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DREAM Is a Critical Transcriptional Repressor for Pain Modulation

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Summary

Control and treatment of chronic pain remain major clinical challenges. Progress may be facilitated by a greater understanding of the mechanisms underlying pain processing. Here we show that the calcium-sensing protein DREAM is a transcriptional repressor involved in modulating pain. *dream*$^{-/-}$ mice displayed markedly reduced responses in models of acute thermal, mechanical, and visceral pain. *dream*$^{-/-}$ mice also exhibited reduced pain behaviors in models of chronic neuropathic and inflammatory pain. However, *dream*$^{-/-}$ mice showed no major defects in motor function or learning and memory. Mice lacking DREAM had elevated levels of prodynorphin mRNA and dynorphin A peptides in the spinal cord, and the reduction of pain behaviors in *dream*$^{-/-}$ mice was mediated through dynorphin-selective kappa (κ)-opiate receptors. Thus, DREAM appears to be a critical transcriptional repressor in pain processing.

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Introduction

The experience of pain in response to noxious stimuli serves a crucial biological purpose: it alerts a living organism to environmental dangers, inducing behavioral responses that protect the organism from further damage. In contrast, chronic pain arising from disease states and/or pathological functioning of the nervous system offers no advantage and may be debilitating to those afflicted. Despite recent advances in our understanding of pain mechanisms (Woolf and Salter, 2000), the satisfactory management of pathologic pain eludes current treatment strategies.

Since the discovery of an endogenous opiate system (Akil et al., 1976) and the subsequent cloning of endogenous opioid ligands (Dores et al., 1990) and their cognate opiate receptors (reviewed in Akil et al., 1996), which mediate the effects of the prototypic opioid morphine, intense effort has been made to characterize the endogenous opiate system and to understand the physiological and pathophysiological roles of each of its components. The classical opiate system is comprised of three receptor types (μ, δ, and κ) and their cognate ligands encoded by the endogenous opioid genes, *proopiomelanocortin* (*pomc*), *proenkephalin* (*penk*), and *prodynorphin* (*pdyn*), respectively. Because exogenous opioids such as morphine have traditionally been exploited for their analgesic properties, most studies have focused on the role of endogenous opioids in pain modulation, although participation in locomotive, cognitive, cardiovascular, neuroendocrine, and neuroimmune processes are known (Vaccarino and Kastin, 2000). Within the nervous system, opiate receptors and peptides show broad distribution in the brain, spinal cord, and peripheral neurons (Mansour et al., 1988), including regions important for the relay and processing of sensory information, in particular pain.

DREAM (downstream regulatory element antagonistic modulator) was identified in in vitro studies as a putative transcriptional repressor for the *prodynorphin* gene (Carrion et al., 1999), which contains a consensus DNA sequence, or downstream regulatory element (DRE), required for direct association with DREAM. Prior work had suggested that the DRE is important for the active repression of *prodynorphin* transcription under basal

conditions (Carrion et al., 1998). DREAM possesses direct calcium binding ability (by virtue of four EF-hand motifs) and calcium binding to DREAM blocks its ability to associate with DNA (Carrion et al., 1999). With homology to members of the neuronal calcium sensor (NCS) superfamily, DREAM is the first transcription factor known to be directly regulated via calcium binding. In addition to *prodynorphin*, DREAM was believed to regulate the expression of other DRE-containing genes, for example, *c-fos* (Carrion et al., 1999), and may represent a new paradigm in calcium-dependent gene expression.

Despite evidence suggesting that DREAM is a transcription factor, the physiological function of *dream* has remained unclear. The discovery of two nearly identical EF-hand proteins, calsenilin (Buxbaum et al., 1998) and KChIP3 (Kv channel-interacting protein 3) (An et al., 2000), encoded by the same genomic locus as DREAM, suggested different biological functions for a single gene. As a calcium-regulated binding partner of presenilin, calsenilin/DREAM has been suggested to be involved in the proteolytic processing of presenilins and the formation of amyloid plaques in Alzheimer's disease (Buxbaum et al., 1998; Choi et al., 2001; Jo et al., 2001). KChIP3/DREAM was cloned as a binding partner and direct modulator for A type (Kv4) potassium channels (An et al., 2000), which have been implicated in the pathogenesis of heart failure (Wickenden et al., 1999). Given the potential involvement and identification of DREAM/calsenilin/KChIP3 in three distinct biological systems in in vitro studies, the physiological function of DREAM awaited further clarification.

To elucidate the role of DREAM in vivo, we generated *dream*$^{-/-}$ mice. Here we show that DREAM is essential for transcriptional repression of the *prodynorphin* gene in spinal cord neurons. Lack of DREAM in mutant mice results in marked attenuation in pain behaviors regardless of the modality of the noxious stimuli (thermal, mechanical, or chemical) or the tissue type (cutaneous or visceral) affected. In addition to reduced acute pain, loss of DREAM similarly results in attenuation of both inflammatory and neuropathic pain behavior, the latter incurred by peripheral nerve injury. Activation of the dynorphin-selective κ-opiate receptor was found to be causal to the reduced pain responses in *dream*$^{-/-}$ mice. These findings identify DREAM as a critical transcriptional repressor for pain modulation.

Results

Generation of *dream*$^{-/-}$ Mutant Mice

The *dream* gene was disrupted in murine embryonic stem (ES) cells using a targeting vector in which nucleotides encoding amino acids 58–143, corresponding to EF hands I and II and the proximal 5′ region, were deleted (Figure 1A). The targeting construct was electroporated into ES cells. Two G418-resistant cell lines heterozygous for the mutation at the *dream* locus were used to generate chimeric mice, which were crossed to C57BL/6 to obtain heterozygous *dream*$^{+/-}$ mice. The intercross of *dream*$^{+/-}$ mice produced homozygous *dream*$^{-/-}$ mice, as confirmed by Southern blot analysis (Figure 1B). The null mutation of *dream* was verified by the absence of dream mRNA transcripts and protein (Figures 1C and 1D). *dream*$^{-/-}$ mice were born at the expected Mendelian frequency, were fertile, appeared healthy, and displayed longevity that was indistinguishable from wild-type littermates.

Lack of DREAM Has No Detectable Effect on Heart Function or Presenilin Processing

DREAM mRNA expression is detectable in the brain and nonneuronal tissues including the heart, testis, kidney, bone marrow, and thymus (unpublished data; Buxbaum et al., 1998; Carrion et al., 1999; Spreafico et al., 2001). At 3 and 7 months of age, *dream*$^{-/-}$ mice were indistinguishable from wild-type littermates with regards to structure and morphology of all tissues, as well as hematological and serological parameters. Development, homeostasis, and function of hematopoietic cells were comparable between the two genotypes (data not shown).

DREAM (KChIP3) has been implicated as a potential positive modulator of Kv4 potassium channels (An et al., 2000). Loss of Kv4.2-dependent cardiac I_{AF} currents in transgenic mice overexpressing dominant-negative Kv4.2 results in enlargement of the heart by 10–16 weeks of age, leading to congestive heart failure and premature death (Wickenden et al., 1999). We hypothesized that lack of DREAM might impair Kv4 activity in the heart to produce a phenotype similar to that observed in Kv4.2 transgenic mice. However, we found that at 4 months of age, *dream*$^{-/-}$ mice were indistinguishable from wild-type littermates with regards to the heart-to-body mass ratios (data not shown). While 3- and 14-week-old Kv4.2 transgenic mice displayed altered heart functions by echocardiographic analysis (Wickenden et al., 1999), our studies revealed no significant difference in these parameters between wild-type and *dream*$^{-/-}$ littermates at a similar age. Heart morphology, function, and contractility were likewise indistinguishable between the two genotypes of more advanced age (7 months; data not shown).

DREAM (calsenilin) has been suggested to regulate the proteolytic processing of presenilin 1 (PS1) and presenilin 2 (PS2) (Buxbaum et al., 1998; Choi et al., 2001) and to potentiate presenilin-induced apoptosis in vitro (Jo et al., 2001). Western blot analyses of PS1 and PS2 in membrane-enriched brain extracts revealed no detectable difference in the total expression levels between wild-type and *dream*$^{-/-}$ mice, nor in the occurrence or sizes of differentially processed C-terminal PS1 and PS2 fragments (data not shown). Furthermore, we did not detect structural or neuroanatomical abnormalities, which would otherwise suggest defects in cell survival, in the brains of *dream*$^{-/-}$ mice at 7 months of age. Taken together, we find no evidence to suggest an essential involvement of DREAM in presenilin processing or Kv4-dependent heart functions.

General Behavioral Assessment of *dream*$^{-/-}$ Mice

We subsequently evaluated the behavior of these mutant mice (see Supplemental Table S1 at http://www.cell.com/cgi/content/full/108/1/31/DC1). *dream*$^{-/-}$ animals were indistinguishable from wild-type littermates in locomotor responses under nonstressful (locomotor activity box) and stressful (open-field test) conditions. The

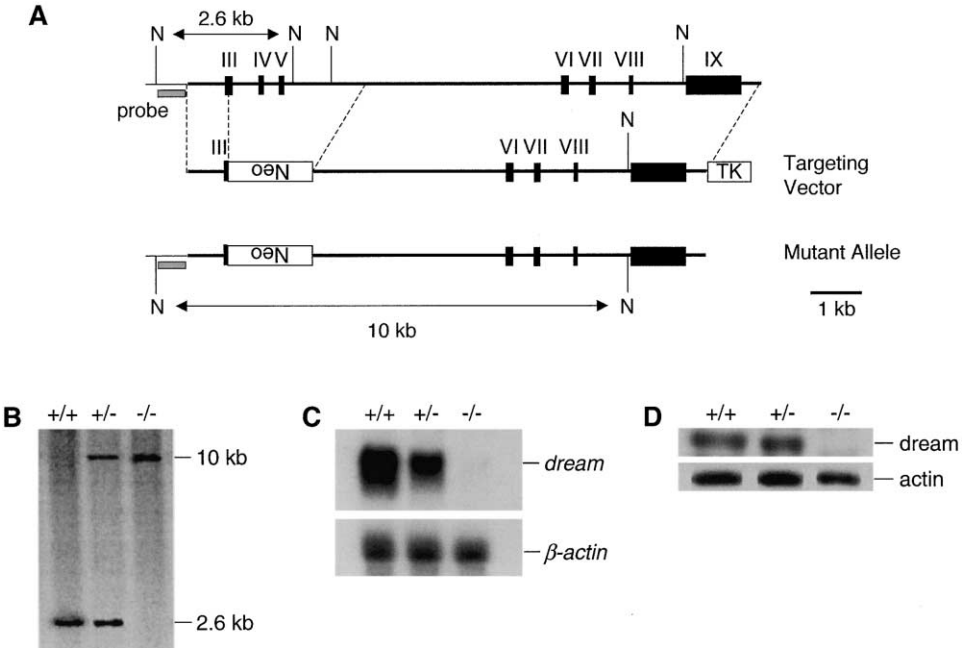

Figure 1. Targeted Disruption of the *dream* Locus by Homologous Recombination

(A) Gene targeting strategy. Top: wild-type *dream* locus showing exons 3–9 (III–IX; filled boxes). Middle: targeting vector in which exons 3–5 were replaced with the *neomycin* (*neo*) resistance marker and the *thymidine kinase* (*TK*) marker was inserted at the 3′ end. Bottom: mutated *dream* locus. "N" indicates NheI. The 5′ flanking probe is shown.

(B) Southern blot analysis of NheI-digested genomic DNA from *dream*$^{+/+}$, *dream*$^{+/-}$, and *dream*$^{-/-}$ mice.

(C) Northern blot analysis of dream mRNA expression in the brains of *dream*$^{+/+}$, *dream*$^{+/-}$, and *dream*$^{-/-}$ mice.

(D) Western blot analysis of DREAM protein expression in the brains of *dream*$^{+/+}$, *dream*$^{+/-}$, and *dream*$^{-/-}$ mice.

degree of anxiety (defecation) was comparable between the two genotypes, as was the performance in the light/dark activity box. Motor coordination and balance were unimpaired in *dream*$^{-/-}$ mice (see also Figure 2A). *dream*$^{-/-}$ mice displayed more hindlimb placement errors relative to wild-type mice in the grid-walking task, indicating a mild impairment of gross motor skills. However, reaching tasks showed that fine motor control of the forelimbs was intact. In the place-learning version of the Morris water maze, acquisition of the spatial task was not different between *dream*$^{-/-}$ and wild-type mice (Figure 2B). These data indicate that lack of DREAM does not result in substantial abnormalities in locomotor activities, motor skills and coordination, anxiety, or spatial learning and memory.

Reduced Thermal and Mechanical Cutaneous and Chemical Visceral Pain in *dream*$^{-/-}$ Mice

During our behavioral screening analysis, we observed that *dream*$^{-/-}$ mice displayed significantly longer response latency compared with wild-type littermates in the tail-flick test of cutaneous thermal nociception (Figure 2C). In a test of cutaneous mechanical pain, the threshold required to elicit hindpaw withdrawal in response to mechanical pressure was greater in *dream*$^{-/-}$ mice than in wild-type littermates (Figure 2D).

To determine whether the reduced pain responses in *dream*$^{-/-}$ mice were generalized to noxious stimulation of other tissues, we assessed acute visceral pain behavior evoked by intraperitoneal injection of $MgSO_4$, which produces an immediate visceral pain response (abdominal writhing) independent of inflammation, and injection of acetic acid, which induces pain secondary to an inflammatory reaction. *dream*$^{-/-}$ mice demonstrated a striking reduction in the number of abdominal writhes relative to wild-type in both tests of visceral pain (Figures 2E and 2F).

As noted above, *dream*$^{-/-}$ mice performed as well as wild-type animals in tests of motor coordination and skill. Furthermore, responses to nonnoxious tactile stimuli, as well as orienting responses to auditory cues, were indistinguishable between the two genotypes (data not shown; see Supplemental Data, Experimental Procedures). Thus, the observed reduction in pain behavior evoked by noxious cutaneous and visceral stimuli appears to be due to an alteration in nociceptive processing, rather than a defect in motor or general sensory functions in *dream*$^{-/-}$ mice.

Lack of DREAM Attenuates Chemical-Induced and Inflammatory Pain

The effect of DREAM ablation on chemical pain behavior was assessed in the formalin test. Intradermal injection of formalin into the hindpaw produced biphasic paw-licking behavior in both wild-type and *dream*$^{-/-}$ mice; however, the magnitude of the response in both phases was significantly reduced in *dream*$^{-/-}$ animals (Figure 3A).

Pain behavior associated with inflammation was subsequently determined in a model of neurogenic inflammation induced by capsaicin injection into the hindpaw, which elicits an acute licking response as well as me-

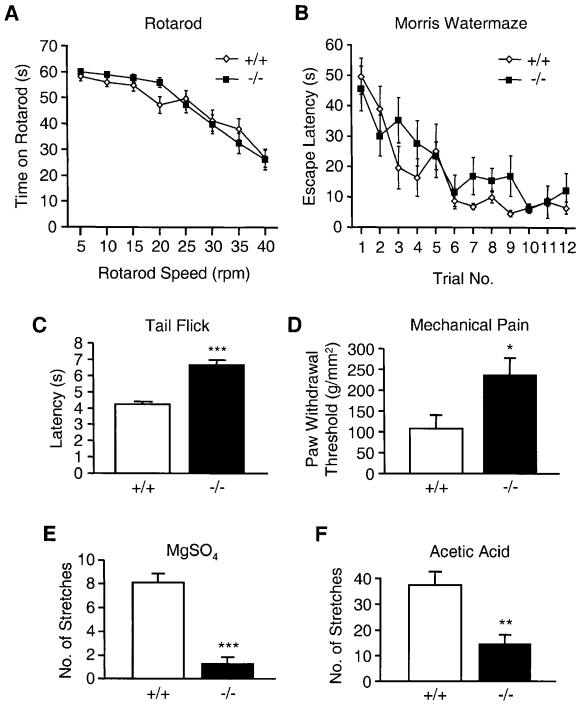

Figure 2. Reduced Pain to Noxious Cutaneous and Visceral Stimuli in *dream*$^{-/-}$ Mice

(A) Rotarod test. Duration (s) that mice remain on the rotating rod at increasing speeds.

(B) Place learning in the Morris water maze. Escape latency indicates the time required to reach the hidden platform at different trials. (A and B) Two-factor ANOVA.

(C) Tail-flick test. Values represent the latency (s) to tail flick from the heat source. Two-tailed Student's t test. Triple asterisk indicates $p < 0.001$ versus wild-type.

(D) Mechanical pain test. Values indicate the threshold (g/mm^2) to elicit a paw withdrawal reflex in response to noxious mechanical stimuli. Single asterisk indicates $p < 0.05$ versus wild-type.

(E and F) Visceral pain in response to (E) $MgSO_4$ (120 mg/kg) or (F) acetic acid (0.6%). Values represent the number of abdominal stretches (writhes). n = 8–12 for each group. All data are presented as mean ± S.E.M. (D–F) Mann-Whitney test. (E) Triple asterisk indicates $p < 0.001$ versus wild-type. (F) Double asterisk indicates $p < 0.01$ versus wild-type.

chanical hypersensitivity (hyperalgesia). The licking response following capsaicin injection was significantly attenuated in *dream*$^{-/-}$ mice compared with wild-type mice (Figure 3B). Whereas sensitivity to innocuous mechanical stimulation was indistinguishable between wild-type and *dream*$^{-/-}$ mice prior to capsaicin injection, capsaicin elicited a profound reduction in paw withdrawal threshold in wild-type animals (Figure 3C). In contrast, capsaicin-induced mechanical hyperalgesia was attenuated in *dream*$^{-/-}$ mice (Figure 3C). The degree of capsaicin-induced inflammation, as indicated by swelling of the injected paw, was not significantly different between the two genotypes (Figure 3D).

Another inflammatory pain model, carrageenan injection into the hindpaw, evokes a mechanical hyperalgesic response. Following carrageenan injection, paw withdrawal threshold in wild-type animals was profoundly reduced at 4 and 24 hr, and gradually recovered to baseline level by 96 hr (Figure 3E). *dream*$^{-/-}$ mice displayed decreased mechanical hypersensitivity relative to wild-type at 4 and 24 hr. The degree of carrageenan-induced inflammation was indistinguishable between the two genotypes at all time points (Figure 3F). Hence, in three models involving inflammation, *dream*$^{-/-}$ mice demonstrated reduced pain behavior. Thus, our data show that DREAM has an essential role in the modulation of chemical and inflammatory pain, without being required for the inflammatory response per se.

Attenuated Neuropathic Pain Behavior of *dream*$^{-/-}$ Mice

We assessed the potential involvement of DREAM in a model of neuropathic pain (chronic constriction of the sciatic nerve) that mimics peripheral nerve injury in humans (Mosconi and Kruger, 1996; Pitcher et al., 1999). The change in paw withdrawal threshold following cuff implantation around the sciatic nerve was determined for 18 days post-cuff implantation (p.c.i.). Wild-type animals displayed a pronounced reduction in paw withdrawal threshold at all time points subsequent to nerve constriction (Figure 4). Moreover, spontaneous pain behavior (characterized by rapid shaking, guarding, and lifting of the hindpaw) was apparent in 10 out of 21 wild-type animals beginning at day 4 p.c.i. In contrast, *dream*$^{-/-}$ mice were significantly less hypersensitive to innocuous mechanical stimuli compared with wild-type animals at all time points following cuff implantation (Figure 4). Spontaneous pain behavior was not observed in any of the nerve-cuffed *dream*$^{-/-}$ animals throughout the 18 day study period. Sham-operated animals did not show any significant changes in mechanical thresholds (data not shown). Thus, following peripheral nerve injury, *dream*$^{-/-}$ mice displayed marked resistance to developing tactile allodynia (hypersensitivity to innocuous mechanical stimuli) and spontaneous pain behavior.

Direct Binding of Murine DREAM to Prodynorphin and c-fos DRE Sequences

Previous studies implicated DREAM as a putative transcriptional repressor for the *prodynorphin* and *c-fos* genes (Carrion et al., 1999). Sequence alignment showed that the *prodynorphin* (Dyn), *c-fos* and *proenkephalin* (Penk) genes harbor intragenic sequences resembling the proposed consensus DRE (Carrion et al., 1998; Figure 5A). Since *dream*$^{-/-}$ mice displayed markedly reduced pain responses, we tested whether murine DREAM could bind to DRE elements of the *prodynorphin* gene.

In electrophoretic mobility shift assays (EMSA), binding of purified recombinant murine DREAM to wild-type prodynorphin DRE occurred in a dose-dependent fashion and was abrogated by the addition of calcium (Figure 5B). Binding of DREAM to wild-type prodynorphin DRE was competed specifically with unlabeled wild-type DRE oligonucleotide (Figure 5C, compare lanes 1 and 2) but not with two different mutant DRE oligonucleotides harboring point mutations in the GTCA consensus binding site (Figure 5C, lanes 3 and 4) or with an irrelevant cAP-1 oligonucleotide (Figure 5C, lane 5). Similarly, c-fos DRE can bind to DREAM (Figure 5D, lane 1); binding was abrogated with unlabeled wild-type c-fos DRE (Figure 5D, lane 2) but not cAP-1 oligonucleotides (Fig-

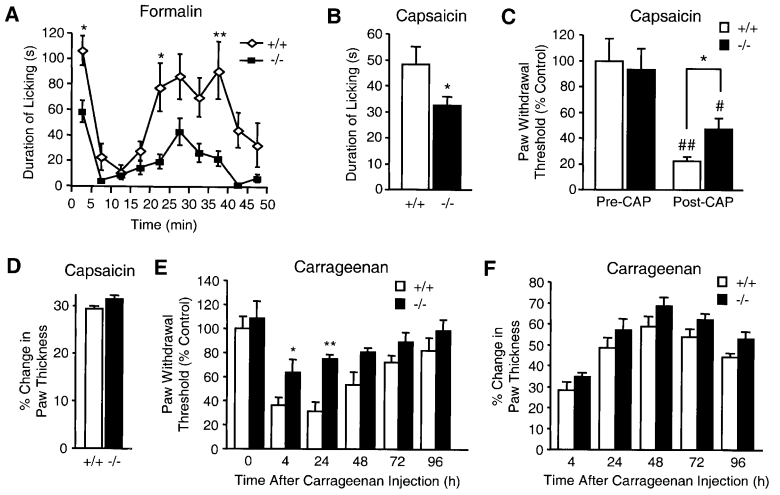

Figure 3. Reduced Chemical and Inflammatory Pain Behavior in *dream*$^{-/-}$ Mice

(A) Formalin test. Values represent time (s) spent in paw licking in each 5 min interval following 2% formalin injection. Neuman-Keuls test. Single asterisk indicates $p < 0.05$; double asterisk indicates $p < 0.01$ versus wild-type.

(B–D) Capsaicin-induced pain behavior.

(B) Time (s) spent in paw licking within the first 5 min following capsaicin (0.3 μg) injection. Two-tailed Student's t test. Single asterisk indicates $p < 0.05$ versus wild-type.

(C) Mechanical sensitivity before (pre-CAP) and 20 min (post-CAP) following capsaicin injection. Values represent the paw withdrawal threshold as a percentage of the baseline (pre-CAP) wild-type response. Tukey-Kramer test. Single asterisk indicates $p < 0.05$ versus wild-type. Single hatch mark indicates $p < 0.05$; double hatch mark indicates $p < 0.01$ versus preinjection values.

(D) Capsaicin-induced paw swelling. Values represent the paw thickness 30 min after capsaicin injection as a percentage of preinjection paw thickness. Two-tailed Student's t test.

(E and F) Carrageenan-induced inflammatory pain.

(E) Mechanical sensitivity 0–96 hr following λ-carrageenan (2%) injection. Values represent the paw withdrawal threshold as a percentage of the baseline (0 hr) wild-type response. Neuman-Keuls test. Single asterisk indicates $p < 0.05$; double asterisk indicates $p < 0.01$ versus wild-type.

(F) Carrageenan-induced paw swelling. Values are represented as described in (E). $n = 8$–11 for each group. All data are presented as mean $\pm$ S.E.M. Two-factor ANOVA.

ure 5D, lane 3). These data show that murine DREAM can associate directly and specifically with DRE elements and that binding of calcium to DREAM abolishes the DREAM-DRE interaction.

Enhanced Basal Expression of Prodynorphin in *dream*$^{-/-}$ Spinal Cord

Nociceptive information is transmitted through a "pain pathway," which begins at the periphery with the stimulus-dependent activation of sensory nerves, through the spinal cord as a result of neurotransmitter release, and eventually to the brainstem and forebrain, where the information is integrated to produce a "pain experience" (Woolf and Salter, 2000). Hence, DREAM may be functioning at multiple levels to modulate pain. While DREAM protein was expressed in the ventral and dorsal horns of the spinal cord as well as the hippocampus, we could not detect DREAM protein in dorsal root ganglia (DRG) (Figure 5E). In keeping with protein expression data, *dream* mRNA was robustly expressed in the ventral and dorsal horns of the lumbar spinal cord of wild-type mice (Figure 6A). These results suggest that the effect of DREAM ablation on pain behavior is not due to a cell-autonomous defect in peripheral sensory neurons. Rather, it is likely that DREAM modulates pain processing at the level of the spinal cord and/or brain.

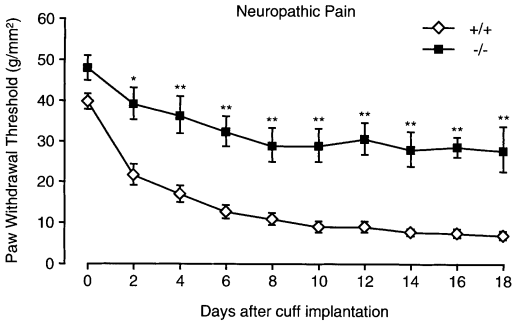

Figure 4. Reduced Neuropathic Pain Behavior in *dream*$^{-/-}$ Mice

Paw withdrawal thresholds (g/mm^2) following cuff implantation around the sciatic nerve. $n = 18$–21 for each group. Data are presented as mean $\pm$ S.E.M. Single asterisk indicates $p < 0.05$; double asterisk indicates $p < 0.01$ versus wild-type (Neuman-Keuls test).

Given the proposed function of DREAM as a transcriptional repressor, we analyzed the abundance of various gene transcripts in the lumbar spinal cords of wild-type and *dream*$^{-/-}$ mice by in situ hybridization. In wild-type mice, the basal expression of prodynorphin mRNA was detected mainly in scattered neurons of the superficial dorsal horn (Figure 6C). In contrast, *dream*$^{-/-}$ mice showed markedly increased basal prodynorphin expression throughout the lumbar spinal cord (Figure 6D). However, we detected no difference in the basal level of proenkephalin (Figures 6F and 6G) or c-fos (Figures 6L and 6M) mRNA between *dream*$^{-/-}$ and wild-type littermate mice. Expression of proopiomelanocortin was comparable in the spinal cord of *dream*$^{-/-}$ and wild-type mice (Figures 6I and 6J). In contrast, the brains and hippocampi of wild-type and *dream*$^{-/-}$ mice did not differ in their abundance of prodynorphin (Figure 7A) or c-fos (data not shown) mRNA by Northern blot analyses. Together, our data support an essential role of DREAM in repressing basal expression of the *prodynorphin* gene in the spinal cord.

DREAM Represses Basal Expression of Prodynorphin in Primary Spinal Cord Cultures

To confirm the elevated basal expression of prodynorphin in the spinal cord, we carried out RT-PCR analysis

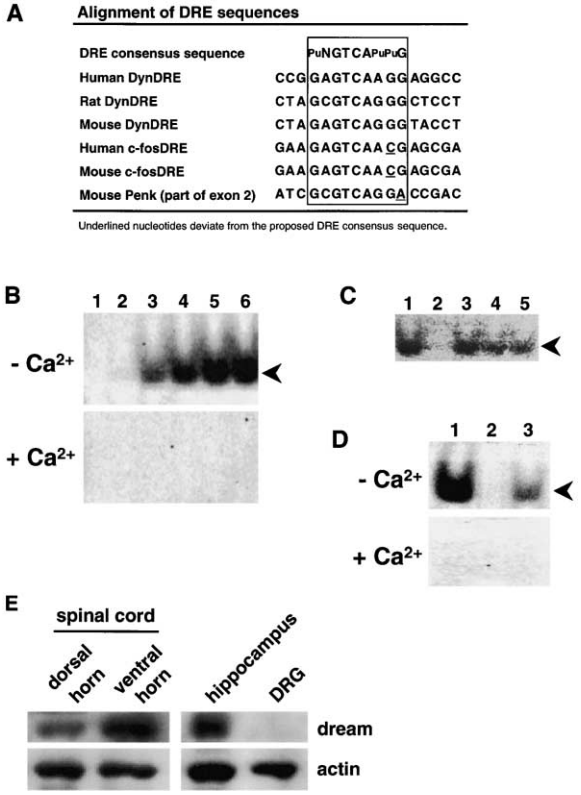

Figure 5. Direct Binding of Murine DREAM to DRE Sequences

(A) Proposed DRE consensus sequence and alignment of human and mouse prodynorphin (Dyn), c-fos, and proenkephalin (Penk) DRE.

(B) Murine DREAM (mDREAM) binding to prodynorphin DRE using EMSA. Binding of mDREAM (lane 1, 0 μM; lane 2, 10 μM; lane 3, 20 μM; lane 4, 30 μM; lane 5, 40 μM; and lane 6, 50 μM) with Cy5-labeled DynDRE (5 nM) with (+) or without (−) 10 mM Ca^{2+}.

(C) Competition of mDREAM (40 μM) binding to DynDRE (5 nM) using wild-type DynDRE (lane 2; 75 nM), DREmut5 (lanes 3; 75 nM), DREmut4 (lane 4; 75 nM), or control cAP-1 (lane 5; 75 nM). (Lane 1) Binding of mDREAM (40 μM) to wild-type DynDRE (5 nM).

(D) mDREAM binding to c-fosDRE using EMSA. (Lane 1) mDREAM (40 μM) was incubated with Cy5-labeled c-fosDRE (5 nM) with (+) or without (−) 10 mM Ca^{2+}. Competition with 50 nM unlabeled c-fosDRE (lane 2) or cAP-1 (lane 3).

(E) Western blot analyses of DREAM expression in the dorsal and ventral horns of the spinal cord, hippocampus, and dorsal root ganglia (DRG).

of prodynorphin mRNA in primary spinal cord cultures established from wild-type and *dream*$^{-/-}$ embryos. The basal level of prodynorphin mRNA was markedly greater in *dream*$^{-/-}$ spinal cord culture compared with that in wild-type (Figure 7B), whereas the basal expression of c-fos mRNA was similar between *dream*$^{-/-}$ and wild-type cultures (Figure 7C). In addition, wild-type and *dream*$^{-/-}$ cultures were stimulated with agonists previously shown to induce *prodynorphin* transactivation (Lucas et al., 1993; Messersmith et al., 1994). In wild-type cultures, serotonin, 8-OH-DPAT (serotonin 1A receptor agonist), and forskolin (adenylate cyclase agonist) each were able to induce prodynorphin mRNA expression (Figure 7D). Under these culture conditions, we did not observe further enhancement of prodynorphin mRNA level beyond basal expression. This is consistent with the notions that (1) spinal cord neurons contain constitutively expressed transcription factor(s) which can transactivate the *prodynorphin* gene in the absence of DREAM protein and (2) DREAM critically determines basal expression of prodynorphin in the spinal cord.

Enhancement of Spinal Dynorphin But Lack of κ-Opiate Receptor Downregulation in *dream*$^{-/-}$ Mice

To assess whether the enhanced basal level of spinal prodynorphin mRNA is accompanied by a concurrent increase in dynorphin peptide content, the amount of dynorphin $A_{(1-17)}$ and dynorphin $A_{(1-8)}$ in the spinal cord was determined by enzyme immunoassay. The level of dynorphin $A_{(1-17)}$ but not dynorphin $A_{(1-8)}$ was greater in the spinal cords of *dream*$^{-/-}$ mice than those of wild-type littermates (Figure 7E). We conclude that elevated prodynorphin mRNA levels are reflected in increased synthesis of dynorphin in *dream*$^{-/-}$ spinal cord. In addition, protein expression of κ-opiate receptor (KOR1), NMDA receptor (NMDAR1), and PSD-95 was not perturbed in *dream*$^{-/-}$ spinal cord (Figure 7F).

Decreased Pain Behavior of *dream*$^{-/-}$ Mice Is Mediated via κ-Opiate Receptors

Endogenous opioids and their cognate receptors have been strongly implicated in pain modulation (Dickenson, 1991; Suzuki et al., 1999). Dynorphins are believed to exert their antinociceptive action predominantly through κ -opiate receptors (Goldstein and Naidu, 1989). Therefore, by using a pharmacological approach, we investigated the functional relevance of enhanced dynorphin expression in *dream*$^{-/-}$ mice on their pain behavior phenotype.

Neither the *pan*-opiate receptor antagonist naloxone nor the κ-selective antagonist nor-BNI altered the tail-flick latency or the threshold to paw withdrawal in response to noxious mechanical stimuli in wild-type animals (Figures 8A and 8B). In contrast, administering either naloxone or nor-BNI produced a significant reduction in each of the acute pain measures in *dream*$^{-/-}$ mice, resulting in nociceptive responses that were indistinguishable from those of wild-type mice (Figures 8A and 8B). Antagonism of peripheral opiate receptors using naloxone methiodide had no significant effect on the tail-flick latencies of either genotype: tail-flick latency (s) following vehicle versus naloxone methiodide injection in wild-type mice, 4.3 ± 0.3 versus 4.3 ± 0.3, $p > 0.05$; in *dream*$^{-/-}$ mice, 6.8 ± 0.4 versus 6.5 ± 0.5, $p > 0.05$.

In the carrageenan model, nor-BNI did not significantly alter the paw withdrawal threshold of wild-type animals (Figure 8C). However, nor-BNI restored the paw withdrawal threshold of *dream*$^{-/-}$ mice to the level observed in wild-type littermates (Figure 8C). Naloxone methiodide did not have a significant effect on the mechanical threshold in either genotype: paw withdrawal threshold (represented as percent wild-type [wt] baseline control) in the absence versus presence of naloxone methiodide in wild-type mice, 31.3 ± 8.4 versus 34.7 ± 4.6, $p > 0.05$; in *dream*$^{-/-}$ mice, 75.2 ± 3.1 versus 61.3 ± 8.2, $p > 0.05$. In the formalin test, nor-BNI eliminated the

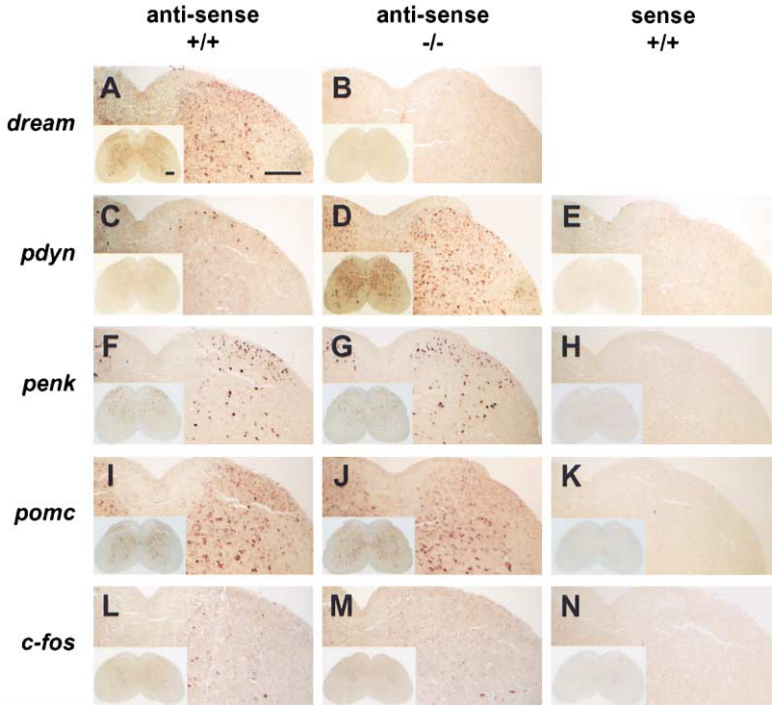

Figure 6. DREAM Represses Basal Prodynorphin Expression in the Spinal Cord

In situ hybridization analyses of the expression patterns of dream, prodynorphin, proenkephalin, proopiomelanocortin, and c-fos mRNA in the lumbar spinal cord. mRNA expression of (A and B) dream, (C and D) prodynorphin (pdyn), (F and G) proenkephalin (penk), (I and J) proopiomelanocortin (pomc), and (L and M) c-fos in the lumbar spinal cord of (A, C, F, I, and L) wild-type and (B, D, G, J, and M) *dream*$^{-/-}$ mice determined using gene-specific antisense cRNA probes. Sense controls for (E) pdyn, (H) penk, (K) pomc, and (N) c-fos in wild-type mice. Insets show the same lumbar spinal cord section under lower magnification. The scale bar equals 200 μm.

difference between genotypes in the licking response in both the acute and tonic phase (data not shown).

In our model of neuropathic pain, nor-BNI had no significant effect on mechanical hypersensitivity of wild-type animals at day 18 p.c.i. (Figure 8D). In contrast, the paw withdrawal threshold of *dream*$^{-/-}$ mice was significantly reduced by nor-BNI, although it did not produce a complete reversal to wild-type hypersensitivity in these animals (Figure 8D). Likewise, naloxone administered at day 18 p.c.i. reduced the paw withdrawal threshold of *dream*$^{-/-}$ animals without producing an effect in wild-type mice: paw withdrawal threshold (g/mm^2) at day 18 in the absence versus presence of naloxone in wild-type mice, 8.0 ± 1.4 versus 7.9 ± 1.4, $p > 0.05$; in *dream*$^{-/-}$ mice, 38.0 ± 7.1 versus 23.5 ± 3.3, $p < 0.05$. In contrast, naloxone methiodide did not significantly alter the paw withdrawal thresholds in either genotype: paw withdrawal threshold (g/mm^2) in the absence versus presence of naloxone methiodide in wild-type mice, 11.6 ± 2.5 versus 12.6 ± 2.4, $p > 0.05$; in *dream*$^{-/-}$ mice, 38.0 ± 5.8 versus 31.8 ± 5.4, $p > 0.05$.

Collectively, the data suggest that there is enhanced activation (either tonic or stimulus-evoked) of κ-opiate receptors in *dream*$^{-/-}$ mice and that this κ-opiate receptor activity is required to mediate the reduced pain behaviors in these animals.

NMDA Receptor Blockade Does Not Alter Pain Behaviors in *dream*$^{-/-}$ Mice

Although the κ-opiate receptor-mediated antinociceptive effects of dynorphins are well established, there are reports indicating that dynorphin may exert pronociceptive effects through an interaction with NMDA receptors (Laughlin et al., 1997; Vanderah et al., 1996). To assess the role of NMDA receptors in the pain behavior of *dream*$^{-/-}$ mice, we investigated the effect of the NMDA receptor antagonist MK-801 on pain responses in the tail-flick test and neuropathic model. We used MK-801 at doses reported to block behavioral effects mediated by NMDA receptors (see also Figure 8E and Jevtovic-Todorovic et al., 1998). Higher doses of MK-801 were not tested because we observed extrasensory behavioral effects, including hyperactivity, head weaving, body rolling, and circling, in pilot studies using this mouse strain.

NMDA receptors are not implicated in mediating acute thermal nociception (Lutfy et al., 1997), making this a suitable model in which to examine NMDA receptor activation (tonic or stimulus-evoked) in *dream*$^{-/-}$ mice. MK-801 had no effect on the tail-flick latency of wild-type animals (Figure 8E). The tail-flick latency of *dream*$^{-/-}$ mice was likewise unaltered by MK-801 (Figure 8E).

The contribution of NMDA receptor activation in neuropathic pain is well established (Woolf and Mannion, 1999). In wild-type animals, MK-801 administered at day 18 p.c.i. produced a dose-dependent increase in paw withdrawal thresholds (Figure 8F). In contrast, at neither of the doses tested did MK-801 alter the already elevated paw withdrawal thresholds of *dream*$^{-/-}$ mice. Therefore, we conclude that NMDA receptor activity is not essential for the reduced mechanical hypersensitivity observed in neuropathic *dream*$^{-/-}$ mice.

Discussion

To elucidate the essential role(s) of DREAM in vivo, we generated *dream*-deficient mice and found these animals to exhibit markedly attenuated pain responses in a battery of pain behavior tests. The reduction in pain responses was observed in the absence of any substantial deficit to nonnoxious sensory and motor function, learning or memory, and immune or cardiac functions. The reduced pain behavior of *dream*$^{-/-}$ mice was corre-

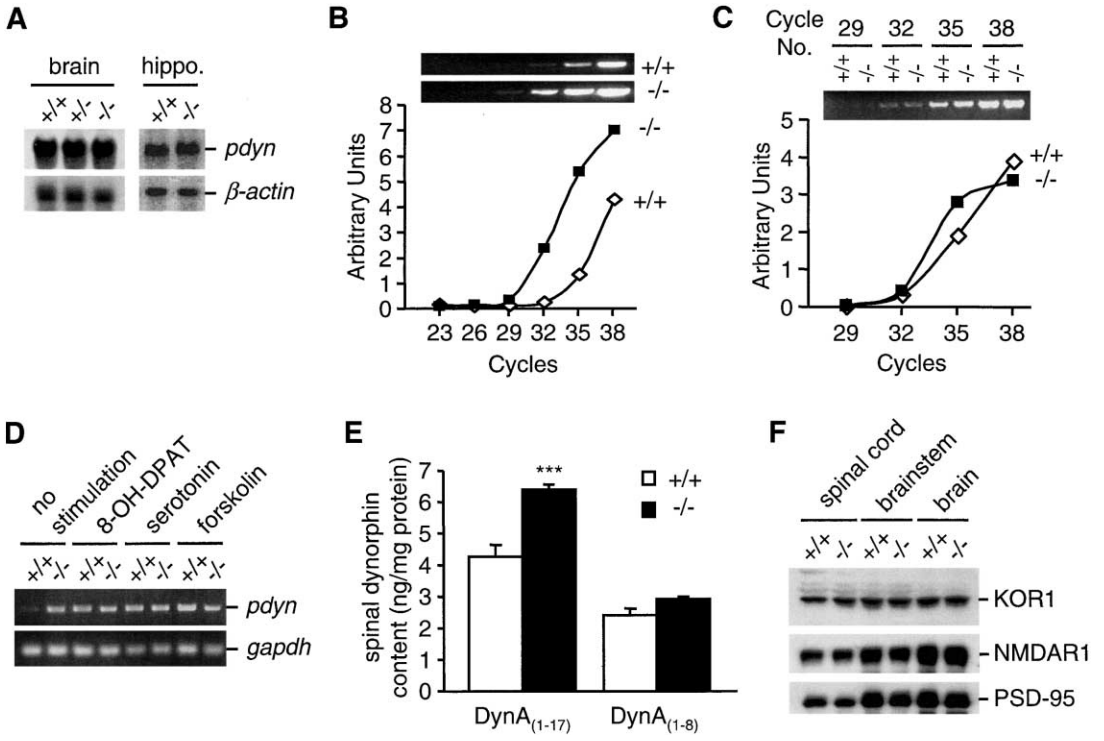

Figure 7. Enhancement of Spinal Prodynorphin mRNA and Peptide Content But Lack of κ-opiate Receptor Downregulation in *dream*$^{-/-}$ Mice

(A) Northern blot analysis of dream mRNA expression in *dream*$^{+/+}$, *dream*$^{+/-}$, and *dream*$^{-/-}$ mouse brain and hippocampus.

(B and C) Basal expression of (B) prodynorphin and (C) c-fos mRNA in cultured wild-type and *dream*$^{-/-}$ spinal cord neurons as assessed by semiquantitative RT-PCR. gapdh mRNA served as the control (data not shown). Graphs represent the relative abundance of prodynorphin- or c-fos-specific PCR products between the two genotypes.

(D) Induction of prodynorphin mRNA expression in primary spinal cord cultures. RT-PCR analysis of prodynorphin mRNA content in cultures without stimulation or stimulated with 8-OH-DPAT (100 nM), serotonin (100 nM), or forskolin (25 μM).

(E) ELISA quantitation of spinal dynorphin $A_{(1-17)}$ and dynorphin $A_{(1-8)}$ levels in wild-type (n = 5) and *dream*$^{-/-}$ (n = 5) mice 3 hr following intrathecal administration of the peptidase inhibitors, *p*-hydroxymercuribenzoate (8 nmol) and phosphoramidon (4 nmol). Triple asterisk indicates $p < 0.001$ versus wild-type (two-tailed Student's t test).

(F) Western blot analyses of the κ-opiate receptor (KOR1), NMDAR1 receptor subunit (NMDAR1), and postsynaptic density-95 (PSD-95) in membrane fractions of the spinal cord, brainstem, and brain of wild-type and *dream*$^{-/-}$ mice.

lated with enhanced basal expression of spinal prodynorphin mRNA and dynorphin peptides. In vitro studies confirmed the specific DNA binding ability of murine DREAM for the prodynorphin DRE sequence. The functional relevance of dynorphin upregulation was apparent from pharmacological studies, where κ-opiate receptor blockade by nor-BNI reversed the pain response of *dream*$^{-/-}$ mice. Together, our data are consistent with an essential role of DREAM both in the transcriptional control of the *prodynorphin* gene and in pain modulation.

In addition to the postulated role of DREAM as a transcriptional repressor, DREAM has also been shown to associate physically with presenilins (Buxbaum et al., 1998) and Kv4 potassium channels (An et al., 2000), and, hence, may modulate their functions. Although our data do not exclude a physiological involvement of DREAM in presenilin and/or Kv4 function, presenilin and/or heart functions dependent upon Kv4 activity do not appear to be compromised in *dream*$^{-/-}$ mice. Notably, the DREAM homologs KChIP1, KChIP2, and KChIP4, as well as another EF-hand protein, frequenin, are able to interact with Kv4 α subunits (An et al., 2000; Nakamura et al., 2001), suggesting compensation by other potassium channel modulators in the absence of DREAM. As DREAM is found in both nuclear and membrane fractions of the mouse brain (data not shown), the physiological function(s) besides its role in transcriptional repression remains to be defined. Interestingly, the EF-hand protein secretagogin has been revealed to have repressor activity on the substance P promoter (Wagner et al., 2000), suggesting the possible existence of a family of calcium binding transcription factors.

Our pharmacological studies indicate that the observed attenuation of pain responses in *dream*$^{-/-}$ mice is mediated by κ-opiate receptor activity. Administering nor-BNI resulted in complete reversal of pain behavior of *dream*$^{-/-}$ mice to that of wild-type animals in models of acute, chemical, and inflammatory pain. Our data are consistent with the notion that κ-opiate receptors are tonically activated in *dream*$^{-/-}$ mice and/or that there is enhanced κ-opiate receptor activation as a consequence of somatosensory stimulation in *dream*$^{-/-}$ animals. The lack of κ-opiate receptor downregulation, at the protein level, in *dream*$^{-/-}$ mice provides additional support that the observed analgesia in *dream*$^{-/-}$ animals is functionally mediated by these receptors.

In the neuropathic model, neither naloxone nor nor-

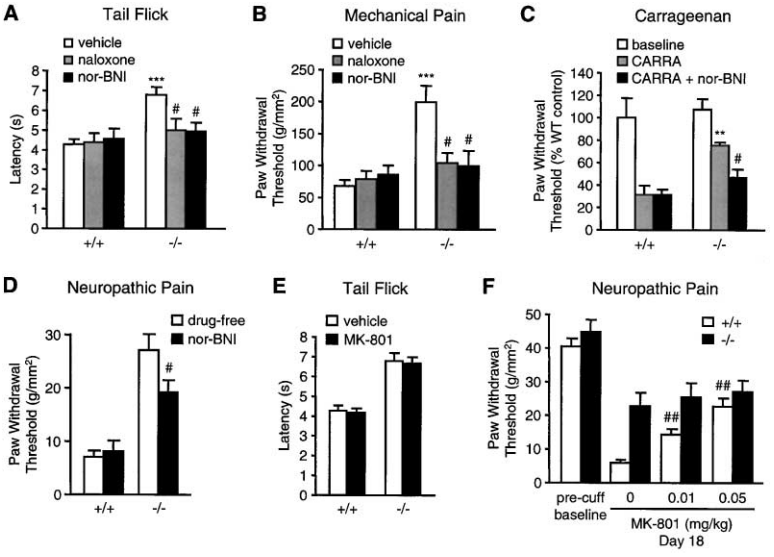

Figure 8. Involvement of κ-Opiate Receptors But Not NMDA Receptors in the Observed Analgesia in *dream*$^{-/-}$ Mice

(A and B) Effects of naloxone (1 mg/kg, i.p.) and nor-BNI (2 mg/kg, s.c.) in (A) the tail-flick test and (B) the mechanical pain test. Two-factor ANOVA and post-hoc tests. Triple asterisk indicates $p < 0.001$ versus wild-type group receiving the same treatment. Single hatch mark indicates $p < 0.05$ versus vehicle (untreated) group of the same genotype.

(C) The effect of nor-BNI on the paw withdrawal threshold in the carrageenan model. Animals received nor-BNI (2 mg/kg, s.c.) 1 hr following 2% carrageenan injection. Values represent the paw withdrawal threshold at 24 hr postcarrageenan injection as a percentage of the baseline (0 hr) wild-type response. Two-factor ANOVA and post-hoc tests. Double asterisk indicates $p < 0.01$ versus wild-type group receiving the same treatment. Single hatch mark indicates $p < 0.05$ versus vehicle (untreated) group of the same genotype.

(D) The effect of nor-BNI (2 mg/kg, s.c.) on the paw withdrawal threshold (g/mm^2) at day 18 p.c.i. in the neuropathic pain model. Two-factor ANOVA and post-hoc tests. Single hatch mark indicates $p < 0.05$ versus vehicle (untreated) group of the same genotype.

(E) The effect of MK-801 (0.05 mg/kg, s.c.) in the tail-flick test. Two-factor ANOVA and post-hoc tests.

(F) The effect of MK-801 (0.01 and 0.05 mg/kg, s.c.) on the paw withdrawal threshold (g/mm^2) at day 18 p.c.i. in the neuropathic pain model. $n = 6$–10 for each group. All data are presented as mean ± S.E.M. Two-factor ANOVA and post-hoc tests. Double hatch marks indicate $p <$ 0.01 versus vehicle (untreated) group of the same genotype.

BNI completely restored the blunted mechanical allodynia of *dream*$^{-/-}$ mice to that of wild-type. This suggests that the attenuated allodynic behavior of *dream*$^{-/-}$ animals is not fully dependent on ongoing opiate receptor activation. One possibility is that other mechanisms not requiring κ-opiate receptors are contributing to the lessened degree of allodynia in *dream*$^{-/-}$ mice. Alternatively, tonic activation of κ-opiate receptors may suppress the induction of neuroplastic changes by nerve injury. Hence, the neuroplastic changes may not reach the full extent observed in wild-type animals and would not be expected to be produced by acutely blocking κ-opiate receptors.

Peripheral opiate receptor blockade using naloxone methiodide did not have a significant effect on acute, inflammatory, or neuropathic pain behavior in *dream*$^{-/-}$ mice. Hence, central κ-opiate mechanisms are required for the attenuated pain responses in these animals. Our evidence of enhanced spinal prodynorphin mRNA and dynorphin contents suggests that the dynorphin/κ-opiate receptor system of the spinal cord is likely mediating the analgesic responses in the absence of DREAM. However, our data do not exclude a possible contribution of supraspinal opiate mechanisms in the observed phenotype.

Many studies provide support for an antinociceptive action of dynorphins. Dynorphin-mediated analgesia has been ascribed to its inhibitory action on neurons at κ-opiate receptors. Electrophysiological evidence supports a κ-mediated inhibitory effect of dynorphins on synaptic transmission of nociceptive neurons in the spinal dorsal horn (Randic et al., 1995). Dynorphins can induce hyperpolarization of neurons (Ogura and Kita, 2000), potentially through a κ receptor-coupled enhancement of potassium conductance (Grudt and Williams, 1993). In addition, dynorphin-mediated activation of κ receptors suppresses calcium currents and calcium-dependent secretion (Rusin et al., 1997; Wiley et al., 1997). Furthermore, dynorphin has been shown to inhibit substance P release in the spinal cord in a κ receptor-mediated manner (Zachariou and Goldstein, 1997). The endogenous dynorphin/κ-opiate system has been suggested to elicit antinociception during inflammation (Schafer et al., 1994) and pregnancy (Dawson-Basoa and Gintzler, 1996) and to mediate cannabinoid-induced antinociception (Mason et al., 1999). These studies support an antinociceptive function of dynorphin by negatively modulating transmission of nociceptive information.

Nevertheless, there are reports suggesting that dynorphins may exert pronociceptive effects, particularly in persistent pain states. Behavioral hyperalgesia as a result of inflammation (Iadarola et al., 1988) or nerve injury (Kajander et al., 1990) is accompanied by elevations in spinal dynorphin content, and while the increase in dynorphin has been considered causative of the hyperalgesia, the alternative (that this represents a negative feedback) has not been eliminated. Administering exogenous dynorphins intrathecally is reported to elicit mechanical allodynia (Laughlin et al., 1997). Recently, *prodynorphin*$^{-/-}$ mice were generated (Sharifi et al., 2001) and reported to recover more rapidly from the allodynic state in one model of neuropathic pain (spinal nerve ligation [SNL]) compared with wild-type animals (Wang et al., 2001). In this study, either MK-801 or antiserum to dynorphin $A_{(1-17)}$ alone rescued the enhanced pain behaviors (i.e., both thermal hyperalgesia and tactile allodynia) of wild-type animals to that of *prodynorphin*$^{-/-}$ mice. In contrast, another study showed that dynorphin $A_{(1-17)}$ antiserum blocked the thermal hyperal-

gesia, but not tactile allodynia, after SNL (Malan et al., 2000). In conjunction with reports that dynorphin directly interacts with NMDA receptors (Tang et al., 1999) and may potentiate NMDA currents (Lai et al., 1998) (although there is also evidence indicating that dynorphin may inhibit NMDA receptors by direct binding [Chen et al., 1995; Chen and Huang, 1998] or indirectly through κ-opiate receptors [Caudle et al., 1994; Ho et al., 1997]), it has been suggested that dynorphin mediates pronociceptive effects through NMDA receptors.

Using MK-801, we showed that NMDA receptor activity is not required for the increased tail-flick latency observed in *dream*$^{-/-}$ mice. In the neuropathic model, while administering MK-801 dose-dependently increased the paw withdrawal thresholds of wild-type animals, MK-801 had no effect on the response of *dream*$^{-/-}$ mice. Hence, NMDA receptors are not implicated in the attenuated neuropathic pain behavior in *dream*$^{-/-}$ mice. One potential explanation for the lack of NMDA receptor involvement in the allodynia that remains in *dream*$^{-/-}$ mice is that there may be insufficient activity of central neurons to permit NMDA receptors to become engaged in synaptic transmission. Alternatively, in the absence of DREAM, the downstream consequences of NMDA receptor activity (for example, the signal transduction events downstream of NMDA receptor activation) may be suppressed or not expressed.

Our finding that attenuated pain responses in *dream*$^{-/-}$ mice are mediated through κ-opiate receptors on a backdrop of elevated spinal dynorphin content is juxtaposed with other studies suggesting that dynorphin may exert pronociceptive effects, in particular the recent report that long-term maintenance of neuropathic pain was not observed in *prodynorphin*$^{-/-}$ mice (Wang et al., 2001). How does one reconcile these apparently paradoxical observations?

First, studies showing that elevation of spinal dynorphin content parallels enhanced pain responses following tissue or nerve damage are correlative. They do not resolve the question of whether dynorphin action is responsible for the hyperalgesia, or whether, in fact, upregulation of dynorphin is a consequence of (and participates in a feedback inhibitory loop to counteract) increased nociceptive input following injury.

Second, dynorphins may preferentially act on one or another type of receptor, depending upon their concentration. Thus, dynorphin concentration may determine the balance between pro- and antinociceptive effects (for review, see Laughlin et al., 2001). It has been proposed that physiological concentrations of dynorphins are antinociceptive and neuroprotective through opiate receptor activation, whereas extremely elevated levels are pronociceptive and even excitotoxic in an NMDA receptor-dependent fashion (Hauser et al., 1999). The lack of κ-opiate receptor downregulation at the protein level in *dream*$^{-/-}$ mice is consistent with the idea that κ-opiate receptor stimulation is neither excessive nor to the extent that would produce appreciable internalization and a net decrease in the steady-state level of receptors.

Third, in our study, genetic ablation of *dream* impacts on the endogenous dynorphin system. Endogenous dynorphins are expected to exert their effects only on a subpopulation of target receptors and cells near the sites of release. In contrast, when dynorphin's pronociceptive effects are inferred from exogenously administering dynorphins (for example, by means of intrathecal delivery), dynorphin may act in a nonselective manner on receptors and cells with which it would not interact under physiological conditions. Moreover, a valid comparison might not be made between the levels of dynorphins achieved in *dream*$^{-/-}$ mice with those following exogenous application.

Given that DREAM can bind to DRE sequences present in multiple genes (our results and Carrion et al., 1999), it is intriguing that *dream*$^{-/-}$ mice showed elevated basal expression of the *prodynorphin* gene but not that of the two other DRE-bearing genes we examined, that is, *c-fos* and *proenkephalin*. Furthermore, from our study, the increased prodynorphin expression in *dream*$^{-/-}$ mice appeared to be restricted to the spinal cord and not to other tissues examined, including the brain, thymus, and heart. Hence, ablating DREAM results in altered gene expression in a manner that is both gene- and tissue-restricted. This points to the importance of additional transcriptional repressor(s), cofactors, and/or differential regulation of basal transcription factors in determining the outcome of lack of DREAM on gene expression in a particular tissue or cell type in vivo.

Conclusions

Our results show that lack of DREAM results in attenuation of pain responses in models of acute, inflammatory, and neuropathic pain. The reduced pain behavior in *dream*$^{-/-}$ mice is attributable to κ-opiate receptor activity. Furthermore, the absence of DREAM results in the enhanced basal expression of prodynorphin in the spinal cord. Biological functions other than pain processing appeared not to be perturbed in *dream*$^{-/-}$ animals. In summary, our results highlight a new aspect of pain modulation, in which DREAM serves as a "transcriptional switch" for repressing and derepressing endogenous modulators of pain processing.

Experimental Procedures

More detailed description of Experimental Procedures is available in the Supplemental Data at http://www.cell.com/cgi/content/full/108/1/31/DC1.

Generation of *dream*$^{-/-}$ Mice

A targeting vector (667 bp short arm and 7.5 kb long arm) was constructed using the pKO Scrambler NTKV-1907 vector (Stratagene). A portion of the *dream* genomic DNA containing *dream* cDNA nucleotides +174 to +429 was replaced with the *neo* cassette. The E14K ES cell line was transfected with this targeting vector to obtain homologously recombined clones. Two independent *dream*$^{+/-}$ ES cell lines were injected into C57BL/6-derived blastocysts to generate chimeric mice, which were crossed to C57BL/6 mice to produce *dream*$^{+/-}$ mice. Wild-type and *dream*$^{-/-}$ littermate mice generated from heterozygous intercrosses were used for all studies. Data reported in this manuscript were consistent between the two mutant mouse lines. Mice were maintained at the animal facilities of the Ontario Cancer Institute in accordance with institutional guidelines.

Behavioral Studies

Unless otherwise specified, all behavior studies were conducted using 4- to 5-month-old mice by observers blinded to the genotypes of the animals. Assessment of sensorimotor reflex, activity, balance

and motor skill, and spatial learning was conducted as previously described (Whishaw et al., 1999). Locomotor activities were assessed in a place-conditioning apparatus (Dockstader et al., 2001).

Pain Behavior Studies

All pain behavior studies were conducted in accordance with the guidelines in "The Care and Use of Experimental Animals" by the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Princess Margaret Hospital, Toronto, Canada. The tail-flick assay was conducted using a tail-flick analgesia meter (Columbus Instruments). The mechanical pain test was performed by applying an ascending series of noxious von Frey hairs to the dorsal surface of each hindpaw until a withdrawal response was observed (Whishaw et al., 1999). In the visceral pain tests, mice were injected i.p. with $MgSO_4$ (120 mg/kg) or acetic acid (0.6%, 5.0 ml/kg) (Cao et al., 1998), and the number of abdominal writhes were counted within the first 5 min ($MgSO_4$) or 20 min (acetic acid) after injection. In the formalin test, paraformaldehyde (2%) was injected into the hindpaw, and the time spent paw licking was recorded in 5 min intervals. In two models of inflammatory pain, capsaicin (0.3 μg) or λ-carrageenan (2%) was injected into the hindpaw. Paw-licking duration within the first 5 min after capsaicin injection was recorded. Paw-withdrawal thresholds were determined prior to and 20 min (capsaicin) or 4–96 hr (carrageenan) after injection using von Frey filaments. Paw thickness was determined using a spring-loaded caliper (Mitutoyo). In a model of neuropathic pain (Mosconi and Kruger, 1996; Pitcher et al., 1999), peripheral nerve injury was induced by cuffing the left sciatic nerve. Paw withdrawal thresholds were determined once every two days after cuff implantation using von Frey filaments. Spontaneous pain behavior was assessed as described (Bennett and Xie, 1988). For pharmacological studies, naloxone hydrochloride (1 mg/kg, i.p.; Sigma), nor-binaltorphimine dihydrochloride (nor-BNI, 2 mg/kg, s.c.; Sigma), naloxone methiodide (2 mg/kg, s.c.; Sigma), and (+)-MK-801 (0.01 and 0.05 mg/kg, s.c.; Sigma) were used. All drugs except for nor-BNI were administered 30 min prior to pain testing. nor-BNI was administered 24 hr prior to testing.

Fluorescent Electromobility Shift Assay (EMSA)

Purified full-length murine DREAM protein was incubated with Cy5-labeled double-stranded oligonucleotides with or without 10 mM $CaCl_2$, and protein-DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. For competition studies, excess unlabeled double-stranded oligonucleotides were added before incubation with wild-type probes. Oligonucleotides were DynDRE, 5′-GAAGCCGGAGTCAAGGAGGCCCCTG-3′; c-fosDRE, 5′-CTGCAG CGAGCAACTGAGAATCCAAGAC-3′; DREmut5, 5′-GAAGCCGGA**AT** CAAGGAGGCCCCTG-3′; DREmut4, 5′-GAAGCCGGA**AA**CAAGGA GGCCCCTG-3′, and the canonical AP-1 sequence from the collagenase gene (cAP-1), 5′-AAGCTTGCATGACTCAGACAG-3′.

In Situ Hybridization

Paraffin sections of lumbar spinal cords of 5-month-old mice were hybridized with digoxigenin-labeled riboprobes generated by in vitro T7/T3 transcription (Roche Molecular Biochemicals) and specific for murine *dream*, *prodynorphin*, *proenkephalin*, *proopiomelanocortin*, and *c-fos*, as described (Hui et al., 1994).

Primary Neuronal Cultures

Spinal cord neuronal cultures were prepared from wild-type and *dream*$^{-/-}$ E15.5 mouse embryos as described (Lucas et al., 1993), with modifications. Cultures (10-day-old) were stimulated with 100 nM serotonin (Sigma), 100 nM 8-hydroxy-DPAT (Sigma), or 25 μM forskolin (Sigma), and total RNA was harvested 6 hr later. For RT-PCR, the following primers were used: 5′-CAAGTGAGTCAGAATGGC GTGG-3′ and 5′-CCATGGAGGGGAAGTGTTATGC-3′ (*prodynorphin*); 5′-GGGTTTCAACGCCGACTACG-3′ and 5′-CAGCTTGGGA AGGAGTCAGC-3′ (*c-fos*); and 5′-ATGTTCCAGTATGACTCCACT CACG-3′ and 5′-GAAGACACCAGTAGACTCCACGACA-3′ (*gapdh*).

Enzyme Immunoassay (ELISA) for Dynorphin A Quantitation

Animals under isoflurane anesthesia received intrathecal administration of *p*-hydroxymercuribenzoate (8 nmol; Sigma) and phosphoramidon (4 nmol; Sigma), as described previously (Hylden and Wilcox, 1980; Tan-No et al., 1996). Three hours later, animals were sacrificed and spinal cords were removed and stored at −70°C until later use. Peptides were extracted as described (Malan et al., 2000). Levels of dynorphin $A_{(1-17)}$ and dynorphin $A_{(1-8)}$ were determined using enzyme immunoassay kits (Peninsula Laboratories).

Western Blotting Analyses of κ-opiate and NMDA Receptor Expression

Membrane fractions were prepared from the brains, brainstems, and spinal cords of wild-type and *dream*$^{-/-}$ mice as described (Appleyard et al., 1997). Antibodies to κ-opioid receptor (KOR-1; Upstate Biotechnology), NMDA receptor (NMDAR1; PharMingen), and PSD-95 (BD Transduction Laboratories) were used.

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