Preface

The neurobiological mechanisms involved in drug addiction have been investigated for several decades with a variety of pharmacological and biochemical approaches. These studies have associated several neuroanatomical and neurochemical mechanisms with different components of drug-addictive processes, and this has led to the identification of possible targets for new treatment strategies. Progress has been accelerated dramatically in the last few years by novel research tools that selectively remove or enhance the expression of specific genes encoding proteins responsible for the biological responses of these drugs. These new models, most of them obtained from the recent advances in molecular biology's technology, have provided definitive advances in our understanding of the neurobiological mechanisms of drug addiction. Classical behavioral, biochemical, and anatomical techniques have been adapted to take a maximum advantage of these new molecular tools. These recent studies have clarified the different molecular and intracellular mechanisms involved in addictive processes, as well as the interactions among these endogenous neurobiological mechanisms; and they have provided new insights toward identifying other genetic bases of drug addiction.

The main purpose of *Molecular Biology of Drug Addiction* is to offer an extensive survey of the recent advances in molecular biology and complementary techniques used in the study of the neurobiological basis of drug dependence and addiction. Ours is a multidisciplinary review of the most relevant molecular, genetic, and behavioral approaches used in this field. The definitive advances given by the new molecular and behavioral tools now available provide a unique opportunity for such an approach. Each chapter in this book is not simply a review of the research activities of the author's laboratory, but rather provides a critical review of the main advances in the corresponding topic. Sixteen different chapters organized in four parts have been included in the book. The first part is devoted to the advances in the knowledge of the neurobiological mechanisms of opioid addiction provided in the last few years using the new available techniques, and some of the new therapeutic perspectives now opening up in this field. The second part addresses the most recent findings on the molecular, genetic, and neurochemical mechanisms involved in psychostimulant addiction, which have changed some of the basic knowledge of the neurobiological substrate of these processes. The third part of the book is focused on cannabinoid addiction. New molecular tools have also been used recently to elucidate the biological substrate of cannabinoid dependence. The behavioral models now available, which allow evaluation of the different components of cannabinoid dependence, have

optimized results in this particular field. The last part addresses several molecular, genetic, and behavioral aspects of alcohol and nicotine addiction, which have provided decisive progress in our understanding of these addictive processes.

Molecular Biology of Drug Addiction addresses the main advances in understanding the molecular mechanisms involved in the complex physiological and behavioral processes underlying drug addiction and will, we hope, serve as a useful reference guide for a wide range of neuroscientists. This book also provides basic information of interest for scientists and clinicians interested in the new therapeutic approaches to drug addiction. The different sections of the book are presented by the most relevant scientific personalities for each area. I deeply thank the authors for their effort and expert contribution in the different chapters, and Elyse O'Grady at Humana Press for offering this rewarding opportunity. Finally, I thank Raquel Martín especially for help in manuscript preparation and administrative assistance and Dr. Patricia Robledo and Dr. Olga Valverde for scientific assistance and help in library research.

Rafael Maldonado

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Molecular Genetic Approaches

Theo Mantamadiotis, Günther Schütz, and Rafael Maldonado

1. Introduction

Genetic influences are pivotal in determining the sensitivity to drugs of abuse. The spectrum of genes involved in the behavioral manifestation of drug dependence or withdrawal has not been fully determined, but there are a number of candidate genes that appear to be important. The complexity of the underlying molecular mechanisms governing the adaptation of the neuronal system has prevented the straightforward study of the genetic influences involved. Animal models have allowed the identification of genes involved in drug-related behaviors and have created tools with which to pursue the pharmacogenetic research necessary for the molecular dissection of biochemical pathways involved. A great leap forward in the development of molecular genetic animal models came with the progress in the field of stem cell research. Mouse embryonic stem (ES) cell technology in the late 1980s became amenable to routine research applications *(1,2)*. The gene of choice could be silenced in the mouse and the consequences of this analyzed in the living organism. More sophisticated techniques allowing for the conditional deletion of genes both temporally and tissue specifically have become available, bypassing either pleiotropic or developmental effects of gene loss *(3)*. These advances will be discussed in detail in this chapter.

Our work has focused on the transcription factor cAMP response element-binding protein (CREB) and the related members CRE response element modulation protein (CREM) and activating transcription factor 1(ATF1). This is of particular significance to the study of drug addiction, because the cAMP signal transduction cascade has been implicated in drug-induced cellular responses *(4,5)* (Fig. 1). CREB activity has previously been shown to be altered in response to a number of drugs, including opiates, both in cells and in vivo *(5–7)*. Here we discuss the use of a number of previously described and novel mouse models, using both the classical and conditional gene knockout approaches, in which CREB protein is either reduced or completely absent, to study the role of this important transcription factor in substance abuse.

2. CREB Function in Brain

CREB is expressed in almost all mammalian cells and is a transcription factor with important functions in many tissues, including brain. It harbors an N-terminal activation domain and C-terminal DNA-binding dimerization domain (Fig. 2) and is a member of the basic leucine zipper (bZIP) protein superfamily. CREB is able to either

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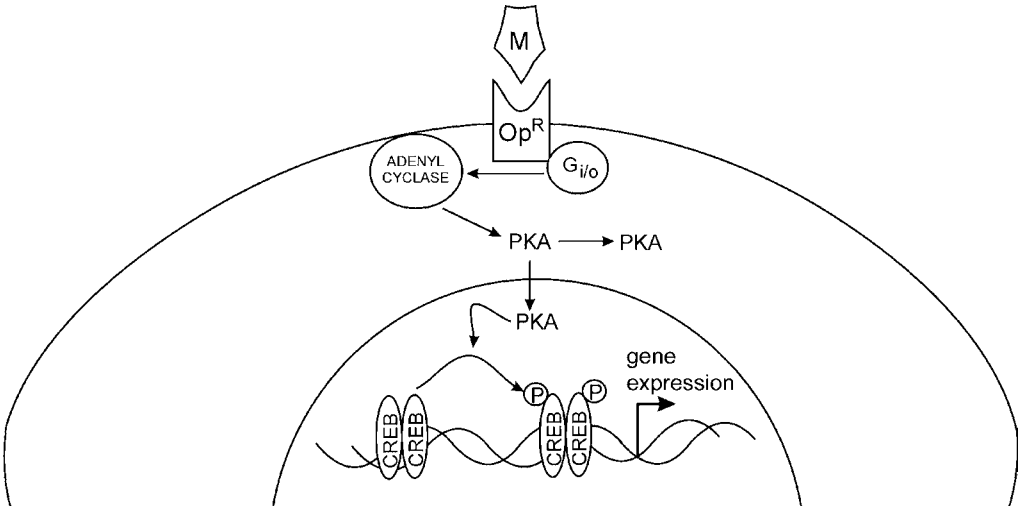

Fig. 1. The opiate signal transduction pathway. Opiates such as morphine bind to G_i- or G_o-coupled opioid receptors. Acute opiate exposure results in adenyl cyclase inhibition, reduction in cAMP levels and cAMP-dependent protein kinase activity and the phosphorylation of both cytoplasmic and nuclear targets, including CREB. On the other hand, chronic opiate exposure increases the levels of these factors.

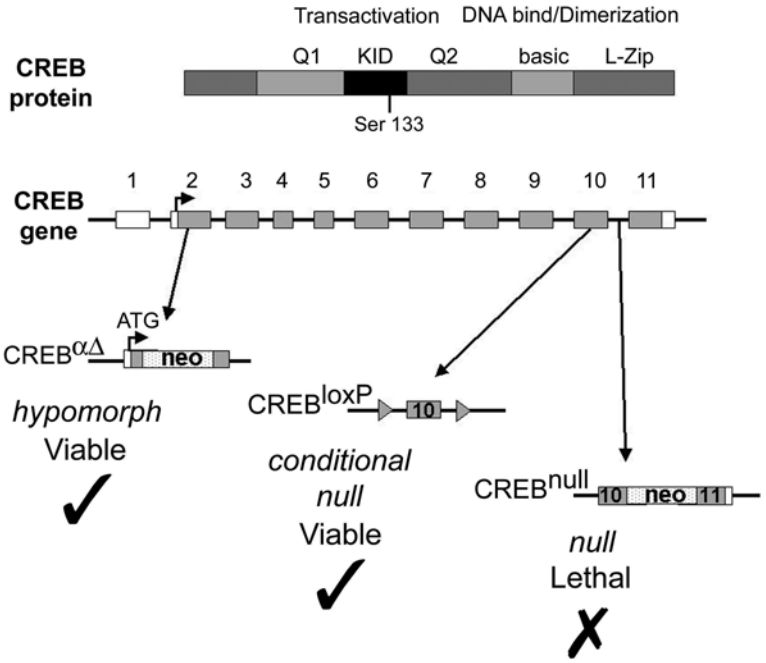

Fig. 2. Functional domains of CREB include the C-terminal transactivation composed of two glutamine-rich domains (Q1 and Q2) flanking the kinase inducible domain (KID), which harbors serine residue 133 which is phosphorylated upon cellular stimulation via the cAMP pathway as well as other signaling pathways. The C-terminal domain harbors the basic leucine zipper (L-Zip) domains, which are involved in DNA binding and dimerization. The CREB gene is comprised by at least 11 exons shown as rectangles (white for untranslated and gray for translated). The three genetically modified CREB mutant mice generated in our laboratory are indicated with the disrupted exons shown. The only genetically modified CREB mutant mice that are viable are the CREBaD and the conditional $CREB^{loxP}$ mice. These have been used in drug studies described elsewhere *(8,9)* and herein.

homodimerize or heterodimerize with its closely related factors, CREM and ATF1 *(10)*. Upon phosphorylation on a critical serine-133 residue, it can bind to cAMP-responsive elements (CREs) and recruit the CREB-binding protein (CBP) and other transcriptional cofactors to transactivate a large number of target genes important for cellular function (Fig. 1). Apart from CREB's role in the cellular responses triggered by drugs of abuse, specific functions attributed to CREB in brain include neuronal survival *(11,12)*, hypothalamic/pituitary growth axis *(13)*, circadian rhythm *(14–16)*, and learning and memory *(17,18)*.

Several molecular changes have been described during exposure to opioids *(19–22)*. Acute opioid administration inhibits adenylyl cyclase activity, whereas chronic opioid treatment leads to a dramatic upregulation of the cAMP pathway at every major step of the cascade between receptor activation and physiological response *(23)* (Fig. 1). This upregulation occurs in discrete brain areas including the locus coeruleus (LC) and the nucleus accumbens (NAc), providing a neuroanatomical link for opioid physical dependence and rewarding effects, respectively *(24–26)* Upregulation of the cAMP pathway also seems to be involved in the addictive mechanisms of other drugs of abuse, such as cocaine *(23,27)*. The phosphorylation state of CREB was shown to be decreased in the LC after acute morphine administration, whereas chronic morphine produces an increase in the phosphorylation and expression of CREB in this structure *(5–7)*. We have previously demonstrated that CREB is an important factor involved in the onset of behavioral manifestations of opiate withdrawal, where the major signs of morphine abstinence were strongly attenuated in CREB$^{\alpha\Delta}$ mutant mice, which lack the major transactivating CREB α and Δ isoforms *(8)* (*see* Section 3.). Work utilizing antisense oligonucleotides has also implicated decreased CREB expression in the LC with attenuated withdrawal and electrophysiological responses *(7)*. CREB has also been implicated in the motivational properties of morphine and cocaine *(27,28)*, although the functional relevance in molecular genetic animal models has not yet been determined.

3. Genetically Altered CREB Mutant Mice

To investigate the role of CREB in drug dependence and motivational responses, we made use of two independent genetically modified CREB mutant mice. On the one hand, we have used CREB mice that lack the two major α and Δ CREB isoforms *(8)* These mice were generated by targeting the second CREB exon, which harbors the first translated ATG codon (Fig. 2) *(29)*. Although the major CREB α and Δ isoforms were ablated, this mutation allowed for the translation of a novel and previously unidentified CREB β isoform, at levels higher than in wild-type mice *(30)*. In essence, these mice carry a hypomorphic CREB allele and are termed CREB$^{\alpha\Delta}$ mice.

To study the consequences of complete CREB loss, a second mouse was generated by targeting the region encoding the entire DNA binding and dimerization domain *(31)* (Fig. 2). Homozygous CREB null mice die at birth, due to the failure of the lungs to inflate. Therefore, until recently the CREB$^{\alpha\Delta}$ mice represented the only viable mouse model with a genetically modified CREB gene.

4. Attenuated Naloxone-Precipitated Withdrawal Response in CREB$^{\alpha\Delta}$ Mice

Various manifestations of somatic signs of naloxone-precipitated withdrawal were evaluated in CREB$^{\alpha\Delta}$ mice. Opioid dependence was induced by repeated morphine

injection. The morphine dose (ip) was progressively increased from 20 to 100 mg/kg over a period of 5 d. Morphine withdrawal syndrome was precipitated by naloxone (1 mg/kg, sc) 2 h after the last morphine administration. Mouse behavior was observed immediately after naloxone administration. Opiate withdrawal syndrome is characterized by a number of behavioral and physiological signs. Some of these responses, such as jumping and teeth chattering, are dependent on the central nervous system, while other responses are mediated by the peripheral nervous system, including diarrhea, weight loss, ptosis, and lacrimation. $CREB^{\alpha\Delta}$ mice exhibited significant attenuation in nine classical withdrawal responses immediately following naloxone injections. All nine responses were significantly attenuated in the mutant animals compared with the control group (Fig. 3). Importantly, the reduction in withdrawal symptoms was due to the reduced CREB levels and not a result of altered opioid receptors, as receptor studies showed that neither the affinity nor number of receptors was changed in mutant mice *(8)*.

Acute administration of morphine was also evaluated in $CREB^{\alpha\Delta}$ mice by assessing the analgesic effects using the hot-plate test. Mutant and wild-type mice exhibited similar nociceptive threshold and analgesic responses to 3, 9, and 20 mg/kg morphine, manifested by increased licking and jumping latencies. As the development of physical dependence is also associated with tolerance, which is the diminishing response to a given drug dose over time, the development of opioid tolerance was examined in $CREB^{\alpha\Delta}$ mice by monitoring antinociceptive responses during chronic morphine treatment (5 mg/kg ip, twice daily for 5 d). There was no difference in antinociceptive responses between mutant and wild-type mice in the hot-plate test upon acute morphine administration (3 and 9 mg/kg ip). However, though both morphine doses generated significant antinociception in mutant mice, the effect is slightly attenuated in naive $CREB^{\alpha\Delta}$ mice for licks and jumps latency (Fig. 4). In summary, $CREB^{\alpha\Delta}$ mice do develop tolerance to morphine analgesia, but to a lesser degree than wild-type mice.

5. Brain-Specific CREB Loss in Mice

As mentioned in Section 3, the only viable genetically modified CREB mutant mouse model available for drug studies to date has been the $CREB^{\alpha\Delta}$ hypomorph mouse. To study the brain-specific loss of CREB in adult mice, free of the complications inherent in classical knockout models, such as pleiotropic effects during embryonic development and postnatal physiology, we employed the Cre/loxP recombination system to conditionally eliminate CREB only in brain, leaving a normal intact CREB gene in all other tissues. To generate the nervous system-specific CREB mutant mice, we used homologous recombination in ES cells to generate a modified CREB allele in which CREB exon 10, encoding the first part of the bZIP domain, was flanked with loxP sites (Fig. 2). Mice harboring the $CREB^{loxP}$ allele were crossed with transgenic mice possessing a transgene for Cre recombinase under the control of the *nestin* promoter and enhancer *(32)* (Fig. 5A). The mice lacking CREB in brain are referred to as $CREB^{NesCre}$ mice.

$CREB^{NesCre}$ mice lacked CREB immunoreactivity in almost all neurons and glia, probing with either of three antibodies recognizing CREB epitopes from the N-terminal half to the C-terminal end, indicating that no CREB protein, including truncated forms, were present (Fig. 5B). Phenotypically, CREBNesCre mice are essentially normal except for a reduction in body size due to a deficiency in growth hormone (T. M., unpublished data).

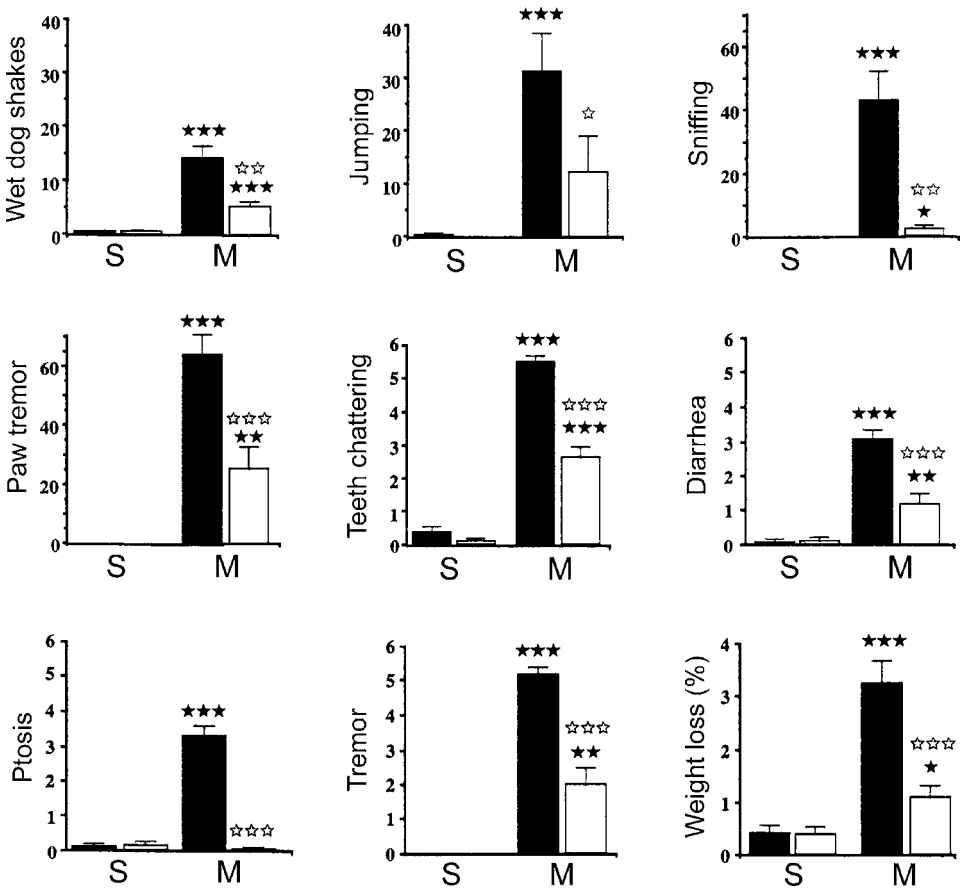

Fig. 3. Behavioral signs measured during naloxone-precipitated morphine withdrawal syndrome in CREB hypomorph mice (white columns), and their wild-type controls (black columns). Opiate dependence was induced by repeated ip injections of morphine-HCl (increasing dose) every 8 h during 3 d. Withdrawal was precipitated once in each mouse by naloxone-HCl injection (1 mg/kg sc) 2 h after the last morphine injection. The mice were placed individually into test chambers 30 min before naloxone injection, and the behavioral signs of withdrawal were evaluated after injection for 30 min. Data were subjected to two-way analysis of variance between animals. The number of animals per group was 12–16. Black stars, comparison between morphine-treated mice (M) and saline-treated mice (S); white stars, comparisons between wild-type and mutant groups receiving the same treatment; one star, $p < 0.05$; two stars, $p < 0.01$; three stars, $p < 0.001$.

6. Ongoing Analyses of CREB Hypomorph and Conditional Mutant Mice

The previous work on $CREB^{\alpha\Delta}$ mice has recently been extended, in parallel with novel studies on $CREB^{NesCre}$ mice. $CREB^{\alpha\Delta}$ mice used in this work were backcrossed for seven generations into a C57/BL6 strain, to determine the contribution of genetic background on the withdrawal behavior. The backcrossed $CREB^{\alpha\Delta}$ mice exhibited almost identical withdrawal responses to those described previously, showing that the attenuated withdrawal syndrome in these mice is a robust phenotype apparently independent of genetic background.

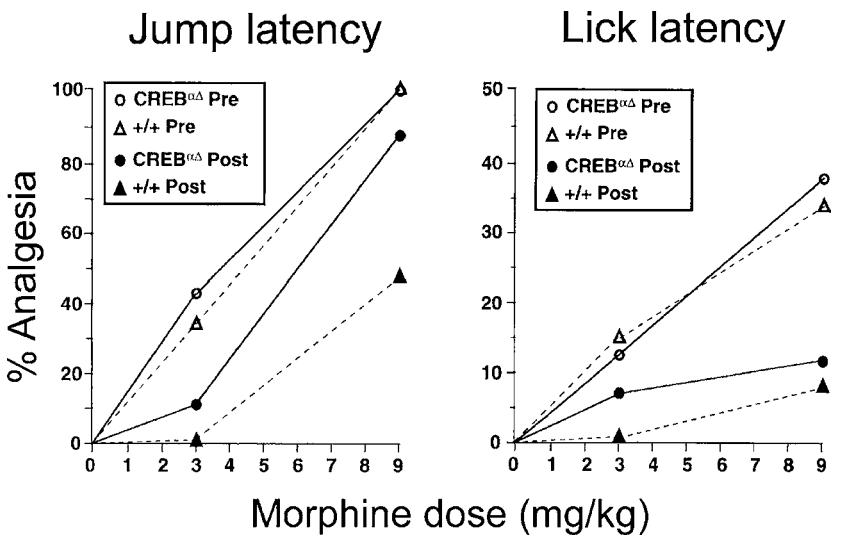

Fig. 4. Development of tolerance to the analgesic effects of morphine. Prior to chronic morphine treatment, mice were examined in the hot-plate test. Fifteen minutes after acute morphine administration (3 or 9 mg/kg ip) the percentage of analgesia was calculated as (test latency minus control latency) divided by (cutoff time minus control latency) × 100. Test latency is the time it takes for the animal to jump off the hot plate after saline injection. Cutoff time is 120 s. Mice were treated with morphine (5 mg/kg ip) for 4 d and reexamined on the hot-plate test 9 h after the last morphine injection. Circles, CREB$^{\alpha\Delta}$ mutant mice; triangles, wild-type mice; open symbols, percentage analgesia before chronic morphine; filled symbols, percentage analgesia after chronic morphine.

Preliminary results indicate that CREB^{NesCre} mice exhibit significantly attenuated withdrawal responses, similar to CREB$^{\alpha\Delta}$ mice, supporting the notion that the phenotype observed in CREB$^{\alpha\Delta}$ mice is primarily a consequence of CREB loss in the nervous system. Furthermore, the CREB α and Δ are probably the major CREB isoforms involved in the expression of morphine withdrawal syndrome. A more detailed analysis of the conditional CREB mutants will allow for the distinction between CNS and peripheral CREB-dependent mechanisms.

An elevated activity of LC neurons has been postulated to contribute to the expression of opiate withdrawal in morphine-dependent rats. Controversial data have been previously reported on the role played by the LC in the expression of morphine abstinence. The firing rate of LC neurons was strongly increased during spontaneous and antagonist-precipitated morphine withdrawal, which seems to contribute to the behavioral expression of the somatic signs of abstinence. Moreover, the LC was the most sensitive brain structure to precipitate the somatic signs of morphine withdrawal by microinjection of opioid antagonists, and its electrolytic lesion strongly inhibited opioid abstinence. Other studies, however, found that morphine treated rats failed to exhibit opiate withdrawal hyperactivity in the LC or that lesions of the noradrenergic brain pathways emanating from the LC failed to attenuate the somatic signs of opioid withdrawal. To examine whether CREB plays a role in this withdrawal-induced hyperactiv-

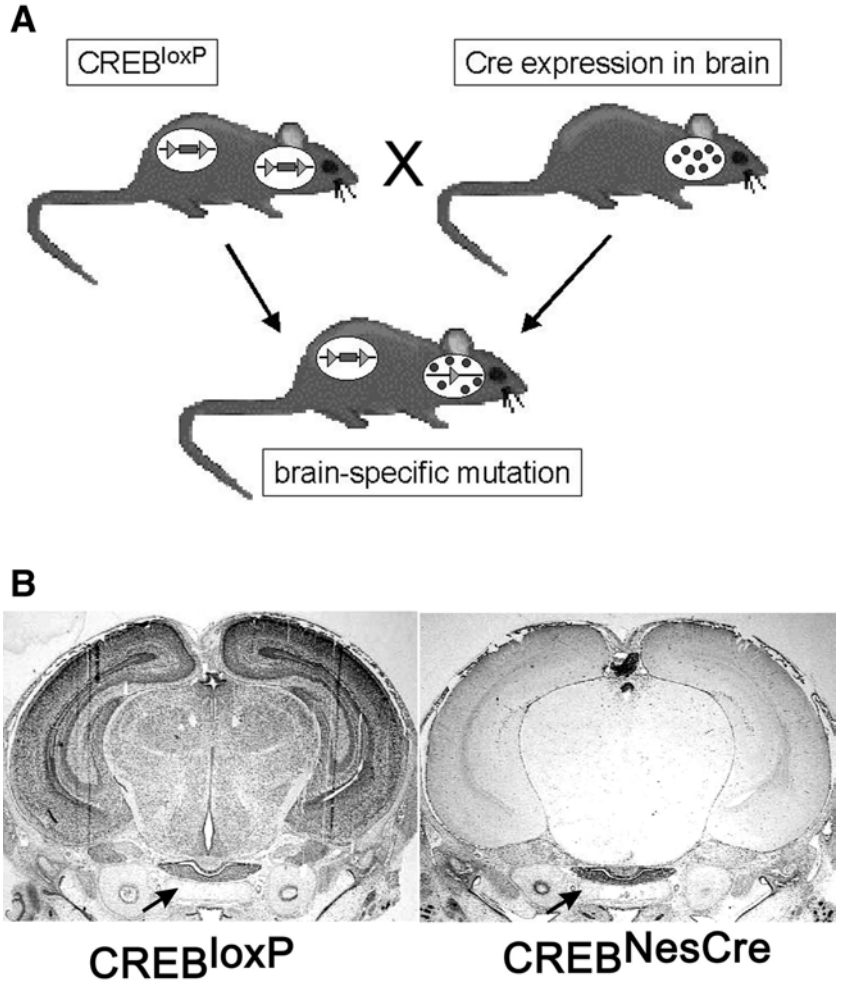

Fig. 5. Disruption of the CREB gene in brain by Cre/loxP-mediated recombination. **(A)** Once mice homozygous for the $CREB^{loxP}$ allele are crossed with mice expressing Cre recombinase specifically in brain, the result is CREB loss restricted to the nervous system. Use of various Cre transgenic lines would result in distinct anatomical and temporal patterns of CREB gene ablation. **(B)** Cre-recombinase expression under the control of the nestin promoter and enhancer results in almost complete CREB loss in brain. $CREB^{loxP}$ brains show normal widespread nuclear protein expression revealed by using anti-CREB antibodies, while $CREB^{NesCre}$ mutant mice exhibit almost complete loss of CREB protein. The anatomical specificity of CREB loss is highlighted by the failure of CREB recombination in the pituitary cells of $CREB^{NesCre}$ mice.

ity, single-unit extracellular recordings of LC neurons in brain slices from wild-type, $CREB^{NesCre}$, and $CREB^{\alpha\Delta}$-deficient mice will be performed following chronic morphine treatment.

Interesting studies focussing on the role of CREB in rewarding behavior have recently been reported. Using rats in the conditioned place preference paradigm, where a herpes simplex virus vector expressing dominant-negative CREB was injected into the NAc of rat brain, a significant enhancement in cocaine rewarding effects was seen,

while overexpression of wild-type CREB had an aversive effect *(27)*. More recently, studies using $CREB^{\alpha\Delta}$ mice suggest that there may be differences in the way CREB modulates downstream target genes, depending on whether morphine or cocaine is used to induce reward. In this study, $CREB^{\alpha\Delta}$-deficient mice do not respond to the reinforcing properties of morphine but do show an enhanced response to cocaine *(9)*. We are currently using both our hypomorph and conditional knockout CREB mutant models to investigate these reward responses. In contrast to this last study, our preliminary data suggest that both $CREB^{\alpha\Delta}$ mice and $CREB^{NesCre}$ mice show a reward response to morphine.

The conditional CREB mutant mice will prove to be useful in further studies as more Cre transgenic mice become available, allowing for more precise anatomical and temporal control over CREB ablation. For example, we now have Cre transgenic mice that will allow for the selective postnatal loss of CREB in either all neurons or dopamine D1 receptor-positive neurons, further refining the neuroanatomical and developmental molecular dissection of CREB function in mouse behavioral studies. The conditional disruption of CREB in either the peripheral or central nervous system will also allow us to distinguish between effects dependent on either or both the central or peripheral nervous system.

7. Conclusions

Neuroadapatations arising during prolonged exposure to opioids and the development of addiction are complex. Using established and emerging techniques in the manipulation of the mouse genome, we have been able to disrupt the CREB gene in the whole organism or specifically in the nervous system. These evolving technologies will bring forward the understanding of the molecular mechanisms involved in the development of drug addiction. As CREB plays a pivotal role in drug addiction, the ongoing studies described here may provide a handle on how to intervene pharmacologically in the biochemical pathways involved in opioid withdrawal syndrome and drug addiction in general.

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