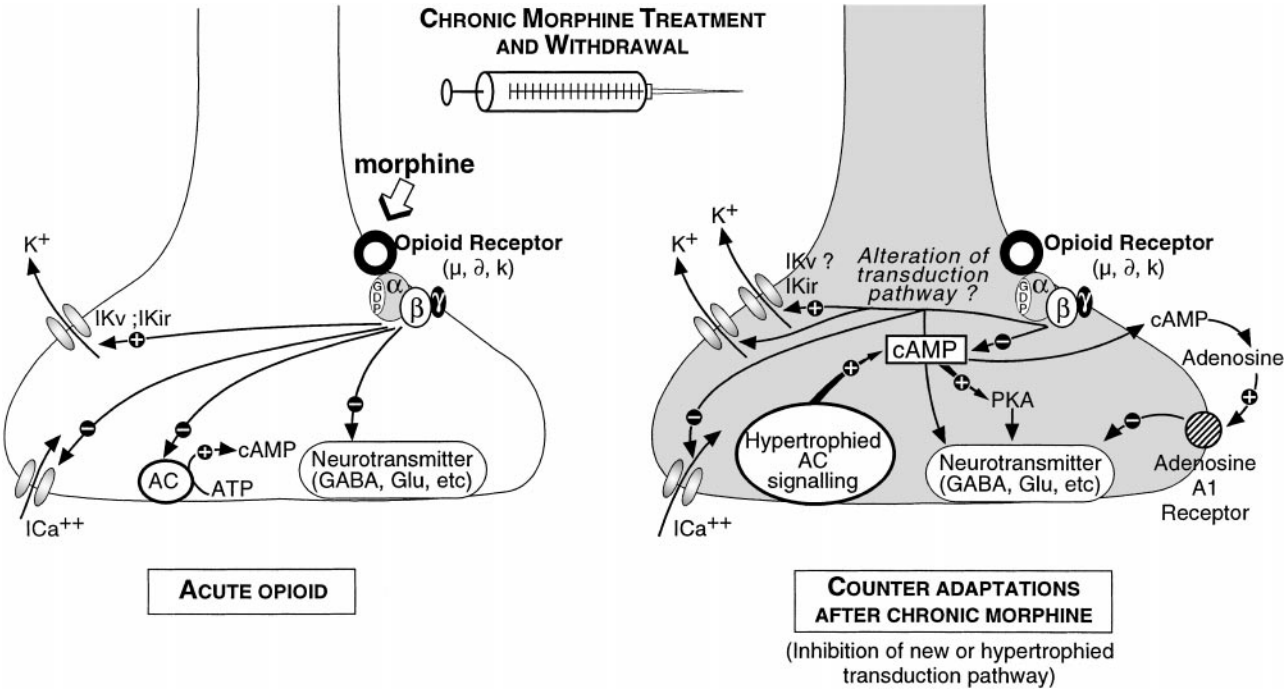

FIG. 7. The regulation of transmitter release from terminals is changed after chronic opioid treatment. *Left*: when applied acutely, opioids inhibit transmitter release by several potential mechanisms. These mechanisms include the activation of potassium conductance, inhibition of calcium conductance, or an inhibition of the release process that has not been well characterized. It is also known that opioids inhibit adenylyl cyclase; however, this mechanism does not appear to play a role in the acute inhibition of transmitter release by opioids. *Right*: after withdrawal from chronic treatment with morphine, the inhibition of transmitter release by opioids is changed in several ways. *1*) Opioids no longer activate voltage-dependent potassium currents to inhibit release. *2*) There is an upregulation of adenylyl cyclase that increases transmitter release by activation of PKA. *3*) The upregulated adenylyl cyclase is sensitive to inhibition by opioids and represents a new, morphine-induced effector. *4*) The increased adenylyl cyclase activity increases the production of cAMP that is metabolized to adenosine such that adenosine tone and thus presynaptic inhibition mediated by A_1 adenosine receptors is enhanced at some synapses.

slices, and this increase was blocked with inhibitors of PKA (42). Second, the increased GABA release caused by forskolin was significantly greater in withdrawn tissues (42, 86). Finally, in withdrawn tissues under conditions where there was significant cAMP-induced release, either with and in some cases without forskolin, the inhibition of GABA release mediated by opioid receptors was significantly increased (86, 203, 430). The interpretation of this observation centered on the emergence of a new effector. In withdrawn tissues, opioids caused inhibition by a cAMP-dependent mechanism that was induced by chronic morphine treatment.

The increase in cAMP-dependent transmitter release and the ability of opioids to inhibit this increase was not unexpected based on the work done on the upregulation of opioid-sensitive adenylyl cyclase isoforms in cell lines. Some of these results were obtained in tissues where adenylyl cyclase activity was reported to be unaffected by chronic morphine treatment [VTA, PAG, dorsal raphe (DR)]. Not all opioid-sensitive synapses were affected by this mechanism. Similar experiments in both the PAG and VTA showed that the regulation of glutamate-mediated synaptic transmission was not affected by a cAMP-dependent mechanism in withdrawn tissues (203, 311). Interestingly, in the shell of the nucleus acumbens, GABA synapses to cholinergic interneurons showed an increase in cAMP-dependent neurotransmitter release by GABA synapses onto medium spiny projection neurons were unaffected by chronic morphine treatment (86; J. M. Brundege and J. T. Williams, unpublished data). It thus appears that changes in adenylyl cyclase activity can differentially affect different synapses even on the same cell. Given the highly localized distribution of adenylyl cyclase at synapses, a high degree of synaptic specificity resulting from morphine-induced adaptive processes seems plausible.

5. Adenosine

Early behavioral experiments suggested increased cAMP-mediated signs of withdrawal (93–95). The effect of cAMP was called the "quasi-morphine-abstinence syndrome." This behavioral response consisted of a series of behavioral signs and symptoms that were in many ways similar to those observed during acute morphine withdrawal. The cAMP-dependent component of the quasi-morphine-abstinence syndrome was based on increasing

its concentration by blocking its metabolism with the phosphodiesterase inhibitor IBMX. It is now known that IBMX is also a potent antagonist of adenosine receptors. Recent work with selective adenosine receptor antagonists indicate that these compounds alone exacerbate the signs and symptoms of opioid withdrawal (122, 240, 408). This recent work suggests that the quasi-morphine-abstinence syndrome may result from block of adenosine receptors but does not rule out an additional component mediated by cAMP. In fact, increased levels of adenosine, resulting from metabolism of cAMP, may be one consequence of the upregulation of adenylyl cyclase (41, 86, 430).

The activation of adenylyl cyclase, by either G_s-coupled receptors, such as D_1 or β-adrenergic receptors, or with forskolin, increases the extracellular concentration of cAMP (459). Once in the extracellular space, cAMP is metabolized by phosphodiesterases to AMP and is rapidly converted to adenosine by ecto-nucleotidases (107, 127). Presynaptic A_1 adenosine receptors that inhibit transmitter release have been identified at many central synapses (53, 128). Under normal conditions, the extracellular concentration of adenosine is ~200 nM, which is just above the threshold for activation of adenosine receptors (126). The basal level of adenosine can therefore be detected with the use of an adenosine receptor antagonist as an increase in the size of the synaptic potential and can, thus, be used as an assay for adenosine tone (126). Activation of adenylyl cyclase with forskolin has been shown to increase adenosine tone measured in this way in the hippocampus, VTA, and nucleus accumbens (52, 86, 287). In the VTA, there is a potent presynaptic inhibition of the $GABA_B$ inhibitory postsynaptic potential (IPSP) measured in dopamine cells that is mediated by A_1 adenosine receptors (532). The increase in the $GABA_B$ IPSP caused by an adenosine antagonist was larger in withdrawn tissues than in controls, indicating an increase in adenosine tone (41, 430). The increased adenosine was suggested to result from the metabolism of cAMP based on two experiments. Both blockade of cAMP-dependent phosphodiesterase and blockade of cAMP transport out of cells with probenicid increased the IPSP to the same level as the adenosine receptor antagonist (41). The adenosine antagonist had a similar effect on the $GABA_A$ inhibitory postsynaptic current (IPSC) in presumed cholinergic interneurons in the nucleus accumbens from morphine-treated animals (86).

The increased role of adenosine during withdrawal was very synapse specific. Glutamate-mediated fast EPSCs in the VTA were completely unaffected by adenosine antagonists during opioid withdrawal (203, 311). This form of regulation would be dependent on the presence of presynaptic A_1 receptors, the highly localized metabolism of cAMP to adenosine, and potentially the activity of the adenosine reuptake proteins. Synapse specificity could result from the localized release of cAMP or from local metabolism of cAMP by resident phosphodiesterases at individual synapses. Increased adenosine-mediated synaptic inhibition resulting from the metabolism of cAMP during withdrawal from opioids would constitute a physiological brake. This mechanism could account for the quasi-morphine-abstinence syndrome mediated by adenosine antagonists that was first described by Collier et al. (95).

6. Other adaptations at opioid-sensitive synapses

Adaptations mediated through the adenylyl cyclase cascade are not universal at opioid-sensitive synapses. There are several synapses where the regulation of transmitter release mediated by adenylyl cyclase after chronic morphine treatment was not affected. Although most of these synapses were glutamatergic, it does not mean that this form of adaptation does not occur at excitatory synapses. Glutamate EPSCs in the VTA, PAG, and the core of the nucleus accumbens were all opioid sensitive, but neither the sensitivity to opioids nor the increase in glutamate release induced by forskolin was significantly affected by chronic morphine treatment (203, 311; Brundege and Williams, unpublished data).

Morphine withdrawal did, however, change receptor-mediated presynaptic regulation of glutamate EPSCs in the VTA. In the VTA, μ-receptors caused a presynaptic inhibition of glutamate EPSCs measured in dopamine cells (311). The inhibition was sensitive to both 4-aminopyridine (4-AP), a potassium channel blocker, and baicalein, a 12-lipoxygenase inhibitor, suggesting that opioids acted via a transduction pathway involving activation of a voltage-dependent potassium conductance by lipoxygenase metabolites as has been shown in the PAG (499). During withdrawal, the inhibition caused by DAMGO was unchanged, but 4-AP and baicalein were significantly less effective. The decreased sensitivity to 4-AP was the same as that found in the PAG, where in control 4-AP completely blocked opioid inhibition of GABA IPSCs but was ineffective in withdrawn slices (203). Thus it was concluded that the normal 4-AP-sensitive transduction mechanism mediating the inhibition of transmitter release by opioids was downregulated, or completely eliminated, and replaced by a different mechanism in withdrawn slices. The inhibition of GABA release in the PAG in withdrawn slices was replaced by inhibition through adenylyl cyclase (203). The mechanism responsible for presynaptic inhibition of glutamate release in the VTA during withdrawal is as yet uncharacterized, but was not through the inhibition of adenylyl cyclase (311).

Although the sensitivity to opioid agonists was not changed in VTA in withdrawn slices, the presynaptic inhibition of glutamate EPSCs to both $GABA_B$ and metabotropic glutamate receptor agonists was increased (311).

The same increased sensitivity to metabotropic glutamate agonists has been observed at the excitatory glutamate synapse on medium spiny cells in the nucleus accumbens (313). These results suggest that one of the consequences of withdrawal from chronic morphine is an enhanced presynaptic inhibition mediated by nonopioid G protein-linked receptors at opioid-sensitive synapses. The mechanism and significance of this observation remain to be determined. The observation alone suggests that there has been a fundamental change in regulation of transmitter release at these synapses; that is, in the presence of what would otherwise be normal activity, the activation of nonopioid presynaptic receptors results in an exaggerated response. These results indicate that the adaptive responses to chronic morphine treatment can encompass unexpected and wide-ranging network responses that may not be directly affected by opioids.

C. Synaptic Plasticity and Chronic Opioids

The physiological mechanisms that mediate compulsive self-administration of drugs as well as the adaptations responsible for opioid dependence represent pathological forms of memory. At the cellular and synaptic level, it is likely that synaptic adaptation induced by drugs of abuse have common mechanisms with other forms of activity-dependent plasticity, such as LTP or LTD. This activity-dependent plasticity may result from the direct effect of morphine on the excitability or transmitter release of individual cells. However, the indirect effects of opioids are also important. For example, although pyramidal cells of the hippocampus are not directly affected by opioids, the disinhibition mediated by the opioid inhibition of GABA interneurons increases pyramidal cell excitability, which would be expected to facilitate some forms of LTP. The recent advances in understanding the molecular mechanisms underlying synaptic plasticity in the mammalian CNS are directly relevant to gaining an understanding of the effects of drug self-administration.

Activity-dependent adaptations of synaptic efficacy are thought to be essential to memory formation and storage and the development of neural circuits. The first demonstration that high-frequency electrical stimulation can induce LTP of synaptic transmission was made at the glutamatergic synapses between the perforant path fibers and granule cells of the dentate gyrus of the hippocampus (36). Since then, similar forms of durable synaptic enhancement have been described at the Schaffer collateral to CA1 pyramidal cell synapses and at the mossy fiber synapses in the CA3 region of the hippocampus. Distinct mechanisms mediate the plasticity at these two hippocampal synapses, and examples of each mechanism have been observed at numerous central synapses. Thus LTD has been observed both at the mossy fiber synapse and at the Schaffer collateral-CA1 synapse (303, 305). This form of plasticity is observed with protocols and by mechanisms distinct from those that induce potentiation.

1. cAMP-dependent LTP and LTD

The long-term regulation of transmitter release by the cAMP cascade has been observed in many organisms from *Drosophila* to vertebrates (140) and may well be one of the most conserved mechanisms that regulate synaptic efficacy. One site where synaptic plasticity has been shown to be dependent on cAMP is the glutamate synapse between the axons of granule cells from the dentate gyrus (mossy fibers) and the dendrites of the pyramidal cells of the CA3 region. High-frequency stimulation of the mossy fibers causes a long-lasting increase in the size of the EPSCs measured in the CA3 pyramidal cells. This LTP is thought by most to be independent of postsynaptic activity. Blocking postsynaptic DL-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) or NMDA receptors, chelation of postsynaptic calcium and hyperpolarization of the postsynaptic neurons were without effect on the induction of LTP at this synapse (545). Experiments examining the frequency of synaptic failures and reduced paired-pulse facilitation were consistent with a presynaptic mechanism for the maintenance of LTP (534, 545). Finally, results using the progressive block of NMDA EPSCs with the open-channel blocker MK801 indicated that the release probability of glutamate was increased after the induction of LTP (512).

The presynaptic signaling pathways responsible for LTP at the mossy fiber synapse are thought to be initiated by an increase in terminal calcium. Buffering extracellular calcium to a low concentration blocked the induction of LTP (69). The rise in terminal calcium is thought to activate a calcium-sensitive isoform of adenylyl cyclase (101). Forskolin and agents that directly activated PKA caused a long-lasting potentiation of synaptic transmission and occluded further induction of LTP. Inhibitors of PKA reduced LTP (196, 197, 511). It was therefore concluded that LTP at the mossy fiber synapse required presynaptic activation of a calcium/calmodulin-dependent isoform of adenylyl cyclase. Recent experiments in mice where the gene coding for AC1 and AC8 were disrupted showed a complete blockade of LTP, confirming earlier interpretations (531).

Synaptic plasticity mediated by a cAMP-dependent pathway has also been described at the parallel fiber synapse onto Purkinje cells in the cerebellum (78, 409) and in the amygdala (195). Thus the cAMP cascade appears to be a common mechanism that regulates the strength of synaptic transmission

In addition to the cAMP-dependent LTP, mossy fiber synapses also display a presynaptic form of LTD. A prolonged low-frequency stimulation protocol is generally

most effective (15 min at 1 Hz). An important component of LTD is the dependence on the activation of presynaptic metabotropic glutamate receptors (mGluRs) that are negatively coupled to adenylyl cyclase (254, 491, 538). The inhibition of adenylyl cyclase was thought to decrease PKA activity and reduce the cAMP-dependent component of glutamate release. Thus synaptic strength at the mossy fiber synapse onto CA3 pyramidal cells can be increased and decreased by cAMP-dependent mechanisms.

2. Opioids and cAMP-dependent LTP and LTD

Interactions between chronic morphine treatment and synaptic plasticity at the mossy fiber synapse in the CA3 region of hippocampus are of particular interest because *1*) opioid receptors inhibit glutamate release at this synapse (436, 513), *2*) the cAMP cascade plays a central role in plasticity at this site (197, 511), and *3*) there has been considerable effort directed toward the molecular mechanisms that mediate LTP at this synapse.

Considering the role of adenylyl cyclase in LTP and LTD at the mossy fiber-CA3 synapse in the hippocampus and the fact that AC1/8 are both upregulated by chronic morphine, it is possible that this is one site where the long-term effect of opiates will affect plasticity. Biochemical analysis of adenylyl cyclase activity has failed to observe a significant change in this area (479); however, detection of a selective change in the mossy fiber terminals may be limited in this heterogeneous structure. Chronic morphine treatment did, however, result in a decrease in prodynorphin mRNA and peptide levels of dynorphin-(1—13) in the hippocampus, the striatum, and the hypothalamus (390, 400). This is a sensitive assay in the hippocampus because it is a measure of a peptide that is expressed and released selectively from mossy fibers (334).

In slices from control guinea pigs, stimulation of the mossy fibers is known to release dynorphin that acts to inhibit further glutamate release (504, 513). The corelease of dynorphin under some conditions is capable of limiting the initiation (513) or the extent of LTP (J. M. Harrison, R. Allen, J. T. Williams, and O. J. Manzoni, unpublished data). Naloxone, norbinaltorphimine (nor-BNI), and H-D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2 (CTAP) all resulted in an increase in the amplitude of LTP, suggesting that the release of endogenous opioids, probably dynorphin, activated both μ- and κ-opioid receptors on the mossy fiber terminals (Harrison et al., unpublished data).

3. Chronic morphine and cAMP-dependent LTP and LTD

In acutely withdrawn slices from morphine-treated guinea pigs, LTP at the mossy fiber synapse was enhanced, but the inhibition caused by DAMGO and U69593 were unchanged (Harrison et al., unpublished data). Whereas opioid antagonists increased the amplitude of LTP in control slices, there was no effect in withdrawn slices. One potential mechanism that would explain the enhancement of cAMP-dependent LTP at mossy fiber synapses and the lack of an augmentation by opioid antagonists is a reduction in dynorphin release during withdrawal. In fact, there are reports that indicate that the content of dynorphin in hippocampus was reduced in animals treated chronically with morphine (390, 400). Somewhat surprisingly there was no upregulation of adenylyl cyclase in withdrawn slices. The prolonged cAMP-dependent inhibition caused by activation of group 2 mGluR receptors with LCCC1 (491) and the effect of forskolin were not changed by morphine treatment. Thus a change in the role of adenylyl cyclase on the regulation of synaptic plasticity at this synapse after chronic morphine treatment was not apparent, confirming biochemical measurements (479).

A cAMP-dependent increase in transmitter release during withdrawal has been described in several areas (42, 86, 203, 430). The effects of this upregulation on LTP or LTD have not been investigated, and the interaction between the actions of opioids and activity-dependent plasticity could be critical in areas such as the amygdala and nucleus accumbens. Both these areas have been shown to be important in context-dependent learning (201, 325, 396, 515), have opioid-sensitive synaptic input (see below), and are sites where LTP and LTD have been observed (195, 259). The coincidence of an upregulation of adenylyl cyclase and context-dependent activity at specific synapses in these areas may be a potent activator of long-term synaptic plasticity associated with morphine treatment.

4. NMDA-dependent LTP and LTD

The most widely studied form of synaptic plasticity is NMDA-dependent LTP. It is the subject of many extensive reviews, and anything but a brief description is outside the scope of this review. It is, however, critical to consider the mechanisms that mediate this form of synaptic plasticity when trying to understand tolerance to morphine, particularly associative tolerance. Most of what is known about the mechanisms underlying NMDA-dependent LTP and LTD is based on the glutamate synapse between the axons from CA3 pyramidal cells (Schaffer-collaterals) and CA1 pyramidal cells in the hippocampus. Although similar observations have been made in a variety of excitatory central synapses in areas such as the perforant path-dentate gyrus in the hippocampus (253, 348, 535), visual (253) and neocortex (489), thalamocortical synapses (206) as well as synapses in the VTA (40) and nucleus accumbens (259), none of these sites has been as well characterized as in CA1 cells of the hippocampus.

Fast excitatory glutamate transmission is principally mediated by AMPA subtype receptors that are activated at the resting membrane potential. At most mature excitatory synapses, AMPA receptors coexist with NMDA receptors that are normally blocked by magnesium at the resting membrane potential. To be activated, NMDA receptors require both glutamate binding and membrane depolarization. LTP is induced by high-frequency stimulation of the Schaffer collaterals (typically, 3 times 1 s at 100-Hz tetanus). The repetitive stimulation results in postsynaptic summation of AMPA-EPSCs depolarizing the dendritic spine to relieve the voltage-dependent block of NMDA channels by magnesium. Calcium entry through the activated NMDA channel increases the intracellular level of calcium. This postsynaptic rise in calcium is thought to activate a variety of calcium-dependent enzymes and is an absolute requirement for LTP induction. Pharmacological and knock-out techniques (433, 434) have shown that activation of calcium/calmodulin kinase is required for LTP. Strains of mice with disrupted genes for Fyn, PKC-γ, or α-calmodulin kinase II have also confirmed the importance of these kinases in CA1-LTP. The phosphorylated (activated) form of calmodulin kinase II can modulate postsynaptic neurons in at least two ways (282): *1*) it can phosphorylate AMPA receptors and augment their conductance (25, 31), and *2*) it could modulate the slow afterhyperpolarization and increase neuronal excitability (340). It should be noted that a long-lasting decrease in the afterhyperpolarization induced by a protocol that induces LTP has not been generally observed.

In contrast to the consensus about the mechanisms of induction, there is some controversy about the site of expression of LTP (presynaptic vs. postsynaptic). Presynaptic modifications of transmitter release might be involved, but there is mounting evidence pointing to a predominant postsynaptic mechanism (reviewed in Refs. 270, 304, 352). One recent hypothesis is that LTP is caused by the recruitment of AMPA receptors at synapses that originally express only NMDA receptors. These NMDA-only synapses have been termed "silent synapses" (207, 280). This major structural/functional change has recently been supported by experiments showing the increased expression of AMPA receptors during postnatal development in hippocampus and visual cortex (281, 376, 405) and after LTP-inducing stimuli in the hippocampus (297, 429).

Two types of LTD have been found on the Schaffer collateral synapse on CA1 pyramidal neurons. One is dependent on NMDA receptors, while the other depends on mGluRs (361). NMDA-dependent LTD is induced by prolonged low-frequency stimulation of the afferent fibers (typically 1 Hz for 15 min). Summarized briefly, a small postsynaptic increase in intracellular calcium is thought to activate a phosphatase cascade involving calcineurin and protein phosphatase 1 (106). Functionally this form of LTD reverses NMDA-dependent LTP mediated by calcium/calmodulin kinase. Thus it appears that there is a balance between kinase and phosphatase activity that can be regulated by internal calcium. A second form of LTD has been described that is dependent on activation of mGluR5 receptors and calcium entry and depends on the activation of PKC. This form of LTD is distinct in that it appears to be phosphatase independent (361).

5. Opioids and NMDA-dependent synaptic plasticity

Morris and Johnston (334) proposed that the effect of opioids on plasticity in one pathway in the hippocampus could affect downstream circuitry (334). Thus the effect of opioids could regulate two or more synapses in series by a domino effect. This concept has been examined at the glutamate synapses between perforant path fibers and granule cells of the dentate gyrus where opioids act by disinhibition to facilitate excitatory transmission (348, 535). In fact, μ-receptor antagonists were found to block LTP at the synapses of the lateral perforant path, suggesting that endogenous opioids were released by the stimulus protocol (334, 535). By altering excitatory input to the dentate granule cells, opioids affect the mossy fiber input to CA3 pyramidal cells and therefore the Schaffer collateral input to CA1 pyramidal cells. Thus the output of the hippocampus into areas such as the nucleus accumbens can be regulated by a very indirect mechanism.

There are several examples of synaptic plasticity that are similar in mechanism to the extensive studies done in the hippocampus. Such examples include regions that are critically important in the long-term actions of drugs of abuse such as the nucleus accumbens (259, 372), the VTA (40), and amygdala (75, 91, 195). The knowledge acquired on synaptic plasticity in the hippocampus will be useful to address the effects of chronic drug use on synaptic physiology. For example, it has recently been shown in both nucleus accumbens and VTA that mGluR-mediated presynaptic inhibition of EPSCs was enhanced after chronic morphine treatment (311, 313). These observations are particularly interesting in light of the role of mGluRs in LTD that has been established at the mossy fiber (254, 491, 538) and Schaffer collateral synapses (361).

Brain structures responsible for adaptations in response to chronic opioids may exhibit forms of synaptic plasticity that are remarkably similar to those observed in the hippocampus. In fact, the list of "molecules implicated in hippocampal LTP" presented by Sanes and Lichtman (411) includes an impressive number that are known targets of chronic opioids. Thus it appears that synaptic plasticity initiated by opioids and activity-dependent processes may be linked by common mechanisms. The systematic study of the effects of chronic drug treatment on LTP and LTD-like phenomena in structures relevant to drug addiction will be crucial to the understanding of the

synaptic consequences of chronic drug use. Assuming that LTP and LTD are potentially important in mechanisms of learning and memory, morphine may have far-reaching actions affecting synaptic plasticity by both direct and indirect mechanisms. This may be particularly important in areas thought to be involved in associative tolerance such as the amygdala (325).

IV. NEURONAL SYSTEMS INVOLVED IN ADDICTION

Early models of opioid dependence focused on counteradaptations to the euphoric, or positively reinforcing, aspects of opioid use. Cessation of chronic opioid use is associated with an intensely dysphoric withdrawal syndrome, or a negative drive to reinstate drug use. This was once thought to be sufficient to explain the persistence of opioid addiction (e.g., Ref. 93). As discussed below, there is now some understanding of the core neural adaptations responsible for the opioid withdrawal syndrome. There is no doubt that they play an important role in maintaining episodes of opioid abuse, for example, rates of relapse to opioid use during unassisted detoxification (i.e., acute withdrawal) are generally greater than 80% (see Ref. 316) presumably due to the intensely dysphoric withdrawal syndrome. However, it has also become clear that opioid addiction cannot be explained solely on this basis (e.g., Ref. 260). Long-term adaptations in neural systems responsible for positive aspects of addiction, such as craving, are now thought to play an important role in the chronic relapsing-remitting nature of the disorder.

A. Systems Involved in Negative Aspects of Opioid Addiction

Negative aspects of the opioid withdrawal syndrome include those referred to as somatic or vegetative signs and aversive signs. In rats and mice, the former include jumping, burrowing, wet-shakes, hyperreactivity, vocalization, teeth chatter, piloerection, ptosis, lacrimation, rhinorhea, diarrhea, penile erection, and ejaculation (e.g., Ref. 276). Experimental models of the aversive nature of opioid withdrawal involve tests of conditioned place aversion (e.g., Ref. 60). Efforts to identify neural systems involved in expression of the opioid withdrawal syndrome have used microinjections of opioid antagonists to find anatomical loci mediating somatic signs of withdrawal. These have often been complemented with lesion studies and biochemical markers of changed neural activity. Unfortunately, both methods are potentially flawed. False-positive results occur in microinjection studies because relatively large doses and volumes of drugs are injected into brain regions so that spread to neighboring regions or the ventricular system cannot be ruled out. Although spread (but not specificity) of microinjections can be controlled for by examining effects on loci around the region of interest, this is rarely done systematically in practice. Similarly, false positives occur in lesion studies (but less so with excitotoxic lesions) because of uncontrolled damage to surrounding structures and fibers of passage. False negatives can readily occur because the dose, extent, or sphere of influence of manipulations (injections or lesions) may not fully encompass the relevant neural populations. This is particularly true for anatomically complex or highly nonspherical structures.

Early microinjection studies implicated neurons accessible from the fourth ventricle, particularly the midbrain (PAG) and pontine central gray (including the LC) to mediate most of the somatic signs of opioid withdrawal (276, 508–510). More recent work (see sect. IV*A2*) has substantiated this general localization, but there has been some controversy concerning specific groups of neurons involved in the initiation and expression of withdrawal behavior (Fig. 8; Ref. 90).

1. Noradrenergic systems

The ability of drugs that reduce the activity of noradrenergic neurons, particularly α_2-adrenoceptor agonists such as clonidine, to inhibit withdrawal focused attention on the role of the noradrenergic nucleus LC in expression of somatic and aversive signs of withdrawal (159; reviewed in Refs. 298, 346; for a contrary view, see Ref. 90). Following Aghajanian (7), many studies confirmed that LC action potential activity is profoundly increased in vivo during opioid withdrawal (but not in vitro, reviewed in Ref. 90), and an extensive CNS survey of signs precipitated by microinjection of the opioid antagonist methylnaloxonium found the LC to mediate expression of more somatic signs of withdrawal at lower doses than in other brain regions (301). Although electrolytic lesion studies seemed to confirm these observations (299), 6-hydroxydopamine lesions of the dorsal noradrenergic bundle (16, 48, 112), which conveys the noradrenergic projections from the LC to the midbrain and forebrain, and complete 6-hydroxydopamine lesion of the LC itself (60) had no effect at all on the somatic or aversive aspects of opioid withdrawal. The latter observations unequivocally ruled out a role for the LC in expression of somatic and aversive signs of opioid withdrawal. Profound activation of LC neurons during withdrawal is, therefore, an epiphenomenon, and withdrawal signs evoked by microinjections of opioid antagonists in the vicinity of the LC are presumably affecting nearby brain regions such as the PAG and pontine tegmental regions such as Barrington's nucleus (see sect. IV*A2*).

Other evidence has implicated noradrenergic neurons arising from medullary sites, including the nucleus tractus solitarius and the ventral medullary A_1 noradren-

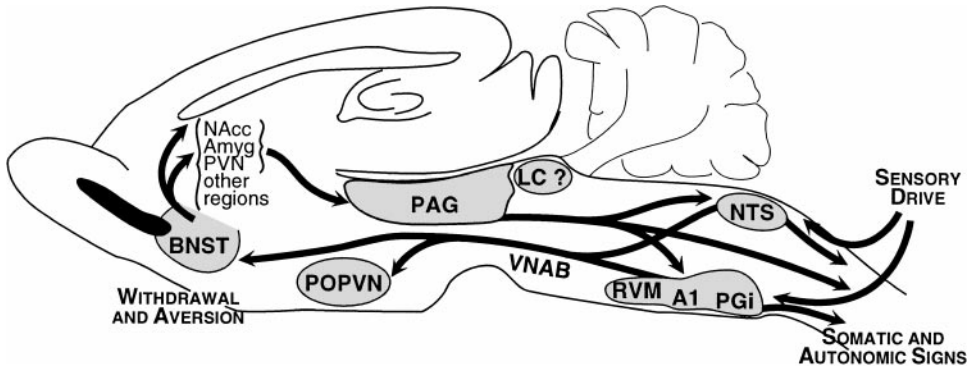

FIG. 8. The anatomical substrate mediating opioid withdrawal. The bed nucleus of the stria terminals (BNST) is a major target of noradrenergic projections from the ventral noradrenergic bundle (VNAB) that has been clearly demonstrated to be necessary for expression of aversive behavior associated with opioid withdrawal. The VNAB also influences other brain areas during withdrawal such as at the paraventricular hypothalamic nucleus (PVN) and preoptic area (PO). Activation of noradrenergic cells in the A1 region and the nucleus tractus solitarius (NTS) is probably associated with VNAB activation, since complete neurotoxic ablation of the locus coeruleus (LC) has demonstrated that this group of neurons is not involved in expression of somatic or aversive signs of opioid withdrawal. The periaqueductal gray (PAG), a crucial site for initiation and expression of somatic signs of opioid withdrawal, receives major innervation from forebrain regions [many of which are innervated by BNST, i.e., nucleus accumbens (Nacc), amygdala (Amyg), and PVN]. The PAG innervates medullary sites including the rostral ventromedial medulla (RVM), particularly the nucleus raphe magnus and the nucleus paragigantocellularis (PGi) and NTS that are probably responsible for the expression of many of the somatic and autonomic signs of opioid withdrawal via their efferent projections to autonomic and somatic motor neurons. The PGi in particular provides excitatory input to sympathetic premotor neurons of the intermediolateral column and is the major source of excitatory drive to the LC during withdrawal. It is not clear yet whether sensory drive contributes to excitation of these medullary regions during withdrawal.

ergic group. These neurons innervate the forebrain via the ventral noradrenergic bundle, and there is particular interest in the bed nucleus of the stria terminalis, which was shown to mediate the aversive but not somatic components of opioid withdrawal (16, 112). Noradrenergic innervation of supraoptic and paraventricular magnocellular hypothalamic neurons may also modulate endocrine disturbances that occur during opioid withdrawal (51). The effects of α_2-adrenergic drugs on opioid withdrawal may involve these projections and/or various other brain regions that express both opioid and α_2-adrenoceptors. Indeed, α_2-adrenoceptor agonists overcome sympathetic nervous system-mediated signs of opioid withdrawal when injected intrathecally (56) and the excitation of neurons in the PAG during opioid withdrawal (84, 203).

2. Descending systems involved in opioid withdrawal

Microinjection studies (44, 276, 301, 508–510) have also strongly implicated the midbrain PAG in expression of somatic signs of opioid withdrawal. Electrical or chemical stimulation of the PAG modulates responses to noxious stimuli in drug-naïve animals and evokes somatic and autonomic responses associated with defensive behaviors that bear some resemblance to opioid withdrawal (23, 283). Major projections arising from the PAG innervate ventral and dorsal medullary regions that are also implicated in expression of opioid withdrawal (see below).

In vitro, opioids acting on μ-receptors in PAG increase potassium conductance (82, 365), inhibit voltage-gated calcium channel currents (98), and presynaptically inhibit both glutamate- and GABA-mediated neurotransmission (83, 498, 499). Although excitatory neurotransmission is inhibited, the dominant acute effect of opioids is thought to be disinhibition of descending projections to medullary sites including the nucleus raphe magnus, nucleus paragigantocellularis, and the nucleus tractus solitarius via direct inhibition of GABAergic neurons and GABA neurotransmission (26).

In vitro electrophysiological studies (84, 203) have also established that opioid-sensitive neurons and GABAergic synapses in PAG display rebound hyperactivity during opioid withdrawal that is consistent with expression of opioid withdrawal. As discussed above, a cAMP-modulated cation conductance contributes to hyperexcitability of PAG cell bodies, some of which are GABAergic (236), during opioid withdrawal (84; Bagley and Christie, unpublished data). In addition, a switch from presynaptic modulation of GABA transmission via arachidonic acid metabolites to mediation via adenylyl cyclase activity is responsible for rebound enhancement of GABA synaptic activity in PAG during withdrawal (203). Microinjection of PKA inhibitors into PAG attenuated withdrawal signs (302, 385), suggesting that these adaptations observed in vitro contribute to the expression of withdrawal. Other forebrain and midbrain regions implicated in opioid withdrawal, for example, the nucleus accumbens and central nucleus of the amygdala (179, 458), also innervate the PAG and may thereby influence expression of some signs of opioid withdrawal.

It is not yet clear whether or not similar adaptations develop at other sites in descending systems involved in autonomic and somatic responses to threatening stimuli. Indirect indices of withdrawal activation of neurons, e.g., c-*fos* expression, indicate that neurons in the nucleus paragigantocellularis, nucleus tractus solitarius, and dorsal horn of the spinal cord are activated during opioid withdrawal (395, 460). Increased activity of (presumed) adrenergic nucleus paragigantocellularis (PGi) neurons has been reported during opioid withdrawal, but it is not yet clear whether this also occurs in vitro (24). When stimulated, the PGi activates both ascending alerting systems including LC and sympathetic neurons (284), and direct stimulation of the PGi in behaving animals induces withdrawal-like behavior (173).

3. Ascending sensory systems in opioid withdrawal

There is no doubt that the LC is strongly activated in vivo by opioid withdrawal and that this largely arises from excitatory afferent drive. The major excitatory afferents to the LC arise from the PGi, which may be activated by intrinsic counteradaptations of enhanced afferent drive arising from sensory neurons or ultimately from other brain regions such as the PAG. There is little evidence available regarding whether or not afferent neurons or their central terminations in the superficial dorsal horn exhibit intrinsic rebound excitation during opioid withdrawal. In vivo extracellular recordings (220) and indirect measures of neural activation have suggested that superficial dorsal horn neurons are hyperactive during opioid withdrawal, but neurochemical lesion studies have suggested that this is dependent on descending noradrenergic neurons (395).

It is tempting to speculate that the PAG, PGi, and NTS form part of a descending (and ascending) network that plays a core role in the generation and expression of negative aspects of opioid withdrawal with diverse influences on somatic, autonomic, and aversive components of the phenomenon. Other brain regions including the nucleus accumbens, amygdala, and paraventricular hypothalamus contribute to the aberrant activity of this system during acute opioid withdrawal.

B. Systems Involved in Positive Aspects of Opioid Addiction

The mesolimbic system is thought to play a key role in endogenous reward (45, 528). This system is of obvious survival value as all species require strong motivation for items such as food, water, and sex. Many drugs of abuse, including opioids, are capable of efficiently activating this system. One of the theories explaining the basis of drug abuse is dependent on the effects of drugs in this reward pathway. Both animals and humans will work for drugs, sacrificing other forms of reward simply because the drug is more efficient at producing satisfaction. Thus the acute and chronic actions of abused drugs in the various components of this system have been intensely examined. The best-recognized components of this pathway include the VTA, nucleus accumbens, ventral pallidum, and prefrontal cortex.

It appears that the connections between these areas are critical for many of the adaptive changes induced by morphine, and other drugs, since no single area can account for all effects. For example, rats will lever press for microinjection of μ-opioid agonists into the VTA, suggesting that the VTA is involved in the reinforcing properties of opioids (113). With repeated microinjection of opioids into the VTA, animals become sensitized to the locomotor stimulant effects; that is, it takes less morphine to cause an equal locomotor response (230, 233, 234, 502). Such sensitization persists for weeks after the termination of the treatment, indicating that opioid actions in the VTA can lead to long-term effects. Both the expression of sensitization (234) and the reinforcing properties of opioids under some conditions (247) are also dependent on the nucleus accumbens. Thus the reward pathway is dependent on the interaction between the VTA and other nuclei.

1. VTA

The VTA is a heterogeneous group of cells made up of dopamine and GABA-containing neurons. Until recently, the dopamine cells were thought to be the output neurons, and GABA cells were interneurons. The recent discovery of GABA projection neurons extends the potential role of the VTA (452, 496). Electrophysiological studies of the substantia nigra pars compacta and the VTA in both rats and guinea pigs (162, 224, 272, 273, 543) have demonstrated neurons that fall into two or three categories (162, 224, 272, 273, 543). Two groups of neurons are directly hyperpolarized by opioids. The best characterized are presumed GABAergic interneurons (225). The second group of cells is distinct in that they are hyperpolarized by opioids, 5-hydroxytryptamine (5-HT), and dopamine (62). The third group is the dopamine cells. Although the membrane potential of dopamine cells was not directly affected by opioids, a substantial portion of dopamine cells expresses both μ- and κ-opioid receptor mRNA (10) and immunoreactive protein (Wessendorf, unpublished observations). The release of dopamine in the nucleus accumbens in vivo and in cultures of midbrain neurons is decreased by activation of κ-receptors (3, 110, 443, 448, 533). Thus a functional role of receptors found on dopamine cell terminals has been established. The function of opioid receptors expressed on the cell body and dendrites of dopamine cells remains to be elucidated, since no direct effects on membrane excitability have been observed.

The most robust response to opioids measured in dopamine cells results from the presynaptic inhibition of GABA release, which through disinhibition increased the firing frequency (171, 225).

A) PRESYNAPTIC REGULATION OF GABA RELEASE. Both $GABA_A$- and $GABA_B$-mediated synaptic potentials in dopamine cells of the VTA were inhibited presynaptically by opioids. The $GABA_A$-mediated synaptic potential is thought to arise from interneurons that are hyperpolarized by opioids (225). The inhibition of spontaneous activity recorded from interneurons correlated with the inhibition of tetrodotoxin-sensitive GABA-mediated IPSPs recorded in dopamine cells (225). It was concluded that cells that were hyperpolarized by μ-opioid receptors in the VTA were GABA interneurons.

The $GABA_B$ IPSP is thought to arise from fibers originating in the nucleus accumbens or ventral pallidum. On the basis of selective effects of 5-HT and dopamine on $GABA_A$ and $GABA_B$ IPSPs, separate terminals are thought to mediate these synaptic responses (63, 223). The $GABA_B$ IPSP was increased by D_1-dopamine agonists and decreased by $5\text{-}HT_{1B}$ agonists, whereas the $GABA_A$ IPSP was insensitive to both agonists. Unlike the inhibition of the $GABA_A$ IPSP, both μ- and κ-opioid agonists decreased the $GABA_B$ IPSP by a presynaptic mechanism (225, 430). Thus the activation of opioid receptors on at least two types of GABA-releasing terminals decrease GABA-mediated inhibition, allowing an increase in activity through disinhibition (171, 225).

B) PRESYNAPTIC REGULATION OF GLUTAMATE RELEASE. Excitatory synaptic input mediated by glutamate is a key component of the regulation of dopamine cells. The glutamate afferents arise from three primary sources: the medial prefrontal cortex, the pedunculopontine region, and the subthalamic nucleus (133). Glutamate acts on AMPA, NMDA, and mGluRs to depolarize dopamine neurons (321, 428). In addition, glutamate mediates a slow IPSP through activation of mGluR receptors (134). Synaptically released glutamate can therefore cause rapid and slow changes in the activity of dopamine cells. One role of the glutamate innervation of the VTA is to mediate a switch from pacemaker-like firing in dopamine cells to burst-firing pattern (148, 341, 465).

Presynaptic inhibition of glutamate release by μ-receptors caused inhibition of both AMPA- (311) and mGluR-mediated (134) synaptic responses. Taken together, the acute effects of opioids reduce both excitatory and inhibitory afferent inputs onto dopamine cells such that the firing rate and pattern would be more dependent on the intrinsic membrane properties that sustain spontaneous activity.

C) CHRONIC MORPHINE TREATMENT AND THE REGULATION OF EXCITABILITY. The activity of dopamine cells recorded in vivo during acute withdrawal from repeated morphine was depressed for at least 7 days (118, 116). Although the basal firing rate returned to control values after 14 days of withdrawal, the sensitivity to morphine was significantly increased. Adaptations in synaptic regulation of dopamine cells within the VTA may account at least in part for these observations. For example, the prolonged inhibition of dopamine cells could result from the increased probability of GABA release that has been observed when measuring $GABA_A$ IPSCs during withdrawal (42). An increased sensitivity to the presynaptic inhibition of glutamate release to both $GABA_B$ and mGluR agonists could also reduce the release of glutamate (311). In fact, a decrease in the burst rate of dopamine cells recorded in vivo, which is a measure of glutamate release, was the measure that was affected the most during withdrawal (116).

A small upregulation of GluR1 AMPA receptor subunit expression in homogenates of VTA was found after repeated morphine treatments (138). Such an upregulation could be an adaptive response resulting from a tonic decrease in glutamate release (311). Given the results indicating that the activity of dopamine cells was reduced during withdrawal, it may be that the increased expression of AMPA subunits occurs in interneurons. Physiological experiments examining AMPA EPSCs in the VTA, particularly on interneurons, have not been done in animals treated chronically with morphine.

The increased sensitivity of dopamine cells to morphine in animals withdrawn for 14 days demonstrates sensitization at the single-cell level. Although it is not possible to determine where the increased sensitivity occurs in this experiment, under some conditions, the inhibition of the $GABA_B$ IPSP caused by DAMGO and [Met]enkephalin in the VTA was greater in morphine-withdrawn slices (430). Another lasting effect of withdrawal from morphine treatment was an increase in presynaptic inhibition of the $GABA_B$ IPSP mediated by A_1 adenosine receptors (41, 430). The increase in adenosine tone was determined by examining the effect of an adenosine antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), on the amplitude of the $GABA_B$ IPSP. In slices from withdrawn animals, the antagonist caused a significantly larger increase in the $GABA_B$ IPSP than in controls. It was concluded that there was an increase in the level of endogenous adenosine since the sensitivity of the A1 receptor was not affected by morphine treatment. This effect was persistent, since it was also found in slices taken from animals that were withdrawn for more than 7 days (136). The increase in adenosine tone would reduce inhibition of dopamine cells by GABA released from sites that mediate the $GABA_B$ IPSP. The significance of adenosine-mediated presynaptic inhibition of $GABA_B$ IPSPs on the excitability of dopamine cells is not known.

D) CHRONIC MORPHINE TREATMENT AND POSTSYNAPTIC ADAPTATIONS OF DOPAMINE CELLS. Various neurochemical, morphological, and physiological changes in slices of VTA result

from chronic morphine treatment. Some of these changes have been shown to occur on dopamine cells. For example, the amount of immunoreactive tyrosine hydroxylase (TH) was increased at the same time as a small decrease in mRNA expression was observed (43). The size of dopamine cells also declined by 25% (440). These effects selectively affected dopamine cells, since the TH expression in LC was increased and the morphology of TH-negative cells in the VTA was not affected. It is not known if these changes result from direct or indirect actions of opioids on the dopamine cells. Many other changes in the VTA have been identified based on experiments done with tissue homogenates or microinjections. These experiments, however, do not distinguish effects on dopamine cells from other cell types in the area (neurofilament proteins, Ref. 28; axoplasmic transport, Ref. 29; phospholipase C-γ, Ref. 529; G proteins, Ref. 479; ERK, Ref. 33; glutamate receptor subunits, Refs. 68, 138).

Dopamine release is decreased by the κ-opioid agonist U69593, in dissociated cultures from rat midbrain (110). With prolonged treatment (6 days), tolerance to the inhibition by U69593 and an increase in release upon withdrawal was observed (110). Thus continuous exposure of dopamine cells in primary culture results in adaptations at both the receptor (tolerance) and the effector. Upon the induction of withdrawal there was a long-lasting increase in the amount of spontaneously released dopamine. The early component of the augmented release was thought to result from accumulation of dopamine in stores simply as a result of the inhibition of release. The augmented release seen after longer incubation periods may represent the induction of processes that facilitate transmitter release, as has been observed in brain slice experiments at GABA synapses (42, 86, 203, 430).

It appears that the inhibition of dopamine release induced by activation of κ-receptors is less effective than the increased excitability of dopamine cells induced by systemically administered morphine. Acute administration of morphine increased dopamine release in the ventral striatum measured using microdialysis (3). During acute withdrawal from chronic morphine treatment, both basal and morphine-stimulated release were depressed. The decrease in basal dopamine correlates with the depression of dopamine cell firing during the first week of withdrawal (116). In addition, during withdrawal, the morphine-induced increase in dopamine release was depressed in absolute measures, but significantly greater when expressed as the increase over basal (3). Thus it was concluded that the sensitivity to morphine increased after the first 3 days of withdrawal, but returned to control after 1 wk. The increased sensitivity to morphine is similar to, although not as long lasting as, that found when measuring the effect of morphine on the firing rate of dopamine cells (116).

In summary, the effects of chronic morphine treatment on the activity of dopamine cells are made up of both pre- and postsynaptic adaptations. Presynaptic adaptations involve cAMP-dependent and -independent mechanisms that include the regulation of both GABA and glutamate release. Many of the postsynaptic adaptations may result from the direct activation of κ-opioid receptors or indirectly from altered afferent input. The primary result is a long-lasting decrease in dopamine cell activity. This decreased activity results in part because of an increase in GABA-mediated inhibition and possibly augmented presynaptic inhibition of glutamate release.

2. Nucleus accumbens

The nucleus accumbens is a site of convergence of many limbic areas and is thought to be critical for both the acute reinforcing and withdrawal aversive stimulus properties of opioids (185, 261, 422). Dopamine projections to the nucleus accumbens have both pre- and postsynaptic actions (182, 189, 349, 373, 493). Excitatory glutamate projections from hippocampus, amygdala, septal nuclei, and prefrontal cortex innervate neurons in the nucleus accumbens (185, 338). Inhibition mediated by GABA is intrinsic to the nucleus or arises from projections from other parts of the basal ganglia (185, 263). The nucleus accumbens is a complex area having two major subdivisions and several distinct cell types (185). The core and shell regions make up the primary subdivisions (185). The more medial shell region tends to project to midline regions, for example, the ventromedial aspect of the ventral pallidum and the VTA. Projections from the core innervate the dorsolateral ventral pallidum and the substantia nigra.

Medium spiny cells make up the vast majority of neurons in the nucleus accumbens. Much of what is assumed about the properties and local connections between neurons in the nucleus accumbens is based on experiments done in the dorsal striatum. The conductance of these cells is dominated by an inwardly rectifying potassium current (492). This strong inward rectification is responsible in part for the ability of these cells to have a membrane potential that has two stable states, one at very hyperpolarized potentials (-80 mV, down state) and the other near threshold for action potentials (-50 mV, up state). The shift from one state to the other is critical for the output of the cells and is dependent on excitatory synaptic input (357, 453, 454, 520, 524). Medium spiny neurons can be subdivided into two very general groups based on their receptor expression, peptide neurochemistry, and projections (185). A very simplified description is that cells projecting to the VTA contain substance P, have D_1 receptors, and are more often found in the shell of the nucleus accumbens (288). Cells having D_2 receptors and enkephalin more often project to the ventral pallidum. Although this description is a starting point, it is

also a gross simplification because there is probably considerable overlap among these two basic populations similar to that described in the striatum (464).

The remaining neurons are made up of several groups of interneurons. Interneurons play a key role in the integration of afferent input and regulation of the output of the striatum and nucleus accumbens (185, 243, 244, 263). Anatomical and physiological data indicate that the fast-spiking interneurons innervate the GABAergic medium spiny projection neurons (244, 263, 320). Based on dual cell recordings from medium spiny and GABA interneurons in brain slices, a single interneuron was capable of evoking an IPSP in medium spiny cells that would delay the activation of action potentials (263). It was estimated that each medium spiny cell receives innervation from 4–27 interneurons, and each interneuron innervates 135–540 medium spiny. Thus the activity of a single interneuron has an important influence on the regulation of the output neurons in this area. Koos and Tepper (263) also conducted dual recordings from pairs of interneurons, which indicated there could be substantial electrotonic coupling between interneurons that would further extend the local regulation of the medium spiny cells.

Another potential source of GABA input to medium spiny neurons is recurrent collateral innervation between these cells. Although spiny neuron to spiny neuron synaptic connections have been proposed for some time, physiological experiments have failed to provide any evidence for such connections (210, 263). There is, however, considerable evidence that medium spiny neuron recurrent collaterals synapse onto accumbens interneurons, suggesting that spiny neurons and interneurons are reciprocally connected (38, 39, 244).

a) acute effects of morphine. Morphine can have variable effects in the nucleus accumbens when administered systemically (174, 175). The firing rate of a small proportion of neurons was inhibited, but most cells were unaffected by morphine. This observation was the same in experiments where animals were trained to self-administer heroin, although the percentage of cells that responded increased when the dose of heroin was increased (278). At least part of the inhibition observed in these animals may have been mediated through the VTA, since microinjection of morphine into the VTA had been previously found to inhibit activity in the nucleus accumbens (176). Local application of morphine (iontophoresis) also inhibited the firing of a percentage of cells, indicating that both local and long-distance pathways were involved in the acute actions of morphine in the nucleus accumbens (176).

Opioid receptors are present within the nucleus accumbens postsynaptically (307) and presynaptically on both glutamate and GABA afferents (163, 466–469). Opioids presynaptically inhibit EPSCs recorded in medium spiny neurons of the nucleus accumbens (312). This inhibition is thought to be critical since the glutamate input is required not only to bring the membrane potential into the relatively excitable "up state," but also to drive action potentials. A direct hyperpolarization of a subpopulation of neurons presumed to be some type of interneuron has been observed in the striatum (214). Presynaptic inhibition of $GABA_A$ IPSCs has also been observed in the nucleus accumbens, striatum, and globus pallidus (86, 214, 450, 542).

Several acute actions of opioids have been described in the nucleus accumbens, ventral pallidum, and associated striatal structures; however, these observations have not been developed sufficiently to reach any meaningful conclusions about the overall influence of opioids on neuronal activity. The complexity of the excitatory afferent input and intrinsic inhibitory innervation as well as the heterogeneity of cells make it impossible at this time to reach a consensus on the overall effect of opioids in the nucleus accumbens. In fact, the same situation exists for the actions of dopamine in this region. Despite considerable effort and multiple experimental approaches, the mechanisms and overall actions of dopamine in the striatum and nucleus accumbens are contentious and unresolved (182, 349, 350). Experiments using selective activation of excitatory afferents are critical. Given that the activity of GABA interneurons has a dramatic influence on the excitability of projection neurons (263), a study of postsynaptic effects of opioids on these cells is also relevant. Dual cell recording experiments between interneurons and medium spiny neurons could be extended to examine opioid actions at a defined synapse.

b) withdrawal from chronic morphine treatment. Evidence that the nucleus accumbens plays an important role in various aspects of withdrawal from morphine is based on behavioral studies using microinjection of agonists and antagonists (262, 458). Microinjection of D_2 agonists into nucleus accumbens also reduced several components of withdrawal (179). Thus it appears that the withdrawal-induced decrease of dopamine release in the nucleus accumbens may be an important aspect of acute withdrawal. Non-dopamine-dependent withdrawal was also demonstrated in experiments in mice lacking D_2 dopamine receptors. These animals did not demonstrate place preference for morphine, suggesting that morphine lacked rewarding properties, but did exhibit robust withdrawal signs (300). Taken together, these studies indicate an important but not exclusive role for dopamine in the nucleus accumbens, mediating both the acute and chronic actions of morphine.

The nucleus accumbens is one of many sites in which an upregulation of adenylyl cyclase following chronic morphine treatment has been observed (479). The nucleus accumbens is an area enriched in type V adenylyl cyclase (156, 329), which is an isoform that was acutely

inhibited by opioids and upregulated with chronic morphine treatment in cell-culture expression systems (19, 20). Microinjection of two PKA inhibitors, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and N-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide (H-8), into the nucleus accumbens blocked place aversion associated with morphine withdrawal, suggesting that PKA is activated as a result of the increased adenylyl cyclase (495).

Adenylyl cyclase was found to play a prominent role in the regulation of GABA IPSCs in the large cholinergic interneurons of the nucleus accumbens during withdrawal from chronic morphine treatment (86). In withdrawn tissues, an activator of adenylyl cyclase (forskolin), a D_1 dopamine agonist (SKF-82958), and a β-adrenergic agonist (isoproterenol) all caused a significantly greater increase in the GABA IPSC than in control. Thus it was concluded that an upregulation of adenylyl cyclase during withdrawal led to augmented GABA release. The opioid inhibition of GABA IPSPs was unchanged in withdrawn tissues under basal conditions. However, when release was augmented by the addition of forskolin, the inhibition caused by opioids was significantly increased. Taken together, these observations suggest that the upregulation of adenylyl cyclase and resulting increase in GABA release is a cellular correlate to withdrawal. The increased sensitivity to forskolin was present 1–2 days after the onset of withdrawal, but declined to control values within 7 days, indicating that it was a relatively acute change. The increased sensitivity to opioids may be an early sign of sensitization at the synaptic level; however, the effect of opioids was not examined at time points beyond the first hours of withdrawal. The significance of these observations in the regulation of activity within the nucleus accumbens is unknown.

These results predict that acetylcholine release would be increased acutely by opioids through disinhibition and reduced during withdrawal. However, dialysis experiments done in vivo indicate that opioids acutely decreased acetylcholine release (387). The acute inhibition caused by morphine was absent, and an increase in acetylcholine release was found following prolonged withdrawal from morphine (137, 345, 480). Although no postsynaptic actions of opioids were observed, all experiments were done with whole cell recordings such that potential postsynaptic actions could be missed due to technical limitations. In fact, preliminary results using perforated patch recordings indicate that opioids changed the spontaneous firing rate of these neurons from a steady pacemaker pattern to a robust bursting pattern (B. Chieng and J. T. Williams, unpublished data). The mechanism of this acute effect and the potential modulation of that effect induced by chronic morphine treatment remain to be determined.

Opioids also inhibited both excitatory and inhibitory synaptic currents in medium spiny projection cells (Brundege and Williams, unpublished data). In contrast to the GABA inputs to cholinergic interneurons, the opioid inhibition of GABA IPSCs to medium spiny neurons was unchanged after chronic morphine treatment. Furthermore, the ability of forskolin to potentiate GABA release at these synapses was unaffected by chronic morphine. Chronic morphine also had no effect on opioid inhibition after forskolin potentiated glutamate EPSCs in medium spiny neurons of the core. It thus appears that changes in synaptic physiology after chronic morphine treatment are localized to specific synapses within the nucleus accumbens and that alterations in the synaptic input to interneurons rather than medium spiny projection neurons may play a role in mediating the effects of opiate addiction and withdrawal. Further definition of the local circuitry within the nucleus accumbens is necessary to appreciate the significance of the specificity of this adaptive response.

Excitatory synaptic inputs to the accumbens were potently depressed by activation of A_1 adenosine and metabotropic glutamate receptors (309, 310, 313; Brundege and Williams, unpublished data). Not only was the level of endogenous adenosine sufficient to cause a small tonic inhibition of the glutamate EPSC (310; Brundege and Williams, unpublished data), presynaptic inhibition caused by D_1 dopamine receptor activation has been reported to be mediated by adenosine (182). Thus the control of extracellular adenosine can play an important role in the excitability of medium spiny neurons. It is also true, however, that the interaction between adenosine and D_1 receptors is a contentious observation (349, 350). When adenosine tone was increased by blocking its reuptake with NBTI and dipyridamole, the EPSC was potently inhibited, and this inhibition was reversed with DPCPX (310). It was therefore suggested that the extracellular adenosine resulted from the metabolism of cAMP. Under basal conditions, the inhibition of cAMP-dependent phosphodiesterase with RO-20–1724 increased the EPSP to the same extent as the adenosine receptor antagonists and occluded the increase by these antagonists (310). In addition, RO-20–1724 also significantly reduced the inhibition of the EPSP caused by inhibition of adenosine reuptake (310).

The role of adenosine during withdrawal in the nucleus accumbens has been examined in medium spiny cells (Brundege and Williams, unpublished data) and interneurons (86). The only site where adenosine tone was increased was at the GABA IPSC measured in interneurons (86). In those experiments the adenosine receptor antagonist DPCPX produced a larger augmentation in the IPSC in withdrawn slices than in controls. In medium spiny cells, withdrawal caused the concentration response curve for adenosine-mediated inhibition of EPSCs to shift to the left (Brundege and Williams, unpublished data). The increased sensitivity appeared to be dependent

on reuptake mechanisms because the same concentration-response curve to N^6-cyclopentyladenosine, an adenosine agonist that is not a substrate for uptake, was not changed by morphine treatment. Thus the sensitivity to presynaptic inhibition by adenosine was increased during withdrawal, perhaps resulting from altered adenosine reuptake. This observation, although different in mechanism, is consistent with an increased presynaptic inhibition found at this same synapse (313) and at the excitatory glutamate synapse in the VTA that is mediated by mGluRs (311). The increase in presynaptic inhibition suggests that multiple events downstream from opioid receptors can be affected by chronic morphine treatment. An increased presynaptic inhibition of glutamate release would presumably decrease the glutamate drive of medium spiny neurons during withdrawal. Although opioids cause inhibition at this synapse, the amplitude of the inhibition is small (maximum 30%). The added inhibition resulting from adenosine and mGluR activation, both of which can cause potent inhibition (60–90%), may contribute to mechanisms involved in sensitization to opioids. The significance of this adaptive mechanism on the overall function of the nucleus accumbens remains to be determined.

In summary, there is no general conclusion on the changes in function in the nucleus accumbens or associated basal ganglia resulting from chronic morphine treatment. Although there have been a number of observations made at selected synapses, these isolated observations have not been developed sufficiently to allow any predictions about short- or long-term adaptations. Given the emphasis that this area has received based on behavioral studies, the cellular, synaptic, and network mechanisms that account for these results are worthy of continued effort.

V. CORE ADAPTATIONS COMMON TO OTHER DRUGS

A. Common Network Actions: The Mesocorticolimbic System

The direct and indirect pharmacological effects of drugs acting through the dopamine system have established its role in initiating drug abuse as well as craving and relapse (119, 120, 518). The interactions of drugs of abuse with the dopamine system has been studied with dopamine receptor agonists and antagonists, lesions, and stimulation of dopamine pathways and various dopamine receptor and transporter knockout animals. Although different drugs of abuse act at distinct receptors and through separate transduction mechanisms, many promote the release and/or prolong the duration of action of dopamine in the mesocortical and/or mesolimbic systems. Drugs that directly increase the firing rate of dopamine cells include nicotine (61, 377, 404) and alcohol (49, 50, 322). Opioids and cannabinoids inhibit GABA release in the substantia nigra and VTA resulting in an increase in dopamine cell firing through disinhibition (73, 141, 142, 154, 225). Psychostimulants, such as cocaine and amphetamine, prolong the duration of synaptically released dopamine in the extracellular space (274, 319, 413). The fact that drugs such as cocaine and morphine affect dopamine cells by separate mechanisms would explain the synergistic effect of these two agents on dopamine release in the nucleus accumbens (186). An increase in firing caused by disinhibition in the VTA in combination with inhibition of dopamine uptake work together to augment dopamine tone in many projection areas. The synergistic release of dopamine may underlie the popularity of this combination of drugs (speedballs) and may also explain the use of other combinations of drugs.

A reliable and commonly used measure of the reinforcing value of many drugs employs intracranial self-stimulation (528). Many reinforcing drugs including opiates, psychostimulants, nicotine, alcohol, and cannabinoids lower the threshold for self-stimulation, whereas withdrawal from chronic treatment increases the threshold (528). Thus the circuitry involved in stimulus-induced reward seems to overlap with many but not all drugs of abuse. Part of this circuitry includes the activation of dopamine cells in the VTA. Stimulating electrodes placed in, but not outside, the VTA were capable of evoking dopamine release in the nucleus accumbens and supporting self-stimulation (149). Once animals had acquired self-stimulation, however, the release of dopamine in nucleus accumbens was rarely observed, even though stimulation induced by the experimenter was effective. The conclusion suggested that dopamine release was necessary for acquisition of self-stimulation, potentially indicating novelty or expectation, but was less important than the reward itself.

The activity of dopamine and striatal cells measured with more natural reward has been examined in an elegant series of studies in awake behaving monkeys by Schulz and colleagues (192, 418, 487, 488). Briefly summarized and oversimplified, it appeared that the firing rate and pattern of dopamine cells could be predicted. Initially, the firing rate would increase upon the presentation of a juice reward for a correct behavioral response. When the behavioral response was learned and a sensory stimulus (conditioning stimulus) was paired with the juice reward, dopamine cells began to respond with an increase in firing in response to the conditioning stimulus and not the subsequent juice reward. If the conditioning stimulus was given but no juice reward was presented, the firing rate of dopamine cells decreased transiently at the time when the juice would normally have been administered. Thus it was concluded that the firing rate of dopamine

cells could be a predictor of reward, an interpretation consistent with that based on the measurement of dopamine release in the nucleus accumbens in the self-stimulation protocol. The inhibition of dopamine cell firing may be a signal of disappointment when an expected reward did not appear and is particularly interesting in relationship to the depressed firing rate of dopamine cells during withdrawal from morphine (116, 114), alcohol (117), nicotine (132), cannabinoids (115), and cocaine (prolonged withdrawal, Ref. 1).

The changed activity of dopamine cells during withdrawal from drugs of abuse is assumed to result in modulation of dopamine release in projection areas. In fact, decreased dopamine release has been observed in the nucleus accumbens with microdialysis after withdrawal from ethanol, morphine, cocaine, and amphetamine (403). The acute response of cells in both the nucleus accumbens and prefrontal cortex during self-administration of reinforcing drugs is more complex than in dopamine cells. For example, the change in firing rate of individual neurons during self-administration of cocaine and heroin were often in the opposite direction (74). Similar results were obtained with self-administration of cocaine or water while recording activity in the nucleus accumbens (67). These studies conclude that the regulation of activity in response to different reinforcers utilized heterogeneous but overlapping neuronal circuits and that the firing rate and pattern is dependent on the behavioral state of the animal or the specific cues associated with administration of the reinforcer. The activity of dopamine cells seems predictable, suggesting that the phasic activity of dopamine cells is more or less synchronous. It is therefore possible that a global rise in dopamine resulting from a burst of activity in the VTA has by itself little or no long-term effect in projection areas. The important and potentially lasting effect(s) of dopamine may be dependent on the activity in projection areas that coincides with the release of dopamine. Thus dopamine may modify pre- and postsynaptic elements such that the likelihood of inducing synaptic plasticity (LTP and LTD) at active synapses is increased. Interestingly, the activation of adenylyl cyclase through D_1/D_5 receptors induced a long-lasting potentiation of the EPSP in the CA1 region of the hippocampus (194), suggesting a role for dopamine in LTP in other brain regions. Generalized rises and falls in dopamine levels when superimposed on different patterns of afferent input would affect various groups of neurons in different ways. Given the structure of the dorsal striatum and nucleus accumbens where the activity of groups of output neurons is highly dependent on the activity of inhibitory interneurons (13, 164, 263), drugs and experimental conditions could affect synaptic activation of groups of neurons differentially.

B. Cellular and Synaptic Adaptations: The cAMP Cascade

The most intensively studied long-term action of cocaine and amphetamine is an increased sensitivity to the locomotor activation induced by cocaine and/or amphetamine (234, 261, 397, 422). This sensitization can be very long lasting, and the cellular and synaptic mechanisms that mediate this persistent change are of obvious significance. Interestingly, psychostimulants can result in cross-sensitization to opioids (230, 233, 235, 456). There is considerable evidence that the VTA is a key site involved in the induction of sensitization (375, 482, 457, 501). First, repeated administration of opioids or psychostimulants directly into the VTA causes sensitization. Second, sensitization caused by systemically applied psychostimulants can be blocked by microinjection of antagonists (glutamate and D_1 dopamine) into the VTA (231, 232, 530).

The induction of sensitization has several components and has been reviewed extensively (234, 261, 397, 422, 518). The activation of the cAMP cascade through D_1 dopamine receptors in the VTA has been suggested to be one such component (482), because blockade of D_1 dopamine receptors (375, 457, 501) and inhibitors of PKA prevented (482) the induction of sensitization. Although D_1 activation of adenylyl cyclase and PKA is a necessary component for the induction of sensitization to psychostimulants, the activation of PKA alone was ineffective. This suggests that the D_1 receptors located on GABA projections from the nucleus accumbens and/or glutamate terminals projecting from the prefrontal cortex are important. The release of transmitter from each is increased by D_1 receptor activation (63, 134, 378). Although opioids inhibit release from both terminals, the increased release of dopamine resulting from the disinhibition of firing would be expected to activate D_1 receptors. Given the role of adenylyl cyclase in sensitization, it would seem that the upregulation of adenylyl cyclase with chronic administration of opioids is a likely mechanism leading to the induction of sensitization (482). In fact, acute withdrawal has been observed to increase presynaptic inhibition by opioids at synapses where adenylyl cyclase activity has been upregulated (54, 86, 430). An increased sensitivity to the opioid inhibition of GABA release was observed in the PAG during opioid withdrawal, as was the inhibition caused by clonidine, an α_2-adrenoceptor agonist, indicating that a mechanism downstream of the opioid receptor was upregulated by chronic morphine treatment (203). From this result it could be predicted that sensitivity to activation of any $G_{i/o}$-coupled receptor, including the cannabinoid receptor, would result in an increased response. It is not known, however, how long after withdrawal this increased sensitivity remains.

One adaptation that lasted for at least 7–10 days following withdrawal from repeated injections of mor-

phine, cocaine, and amphetamine was an increase in presynaptic inhibition caused by endogenous adenosine (41, 136). Originally done in brain slices from guinea pigs treated with either morphine or cocaine, the presynaptic regulation of the $GABA_B$ IPSP measured in dopamine cells of the VTA was significantly changed in drug-treated animals. This work showed that the activation of adenylyl cyclase by either D_1 receptors or forskolin had two opposing actions. One was to augment GABA release through the activation of PKA, and the second was to inhibit GABA release by activation of a presynaptic A_1 adenosine receptor. In slices from drug-treated animals, the enhancement of inhibition by adenosine was so great that the effect of D_1 receptor activation reversed direction and inhibited, rather than augmented, $GABA_B$ IPSPs. This inhibition was blocked by adenosine receptor antagonists and by agents that blocked the transport (probenicid) or metabolism (a phosphodiesterase inhibitor) of cAMP. The opposing actions of forskolin have been reported in several brain areas (41, 53, 86, 287, 310, 401). It remains to be determined, however, how general the persistent increase in extracellular adenosine is and by what mechanism it occurs. It appears to be a synapse-selective effect in that it is not observed at all opioid-sensitive synapses. In fact, different synapses on an individual cell can be differentially affected. In the VTA, GABA synapses are regulated by endogenous adenosine during withdrawal, but glutamate synapses are not (311, 430). Likewise in the PAG, GABA synapses were unaffected, despite the fact that there is a marked increase in cAMP-dependent GABA release (22, 203). The selectivity could result from differential sensitivity to adenosine, but more likely results from a localized metabolism of cAMP to adenosine. A cAMP-dependent phosphodiesterase is the presumed candidate for an increased metabolism, since ectonucleotidases have been reported to be ubiquitously expressed and highly active in brain slices (127).

VI. CONCLUSIONS

The understanding of the acute and chronic effects of opioids has expanded tremendously in the past 25 years. Three major subtypes of opioid receptor were identified pharmacologically using highly selective agonists and antagonists, and these have been confirmed at the molecular level. Although agonists at each receptor had different effects measured in behavioral assays, the consequences of receptor activation were similar, if not identical, at the cellular level. Historically, opioid-sensitive effectors included the inhibition of adenylyl cyclase, activation of potassium conductance, inhibition of calcium conductance, and the inhibition of transmitter release. This list of effectors has grown considerably in recent years, in part as a result of studies on the effects of chronic treatments with opioids. However, the potential roles of these diverse cellular opioid effectors in adaptive responses to opioids have yet to be fully elucidated.

The recent molecular characterization of the three subtypes of opioid receptor was a critical step in furthering the knowledge of events leading to tolerance at the cellular level. Issues of receptor subunit stoichiometry and composition, sites of interaction with G proteins, the role of phosphorylation sites, and mechanisms of trafficking are currently areas of intense investigation. The results from these studies will most certainly facilitate the understanding of the mechanisms responsible for desensitization, downregulation, and tolerance at the cellular level. Thus the passive adaptations underlying opioid tolerance are becoming clearer due to advances in the molecular understanding of the receptor and its interaction with proximal signaling elements. However, the molecular mechanisms critical for tolerance development, such as GRK-dependent desensitization of coupling to effectors, phosphorylation events and internalization, or nuclear modulation of receptor signaling and turnover have still not been clearly resolved. The role of these processes in the development of tolerance within functioning neural systems, whole animals, and humans remains virtually unknown but will come from examination and manipulation of the relevant signaling cascades in specific neural systems and whole animals.

Potential counteradaptive mechanisms in cellular and synaptic physiology following chronic opioid treatment have only recently been examined. Some of these studies were based on knowledge gained from biochemical work done in cell lines but have recently been confirmed in neurons thought to be important for mechanisms of opioid dependence. Upregulation of adenylyl cyclase has long been recognized as a model for counteradaptation in cell lines, but identification of its physiological consequences in central neurons is relatively recent. Both the excitability of nerve cell bodies and individual synapses are dramatically altered by this process. Counteradaptations of this kind are restricted to specific groups of opioid-sensitive nerve cell bodies and synapses, contingent on the nature of signaling elements such as sodium channel subtypes and adenylyl cyclase isoforms expressed. The specific neural systems that appear to be involved in mediating different aspects of opioid dependence such as somatic and aversive components of opioid withdrawal, are also becoming clear. Our understanding of the mechanism(s) involved in these counteradaptations is still in its early stages, but the molecular tools that are presently being developed to manipulate signaling elements in functioning central and peripheral neurons will greatly facilitate this process.

Counteradaptations in the function of individual synapses are particularly significant. Although adaptations in the excitability of nerve cell bodies are well recognized,

the probability of transmitter release at some central synapses is exquisitely sensitive to changes that occur in the activity of second messenger systems, e.g., adenylyl cyclase. These changes in individual synapses are reminiscent of some of the molecular mechanisms crucial for synaptic memory formation in the CNS. Thus drug-induced changes may represent a pathological form of memory at the level of individual synapses. Considerable effort and progress have been made in understanding synaptic plasticity, particularly at two synapses in the hippocampus. To date, similar studies in other brain regions have been limited, and studies on the interaction between acute or chronic opioid treatment have been almost nonexistent. One of these regions, the mesolimbic dopamine system, appears to be important in the formation of associations between salient environmental stimuli and internal cues. Disruption or distortion of synaptic plasticity in this system resulting from chronic opioid (or other drug) use may be one of the keys to understanding the induction of compulsive drug-seeking behavior, a core feature of addiction. Adaptive responses resulting from chronic opioid administration are also dependent on the context in which the drug is taken. This learned component to drug dependence implies that mechanisms involved in activity-dependent synaptic plasticity are an important and as yet little explored avenue.

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