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Reduction of Morphine Abstinence in Mice with a Mutation in the Gene Encoding CREB

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Chronic morphine administration induces an up-regulation of several components of the cyclic adenosine 5′-monophosphate (cAMP) signal transduction cascade. The behavioral and biochemical consequences of opiate withdrawal were investigated in mice with a genetic disruption of the α and Δ isoforms of the cAMP-responsive element-binding protein (CREB). In CREBαΔ mutant mice the main symptoms of morphine withdrawal were strongly attenuated. No change in opioid binding sites or in morphine-induced analgesia was observed in these mutant mice, and the increase of adenylyl cyclase activity and immediate early gene expression after morphine withdrawal was normal. Thus, CREB-dependent gene transcription is a factor in the onset of behavioral manifestations of opiate dependence.

Adaptations in the cAMP signal transduction pathway underlie the mechanisms of opiate tolerance and dependence, and up-regulation of these components plays an important role in the onset of the withdrawal syndrome (*1–3*). In particular, the activation of the transcription factor CREB is implicated in naloxone-precipitated withdrawal syndrome (*4*). Here, the behavioral manifestations of abstinence were investigated during naloxone-precipitated morphine withdrawal in mice with a targeted mutation of the gene encoding CREB (*5*). This mutation causes a hypomorphic allele of the CREB gene such that two of the three known transcriptionally active isoforms are disrupted, CREB α and Δ (*6*). A minor isoform of the CREB gene, CREBβ, is up-regulated in these mutant mice. However, given that the transcriptional activity of CREBβ is lower than that of CREBΔ and that the overall level of CREB protein, based on protein immunoblot and immunohistochemical analysis, is reduced (*6*), we estimate that the homozygous mutants have about 10 to 20% of residual CREB activity. In vivo studies have shown that this reduction in CREB activity leads to impairment of memory consolidation in these mice (*7*).

Opiate dependence was induced by repeated morphine injection, and the behavior of the mice was observed in transparent round plastic boxes with white floors before and after naloxone administration. The mutant mice treated with saline appeared healthy and their spontaneous behavior nearly normal, except for the presence of a lower number of rears (14.54 ± 4.68, mean ± SEM) compared with control wild-type animals [44.61 ± 7.43, $t(1,22) = 3.27$, $P < 0.005$, two-tailed Student's t test]. To further characterize the behavior of mutant mice, we also evaluated their responses in the open-field test (*8*). No significant difference was observed between wild-type and mutant mice for any of the behavioral parameters quantified in this test (only a tendency of mutant mice to cross more squares was observed: 317.9 ± 30.8 squares compared with 259.9 ± 10.7 in wild type, mean ± SEM). This result indicates that mutant mice elicit a normal behavioral response to a stressful situation. Analgesia was measured after the first morphine administration [20 mg per kilogram of body weight, administered intraperitoneally (i.p.)] with the hot plate test in which the animal is placed on a hot plate and the elapsed time for several behavioral responses is recorded. The hot plate was set to shut off after 90 s (*9*). There was no difference between the mutant and the wild-type mice in the latency of either morphine-increased licks (wild type = 64.9% analgesia, mutant = 54.4% analgesia) or jumps (wild type = 95.1% analgesia, mutant = 100% analgesia) (*10*). Morphine treatment induced classical responses in both mutant and wild-type mice, such as the Straub reflex (reflex posture of the tail exhibited by rodents after opiate administration) and increased horizontal locomotor activity. Thus, the acute analgesic responses and the changes in locomotor activity induced by opiates were not affected in CREBαΔ mutant mice.

In chronically treated wild-type mice, naloxone administration [1 mg/kg, administered subcutaneously (s.c.)] precipitated the standard behavioral signs of withdrawal in morphine-treated animals and did not trigger behavioral changes in saline-injected control groups (Fig. 1). In contrast, a dramatic decrease in all nine classical signs of withdrawal was observed in the mutant mice. Sniffing and ptosis (drooping of the upper eyelid) were almost completely absent in the CREBαΔ mutant mice. All seven other parameters an-

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alyzed were present in the mutant mice, but to a much reduced extent compared with the control groups (Fig. 1). The decrease observed in the number of rears presumably did not affect the behavioral expression of withdrawal in mutants because signs not dependent on locomotor activity, such as ptosis, weight loss, and diarrhea, were also strongly attenuated. The time course of the global abstinence score plot, which assesses all somatic responses in the withdrawal syndrome, was dramatically reduced in the CREBαΔ mutant mice during all 5-min intervals (Fig. 2).

This strong attenuation of morphine withdrawal in CREB mutant mice was not the result of a change in the total amount of opioid receptors. The binding properties of the nonselective (μ, δ, and κ) opioid ligand [^{3}H]diprenorphine was investigated in brain homogenates (*11*). The binding of [^{3}H]diprenorphine at two concentrations, 0.3 nM (value of the dissociation constant) and 3 nM, was not significantly modified in the brains of mutant mice (decrease of less than 10%).

The development of tolerance to the analgesic effects of morphine was evaluated in a different group of CREBαΔ mutant mice before and after a treatment of repeated morphine injections (*12*). Similar antinociceptive responses during the hot plate test were observed in wild-type and CREBαΔ mutant mice after acute morphine administration (*10*). After chronic morphine treatment, the degree of analgesia at the highest acute dose of morphine (9 mg/kg) was reduced in wild-type mice (47.7% compared with 100% in naïve animals) as well as in CREBαΔ mice (87.7% compared with 100% in naïve animals), but not to the same extent. At a lower acute dose similar results were obtained (*13*). Together these data indicate that CREBαΔ mutants develop tolerance to morphine analgesia, but to a lesser degree than wild-type mice.

Because changes in the levels of expression of immediate early genes have been observed after opiate withdrawal (*14, 15*), we examined the expression of c-*fos* and c-*jun* mRNA by in situ hybridization in brains from wild-type and CREBαΔ mutant mice after naloxone-precipitated withdrawal. Induction of mRNA for c-*jun* as well as for c-*fos* was observed during withdrawal in both wild-type and mutant mice (*16*). At the protein level, an identical increase in immunoreactivity for c-FOS (Fig. 3) and c-JUN (*16*) was observed 2 hours after naloxone-precipitated morphine withdrawal, in the locus coeruleus and the amygdala of both wild-type and CREBαΔ mutant animals. Thus, the increase in immediate early gene expression after morphine withdrawal was not affected by a reduction in the amount of CREB activity. Furthermore, these increases may not be related to the associated behavioral manifestations of the withdrawal syndrome, because in the CREBαΔ mice enhancement in the expression of immediate early genes occurred despite the lack of these behavioral symptoms.

Adenylyl cyclase activity is increased in the cortex of animals after opiate withdrawal (*17*). Total adenylyl cyclase activity was measured in crude membrane preparations from cortex (*18*). Naloxone-precipitated morphine withdrawal syndrome in wild-type animals resulted in a slight increase (10.4%) of adenylyl cyclase activity. In CREBαΔ mutant mice, a similar increase in adenylyl cyclase activity (28%) was observed after morphine withdrawal syndrome, but it should be noted that in saline-treated CREBαΔ mice there was also a slight increase (14%) of adenylyl cyclase activity. However, the relevance of these results is limited because the regions selectively implicated in opiate withdrawal, such as the locus coeruleus, ventral tegmental area, and amygdala (*3, 19*), could not be investigated separately on an individual animal basis.

The CREBαΔ mutation did not affect the normal up-regulation of the immediate early genes c-*jun* and c-*fos* nor did it affect the increase in adenylyl cyclase activity produced by morphine abstinence. Because no cyclic AMP response element (CRE) binding sites have been reported in the promoter of c-*jun*, it is not surprising that the CREBαΔ mutation did not affect up-regulation induced by morphine withdrawal. However, one reason for the continued induction of c-*fos* after abstinence in mutant mice could be that this effect is not mediated solely by CREB binding to CREs. It has been established that other elements in the promoter region of this gene, such as the serum response element, are important and necessary for its induction after stimulation by nerve growth factor (*20, 21*).

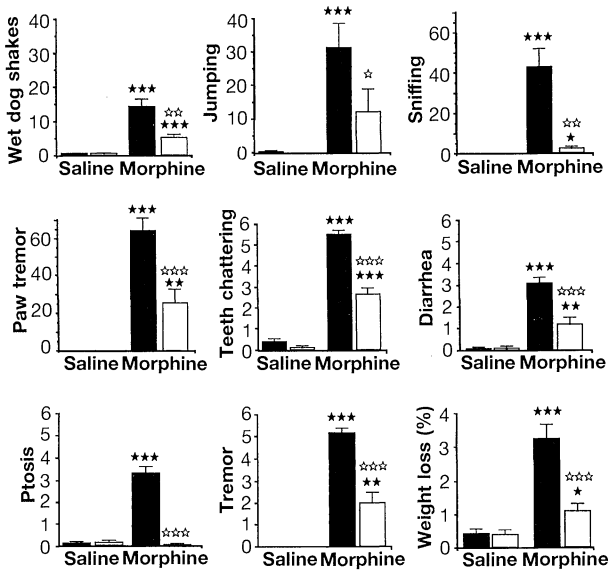

Fig. 1. Incidence of behavioral signs of abstinence and weight loss (mean ± SEM) measured during naloxone-precipitated morphine withdrawal syndrome in mutant mice with a mutation of the gene encoding CREB (white columns), and their wild-type littermates (black columns). Opiate dependence was induced in mice by repeated injection i.p. of morphine HCl every 8 hours during 3 days. The morphine dose was progressively increased as follows: first day, 20, 40, and 60 mg/kg; second day, 80, 100, and 100 mg/kg; third day, 100 mg/kg. Control mice were treated with saline under the same conditions. Before the behavioral experiments, the genotype of the mice was determined by Southern (DNA) blot analysis as previously described (*5*). Withdrawal was precipitated only once in each animal by injecting naloxone HCl (1 mg/kg, s.c.) 2 hours after the last morphine administration. The animals were placed individually into test chambers 30 min before naloxone injection, and the behavioral signs of withdrawal were evaluated after injection for 30 min (*23*). Observations were quantified and values were analyzed by two-way analysis of variance (ANOVA) (mutation and treatment) between animals. Individual comparisons were made by the two-tailed Student's *t* test. The number of animals per group ranged from 12 to 16. The black stars indicate comparisons between morphine-treated mice and their respective saline groups. The white stars show comparisons between wild-type and mutant groups receiving the same treatment. One star: $P < 0.05$; two stars: $P < 0.01$; and three stars: $P < 0.001$.

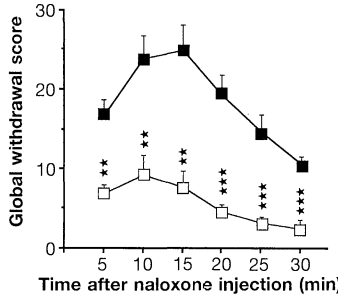

Fig. 2. Evolution of the severity of the withdrawal syndrome in wild-type (black squares) and mutant (white squares) mice measured at 5-min intervals during the 30 min of observation. A global withdrawal score was calculated for each animal by using a range of possible scores from 0 to 100 for the entire observation period as previously reported (*24*). Values are mean ± SEM and were analyzed by two-way ANOVA (mutation, between animals; time, within one animal). Individual comparisons were made with the two-tailed Student's *t* test. The stars indicate comparisons between wild-type and mutant groups at each time point. Two stars: $P < 0.01$; three stars: $P < 0.001$. See legend to Fig. 1 for details concerning methods.

Withdrawal most likely activates a number of signal transduction pathways in the brain that can in turn enhance gene transcription by a variety of elements within the promoter region of the immediate early genes. Reduction in the amount of CREB activity probably leads to an alteration in the expression of genes other than c-*fos* and c-*jun*, which forms the basis of an altered behavioral response upon opiate withdrawal. The results reported here only reflect the physical component of opiate abstinence. The effects of chronic morphine treatment on other components of dependence, such as the dysphoric-aversive properties of withdrawal and the rewarding effects of opiates, still remain to be elucidated in the CREB mutant mice.

Here, alterations in the activity of the transcription factor CREB occurring during morphine treatment probably cause the adaptive changes responsible for the behavioral expression of opiate withdrawal. In particular, mice that lack two of the three functional isoforms of CREB already show dramatic alterations in the classical withdrawal syndrome and a lower development of tolerance to the analgesic response. The ideal experimental animal for these studies is of course a mouse lacking all three functional isoforms of CREB. However, these mice (CREB null) die at birth (*22*), which prohibits these types of analyses. Changes in the various components of the cAMP signal transduction cascade during morphine treatment and withdrawal are known. Here, a causal link between a reduction in the amount of one member of this cascade, namely CREB, and the behavioral manifestations of the withdrawal syndrome is demonstrated. Thus, these CREB mutant mice can be used to investigate the molecular mechanisms underlying physical and psychological aspects of drug addiction.

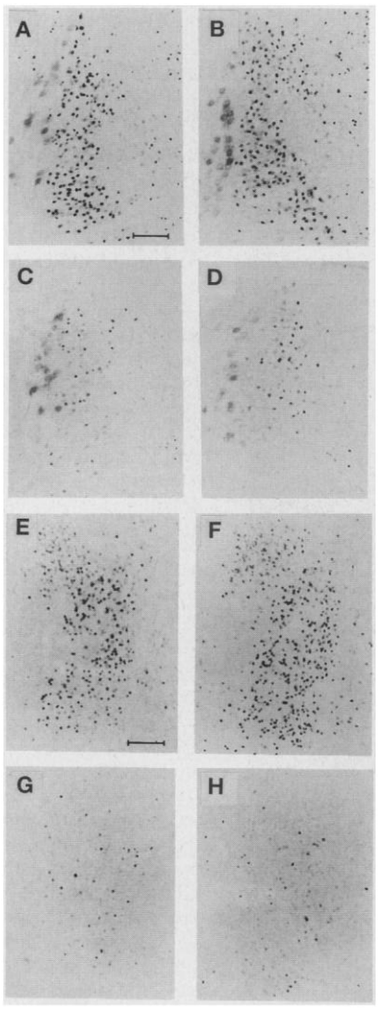

Fig. 3. Expression of c-FOS immunoreactivity in the locus coeruleus [panels (A) to (D)] and amygdala [panels (E) to (H)] revealed by immunocytochemistry (*25*). (**A** and **E**) Morphine-treated wild-type mice; (**B** and **F**) morphine-treated CREBαΔ mutant mice; (**C** and **G**) saline-treated wild-type mice; (**D** and **H**) saline-treated CREBαΔ mutant mice. Scale bar = 100 μm.

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13. Analgesia was also reduced when an acute morphine dose of 3 mg/kg was administered after chronic morphine treatment in wild-type mice (0.2% compared with 34.2% in naïve animals) as well as in CREBαΔ mice (11.1% compared with 43.3% in naïve animals), but again, not to the same extent.
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22. We have recently generated a mouse mutant in which the entire DNA binding domain of the CREB gene has been deleted, resulting in a null allele. These mice die at birth and are currently being investigated in our laboratory (D. Rudolph and G. Schütz, unpublished observations).
23. Test chambers consisted of transparent round plastic boxes (30 cm in diameter by 50 cm in height) with a white floor. Behavior was observed in two sessions: the first session was during the 15 min preceding naloxone injection, and a second session was during the 30 min after this injection. All animals were scored by an observer who did not know either the genetic constitution or the pharmacological treatment. The number of wet dog shakes, jumping, paw tremor, and sniffing were counted. Teeth chattering, diarrhea, tremor, and ptosis were evaluated over 5-min periods, one point being given for the presence of each sign during each period. The number of periods showing the sign was then counted (maximum score: 6). Body weight was determined before and 30 min after naloxone injection.
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25. Animals were killed by transcardiac perfusion with 4% (w/v) paraformaldehyde 2 hours after naloxone-precipitated withdrawal. Their brains were then fixed overnight in the same fixative before vibratome sectioning. Expression of the c-FOS and c-JUN proteins was examined in coronal 50-μM vibratome sections at the level of the locus coeruleus (Fig. 3, A through D) and amygdala (Fig. 3, E through H). All antibodies were generated in rabbits immunized with bacterially expressed fusion proteins. All cDNAs used to construct the fusion proteins were of mouse origin. Specificity and characterization of the antibodies had been described previously [K. Kovary and R. Bravo, *Mol. Cell Biol.* **11**, 4466 (1991)]. Sections were incubated in normal goat serum [2% in phosphate-buffered saline (PBS) and 0.2% Triton X-100] for 1 hour, and then in the primary antisera for 36 hours at 4°C. The primary antisera were diluted as follows: anti–c-FOS, 1:40,000; and anti–c-JUN, 1:40,000 (*16*). Immunoreactivity was visualized by the avidin biotin complex method (Vectastin, Vector Labs, Burlingame, CA). Sections were developed in 0.02% diaminobenzidine with 0.02% hydrogen peroxide, and a subset of sections were counterstained with hemalum.

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