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Neuroadaptive Responses in Brainstem Noradrenergic Nuclei Following Chronic Morphine Exposure

Elisabeth J. Van Bockstaele,[1]* A. Sue Menko[1] and Guy Drolet[2]

[1]Thomas Jefferson University, Department of Pathology, Anatomy, and Cell Biology, Philadelphia, PA 19107; [2]CHUL Research Centre, Neuroscience Unit and Faculté de Médecine, Université Laval, Québec, Canada

Abstract

Opiate dependence and withdrawal involve neuroadaptive responses in the central nervous system. A host of studies have previously implicated the A6 noradrenergic neurons of the pontine nucleus locus coeruleus (LC) as an important mediator of somatic signs observed upon withdrawal from opiates. Recent studies, however, are showing that noradrenergic neurons of the LC may not be solely involved in mediating somatic signs of withdrawal. The A2 noradrenergic neurons of the nucleus of the solitary tract (nucleus tractus solitarius [NTS]) in the caudal brainstem may be another possible site. Neurons in the nucleus paragigantocellularis lateralis (PGi), located in the rostral ventral medulla, which are known to send collateral projections to both the LC and the NTS, may co-modulate both noradrenergic nuclei in a parallel fashion, which may represent an anatomical substrate underlying the behavioral expression of opiate withdrawal. The PGi provides glutamatergic and opioid innervation to LC neurons. Hyperactivity of LC during opiate withdrawal arises, in part, from increased glutamate transmission in this pathway. The authors have recently shown that the excitatory transmitter, glutamate, co-exists with the endogenous opioid peptide, enkephalin, in a subset of axon terminals in the LC. Decreases in endogenous opioids in afferents to LC and NTS, following chronic opiate administration, may be equally important in modulating noradrenergic neurons following chronic opiate exposure, by removing a neurochemical system that would inhibit noradrenergic neurons. A persistent decrease in opioid peptide release from afferents during withdrawal would result in glutamate acting on postsynaptic targets, in an unopposed fashion. A parallel effect in opioid projections from PGi to the NTS would potentially support similar actions in this noradrenergic nucleus. The authors' recent data show that opioid-containing neurons in the PGi project to the NTS, and that enkephalin levels are decreased in opioid afferents to the NTS. This review summarizes data that the authors have collected regarding opioid expression changes in brainstem circuits (PGi–LC and PGi–NTS), following chronic morphine treatment, which may represent a model for understanding of adaptations in endogenous opioid circuits during drug dependence and withdrawal.

Index Entries: Opiates; locus coeruleus; nucleus of the solitary tract; enkephalin; opioid receptors; glutamate.

*Author to whom all correspondence and reprint requests should be addressed. E-mail:elisabeth.vanbockstaele@mail.tju.edu

Introduction

Opiate withdrawal is characterized by behavioral and physiological disturbances that involve numerous regions in the central nervous system *(1–4).* In particular, the noradrenergic system has long been implicated in mediating symptoms of the opiate withdrawal syndrome. Evidence in support of this comes from studies showing that the α_2-adrenergic agonist, clonidine, is an effective pharmacotherapy for treating physical symptoms of opiate withdrawal *(5).* Furthermore, alterations in levels of platelet α_2-adrenoceptors in heroin addicts are found to correlate with the severity of the opiate withdrawal syndrome *(6).* However, elucidating the noradrenergic loci in the central nervous system mediating mechanisms of withdrawal has been challenging. Initially, support for the involvement of the brainstem noradrenergic nucleus locus coeruleus (LC), in mediating somatic aspects of the opiate withdrawal syndrome, was based on observations that, in opiate dependent rats, there is a marked increase in the discharge rate of LC neurons during antagonist-induced withdrawal *(7).* The increase in LC neuronal activity was shown to correlate with the behavioral expression of the opiate withdrawal syndrome, and was prevented by clonidine treatment *(8–27).* Thus, Aghajanian et al. postulated that the efficacy of clonidine stemmed from its ability to reduce noradrenergic activity of LC neurons via presynaptic stimulation of α_2-adrenergic receptors *(27).* Moreover, biochemical studies indicated that norepinephrine levels in the hippocampus and cortex, which arise solely from LC afferents, are increased following opiate withdrawal *(28),* and could be reversed by clonidine preadministration *(29).*

More recently, however, it is being recognized that additional brain nuclei, other than the LC, may be more involved in mediating somatic signs of opiate withdrawal *(30–32).* Evidence in support of this comes from experiments showing that chemical lesions of the LC prior to the induction of dependence do not attenuate somatic signs observed following systemic naloxone *(17,31,32),* suggesting that efferent projections of the LC are not necessarily involved in mediating the physical signs of opiate withdrawal. Thus, profound activation of LC neurons during withdrawal, observed following microinjections of opioid antagonists into the region, may result from modulation of neighboring nuclei, such as the periaqueductal gray or Barrington's nucleus *(4).* Moreover, detection of Fos (protein or mRNA), a marker of neuronal activation *(33),* has indicated several other brainstem nuclei, including the nucleus of the solitary tract (nucleus tractus solitarius [NTS]), caudal and rostral ventrolateral medulla, and periaqueductal gray among others, which are activated following precipitated withdrawal *(25,34–38).*

The circuit linking the NTS to the bed nucleus of the stria terminalis (BNST) has recently received attention as an important pathway in mediating the behavioral expression of opiate withdrawal *(32).* Local microinjections of the β-receptor antagonist, propranolol, into the BNST, attenuated withdrawal-induced aversion, as well as reduced teeth chatters, eye twitches, and wet dog shakes *(35).* The presence of c-fos protein and Fos-related antigens, which is used as a measure of cell activation in brain *(39–41),* has shown an increase in the BNST, which is significantly reduced in rats pretreated with propranolol *(32).* The noradrenergic innervation of the BNST arises primarily from the A2 cell group, which is located in the NTS. Delfs et al. *(32)* also demonstrated that many A2 neurons, projecting to the BNST, were activated following precipitated withdrawal. Thus, there exists anatomical and behavioral experiments to support activation of noradrenergic A2 neurons in the NTS (which project to the BNST), in mediating somatic aspects of the opiate withdrawal syndrome.

Neurons in the nucleus paragigantocellularis (PGi), located in the rostral ventral medulla, may be poised for co-activating both noradrenergic nuclei, by virtue of collateralized projections from PGi neurons to the LC and the NTS *(42).* Thus, alterations in the phys-

iological activity of LC neurons during withdrawal may be secondary to activation of afferent neurons, such as from its major excitatory afferent, the PGi *(43–45).* In support of this, in vivo studies show that there is an increase in the release of excitatory amino acids in the LC from extrinsic afferents, following withdrawal from opiates *(18,44–47).* Injections of the broad-spectrum spectrum excitatory amino acid antagonist, kynurenic acid, intraventricularly *(48),* or directly into the LC *(22),* significantly attenuate the activation of LC cells induced by naloxone-precipitated withdrawal. Microdialysis studies in the LC reveal increased excitatory amino acid efflux during naltrexone-precipitated withdrawal *(18,47).* Moreover, lesions of the PGi attenuate the hyperactivity of LC neurons, seen following precipitated opiate withdrawal, suggesting that glutamate afferents from this region participate in the altered physiological state of these noradrenergic neurons *(48).* In addition to glutamate, the PGi also contributes the major opioid innervation to the LC *(49).* Changes in neurochemical expression in opioid afferent projections from the PGi to postsynaptic targets, including the LC and NTS, may also be important in modulating noradrenergic neurons, following chronic opiate exposure. The authors have shown that the excitatory transmitter, glutamate, co-exists with the endogenous opioid peptide, leucine^5-enkephalin (ENK), in a subset of axon terminals in the LC *(50).* Thus, alterations in opioid peptide levels, in medullary neurons projecting to noradrenergic nuclei, following chronic opiate exposure, may contribute to the observed hyperactivity of LC neurons during opiate withdrawal. Decreased release of endogenous opioid peptides from LC afferents would result in the removal of an inhibitory neurochemical that causes hyperpolarization of noradrenergic neurons *(51,52).* Co-existing excitatory transmitters, e.g., glutamate, could then hyperactivate LC neurons in an unopposed fashion. A parallel circuit to the NTS would support similar actions in this noradrenergic nucleus. This review presents evidence of involvement of endogenous opioid peptides in the adaptive cellular mechanisms underlying the behavioral and physiological aspects of the opiate withdrawal syndrome.

PGi as Co-Regulator of Noradrenergic Function

The PGi is a functionally heterogeneous region of the medulla. Neurons in the medial aspect of the PGi have been implicated in pain and analgesia processes *(53);* neurons in the lateral aspect of the PGi subserve functions related to blood pressure control *(54,55).* In addition, a growing body of evidence supports a role for PGi neurons in mediating the behavioral expression of opiate withdrawal. Several lines of evidence point to alterations in PGi neurons during precipitated withdrawal. Increased c-fos expression has been reported in the PGi of morphine-dependent rats that are subjected to naloxone-precipitated withdrawal *(37).* Increased neuronal activity of presumed adrenergic neurons of the C1 cell group, in the rostral ventral medulla, has been reported during naloxone-precipitated withdrawal *(56).* Lesions of the PGi have been shown to attenuate the hyperactivity seen following precipitated opiate withdrawal *(48).* Recent reports in conscious, unrestrained, nonopioid-dependent rats have shown that electrical stimulation of the PGi causes a characteristic series of behaviors, which resemble those seen during naloxone-precipitated withdrawal from dependence on opioid agonists *(57).* These data suggest that activation of the PGi by electrical stimulation can elicit behaviors similar to those observed during opioid withdrawal. Taken together, these data suggest a critical role for circuits originating from the PGi in the behavioral and autonomic expression of withdrawal.

The authors previously provided anatomical evidence that PGi neurons send axonal collaterals to both the LC and to the NTS *(42).* Five representative levels throughout the rostral – caudal extent of the PGi, including the juxtafa-

cial, retrofacial, and caudal PGi subregions, illustrate the pattern of retrogradely labeled neurons observed from the LC and NTS (Fig. 1). Overall, neurons retrogradely labeled from LC were located more ventrally, and were scattered throughout the medial – lateral extent of the PGi, compared to those labeled from NTS. Caudally, at the level of the lateral reticular nucleus, LC- and NTS-projecting neurons are interdigitated; however, no doubly labeled neurons were identified (Fig. 1A). More rostrally, doubly labeled neurons were observed predominantly in the lateral retrofacial PGi ventral to the nucleus ambiguus (Fig. 1B,C,D). In the juxtafacial PGi, although retrogradely labeled neurons, from LC and NTS, were present, few doubly labeled neurons were identified (Fig. 1E). Using a combination of epifluorescence microscopy and bright-field microscopy, a doubly labeled neuron is shown in Fig. 2. Approximately 25% of the neurons projecting to the LC were found to send collaterals to the NTS, demonstrating that activity in a significant number of neurons in the PGi, which project to the LC, will simultaneously influence the NTS, and, conversely, a substantial amount of PGi activity that affects NTS function simultaneously has an impact on the LC.

Hyperactivity of LC neurons, following precipitated opiate withdrawal, may be secondary to activation of afferent nuclei, such as from the PGi *(12,18,22),* which trigger a cascade of reactions via dual modulation of these two brainstem noradrenergic nuclei. To test this hypothesis, elucidating alterations in known neurochemicals in these pathways should provide insight into the neuroadaptations, which may occur following chronic morphine treatment.

Decreases in Endogenous ENK Peptides Following Chronic Morphine Treatment

Although much attention has focused on changes in intracellular messengers in LC neurons *(26,58–61),* and on glutamatergic afferents to the LC *(18,44–47),* little is known regarding adaptations in endogenous opioid peptides in this brain region, following chronic morphine treatment. The authors have previously identified the PGi and nucleus prepositus hypoglossi (PrH), in the dorsomedial medulla, as robust endogenous sources of ENK to the "nuclear" or "core" portion of the LC *(49).* A high proportion (approx 60%) of the neurons in the PGi and PrH, projecting to the LC (as identified by retrograde transport), exhibited immunolabeling for ENK *(49).* The authors also showed, in a separate set of studies, that ENK-immunoreactive axon terminals form heterogeneous-type synapses (excitatory- and inhibitory-type) with noradrenergic dendrites in the LC *(62),* and that a subset of these exhibit immunoreactivity for γ-aminobutyric acid *(63)* and glutamate *(50).* The authors also have generated data to indicate that ENK neurons in the PGi prominently target the commissural portion of the NTS, which supports the authors' hypothesis that PGi neurons, and specifically opioid neurons, collateralize to both the LC and NTS *(64).* Retrogradely labeled neurons from the LC and NTS were also found in the dorsomedial medulla, in the area of the medial PrH (not shown, but see ref. *42*). Labeled neurons in the dorsomedial medulla were most numerous where the medial longitudinal fasciculus meets the fourth ventricle, but could also be seen scattered more ventrally along the lateral borders of the mlf in the perifascicular reticular formation. The percentage of LC-projecting neurons sending collaterals to the NTS was fewer in the dorsomedial medulla, compared to the PGi. Approximately 12% of neurons projecting to the LC sent collateral projections to the NTS *(42).*

To test the hypothesis that chronic morphine treatment alters the expression of endogenous opioids in these brainstem pathways, adult rats were implanted with two morphine pellets each (National Institute of Drug Abuse: 75 mg morphine base) for a period of 5 d prior to transcardial perfusion (for immunocytochemical studies) or decapitation (for Western blot

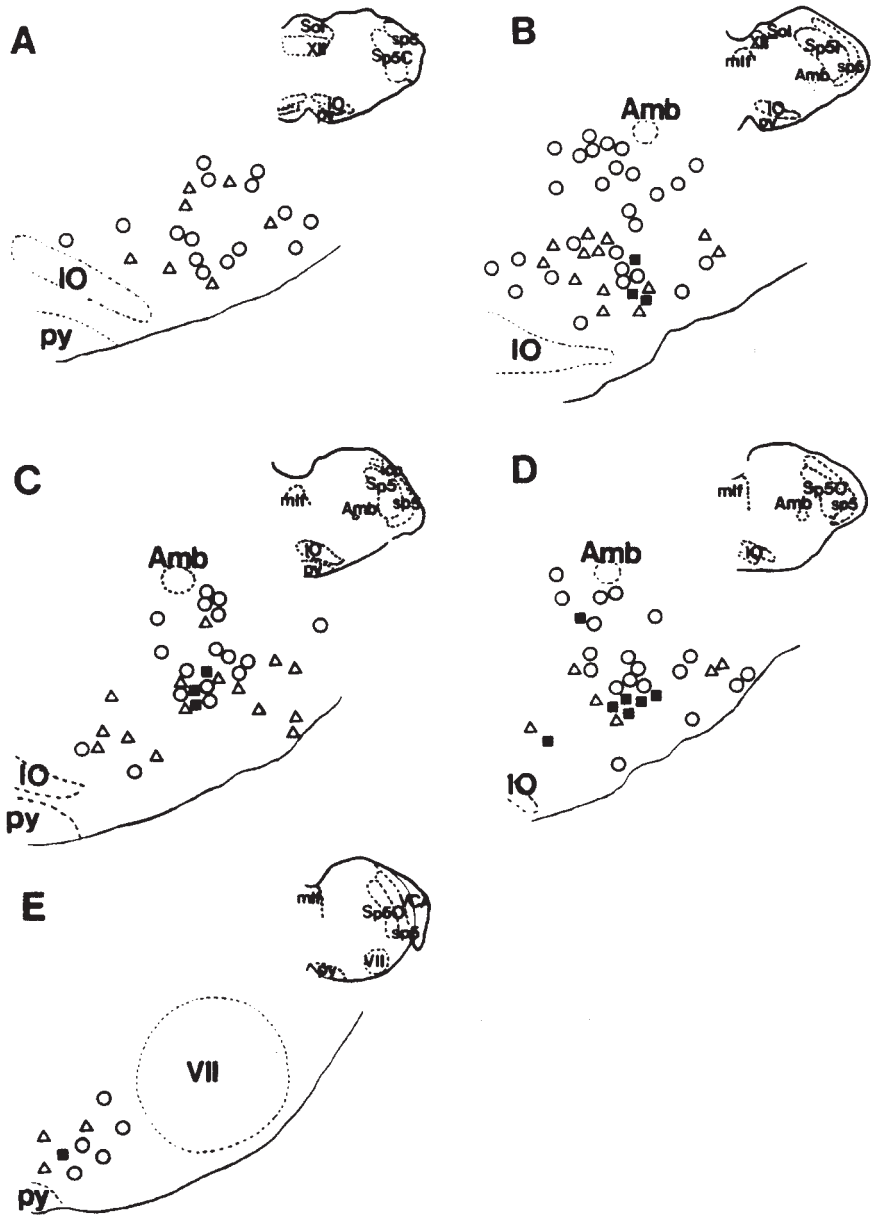

Fig. 1. Camera lucida plots of five representative levels from caudal medullary levels **(A)** to rostral levels **(E),** showing PGi neurons that are retrogradely labeled from the NTS (circles) or LC (triangles). Neurons containing both retrograde labels are illustrated as squares. Insets: The level from which the high-power plot was drawn. Sol, solitary nucleus; IO, inferior olive; py, pyramidal tract; sp5, spinal trigeminal tract of V; Sp5, spinal trigeminal nucleus of V, pars caudalis; icp, inferior cerebellar peduncle; Amb, nucleus ambiguus; mlf, medial longitudinal fasciculus; VII, facial nucleus; VCA, ventral cochlear nucleus, anterior division; Sp5I, spinal trigeminal nucleus of V, pars interpolaris; Spr5O, spinal trigeminal nucleus of V, pars oralis. (Adapted from ref. *42*).

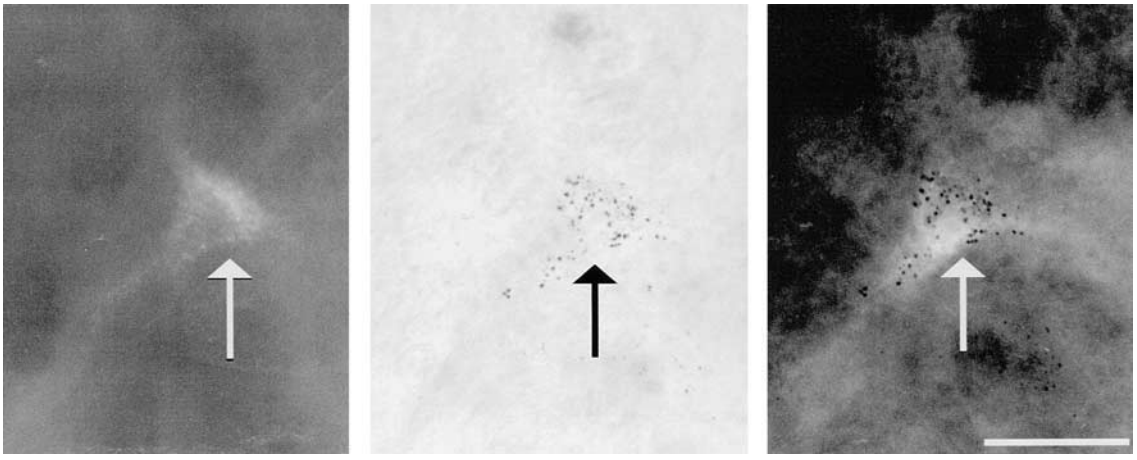

Fig. 2. Photomicrographs showing a single PGi neuron containing two retrograde tracers: FG, which was placed into the LC, and WGA-apoHRP-AU, which was placed into the NTS. Left panel: Ultraviolet epifluorescent-illuminated photomicrograph, showing a PGi neuron that contains FG from an injection placed into the LC. The middle panel shows a bright-field photomicrograph of the same cell containing the protein gold tracer, WGA-apoHRP-AU, which was placed into the NTS. The panel to the right is a double exposure of the same PGi neuron, which is dually labeled. (Adapted from ref. *42*).

analysis). Fresh tissue samples included micropunches of either the LC/peri-LC area (pLC) or NTS regions. Control rats received placebo pellets. This dosage has been shown to yield physical dependence to the drug *(3).* Two antisera, a polyclonal antiserum (directed against Met^5-ENK) that recognizes several opioid derivatives of the preproenkephalin (PPE) A and B precursor, and a monoclonal antiserum (Leu^5-ENK) that selectively recognizes the C-terminal extended Met^5-ENK hexapeptides and the extended heptapeptide (-Arg-Phe-OH), but does not recognize other endogenous opioid peptides (such as β-endorphin and dynorphin), were selected to examine the distribution of ENK, using immunocytochemistry and Western blot analysis in the LC of morphine- and placebo-treated rats. In addition, the NTS region was microdissected and Western blot analysis conducted, to examine ENK protein levels.

Tissue sections were processed for peroxidase localization of Met^5-ENK for light microscopic analysis (Fig. 3). In sections obtained from placebo-treated rat brains, peroxidase labeling for Met^5-ENK (Fig. 3A,B) was moderately distributed within the portion of the LC containing noradrenergic cell bodies (nuclear LC "core"), and was densely distributed in pLC, including the dorsolateral pLC, immediately ventral to the superior cerebellar peduncle, as previously described (*62;* Fig. 3). Immunolabeling of Met^5-ENK, in sections through the LC obtained from morphine-treated rats (Fig. 3C,D), was decreased in both LC and pLC, as seen using bright-field microscopy. Decreases in ENK were quantified using bright-field microscopy densitometry. Light-level densitometric analysis confirmed that immunoperoxidase-labeling for ENK was decreased in sections obtained from morphine-treated rats, and that the core of the LC exhibited a greater decrease in Met^5-ENK immunoreactivity, compared to pLCs *(65).*

To further quantify decreases in ENK levels, microsamples were collected through the

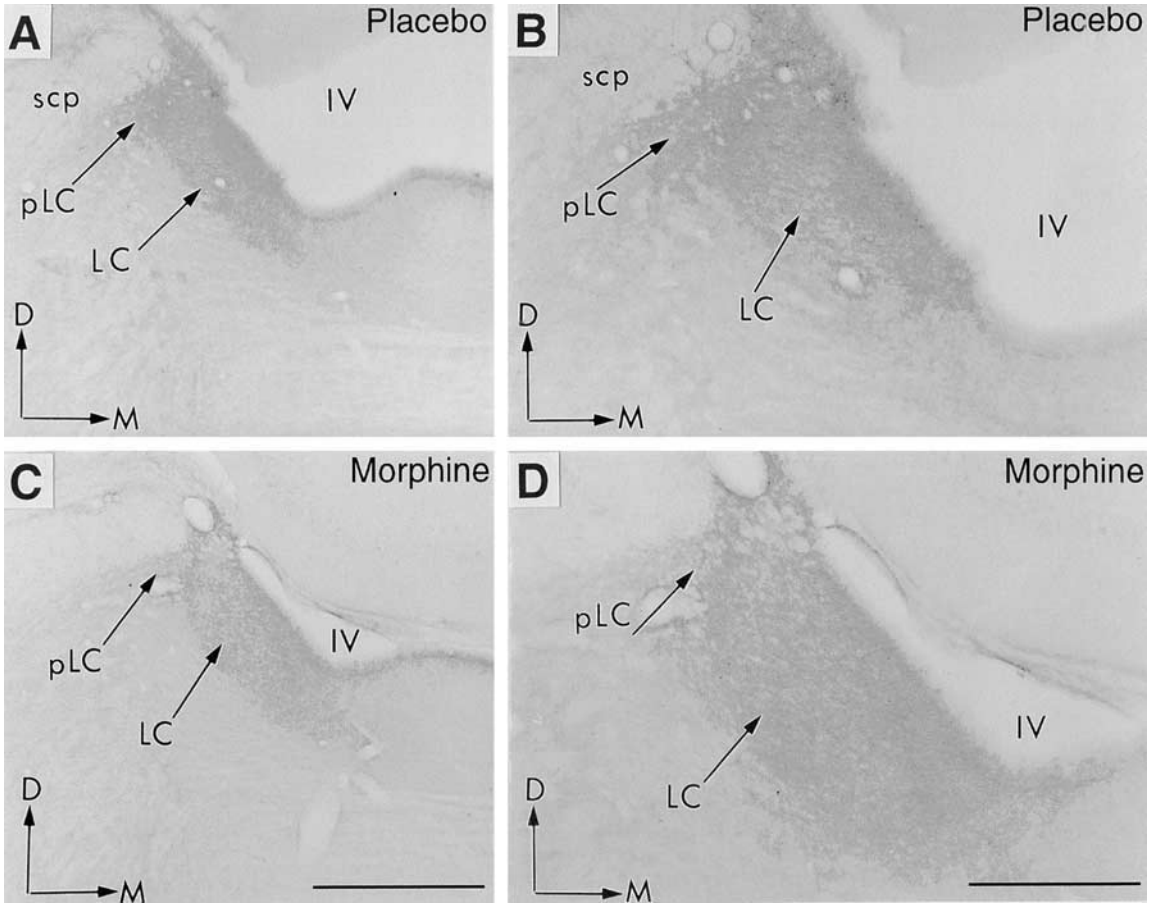

Fig. 3. Photomicrographs showing peroxidase labeling for ENK in the LC cell body (LC) region (also defined as "nuclear" or "core" of the LC) and peri-LC (pLC) areas (region containing noradrenergic dendrites of LC neurons) of placebo- and morphine-treated rats. Low-magnification micrographs showing peroxidase-labeling for ENK in varicose processes in LC and pLC areas in a placebo-treated rat **(A)** and morphine treated rat **(C).** Varicose processes are distributed throughout the region of the LC, as well as in pLC areas, known to contain noradrenergic dendrites of LC neurons. (B and D) High-magnification micrograph showing a decrease in peroxidase immunoreactivity for ENK in the LC region (LC), as well as in the pLC ventral to the superior cerebellar peduncle (scp), in morphine-treated rats **(D),** compared to placebo-treated rats **(B).** Arrows point medially (M) and dorsally (D). IV, fourth ventricle; scp, superior cerebellar peduncle. Bar for A and C = 250 µm; bar for B and D = 175 µm.

LC and NTS, and further processed for Western blot analysis, using antibodies raised against either Met^5- or Leu^5-ENK. The top band (located at approx 40 kDa) probably represents the proenkephalin-like opioid peptide. This peptide exhibited a reduction in morphine-treated rats in the NTS (Fig. 4). Similar results were obtained in the LC *(65).* These data suggest that chronic exposure to morphine decreases the amount of endogenous opioid peptides in both noradrenergic nuclei (*65;* Fig. 4).

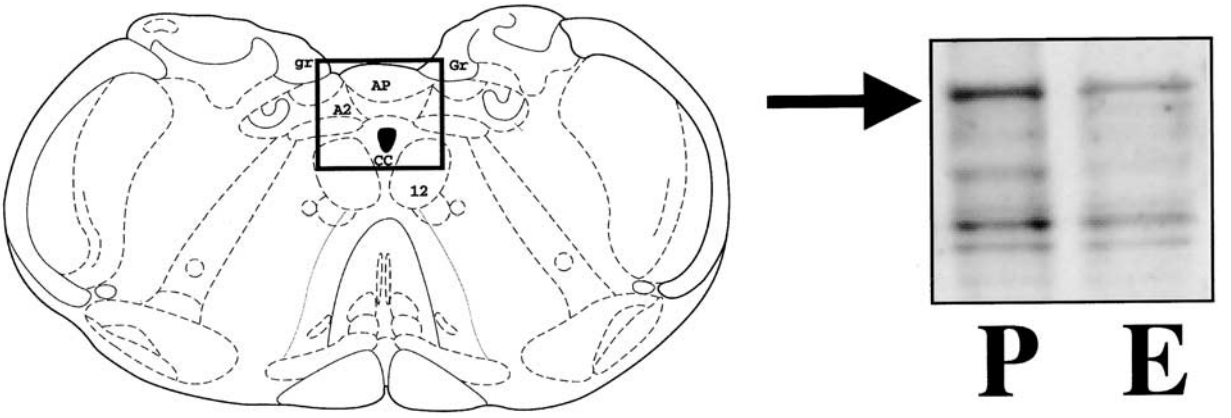

Fig. 4. Western blot analysis of samples obtained from the NTS (boxed region in schematic; adapted with permission from the rat brain atlas of Paxinos and Watson *[88]*) of placebo **(P)** and experimental **(E)** morphine-treated rats. Expression of the primary antibody directed against Met^5-ENK was readily detected in microsamples obtained from the NTS. Multiple bands were detected on the gel; however, proteins that migrated close to 40 Kda (arrow) most likely represent the migration of proenkephalin-like opioid peptides. Expression was reduced in samples of the NTS obtained from morphine-dependent rats (E), compared to placebo (P) rats. gr, nucleus gracilis; AP, area postrema; co, central canal; A2, noradrenergic neurons.

Decreases in Endogenous Opioid Peptides Arise from Alterations in PPE mRNA

To test whether decreases in opioid peptide protein expression were mirrored by decreases in gene expression, Northern blot analysis was conducted using a probe containing the entire coding region of rat PPE cDNA (generous gift of Dr. Sabol, National Institute of Mental Health). Northern blot analysis identified a band of approx 950 bp on samples obtained from the medulla, striatum, and heart (control sample) of rats receiving placebo or morphine pellets for 5 d. In heart and striatum samples obtained from morphine-treated rats, PPE mRNA was similar to that of samples obtained from placebo-treated rats. In contrast, medullary regions obtained from morphine-treated rats exhibited a twofold decrease in PPE mRNA expression, compared to morphine-treated rats *(65).* These results suggested that the effects of morphine on ENK levels in the LC could be the result of changes in gene expression of PPE mRNA.

To further specify the distribution of neurons exhibiting a decrease in PPE mRNA, *in situ* hybridization (ISH) was conducted in medullary regions. ISH revealed a high positive signal within PGi (not shown, but *see* ref. *65*) and PrH (Fig. 5) neurons of saline treated rats. Morphine treatment significantly reduced the expression of PPE mRNA in both the PGi (not shown, but *see* ref. *65*) and the PrH of morphine-treated rats (Fig. 5).

Functional Implications

Convergent lines of evidence show a decrease in opioid peptides in afferents to LC and NTS areas of opiate-dependent rats. The decrease in mRNA for PPE in medullary regions, such as the PGi and PrH, known to provide opioid input to the LC and NTS, sug-

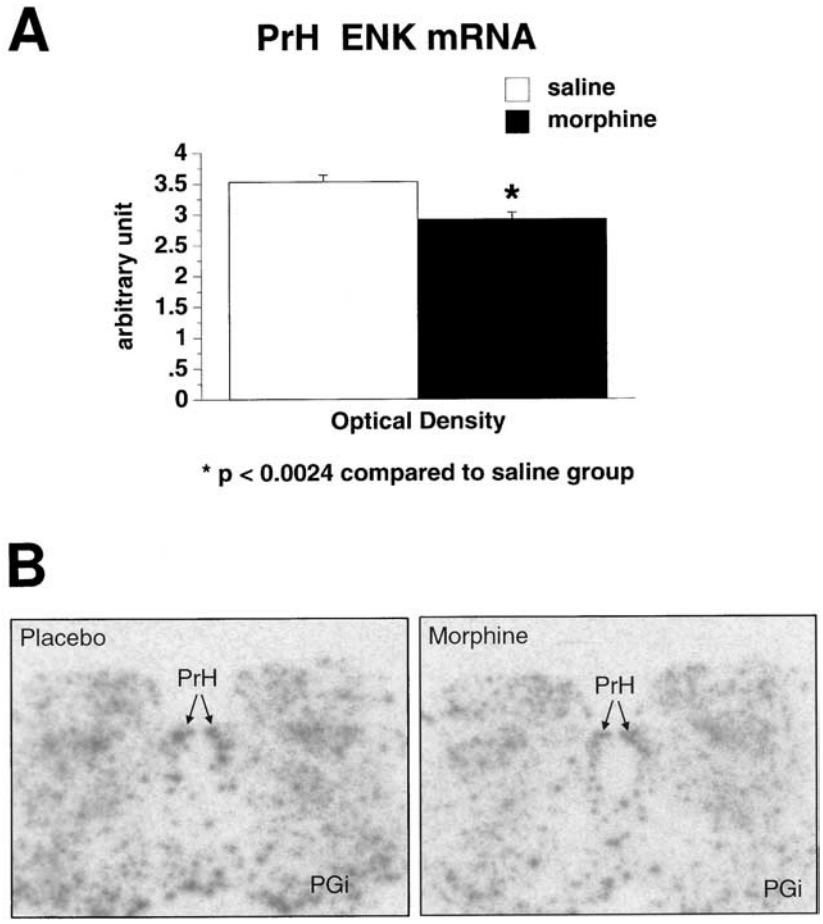

Fig. 5. Brain sections through the medulla oblongata of morphine- and placebo-treated rats were processed for ISH of mRNA for PPE, using a plasmid generously provided by Dr. S. Sabol from NIH. **(A)** Graphic illustration depicting the effects of morphine treatment on average optical density for ENK mRNA hybridization signal in the PrH. **(B)** Representative example of medullary sections containing the PrH and PGi processed for ISH of ENK mRNA expression in placebo- and morphine-treated rats. Arrows indicate location of PrH in the dorsal medial medulla and PGi in the ventral medulla. Expression of mRNA for PPE is reduced in samples obtained from morphine-treated rats.

gests that opioid peptide gene expression is diminished following chronic morphine exposure. Opioids do not tonically regulate LC activity, because pure opiate antagonists have no effect on LC discharge of previously untreated rats *(66).* However, there is evidence for opioid regulation of LC activity in opiate-naïve animals during stress *(51).* In that study, it was demonstrated that systemic naloxone increased LC discharge of opiate-naïve stressed rats, at doses that had no effect in unstressed rats. Recent studies are showing that, in the LC, hypotensive stress, which causes an increase in discharge activity (mediated by corticotropin-releasing factor), followed by a decrease in activity below baseline,

is mediated by endogenous opioid afferents *(67).* Thus, although endogenous opioids may not be tonically released, during a stressful situation such as antagonist-induced withdrawal, decreases in endogenous opioids in PGi efferents are likely to influence the physiological activity of postsynaptic neurons, which may contribute to the observed dramatic physiological hyperactivity seen in the LC, upon withdrawal from opiates.

This data adds to the growing body of literature showing that the endogenous opioid system is altered following a variety of different treatments, e.g., numerous changes have been observed in endogenous opioid systems, following the induction of recurrent limbic seizures *(68).* In the kainic acid model, ENK immunoreactivity is diminished following a 3–6-h interval in the hippocampus, following the onset of seizures. By 24–48 h, ENK immunoreactivity rises above normal, and remains elevated after 72 h. Hippocampal proenkephalin mRNA levels sharply increase, and peak at 6 h, remain elevated at 24 h, and return to normal at 72 h. These findings suggested that seizures initially release ENK from mossy fibers, and subsequenly increase the synthetic rate of opioid-peptide-containing neurons in the hippocampus. Hong et al. *(69)* postulated that changes in opioid peptide levels occurred as a general compensatory response to seizure activity. In other neuronal circuits, chronic administration of morphine has been shown to decrease ENK *(70,71),* PPE mRNA levels *(72,73),* but others have not observed such changes *(74–76).* Although differences in the literature exist, it is also likely that there are regionally specific changes in ENK expression and protein levels, following exposures to opiates *(73).*

The precise mechanism coupling opioid receptor stimulation to alterations in gene expression is being elucidated. Transcription factors of the fos and jun families (constituting the AP-1 proteins), CAMP response element (CRE)-binding protein (CREB) transcription, and the c-EBP family have been identified in various experimental systems as potential transducers of opioid signals. The proenkephalin gene contains an enhancer which is required for cAMPs- and Ca^{2+}-inducible expression in transformed cell lines *(77,78).* This enhancer contains three closely spaced DNA regulatory elements *(79,80):* the CRE-1 and CRE-2 elements and an element binding the AP2 transcription factor. Konradi et al. *(81)* have shown that CREB-like proteins, rather than fos, appear to interact with the proenkephalin CRE-2 site. Thus, the proteins binding to the CRE-2 element appear to be the critical signal transducers in cAMP- and Ca^{2+}-mediated expression. The relationship between PPE mRNA expression and cAMP, in medullary projections to the LC, has not been examined. Although ENK and cAMP are altered during morphine tolerance and dependence in other areas *(26,58–61),* whether cAMP consistently affects brain ENK levels, in the same way, is still not clear, nor are the changes in the levels of cAMP and ENK interrelated, which may affect the development of morphine tolerance and abstinence.

Acute precipitation of opiate withdrawal increases phosphorylation of transcription factors, such as CREB *(82).* Phosphorylation of CREB has been shown to be increased following the onset of withdrawal *(24).* Chronic morphine has also been shown to increase total levels of CRE-binding activity, which in turn should increase expression of the proenkephalin gene, and raise ENK levels. However, in certain brain areas (such as the cortex), it has been shown that ENK levels are decreased in the presence of increased cAMP *(83).* Future studies are required to address whether changes in cAMP levels and CREB occur in PGi neurons, in order to shed light on the mechanism of opiod peptide synthesis in these PGi–LC and PGi–NTS circuits. Additionally, elucidating opioid peptide changes in other PGi efferents is necessary to fully explore adaptations of these neurons, following chronic morphine treatment.

Summary, Conclusions, and Future Directions

Chronic morphine treatment alters the levels of endogenous ENK-related peptides in two brainstem noradrenergic nuclei, as indicated by light-level densitometric analysis of ENK immunolabeling and Western blot analysis. The authors' data, using Northern blot analysis and ISH experiments, further reveal that decreases in ENK peptides in the LC and NTS are probably attributed to decreases in the synthesis of PPE mRNA in neurons afferent to these nuclei, e.g., the PGi and PrH. These data extend previous investigations, which show that the PGi and PrH provide a major opioid innervation to the LC and NTS *(49,64)*, by showing that decreases in opioid peptide mRNA most likely translates to decreased levels of ENK in afferent terminals in these noradrenergic nuclei, following chronic morphine treatment *(65)*. The authors' data also suggest that there are likely to be region-specific differences in opioid gene expression changes in brain nuclei, following morphine dependence and withdrawal, because others have shown alterations in PPE mRNA levels in other brain regions *(72,73,84–86)*.

Recent data from this lab supports a persistent decrease in ENK-related peptides in the LC and NTS of rats subjected to naltrexone-induced withdrawal for 30 min. Microsamples of the LC, obtained from opiate-dependent rats that were processed for endomorphin 1, using Western blot analysis, showed no detectable differences in protein levels. Such data suggest that chronic morphine treatment and early periods of withdrawal are accompanied by selective decreases in ENK-related peptides. Persistent decreases in ENK in LC afferents is likely to have an impact on the physiological activity of neurons, by removing an endogenous inhibitory neurochemical that is known to hyperpolarize neurons *(52)*. Figure 6 summarizes the proposed model, showing neuroadaptations in opioid afferents after chronic morphine, and following withdrawal. This scheme is supported by additional data recently generated in the lab (unpublished observations), indicating that neurons that are activated following precipitated withdrawal (as measured using c-fos immunolabeling) exhibit PPE mRNA (as detected with ISH). Thus, withdrawal from opiates specifically involves the activation of opioid-containing PGi neurons, which are likely to play a major role in efferent circuits from this medullary region.

Although the exact nature of the behavioral and physiological disturbances (observed as a consequence of withdrawal), which are subserved by these circuits, remain unclear, one possibility is that they are involved in the autonomic dysfunctions associated with opiate withdrawal *(37)*. Autonomic symptoms of withdrawal, which include an increase in arterial blood pressure, which is accompanied by an increase in adrenal and lumbar sympathetic nerve activity *(87)*, strongly support a role for these autonomic-related nuclei. Future studies will be required to elucidate the specific manifestations of withdrawal associated with activation of these circuits, as well as other targets of PGi efferents. Additionally, the significance of the observed decreases in PPE mRNA in the PrH remains to be investigated. Finally, characterizing neuroadaptations in these brainstem circuits, following several time-points after withdrawal, is still needed, to shed light on the plasticity in endogenous opioid systems, following exposure to and cessation of morphine.

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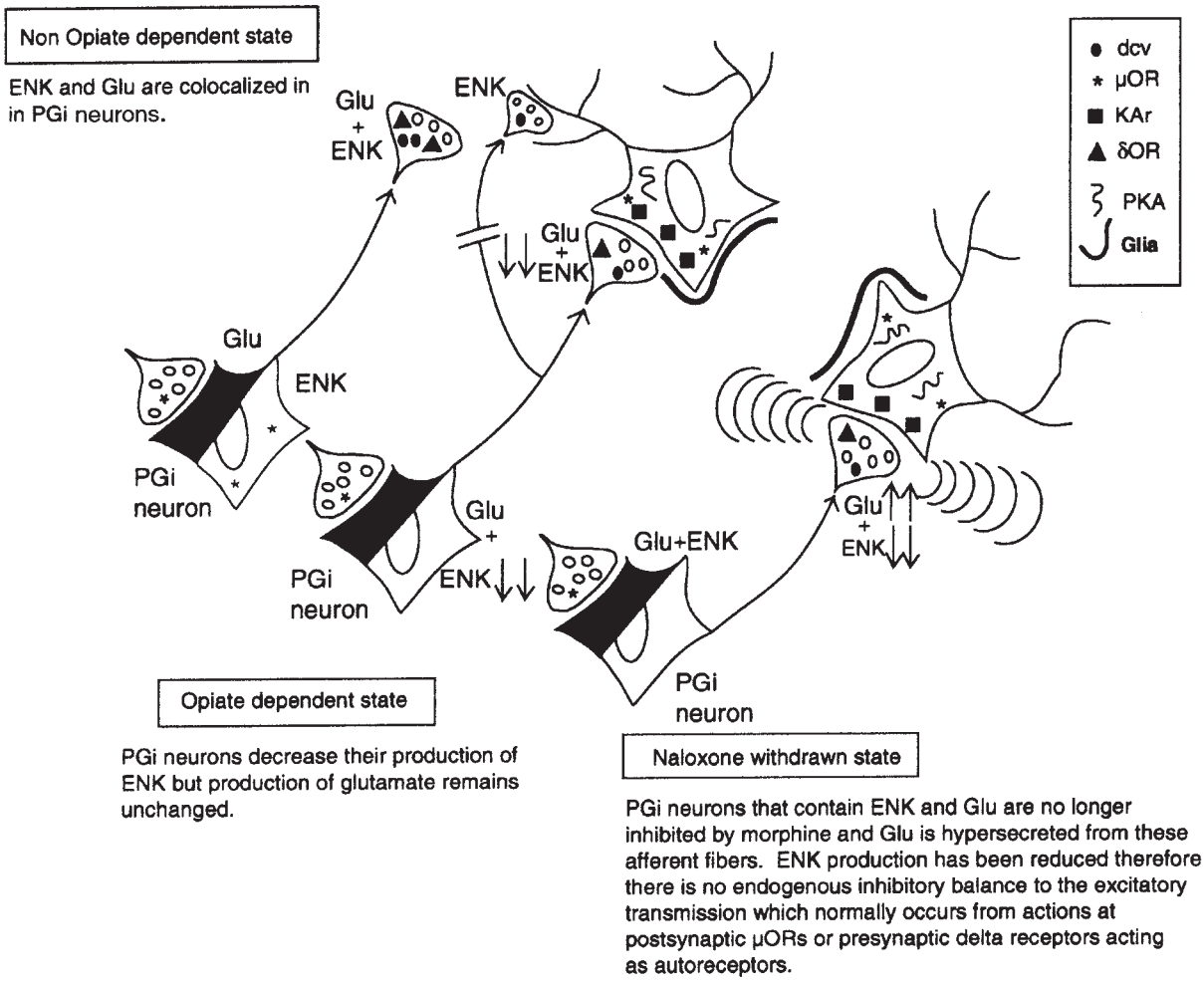

Fig. 6. Schematic illustration summarizing the proposed model regarding neuroadaptive responses in opioid-containing neurons in the PGi, following chronic opiate treatment and withdrawal. Decreases in endogenous opioid synthesis in PGi efferent neurons could contribute to the observed physical disturbances seen upon withdrawal from opiates, by removing an endogenous inhibitory influence on postsynaptic target neurons, including the LC or NTS. In the nonopiate-dependent state, ENK and glutamate co-exist in common afferents *(50),* and a subset of opioid-containing terminals exhibit δ opioid receptors, which may act as autoreceptors *(89).* During chronic morphine treatment, PPE mRNA is decreased in PGi neurons, resulting in decreased production of ENK in afferent terminals. Following cessation of morphine, glutamate is released from PGi afferents, and activates postsynaptic neurons in an unopposed fashion. ENK release, which is blunted by the decrease in PPE mRNA, can no longer efficiently modulate μ opioid receptors present postsynaptically *(90).* Such alterations in neurotransmission could contribute to the hyperactivity observed in LC neurons, following withdrawal. dcv, dense core vesicle; μOR, μ subtype of opioid receptor; Kar, kainate receptor; δOR, delta subytpe of opioid receptor; PKA, protein kinase A.

References

1. Abood L. (1984) Mechanisms of tolerance and dependence: an overview. *NIDA Res. Monogr.* **54,** 4–11.
2. Kantak K. and Miczek K. (1988) Social, motor, and autonomic signs of morphine withdrawal: differential sensitivities to catecholaminergic drugs in mice. *Psychopharmacology* **96,** 468–476.
3. Koob G. F., Maldonado R., and Stinus L. (1992) Neural substrates of opiate withdrawal. *Trends Neurosci.* **15,** 186–191.
4. Williams J., Christie M., and Manzoni O. (2001) Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev.* **81,** 299–343.
5. Gold M. S., Pottash A. C., Extein I. L., and Kleber H. D. (1981) Neuroanatomical sites of action of clonidine in opiate withdrawal: the locus coeruleus connection. *Progr. Clin. Biol. Res.* **71,** 285–298.
6. Garcia-Sevilla J., Ugedo L., Ulibarri I., Elizagarate E., and Guttierez M. (1984) Heroin addicts have increased platelet alpha2-adrenoceptor densities which correlate with the severity of the abstinence syndrome. *Eur. J. Pharmacol.* **100,** 131–132.
7. Aghajanian G. K. and Wang Y. Y. (1986) Pertussis toxin blocks the outward currents evoked by opiate and alpha2-agonists in locus coeruleus neurons. *Brain Res.* **371,** 390–394.
8. Aston-Jones G., Hirata H., and Akaoka H. (1997) Local opiate withdrawal in locus coeruleus in vivo. *Brain Res.* **765,** 331–336.
9. Lane-Ladd S. B., Pineda J., Boundy V. A., Pfeuffer T., Krupinski J., Aghajanian G. K., and Nestler E. J. (1997) CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. *J. Neurosc.* **17,** 7890–7901.
10. Javelle N., Renaud B., and Lambas-Senas L. (1997) Monoamine metabolism in the locus coeruleus measured concurrently with behavior during opiate withdrawal: an in vivo microdialysis study in freely moving rats. *J. Neurochem.* **68,** 683–690.
11. Dossin O., Hanoun N., and Zajac J. M. (1996) Involvement of locus coeruleus projections in opiate withdrawal but not in opiate tolerance in mice. *Eur. J. Pharmacol.* **308,** 271–274.
12. Rasmussen K., Kendrick W. T., Kogan J. H., and Aghajanian G. K. (1996) A selective AMPA antagonist, LY293558, suppresses morphine withdrawal-induced activation of locus coeruleus neurons and behavioral signs of morphine withdrawal. *Neuropsychopharmacology* **15,** 497–505.
13. Krystal J. H., Compere S., Nestler E. J., and Rasmussen K. (1996) Nimodipine reduction of naltrexone-precipitated locus coeruleus activation and abstinence behavior in morphine-dependent rats. *Physiol. Behav.* **59,** 863–866.
14. Maldonado R., Valverde O., Garbay C., and Roques B. P. (1995) Protein kinases in the locus coeruleus and periaqueductal gray matter are involved in the expression of opiate withdrawal. *Naunyn Schmiedebergs Arch. Pharmacol.* **352,** 565–575.
15. Rasmussen K. (1995) The role of the locus coeruleus and N-methyl-D-aspartic acid (NMDA) and AMPA receptors in opiate withdrawal. *Neuropsychopharmacology* **13,** 295–300.
16. Kogan J. H. and Aghajanian G. K. (1995) Long-term glutamate desensitization in locus coeruleus neurons and its role in opiate withdrawal. *Brain Res.* **689,** 111–121.
17. Chieng B. and Christie M. J. (1995) Lesions to terminals of noradrenergic locus coeruleus neurons do not inhibit opiate withdrawal behaviour in rats. *Neurosci. Lett.* **186,** 37–40.
18. Aghajanian G. K., Kogan J. H., and Moghaddam B. (1994) Opiate withdrawal increases glutamate and aspartate efflux in the locus coeruleus: an in vivo microdialysis study. *Brain Res.* **636,** 126–130.
19. Guitart X., Kogan J. H., Berhow M., Terwilliger R. Z., Aghajanian G. K., and Nestler E. J. (1993) Lewis and Fischer rat strains display differences in biochemical, electrophysiological and behavioral parameters: studies in the nucleus accumbens and locus coeruleus of drug naive and morphine-treated animals. *Brain Res.* **611,** 7–17.
20. Maldonado R. and Koob G. F. (1993) Destruction of the locus coeruleus decreases physical signs of opiate withdrawal. *Brain Res.* **605,** 128–138.
21. Kogan J. H., Nestler E. J., and Aghajanian G. K. (1992) Elevated basal firing rates and enhanced responses to 8-Br-cAMP in locus coeruleus neurons in brain slices from opiate-dependent rats. *Eur. J. Pharmacol.* **211,** 47–53.
22. Akaoka H. and Aston-Jones G. (1991) Opiate withdrawal-induced hyperactivity of locus coeruleus neurons is substantially mediated by augmented excitatory amino acid input. *J. Neurosci.* **11,** 3830–3839.

23. Grant S. J., Huang Y. H., and Redmond D. E., Jr. (1988) Behavior of monkeys during opiate withdrawal and locus coeruleus stimulation. *Pharmacol. Biochem. Behav.* **30,** 13–19.
24. Guitart X., Thompson M. A., Mirante C. K., Greenberg M. E., and Nestler E. J. (1992) Regulation of cyclic AMP response element-binding protein (CREB) phosphorylation by acute and chronic morphine in the rat locus coeruleus. *J. Neurochem.* **58,** 1168–1171.
25. Hayward M. D., Duman R. S., and Nestler E. J. (1990) Induction of the c-fos proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. *Brain Res.* **525,** 256–266.
26. Rasmussen K., Beitner-Johnson D. B., Krystal J. H., Aghajanian G. K., and Nestler E. J. (1990) Opiate withdrawal and the rat locus coeruleus: behavioral, electrophysiological, and biochemical correlates. *J. Neurosci.* **10,** 2308–2317.
27. Aghajanian G. (1978) Tolerance of locus coeruleus neurons to morphine and suppression of withdrawal by clonidine. *Nature (Lond).* **276,** 186–188.
28. Silverstone P., Done C., and Sharp T. (1993) In vivo monoamine release during naloxone precipitated withdrawal. *Neuropharmacol. Neurotoxicol.* **4,** 1043–1045.
29. Crawley J., Laverty R., and Roth R. (1979) Clonidine reversal of increased noradrenaline metabolite levels during morphine withdrawal. *Eur. J. Pharmacol.* **57,** 247–250.
30. Christie M. J., Williams J. T., Osborne P. B., and Bellchambers C. E. (1997) Where is the locus in opioid withdrawal? *Trends Pharmacol. Sci.* **18,** 134–140.
31. Caille S., Espejo E. F., Reneric J. P., Cador M., Koob G. F., and Stinus L. (1999) Total neurochemical lesion of noradrenergic neurons of the locus coeruleus does not alter either naloxone-precipitated or spontaneous opiate withdrawal nor does it influence ability of clonidine to reverse opiate withdrawal. *J. Pharmacol. Exp. Ther.* **290,** 881–892.
32. Delfs J., Zhu Y., Druhan J., and Aston-Jones G. (2000) Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature* **403,** 430–434.
33. Bullitt E. (1990) Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. *J. Comp. Neurol.* **296,** 517–530.
34. Nye H. E. and Nestler E. J. (1996) Induction of chronic Fos-related antigens in rat brain by chronic morphine administration. *Mol. Pharmacol.* **49,** 636–645.
35. Aston-Jones G., Delfs J. M., Druhan J., and Zhu Y. (1999) The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal. *Ann. NY Acad. Sci.* **877,** 486–498.
36. Chieng B., Keay K. A., and Christie M. J. (1995) Increased fos-like immunoreactivity in the periaqueductal gray of anaesthetised rats during opiate withdrawal. *Neurosci. Lett.* **183,** 79–82.
37. Stornetta R. L., Norton F. E., and Guyenet P. G. (1993) Autonomic areas of rat brain exhibit increased Fos-like immunoreactivity during opiate withdrawal in rats. *Brain Res.* **624,** 19–28.
38. Beckmann A. M., Matsumoto I., and Wilce P. A. (1995) Immediate early gene expression during morphine withdrawal. *Neuropharmacology* **34,** 1183–1189.
39. Sagar S. M., Sharp F. R., and Curran T. (1988) Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* **240,** 1328–1331.
40. Sagar S. M., Sharp F. R., and Curran T. (1988) Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* **240,** 1328–1331.
41. Bullitt E. (1989) Induction of c-fos-like protein within the lumbar spinal cord and thalamus of the rat following peripheral stimulation. *Brain Res.* **493,** 391–397.
42. Van Bockstaele E. J., and Aston-Jones G. (1992) Collateralized projections from neurons in the rostral medulla to the nucleus locus coeruleus, the nucleus of the solitary tract and the periaqueductal gray. *Neuroscience* **49,** 653–668.
43. Ennis M., Aston-Jones G., and Shiekhattar R. (1992) Activation of locus coeruleus neurons by nucleus paragigantocellularis or noxious sensory stimulation is mediated by intracoerulear excitatory amino acid neurotransmission. *Brain Res.* **598,** 185–195.
44. Ennis M. and Aston-Jones G. (1987) Two physiologically distinct populations of neurons in the ventrolateral medulla innervate the locus coeruleus. *Brain Res.* **425,** 275–282.
45. Ennis M. and Aston-Jones G. (1988) Activation of locus coeruleus from nucleus paragigantocellularis: a new excitatory amino acid pathway in brain. *J. Neurosci.* **8,** 3644–3657.

46. Ennis M. and Aston-Jones G. (1986) A potent excitatory input to the nucleus locus coeruleus from the ventrolateral medulla. *Neurosci. Lett.* **71,** 299–305.
47. Zhang T., Feng Y., Rockhold R. W., and Ho I. K. (1994) Naloxone-precipitated morphine withdrawal increases pontine glutamate levels in the rat. *Life Sci.* **55,** PL25–PL31.
48. Rasmussen K. and Aghajanian G. K. (1989) Withdrawal-induced activation of locus coeruleus neurons in opiate-dependent rats: attenuation by lesions of the nucleus paragigantocellularis. *Brain Res.* **505,** 346–350.
49. Drolet G., Van Bockstaele E. J., and Aston-Jones G. (1992) Robust enkephalin innervation of the locus coeruleus from the rostral medulla. *J. Neurosci.* **12,** 3162–3174.
50. Van Bockstaele E. J., Saunders A., Commons K. G., Liu X. B., and Peoples J. (2000) Evidence for coexistence of enkephalin and glutamate in axon terminals and cellular sites for functional interactions of their receptors in the rat locus coeruleus. *J. Comp. Neurol.* **417,** 103–114.
51. Abercrombie E. D. and Jacobs B. L. (1988) Systemic naloxone administration potentiates locus coeruleus noradrenergic neuronal activity under stressful but not non-stressful conditions. *Brain Res.* **441,** 362–366.
52. Williams J. T., Christie M. J., North R. A., and Roques B. P. (1987) Potentiation of enkephalin action by peptidase inhibitors in rat locus ceruleus in vitro. *J. Pharmacol. Exp. Ther.* **243,** 397–401.
53. Azami J., Llewelyn M. B., and Roberts M. H. (1982) The contribution of nucleus reticularis paragigantocellularis and nucleus raphe magnus to the analgesia produced by systemically administered morphine, investigated with the microinjection technique. *Pain* **12,** 229–246.
54. Ross C. A., Ruggiero D. A., Park D. H., Joh T. H., Sved A. F., Fernandez-Pardal J., Saavedra J. M., and Reis D. J. (1984) Tonic vasomotor control by the rostral ventrolateral medulla: effect of electrical or chemical stimulation of the area containing C1 adrenaline neurons on arterial pressure, heart rate, and plasma catecholamines and vasopressin. *J. Neurosci.* **4,** 474–494.
55. Sun M. K., Yen C. T., and Blum P. S. (1984) Response properties and functional organization of neurons in midline regions of medullary reticular formation of cats. *J. Neurophysiol.* **26,** 961–979.
56. Baraban S. C., Stornetta R. L., and Guyenet P. G. (1995) Effects of morphine and morphine withdrawal on adrenergic neurons of the rat rostral ventrolateral medulla. *Brain Res.* **676,** 245–257.
57. Rockhold R. W., Liu N. S., Coleman D., Commiskey S., Shook J., and Ho I. K. (2000) The nucleus paragigantocellularis and opioid withdrawal-like behavior. *J. Biomed. Sci.* **7,** 270–276.
58. Nestler E. J., Erdos J. J., Terwilliger R., Duman R. S., and Tallman J. F. (1989) Regulation of G proteins by chronic morphine in the rat locus coeruleus. *Brain Res.* **476,** 230–239.
59. Guitart X. and Nestler E. J. (1993) Second messenger and protein phosphorylation mechanisms underlying opiate addiction: studies in the rat locus coeruleus. *Neurochem. Res.* **18,** 5–13.
60. Nestler E. J., Alreja M., and Aghajanian G. K. (1994) Molecular and cellular mechanisms of opiate action: studies in the rat locus coeruleus. *Brain Res. Bull.* **35,** 521–528.
61. Koob G. F. and Nestler E. J. (1997) The neurobiology of drug addiction. *J. Neuropsychiatry Clin. Neurosci.* **9,** 482–497.
62. Van Bockstaele E. J., Branchereau P., and Pickel V. M. (1995) Morphologically heterogeneous met-enkephalin terminals form synapses with tyrosine hydroxylase-containing dendrites in the rat nucleus locus coeruleus. *J. Comp. Neurol.* **363,** 423–438.
63. Van Bockstaele E. J. and Chan J. (1997) Electron microscopic evidence for coexistence of leucine5-enkephalin and gamma-aminobutyric acid in a subpopulation of axon terminals in the rat locus coeruleus region. *Brain Res.* **746,** 171–182.
64. Johnson A., Grunwald G., Peoples J., and Van Bockstaele E. J. (2000) Evidence for opioid projections from the rostral ventral medulla to the nucleus of the solitary tract in rat brain. *Soc. Neurosci. Abstr.* **26,** 1187.
65. Van Bockstaele E. J., Peoples J., Menko A. S., McHugh K., and Drolet G. (2000) Decreases in endogenous opioid peptides in the rat medullocoerulear pathway after chronic morphine treatment. *J. Neurosci.* **20,** 8659–8666.
66. Valentino R. J. and Wehby R. G. (1989) Locus ceruleus discharge characteristics of morphine-dependent rats: effects of naltrexone. *Brain Res.* **488,** 126–134.
67. Valentino R. and Bockstaele E. V. (2001) Opposing regulation of the locus coeruleus by

corticotropin releasing factor and opioids: potential for reciprocal interactions between stress and opioid sensitivity. *Psychopharmacology,* **10,** p. 1007.
68. Hong J. S., McGinty J. F., Grimes L., Kanamatsu T., Obie J., and Mitchell C. L. (1988) Seizure-induced alterations in the metabolism of hippocampal opioid peptides suggest opioid modulation of seizure-related behaviors. *NIDA Res. Monogr.* **82,** 48–66.
69. Hong J. S., McGinty J. F., Lee P. H., Xie C. W., and Mitchell C. L. (1993) Relationship between hippocampal opioid peptides and seizures. *Progr. Neurobiol.* **40,** 507–528.
70. Tejwani G. A., Rattan A. K., Koo K. L., Matwyshyn G. A., and Bhargava H. N. (1994) Methionine-enkephalin concentrations in discrete brain regions, spinal cord, pituitary gland and peripheral tissues of U-50,488H-tolerant and abstinent rats. *Pharmacology* **48,** 216–225.
71. Gudehithlu K. P., Tejwani G. A., and Bhargava H. N. (1991) Beta-endorphin and methionine-enkephalin levels in discrete brain regions, spinal cord, pituitary gland and plasma of morphine tolerant-dependent and abstinent rats. *Brain Res.* **553,** 284–290.
72. Gudehithlu K. P. and Bhargava H. N. (1995) Modulation of preproenkephalin mRNA levels in brain regions and spinal cord of rats treated chronically with morphine. *Peptides* **16,** 415–419.
73. Yukhananov R. Y. and Handa R. J. (1997) Effect of morphine on proenkephalin gene expression in the rat brain. *Brain Res. Bull.* **43,** 349–356.
74. Childers S. R., Simantov R., and Snyder S. H. (1977) Enkephalin: radioimmunoassay and radioreceptor assay in morphine dependent rats. *Eur. J. Pharmacol.* **46,** 289–293.
75. Fratta W., Yang H. Y., Hong J., and Costa E. (1977) Stability of Met-enkephalin content in brain structures of morphine-dependent or foot shock-stressed rats. *Nature* **268,** 452–453.
76. Wesche D., Hollt V., and Herz A. (1977) Radioimmunoassay of enkephalins. Regional distribution in rat brain after morphine treatment and hypophysectomy. *Naunyn Schmiedebergs Arch. Pharmacol.* **301,** 79–82.
77. Comb M., Birnberg N. C., Seasholtz A., Herbert E., and Goodman H. M. (1986) A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* **323,** 353–356.
78. Van Nguyen T., Kobierski L., Comb M., and Hyman S. E. (1990) The effect of depolarization on expression of the human proenkephalin gene is synergistic with cAMP and dependent upon a cAMP-inducible enhancer. *J. Neurosci.* **10,** 2825–2833.
79. Comb M., Mermod N., Hyman S. E., Pearlberg J., Ross M. E., and Goodman H. M. (1988) Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. *EMBO J.* **7,** 3793–3805.
80. Hyman S. E., Comb M., Pearlberg J., and Goodman H. M. (1989) An AP-2 element acts synergistically with the cyclic AMP- and phorbol ester-inducible enhancer of the human proenkephalin gene. *Mol. Cell. Biol.* **9,** 321–324.
81. Konardi C., Cole R. L., Green D., Senatus P., Leveque J. C., Pollack A. E., Grossbard S. J., and Hyman S. E. (1995) Analysis of the proenkephalin second messenger-inducible enhancer in rat striatal cultures. *J. Neurochem.* **65,** 1007–1015.
82. Nestler E. J., Hope B. T., and Widnell K. L. (1993) Drug addiction: a model for the molecular basis of neural plasticity. *Neuron* **11,** 995–1006.
83. Sheu M. J., Sribanditmongkol P., Santosa D. N., and Tejwani G. A. (1995) Inhibition of morphine tolerance and dependence by diazepam and its relation to cyclic AMP levels in discrete rat brain regions and spinal cord. *Brain Res.* **675,** 31–37.
84. Lightman S. L. and Young W. S. D. (1987) Changes in hypothalamic preproenkephalin A mRNA following stress and opiate withdrawal. *Nature* **328,** 643–645.
85. Tempel A., Kessler J. A., and Zukin R. S. (1990) Chronic naltrexone treatment increases expression of preproenkephalin and preprotachykinin mRNA in discrete brain regions. *J. Neurosci.* **10,** 741–747.
86. Uhl G. R., Ryan J. P., and Schwartz J. P. (1988) Morphine alters preproenkephalin gene expression. *Brain Res.* **459,** 391–397.
87. Delle M., Ricksten S., Haggendal J., Olsson K., Skarphedinsson J., and Thoren P. (1990) Regional changes in sympathetic nerve activity and baroreceptor reflex function and arterial plasma levels of catecholamines, renin and vasopressin during naloxone precipitated morphine withdrawal in rats. *J. Pharmacol. Exp. Ther.* **253,** 646–654.
88. Paxinos G. and Watson C. (1986) *The Rat Brain in Stereotaxic Coordinates.* Academic, New York.
89. Van Bockstaele E. J., Commons K., and Pickel V. M. (1997) Delta-opioid receptor is present in

presynaptic axon terminals in the rat nucleus locus coeruleus: relationships with methionine5-enkephalin. *J. Comp. Neurol.* **388,** 575–586.
90. Van Bockstaele E. J., Colago E. E., Cheng P., Moriwaki A., Uhl G. R., and Pickel V. M. (1996) Ultrastructural evidence for prominent distribution of the mu-opioid receptor at extrasynaptic sites on noradrenergic dendrites in the rat nucleus locus coeruleus. *J. Neurosci.* **16,** 5037–5048.