Essential role of the cAMP-cAMP response-element binding protein pathway in opiate-induced homeostatic adaptations of locus coeruleus neurons

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Excessive inhibition of brain neurons in primary or slice cultures can induce homeostatic intrinsic plasticity, but the functional role and underlying molecular mechanisms of such plasticity are poorly understood. Here, we developed an ex vivo locus coeruleus (LC) slice culture system and successfully recapitulated the opiate-induced homeostatic adaptation in electrical activity of LC neurons seen in vivo. We investigated the mechanisms underlying this adaptation in LC slice cultures by use of viral-mediated gene transfer and genetic mutant mice. We found that short-term morphine treatment of slice cultures almost completely abolished the firing of LC neurons, whereas chronic morphine treatment increased LC neuronal excitability as revealed during withdrawal. This increased excitability was mediated by direct activation of opioid receptors and up-regulation of the cAMP pathway and accompanied by increased cAMP response-element binding protein (CREB) activity. Overexpression of a dominant negative CREB mutant blocked the increase in LC excitability induced by morphine- or cAMP-pathway activation. Knockdown of CREB in slice cultures from floxed CREB mice similarly decreased LC excitability. Furthermore, the ability of morphine or CREB overexpression to up-regulate LC firing was blocked by knockout of the CREB target adenylyl cyclase 8. Together, these findings provide direct evidence that prolonged exposure to morphine induces homeostatic plasticity intrinsic to LC neurons, involving up-regulation of the cAMP-CREB signaling pathway, which then enhances LC neuronal excitability.

adenylyl cyclase | firing adaptation | intrinsic plasticity | opiate dependence/withdrawal

Recent findings reveal that brain neurons in primary or slice cultures can develop compensatory homeostatic adaptations in intrinsic excitability or synaptic connections in response to excessive inhibition (1, 2). Homeostatic intrinsic plasticity is a neuronal feedback mechanism by which neurons compensate for a strong stimulus by restabilizing the activity of neurons or neural networks within a physiologically normal range. However, the functional role and molecular mechanisms of how neurons compensate for excessive inhibition are incompletely understood (1).

Locus coeruleus (LC) neurons are implicated in physical dependence to opiate drugs of abuse (3, 4). In vivo investigations show that acute morphine administration decreases the firing rate of LC neurons (5). During chronic opiate exposure, LC firing rates return to normal levels (tolerance) and, on antagonist precipitation of withdrawal, increase dramatically above normal levels (6, 7). Part of this increase is mediated through extrinsic mechanisms (*Discussion*), with the rest proposed to be mediated through up-regulation of the cAMP pathway within LC neurons (3, 4, 8, 9).

In vivo studies also have provided insight into the molecular mechanisms by which chronic opiates up-regulate the cAMP pathway in LC. Chronic morphine administration increases levels of adenylyl cyclase 1 (AC1) and AC8 in LC (10, 11), and knockout of either isoform attenuates the ability of chronic morphine to increase LC neuronal excitability and behavioral features of opiate withdrawal (12). Blockade of CREB (cAMP response-element binding protein) in the LC in vivo prevents the opiate-induced up-regulation of AC8 (11). Such blockade also attenuates the ability of chronic morphine to increase LC neuronal excitability and to induce dependence and withdrawal (11, 13).

Despite the several lines of evidence that support this scheme of opiate-induced homeostatic adaptation in the LC, key features of the model remain poorly defined. All of the investigations, as noted above, have relied on in vivo models of morphine administration, where morphine exerts effects not only on LC neurons but on many other CNS regions, including several that innervate the LC (14–16). This has led to questions as to whether the homeostatic plasticity intrinsic to LC neurons plays a role in mediating morphine's effects on the activity of LC neurons. The lack of an ex vivo system for addressing this issue experimentally has been the major limiting factor.

In the present study, by use of an ex vivo LC slice culture system combined with viral-mediated gene transfer and genetic mutant mice, we provide several important lines of direct evidence to support our model that prolonged exposure to morphine induces homeostatic adaptations intrinsic to LC neurons, involving up-regulation of the cAMP-CREB pathway, which enhance LC neuronal excitability.

Results

Chronic Morphine Treatment of LC Slice Cultures Increases the Intrinsic Excitability of LC Neurons. LC neurons exhibit tonic firing in vivo as well as pacemaker activity in acute brain slices ex vivo (6, 13, 17, 18). However, this feature is dependent on LC development and is sensitive to slice conditions and cytoplasmic changes during cell recordings (19–21) (more information in *LC Slice Cultures* in *Materials and Methods*). In a previous study, we successfully maintained the normal pacemaker activity of LC neurons in adult slice cultures (13). These LC slice cultures provide an ideal system in which to address whether chronic morphine can induce homeostatic changes in LC excitability ex vivo and whether such changes represent adaptations intrinsic to LC neurons that are mediated through the cAMP-CREB pathway.

We first examined the time course of the morphine effect with 5 μM morphine, a concentration that is relevant to the in vivo situation (9, 22). We found that short-term (30–60 min) morphine exposure almost completely blocked the firing rate of LC

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noradrenergic neurons (Fig. 1 *A* and *B*). In contrast, after chronic (2–3 d) morphine treatment, the firing rate of LC neurons partially recovered, suggesting the development of morphine tolerance at the cellular level. Moreover, chronic morphine treatment, followed by the addition of the opioid receptor antagonist naloxone, increased the firing rate of LC neurons above control levels, whereas naloxone had no effect in short-term or 1-d morphine-treated slice cultures (Fig. 1 *A* and *B*) ($F_{6,434} = 9.58$, $P < 0.001$). There was no detectable difference between 2 and 3 d of morphine treatment (Fig. 1 *A* and *B*). Naloxone was used to precipitate morphine withdrawal in the slice cultures as used in withdrawal models in vivo (3, 4). Naloxone itself did not change the baseline firing rate of LC neurons in morphine-naïve slice cultures (Fig. S1*A*) ($t_{98} = 0.05$, $P = 0.96$). As an additional control, rather than adding naloxone, we washed morphine out of the slice over a period of 90 min (8). We observed that, after chronic morphine treatment, this spontaneous withdrawal of morphine led to an equivalent increase in LC firing (Fig. S1*B*) ($t_{134} = -5.06$, $P < 0.001$). Next, we examined the dose response of the morphine effect. We found that at least 1 μM morphine was needed to induce the withdrawal-induced firing increase (Fig. 1 *C* and *D*) ($F_{4,353} = 25.96$, $P < 0.001$). Treatment with 10 μM morphine induced a 3-fold increase in the firing rate of LC neurons over control levels, which is similar to the increased excitability induced in cultured cortical pyramidal neurons in response to the Na^+ channel blocker tetrodotoxin (TTX) (23). These results show that the homeostatic regulation of LC neurons by chronic morphine seen in vivo can be recapitulated in LC slice cultures ex vivo.

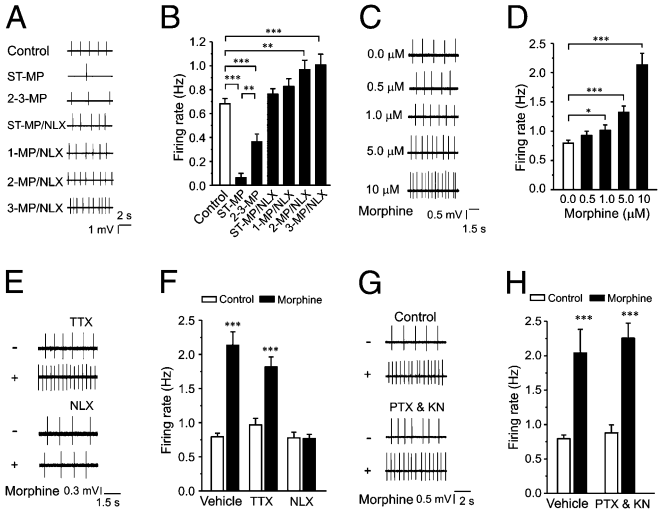

Fig. 1. Increased firing of LC neurons was induced by prolonged treatment of LC slice cultures with morphine, and blockade of synaptic transmission had no effect on this firing increase. (*A* and *B*) Time course: slice cultures were treated with 5 μM morphine for varying periods of time, and recordings from LC neurons were obtained in the presence or absence of 1 μM naloxone (NLX). ST-MP, short-term (30–60 min) morphine treatment; 1-MP, 1-d morphine treatment; 2-MP, 2-d morphine treatment; 3-MP, 3-d morphine treatment. (*C* and *D*) Dose response: slice cultures were treated for 2–3 d with varying concentrations of morphine, and recordings from LC neurons were obtained immediately after addition of NLX. (*E*) Sample traces recorded from 1 μM TTX- or 1 μM NLX-incubated slices with (+) or without (–) 10 μM morphine treatment for 2–3 d. (*F*) Blockade of action potential-dependent synaptic transmission by TTX had no effect on the morphine-induced firing increase, whereas the morphine effect was blocked by NLX. (*G*) Sample traces were recorded from LC neurons in slice cultures treated with 100 μM PTX and 1 mM KN for 2–3 d. (*H*) Blockade of $GABA_A$ and glutamate receptor-mediated synaptic transmission by PTX and KN for 2–3 d did not affect the 10 μM morphine-induced increase in LC firing in slice cultures. Treatment of morphine-naïve slices with PTX and KN for 2–3 d had no effects on baseline LC firing. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Consistent with previous observations (24), residual spontaneous synaptic activity mediated by $GABA_A$ and glutamate receptors was seen in our LC slice cultures (Fig. S1*C*). To exclude the possibility that residual synaptic inputs to LC neurons contribute to the morphine withdrawal-induced firing increase seen in the slice cultures, we examined morphine effects on LC firing rate when residual synaptic inputs were abolished with pharmacological tools. First, any action potential-dependent spontaneous synaptic activation of LC neurons was blocked by incubation with TTX for 2–3 d with or without concomitant morphine. TTX did not affect the ability of morphine withdrawal to increase LC firing (Fig. 1 *E* and *F*) ($t_{111} = -4.80$, $P < 0.001$). TTX itself, in the absence of morphine, did not significantly change the baseline spontaneous firing rate of LC neurons (Fig. 1*F*) ($t_{175} = -1.70$, $P = 0.09$), indicating that LC pacemaker neurons are different from other neurons. In contrast, the ability of morphine to increase LC firing was blocked completely by 2–3 d of concomitant incubation with naloxone (Fig. 1 *E* and *F*) ($t_{50} = 0.11$, $P = 0.91$), verifying that morphine's effects are mediated through activation of opioid receptors.

To further exclude a contribution from residual synaptic activity, we made whole-cell voltage-clamp recordings from LC neurons in slice cultures and observed a low level of spontaneous postsynaptic currents (sPSCs) as mentioned above. This activity was abolished completely by coapplication of the $GABA_A$ receptor antagonist picrotoxin (PTX) and the nonselective ionotropic glutamate receptor antagonist kynurenic acid (KN) or competitive AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) in all cells tested (Fig. S1*C*) ($n = 9$). Consistent with our TTX data, the blockade of sPSCs with PTX and KN for 2–3 d did not affect the morphine-induced firing increase in LC slice cultures (Fig. 1 *G* and *H*) ($t_{60} = -5.47$, $P < 0.001$). PTX and KN incubation for 2–3 d had no effect on LC firing in control-slice cultures (Fig. 1 *G* and *H*) ($t_{148} = -0.69$, $P = 0.49$). These data further show that the morphine-induced increase in LC neuronal excitability in slice cultures is mediated by direct activation of opioid receptors on LC neurons and regulation of intrinsic properties of these neurons.

Involvement of the cAMP Pathway in the Morphine-Induced Increase in LC Firing. To examine the downstream signaling pathway that mediates the induction of LC firing after chronic morphine, we focused on the cAMP pathway based on the evidence that it mediates chronic morphine-induced increases in LC neuronal excitability in vivo (as stated above). We chronically activated the cAMP pathway in the slice cultures by adding the adenylyl cyclase activator forskolin or the membrane permeable cAMP analog 8-Br-cAMP to the culture medium in the presence or absence of morphine. We found that 2–3 d of forskolin or 8-Br-cAMP treatment increased the firing rate of LC neurons in morphine-naïve slices (Fig. 2 *A* and *B*) (forkolin: $t_{151} = -9.41$, $P < 0.001$; 8-Br-cAMP: $t_{158} = -8.47$, $P < 0.001$) and that this effect completely occluded the actions of morphine withdrawal, which had no further effect on LC firing rate in forskolin- or 8-Br-cAMP–treated slice cultures (Fig. 2 *A* and *B*) (forskolin: $t_{74} = -0.01$, $P = 0.99$; 8-Br-cAMP: $t_{72} = -1.69$, $P = 0.09$). These data suggest that morphine and cAMP may act through a shared signaling pathway. Moreover, we found that, although the protein kinase A inhibitor [(Rp)-adenosine 3′, 5′-monophosphorothioate (Rp isomer)] (RP-cAMP) had no detectable effect on LC firing when it was applied to morphine-naïve slice cultures for 2–3 d ($t_{158} = 0.95$, $P = 0.35$), it significantly diminished the chronic morphine/withdrawal-induced firing increase (Fig. 2 *A* and *B*) ($t_{89} = -4.54$, $P < 0.001$). However, RP-cAMP did not completely block the morphine effect (Fig. 2 *A* and *B*) ($t_{76} = -3.81$, $P < 0.001$), suggesting the involvement of an unknown, non-protein kinase A (PKA) mech-

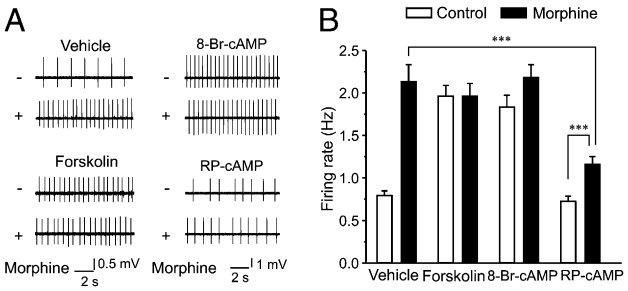

Fig. 2. Effect of the cAMP pathway on the morphine-induced increase in LC firing in slice cultures. (*A*) Sample traces of LC firing in control culture medium or in the presence of 10 μM forskolin, 2 mM 8-Br-cAMP, or 100 μM RP-cAMP, with or without 10 μM morphine in slice cultures. Recordings were obtained immediately after the addition of 1 μM naloxone. (*B*) Forskolin or 8-Br-cAMP significantly increased LC neuronal firing, and chronic morphine treatment did not induce a further increase in firing in forskolin- or 8-Br-cAMP–pretreated LC slice cultures. RP-cAMP itself did not affect the baseline firing of LC neurons. RP-cAMP partially decreased the chronic morphine-induced increase in LC firing. Asterisks indicate statistically significant differences (***$P < 0.001$).

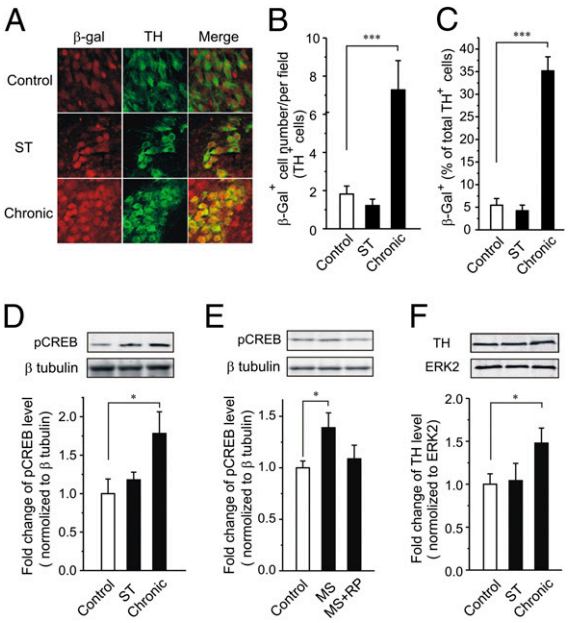

Fig. 3. Chronic morphine treatment increased CREB activity and TH expression in LC slice cultures. (*A* and *B*) Slice cultures were obtained from *CRE-LacZ* transgenic mice; 10 μM morphine treatment for 2–3 d dramatically increased the number of TH-positive cells that are also β-galactosidase (β-gal)–positive. (*C*) The percentage of TH-positive LC neurons that are also β-gal–positive was increased 7-fold by chronic morphine administration, whereas this effect was not seen in slice cultures exposed to short-term (ST) morphine. (*D*) Chronic, but not ST, morphine treatment also increased phospho-CREB levels in LC. (*E*) The chronic morphine (MS)-induced increase in phospho-CREB was blocked by RP-cAMP (MS + RP). (*F*) Chronic, but not ST, morphine treatment also increased TH protein levels. Asterisks indicate statistically significant differences (*$P < 0.05$; ***$P < 0.001$).

anism. These results show that the cAMP pathway is involved in inducing the adaptive firing increase of LC neurons in response to chronic morphine ex vivo.

Chronic Morphine Treatment Increases CREB Activity in LC Slice Cultures. Chronic administration of morphine to rodents has been shown to induce CREB transcriptional activity in LC (25). To determine whether chronic morphine produces a similar effect in slice cultures, we first used slice cultures obtained from *CRE-LacZ* reporter mice, where cAMP response elements (CREs) drive the *LacZ* gene [which encodes β-galactosidase (β-gal)]. It was observed that chronic morphine treatment of these LC slice cultures increased the number of noradrenergic neurons marked by tyrosine hydroxylase (TH), the rate-limiting enzymes in the synthesis of norepinephrine, which are also β-gal–positive (Fig. 3 *A* and *B*) ($F_{2,40} = 9.32$, $P < 0.001$). In fact, chronic morphine treatment induced a 7-fold increase in the percentage of TH-positive cells in LC that are β-gal–positive (Fig. 3*C*) ($F_{2,40} = 55.29$, $P < 0.001$). In contrast, short-term morphine treatment had no detectable effects on CRE-mediated transcription (Fig. 3 *B* and *C*). It must be emphasized that the induction of CREB activity was observed in the absence of naloxone: in other words, when LC firing was not elevated above normal levels (Fig. 1 *A* and *B*). These results indicate that chronic morphine treatment per se induces CREB activity in LC noradrenergic neurons in slice cultures.

Second, we measured levels of phospho-CREB, the active form of the protein, in LC slice cultures using Western blotting. We exposed slice cultures to morphine for 2–3 d, after which time we excised LC from the slice by punch dissection. We observed that LC punches chronically exposed to morphine had significantly higher levels of phospho-CREB compared with control slices (Fig. 3*D*) ($F_{2,15} = 4.00$, $P < 0.05$), with no change observed after short-term morphine treatment (Fig. 3*D*). In contrast, the level of total CREB protein was not altered by either short-term or chronic morphine administration (Fig. S2*A*) ($F_{2,15} = 0.87$, $P = 0.44$). In addition, we observed no change in levels of phospho-ERK or total ERK in the LC after chronic morphine exposure, although phospho-ERK levels were induced by short-term morphine (Fig. S2*B*) ($F_{2,15} = 5.43$, $P < 0.05$). These latter findings support the view that the chronic morphine-induced increase in phospho-CREB levels is not mediated by the ERK signaling pathway.

Third, we investigated the effects of RP-cAMP on levels of phospho-CREB in LC slice cultures. Chronic morphine treatment increased the level of phospho-CREB in the LC as shown above (Fig. 3*E*) ($t_{14} = -2.45$, $P < 0.05$), and this morphine effect was blocked completely when the LC slices were incubated concomitantly with RP-cAMP for 2–3 d (Fig. 3*E*) ($t_{14} = -0.59$, $P = 0.56$). This finding is consistent with the observation that RP-cAMP also decreased the chronic morphine/withdrawal-induced increase in LC firing (Fig. 2).

TH has been shown to be a target gene for CREB in numerous tissue preparations (26) and, accordingly, we have shown that it is induced in the LC in vivo in response to chronic morphine administration (27). We found that chronic treatment of slice cultures with morphine for 2–3 d (without naloxone exposure) significantly increased levels of TH expression in LC (Fig. 3*F*) ($t_{10} = -2.25$, $P < 0.05$). This effect was not seen in slices exposed to short-term morphine (Fig. 3*F*) ($t_{10} = -0.18$, $P = 0.86$). These findings further validate our slice culture as a bona fide model of morphine dependence observed in LC in vivo.

CREB Inhibition Blocks the Morphine- and cAMP-Pathway–Induced Increase in LC Firing. We next investigated the possible involvement of CREB in mediating the increase in LC firing induced by chronic morphine/withdrawal in LC slice cultures. We first used floxed CREB mice to knockdown CREB in LC by use of viral vectors. Overexpression of Cre-GFP in LC of naïve-slice cultures decreased the level of CREB mRNA (Fig. S3*A*) ($t_5 = 3.33$, $P < 0.05$). Moreover, Cre-GFP expressing LC neurons had much lower firing rates compared with control neurons expressing GFP alone (Fig. 4*A*) ($t_{67} = 7.27$, $P < 0.001$). These findings show that endogenous CREB activity is required for the maintenance of normal LC firing rates. Second, we virally overexpressed a dominant negative CREB mutant (dnCREB) in LC slice cultures treated with or without morphine for 2–3 d and compared the firing rate of

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LC neurons between infected (dnCREB-expressing) and noninfected control neurons. Consistent with the CREB genetic knockdown data (Fig. 4*A*) and our earlier observations (11, 13), dnCREB-expressing LC neurons showed dramatically lower firing rates in morphine-naïve slice cultures compared with nearby noninfected neurons in the same slices (Fig. 4*B*) ($t_{28} = 3.17$, $P < 0.01$). As expected, 2–3 d of morphine treatment of slice cultures followed by withdrawal induced a firing increase in noninfected neurons (Fig. 4*B*) ($t_{38} = -2.28$, $P < 0.05$), an effect blocked completely in dnCREB-expressing LC neurons in the same slices (Fig. 4*B*) ($t_{26} = 1.12$, $P = 0.27$). These findings indicate that CREB activity is required for the normal pacemaker activity of LC neurons and the ability of morphine withdrawal to induce LC neuronal hyperactivity.

Similarly, we found that chronic exposure of slice cultures to forskolin or 8-Br-cAMP increased the firing rate of noninfected LC neurons (Fig. 4 *C* and *D*) ($F_{2,51} = 5.57$, $P < 0.01$), effects completely absent in dnCREB-expressing LC neurons in the same slices (Fig. 4 *C* and *D*) ($F_{2,33} = 0.44$, $P = 0.65$). These results suggest that CREB is also required for the increase in LC firing induced by sustained activation of the cAMP pathway.

CREB- and Morphine-Induced Changes in LC Firing Are Blocked by AC8 Knockout. As stated above, chronic morphine induces AC1 and AC8 in LC in vivo. Moreover, previous studies implicate AC8, but not AC1, as one of CREB's target genes in LC (11, 28). In slice cultures obtained from AC1 knockout mice, we found that dnCREB-expressing LC neurons showed significantly lower firing rates (Fig. 5*A*) ($t_{25} = 2.16$, $P < 0.05$), whereas LC cells expressing a constitutively active CREB (caCREB) had significantly higher firing rates (Fig. 5*A*) ($t_{49} = -2.63$, $P < 0.05$). These responses to dnCREB and caCREB in AC1 knockouts are similar to those seen in wild-type mice (Fig. 4 *B*–*D*) (13). In contrast, these same manipulations of CREB failed to alter LC firing in slice cultures prepared from AC8 knockout mice (Fig. 5*B*) (dnCREB: $t_{42} = -0.17$, $P = 0.86$; caCREB: $t_{25} = -0.53$, $P = 0.60$). These results directly implicate AC8, but not AC1, in mediating CREB regulation of LC neuronal excitability. It would be interesting in future studies to determine whether knockout of AC8 similarly blocks the ability of forskolin or 8-Br-cAMP to induce LC hyperactivity in slice cultures.

We next determined whether AC1 or AC8 mediates the morphine withdrawal-induced hyperactivity of LC neurons observed in slice cultures. Global knockout of AC1 or AC8 attenuates some of the behavioral symptoms of morphine withdrawal and the morphine-induced increase in LC neuronal activity induced by chronic in vivo morphine administration (12). Here, we observed that 2–3 d of morphine treatment followed by withdrawal significantly increased LC firing in slice cultures taken from AC1 knockout (KO) mice or their wild-type (WT) littermates (Fig. 5 *C* and *D* and Fig. S3*B*) (KO: $t_{59} = -3.16$, $P < 0.01$; WT: $t_{40} = -2.28$, $P < 0.05$). In contrast, the chronic morphine/withdrawal-induced effect on LC firing was not seen in slice cultures taken from AC8 KO mice, although we saw the expected firing increase in their WT littermates (Fig. 5 *C* and *E* and Fig. S3*C*) (KO: $t_{50} = 0.60$, $P = 0.55$; WT: $t_{80} = -2.51$, $P < 0.05$). These results show that AC8, but not AC1, is required for the morphine-induced adaptive firing increase seen in LC neurons in slice cultures (Fig. 5*F*).

Discussion

We have proposed a scheme, based solely on information obtained from in vivo morphine-treatment models over two decades, that chronic morphine administration induces a homeostatic hyperactivity of LC neurons through up-regulation of the cAMP pathway

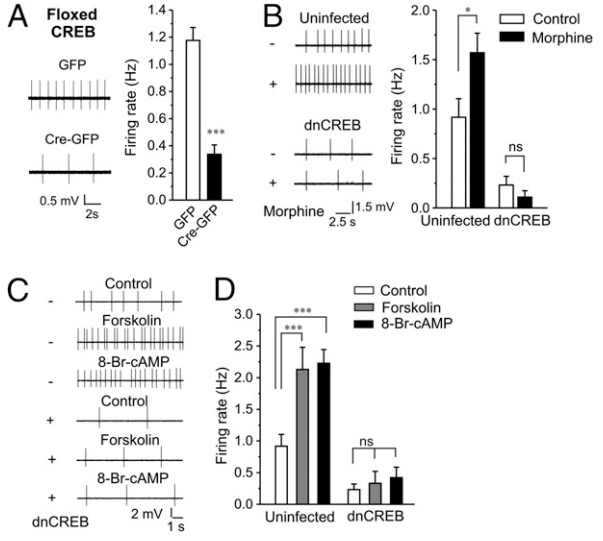

Fig. 4. dnCREB completely blocked the increase in LC firing induced by chronic morphine or cAMP-pathway activation in slice cultures. (*A*) Cre-GFP–expressing LC neurons in slice cultures taken from floxed CREB mice had dramatic lower firing rates. (*B*) Sample traces were recorded from dnCREB-expressing GFP^+ cells and control (uninfected GFP^- neighboring) cells in the same slices. Overexpression of dnCREB in LC neurons decreased baseline firing in control slices and dramatically prevented the 10-μM morphine-induced increase in LC firing. Recordings were obtained immediately after addition of 1 μM naloxone. (*C*) Sample traces were obtained from dnCREB-expressing or uninfected cells in control slices, and slices were treated chronically with 10 μM forskolin or 2 mM 8-Br-cAMP. (*D*) Overexpression of dnCREB completely blocked the forskolin- or 8-Br-cAMP–induced increase in LC firing. Asterisks indicate statistically significant differences (*$P < 0.05$; ***$P < 0.001$; ns, no significant difference).

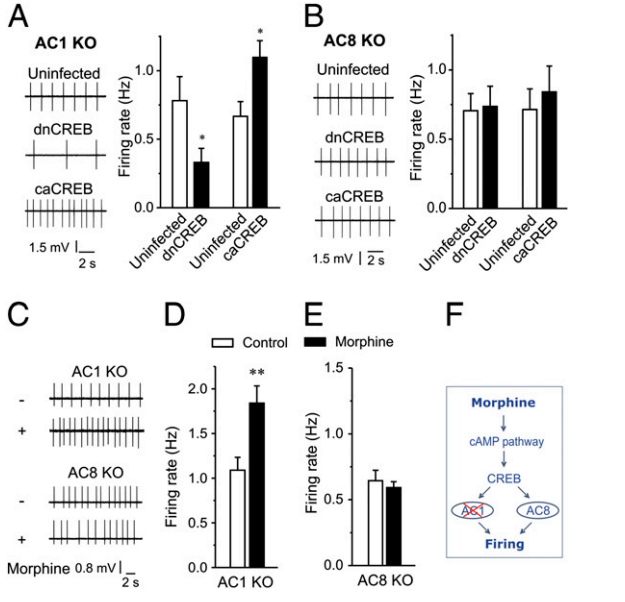

Fig. 5. CREB or morphine regulation of LC firing was absent in slice cultures from AC8, but not AC1, knockout mice. (*A*) The baseline firing rate of LC neurons was decreased by overexpression of dnCREB in slice cultures taken from AC1 knockout mice. Conversely, the baseline firing rate of LC neurons was increased by caCREB expression in slice cultures from these mice. (*B*) In contrast, both the dnCREB- and caCREB-induced changes in LC firing were absent in slice cultures obtained from AC8 knockout mice. (*C*) Sample traces were recorded from LC neurons in slice cultures obtained from AC1 or AC8 knockout mice with or without 10-μM morphine treatment. Recordings were obtained immediately after the addition of 1 μM naloxone. (*D*) Chronic morphine treatment increased LC firing in slice cultures taken from AC1 knockout mice. (*E*) In contrast, this morphine effect was absent in slices from AC8 knockout mice. (*F*) Schematic: together these data indicate that the ability of CREB and morphine to regulate LC firing requires AC8 but not AC1. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$).

and CREB (3, 4). Using an ex vivo morphine-dependence model in LC slice cultures combined with the use of viral-mediated CREB gene transfer, floxed CREB mice, and AC1 and AC8 KO mice, we provide here several important lines of direct evidence to further support this molecular scheme of opiate dependence and propose an important role for homeostatic intrinsic plasticity in the morphine withdrawal-induced hyperactivity of LC neurons seen in vivo.

There have been divergent views about the role of intrinsic changes in LC neurons vs. extrinsic alterations in mediating the hyperactivity of these neurons observed during opiate withdrawal in vivo (3, 9, 24, 29, 30). There is no question that extrinsic factors, particularly, increased glutamatergic transmission to LC neurons, contribute to this LC activation. An in vivo microdialysis study showed that extracellular levels of glutamate and aspartate are increased in LC on morphine withdrawal (31). The withdrawal-induced hyperactivity of LC neurons is reduced by AMPA receptor antagonists or lesions of the nucleus paragigantocellularis, a major glutamatergic nucleus that innervates the LC (14, 29, 32). The molecular basis of this elevated glutamatergic transmission remains unknown.

There also is evidence for the involvement of factors intrinsic to LC neurons. LC neurons in acute slices taken from morphine-dependent animals show a 2-fold increase in firing when morphine is washed out of the slice or an opioid receptor antagonist is added (8, 9). Moreover, a mixture of synaptic transmission blockers, including glutamate receptor antagonists, does not affect this firing increase (9). Further evidence for intrinsic alterations comes from molecular studies, where chronic in vivo morphine administration has been shown to up-regulate the cAMP pathway and CREB in LC, and blockade of these signals, in LC, attenuates withdrawal activation of LC neurons as well as the severity of opiate withdrawal (3, 4, 11–13, 33). Still, because all of these studies involved the administration of morphine in vivo, any changes observed in LC could be mediated through other CNS regions.

To address this possibility, we used, in the present study, a slice-culture preparation where afferent fibers from other brain regions to LC neurons are cut, and we succeeded in recapitulating the morphine withdrawal-induced hyperactivity of LC neurons seen with in vivo models. Furthermore, we show that this hyperactivity requires neither local action potential-dependent synaptic neurotransmission nor $GABA_A$ or glutamate receptor-mediated sPSCs, the only sPSCs seen in our LC slice culture preparation. We also observed that the morphine-induced increase in LC firing was abolished completely by the opioid receptor antagonist naloxone, establishing that opioid receptor-mediated mechanisms are involved. These data strongly support the view that the morphine withdrawal-induced hyperactivity of LC neurons seen ex vivo is independent of synaptic inputs and can indeed be induced by direct activation of opioid receptors on LC neurons. Importantly, we further validated this slice-culture model of opiate dependence by showing that it exhibits several other features of chronic morphine exposure first established in LC in vivo, including up-regulation of the cAMP pathway, activation of CREB, and induction of TH.

We next used pharmacological tools, viral vectors, and genetic mutant mice to directly probe the detailed molecular steps underlying chronic morphine action in LC slice cultures. We show that sustained activity of the cAMP pathway is both necessary and sufficient for the morphine induction of CREB activity and increased LC firing seen in the slice cultures. In turn, we show that CREB activity is both necessary and sufficient for the morphine withdrawal-induced increase in LC firing in slice cultures and that the ability of morphine and CREB to induce LC firing requires AC8, which depends on CREB for its induction. Together, these data suggest a functional feed-forward loop where chronic activation of opioid receptors on LC neurons triggers activation of the cAMP pathway, which leads to sustained activation of CREB. The sustained activation of CREB induces expression of AC8, which then further augments the activity of the cAMP pathway and CREB. Importantly, results of the present study firmly link this cAMP-CREB-AC8 pathway to the chronic morphine/withdrawal-induced increase in LC neuronal excitability. Induction of AC8 may be particularly significant, because it, unlike many other adenylyl cyclase isoforms, is not efficiently inhibited by Gαi/o, the predominant G protein coupled to opioid receptors in LC (18, 34). This means that, during a course of chronic opiate administration, the cAMP pathway in LC neurons gradually and progressively escapes its initial inhibition by opioid receptor activation, further increasing the net activity of this pathway. This explains how activation of the cAMP-CREB pathway (e.g., induction of CRE activity, phospho-CREB, and TH) and the recovery of LC firing to normal levels occur during the course of chronic opiate exposure (in other words, in the absence of withdrawal). This phenomenon occurs in the LC in vivo (25, 27) as well as in slice cultures, as shown here.

In summary, results of the present study provide compelling evidence to support a molecular model of opiate-induced homeostatic intrinsic plasticity, where prolonged exposure to opiates triggers activation of a cAMP-CREB-AC8 loop that underlies increased electrical excitability of LC neurons. According to this model, chronic opiate exposure increases the activity of the cAMP pathway and CREB, which then induces expression of AC8, further contributing to cAMP-pathway activation. We show that each step in this feed-forward loop, cAMP-pathway activation, CREB activation, and AC8 induction, is required for the ability of chronic opiates to increase LC neuronal excitability. Importantly, our success in establishing this opiate-dependence model in LC slice cultures ex vivo shows a key role of homeostatic intrinsic plasticity in the development of opiate dependence and provides a system in which further molecular insight into the neurobiological mechanisms of opiate dependence can now be explored.

Materials and Methods

Animals. Animal procedures were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern and Mount Sinai. Sprague–Dawley rats (Charles River) were used at 25–30 d of age for LC slice cultures. *CRE-LacZ* transgenic mice, which show low levels of transgene expression at baseline (35), were used at 6–12 wk of age. Floxed CREB mice, AC1 and AC8 KO mice, and WT littermates (12) were also used at 6–12 wk of age.

LC Slice Cultures. Slice cultures were prepared as described previously (13, 36). Acute coronal brain slices were trimmed as small as possible (Fig. S4*A*) and maintained for up to 3 d in a 34 °C incubator. We used Gibco MEM medium containing 30 mM Hepes, 20 mM D-glucose, 5% B27, 5.0 mM L-glutamine, and 25 unit/mL streptomycin/penicillin. We tested several culture media used previously by other investigators (36, 37). However, the pacemaker activity of LC neurons in slice cultures was lost in those media and in the slice cultures obtained from rats before 21 d old, indicating that the pacemaker activity of LC neurons is sensitive to environmental conditions and dependent on brain development (13). In contrast, our culture medium successfully maintained stable firing of these neurons in the LC slice cultures. We further examined the other electrical properties of LC neurons in slice cultures and found that there were no differences between LC slice cultures and acute LC slices in voltage-current relationship, resting membrane potential, and current injection-evoked spike numbers (i.e., inherent excitability) (Fig. S4 *B–D*).

Viral-Mediated CREB Gene Transfer. Acute LC slices were incubated at 34 °C for 1 h, after which time HSV vectors were pipetted onto the LC area of the slice surface. The following vectors were used: HSV-CreGFP, HSV-dnCREBGFP (a dominant negative mutant of CREB that lacks Ser133, also known as mCREB, tagged with GFP), HSV-caCREBGFP (a constitutively active form of CREB that represents a CREB-VP16 fusion protein, also tagged with GFP), and HSV-GFP as a control. These vectors have been used successfully in many previous studies to produce the expected changes in CREB-mediated transcription (13, 28, 35, 38). Infected cells in LC slice cultures were visualized by the GFP under a fluorescence microscope with uninfected cells in the same slices used as controls.

Drug Treatments. In all experiments, brain slices were bisected, with one side used for drug treatment and the other side used as the control. The concentrations of reagents used in this study were: 0.5–10 μM morphine sulfate,

1 μM TTX, 10 μM forskolin, 2 mM 8-Br-cAMP, 100 μM RP-cAMP, 100 μM PTX, 1 mM KN, 10 μM CNQX, and 1 μM naloxone. All reagents were purchased from Sigma except for forskolin (CalBiochem) and TTX (Tocris).

Western Blot Analysis. One hour after the last treatment, bilateral punches of the LC from two slices of the same animals were homogenized by sonication in lysis buffer and stored at −20 °C until determination of protein concentrations by the Lowry method. Aliquots (containing 10 μg protein) of each sample were separated by SDS-polyacrilamide gel electrophoresis using a Tris/Glycine/SDS buffer (Bio-Rad) and transferred to a PVDF membrane (Millipore). Membranes were blocked using Odyssey Blocking Buffer (LI-COR Biosciences) and then, were incubated overnight at 4 °C with primary antibody. Phospho-CREB (Ser133; Cell Signaling), tyrosine hydroxylase (Sigma), phospho- and total ERK2 (Cell Signaling), and β-tubulin (Millipore) were used in the blocking buffer. Detection was performed with secondary IRDye 680 donkey anti-mouse and IRDye 800 donkey anti-rabbit antibodies (LI-COR Biosciences). Membranes were scanned on the Odyssey Infrared Imaging System (LI-COR Biosciences). Integrated intensities from the blots for phospho-CREB, β-tubulin, ERK2, or tyrosine hydroxylase were determined using Odyssey software.

Immunohistochemistry. Brain slices were fixed with 4% paraformaldehyde/PBS and processed for immunohistochemistry. Sections were preincubated in a blocking buffer. *LacZ* expression was detected with a polyclonal antibody to β-gal (Biogenesis). Sections were colabeled for TH (Sigma) to determine whether the β-gal–positive neurons were noradrenergic. After washing, sections were incubated with Cy2 donkey anti-mouse and Cy3 donkey anti-goat–labeled secondary antibodies (Jackson ImmunoResearch) and mounted onto slides in distyrene, plasticizer, and xylene (DPX) mounting media (Fluka). Fluorescent cells were visualized using a confocal microscope LSM510-META (Zeiss).

Electrophysiology. All recordings were performed as described previously (11, 13). Acute brain slices at the level of the LC were cut using a microslicer (DTK-1000; Ted Pella Inc) in sucrose-artificial cerebrospinal fluid (aCSF). LC firing rates from slice cultures were measured by extracellular single-unit potential recording for drug-treated slices and by cell-attached or partial-access recording mode for experiments of viral-mediated CREB gene delivery, in which recordings were obtained from visually identified GFP-positive and -negative LC neurons (13). Whole-cell voltage-clamp recordings were used to obtain sPSCs. All recordings were performed using amplifier AxoClamp 2B (Axon Instruments). Data acquisition was made using DigiData 1322A and pClamp 8 (Axon Instruments).

Statistical Analysis. To determine statistical differences, data were analyzed using one-way ANOVA followed by a Bonferroni posthoc test and two-tailed unpaired Student *t* test with Origin 7.0 (Origin Lab Corporation) and presented as means ± SEM. Significance was determined at $P < 0.05$.

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