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KAP1-Mediated Epigenetic Repression in the Forebrain Modulates Behavioral Vulnerability to Stress

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SUMMARY

KAP1 is an essential cofactor of KRAB-zinc finger proteins, a family of vertebrate-specific epigenetic repressors of largely unknown functions encoded in the hundreds by the mouse and human genomes. Here, we report that KAP1 is expressed at high levels and necessary for KRAB-mediated repression in mature neurons of the mouse brain. Mice deleted for KAP1 in the adult forebrain exhibit heightened levels of anxiety-like and exploratory activity and stress-induced alterations in spatial learning and memory. In the hippocampus, a small number of genes are dysregulated, including some imprinted genes. Chromatin analyses of the promoters of two genes markedly up-regulated in knockout mice reveal decreased histone 3 K9-trimethylation and increased histone 3 and histone 4 acetylation. We propose a model in which the tethering of KAP1-associated chromatin remodeling factors via KRAB-ZFPs epigenetically controls gene expression in the hippocampus, thereby conditioning responses to behavioral stress.

INTRODUCTION

Growing evidence points to a role of epigenetics in translating environmental stimuli into long-lasting changes of gene expression in the brain and to the importance of this process in shaping behaviors and conditioning susceptibility to psychiatric diseases (Tsankova et al., 2007). Chromatin modifications have been noted in animal models of depression, addiction, schizophrenia, and in some neurodevelopmental disorders, and several psychoactive drugs induce histone changes at specific promoters in particular regions of the brain (Tsankova et al., 2007). Stress and stress response neurological circuits have been identified as the trigger and the targets, respectively, of epigenetic modulation. In the early postnatal period, variations in maternal care, known to result in individual differences in

the offspring's behavioral responses to stress in adulthood, were found to correlate with differences in DNA methylation of the glucocorticoid receptor promoter in the hippocampus (Weaver et al., 2004). In the rat chronic social defeat stress model, which mimics many symptoms of human depression, prolonged histone H3K27 dimethylation is induced at the *Bdnf* (brain-derived neurotrophic factor) promoter, eventually leading to repression of *Bdnf* transcripts (Tsankova et al., 2006). Conversely, chronic electroconvulsive seizures, used as antidepressant treatment in humans, increase *Bdnf* production in the mouse hippocampus (Tsankova et al., 2004). Also, cocaine and antipsychotic drugs induce acetylation of histone 4 and phosphoacetylation of histone 3 in the striatum, notably at promoters of immediate-early genes such as *cFos* (Kumar et al., 2005). In rats, chronic exposure to cocaine or stress decreases histone deacetylase 5 (HDAC5) function in the nucleus accumbens, a major brain reward region, and this effect is reverted by long-term treatment with the antidepressant imipramine; inversely, loss of HDAC5 causes hypersensitivity to chronic cocaine exposure or stress (Renthall et al., 2007). In humans, the neurodevelopmental disorder Rett syndrome is caused by mutations in the gene encoding methyl-CpG binding protein 2 (MeCP2), a transcriptional repressor involved in chromatin remodeling and the modulation of RNA splicing (Chahrour and Zoghbi, 2007). Finally, experimental evidence implicates the epigenetic regulation of chromatin structure among molecular mechanisms that underlie memory formation (i.e., learning-triggered transcriptional regulation) (Levenson et al., 2004, 2006; Levenson and Sweatt, 2005; Miller and Sweatt, 2007).

Contrasting with this abundance of data pointing to a key role of epigenetics in animal behavior, including adaptations and pathologies, relatively little is known about the molecular mediators of this process. While significant progress has been made in the understanding of intracellular signaling pathways linking neural activity and synaptic transmission to the activity of chromatin modifying enzymes, notably the calcium- or cAMP-mediated activation of kinases and other histone modifiers or associated proteins (Tsankova et al., 2007), how the resulting epigenetic changes are targeted to specific genes in the brain remains largely unknown.

The human genome contains more than 400 genes coding for Krüppel-associated box (KRAB) zinc finger proteins (KRAB-ZFPs) (Huntley et al., 2006; Sripathy et al., 2006; Urrutia, 2003), and comparative sequence analyses indicate that this family of epigenetic repressors is vertebrate specific. While large variations between organisms suggest a role in speciation (Dehal et al., 2001; Krebs et al., 2005), no gene target of KRAB-ZFPs has been unequivocally identified, and the physiological roles of these regulators remain essentially unknown. Nevertheless, extensive in vitro molecular analyses have revealed that their conserved KRAB domain recruits the KRAB-associated protein 1 (KAP1) corepressor, also known as tripartite motif protein 28 (TRIM28), transcription intermediary factor 1 β (TIF1 β) or KRAB-interacting protein 1 (KRIP-1). KAP1 in turn acts as a scaffold for a multimolecular entity comprising heterochromatin protein 1 (HP1), the histone methyltransferase SETDB1, and the histone deacetylase-containing complex NuRD, which together silence transcription by triggering the formation of heterochromatin (Sripathy et al., 2006; Urrutia, 2003). In some culture systems, KAP1 appears to partake in cell-cycle regulation and DNA-damage response, possibly in a KRAB-ZFP-independent fashion (Wang et al., 2005; Ziv et al., 2006). Here, we aim to define the roles of the KRAB/KAP1 pathway in vivo. Our results suggest that, in the adult forebrain, this epigenetic regulatory system conditions behavioral stress responses.

RESULTS

Functional KRAB/KAP1 Pathway in Adult Forebrain Neurons, and Inactivation Thereof

Quantitative PCR analyses of KAP1 RNA expression in adult mouse tissues revealed high levels in the hippocampus and the cerebellum (Figures 1A and S1A). In situ hybridization data deposited in the Allen Brain Atlas also suggested the presence of KAP1 RNA in the murine central nervous system (Lein et al., 2007). Through immunohistochemical analysis of tissue cryosections with a KAP1-specific antibody, we confirmed the presence of the protein throughout the mouse brain, including the hippocampus, the striatum, the cerebellum, and all parts of the cortex, and noted predominant expression in neurons rather than in glial cells (Figures 1B, 1C, and S1B). Of the 104 KRAB-ZFPs for which in situ hybridization results are available in the Allen Brain Atlas, 45 displayed detectable levels of transcripts in the hippocampus (Lein et al., 2007). We verified expression of 14 of the KRAB-ZFPs by real-time reverse-transcription PCR amplification of hippocampal and cerebellar RNA, although for several of them transcripts levels were low (Figures 1D and S2).

To explore a potential role of KAP1 in higher brain functions, we crossed mice harboring a “floxable” KAP1 gene (Weber et al., 2002) with animals expressing the Cre recombinase from a CamKII α promoter, which induces recombination throughout the forebrain starting at approximately postnatal day 14 (Figure S1C) (Schweizer et al., 2003). Histological analysis of brain cryosections obtained from *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice revealed a normal morphology with no apparent cell loss as visualized by DAPI staining (Figure 1B) and with an antibody against the neuronal marker NeuN (Figure S1D). However, these mice completely lacked KAP1 in the dentate gyrus and the CA1

area of the hippocampus and had a partial loss of KAP1 expression in the striatum, the olfactory bulb and throughout the cortex, while other brain areas including the amygdala and nucleus accumbens exhibited normal levels of the protein (Figures 1B and 1C and data not shown).

KAP1 is a target of posttranslational modifications, including phosphorylation and SUMOylation, which condition its corepressor activity (Ivanov et al., 2007; Lee et al., 2007; Li et al., 2007; Ziv et al., 2006). To confirm that KAP1 is present in an active form in hippocampal neurons, we used a lentiviral vector-based system that contains a *tet* repressor-KRAB (tTRKRAB) fusion protein and a KRAB repression-sensitive GFP reporter (Szulc et al., 2006; Wiznerowicz et al., 2007). In the absence of doxycycline, tTRKRAB binds tetracycline operator (*TetO*) sequences in the vector, leading to recruitment of KAP1 and transcriptional silencing. Conversely, when doxycycline is present, it purges tTRKRAB from its target and the reporter is switched on (Figure 2A). We stereotactically injected high-titer lentiviral vector particles into the hippocampus of *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice and of *KAP1^{flox+/+}-CamKII α Cre^{-/-}* littermates, which were treated or not with doxycycline before microscopic analysis of brain cryosections. A large number of GFP-positive neurons were detected in the hippocampus of doxycycline-treated *KAP1^{flox+/+}-CamKII α Cre^{-/-}* mice, but very few of these cells were seen in the absence of doxycycline, consistent with KRAB-mediated repression of the vector (Figure 2B). In contrast, in *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice, GFP-positive neurons were visible in the hippocampal CA1 area irrespective of doxycycline administration, indicating that KRAB-mediated repression was abrogated in these KAP1-deleted cells (Figure 2B). These results demonstrate that KAP1 functions as essential cofactor for KRAB-mediated transcriptional repression in the adult forebrain.

Forebrain KAP1 KO Mice Exhibit Stress-Related Behavioral and Cognitive Impairments

Since KAP1 is highly expressed in brain areas involved in emotion and cognition, we examined whether KAP1 deficiency affects these functions. We verified that adult *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice and their KAP1-competent littermates (*KAP1^{flox+/+}-CamKII α Cre^{-/-}*) had normal general characteristics such as body weight, posture, locomotion and reflexes (data not shown). The animals were then tested for anxiety-like behaviors in two different paradigms, the elevated plus maze (EPM) and the open field (OF). The EPM test is an elevated four-arm maze with two walled arms (safe compartments) and two open arms (risky areas) linked by a central platform. The *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice spent less time in the unprotected and anxiogenic open arms ($t = 2.15$, $p < 0.05$) and central platform ($t = 3.83$, $p < 0.001$), and therefore more time in the closed arms, than control *KAP1^{flox+/+}-CamKII α Cre^{-/-}* littermates ($t = 3.32$, $p < 0.004$; Figure 3B), while total ambulation was equal for both genotypes ($t = 0.30$, n.s.; Figure 3D). The OF test relies on the use of an open arena with an exposed and anxiogenic center and a less exposed periphery (Figures 3E–3H). In this test, the natural tendency of animals to explore the arena competes with their tendency to avoid a brightly lit open, central area. The *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice made less entries into the center ($t = 2.06$, $p < 0.05$; Figure 3E) and moved less

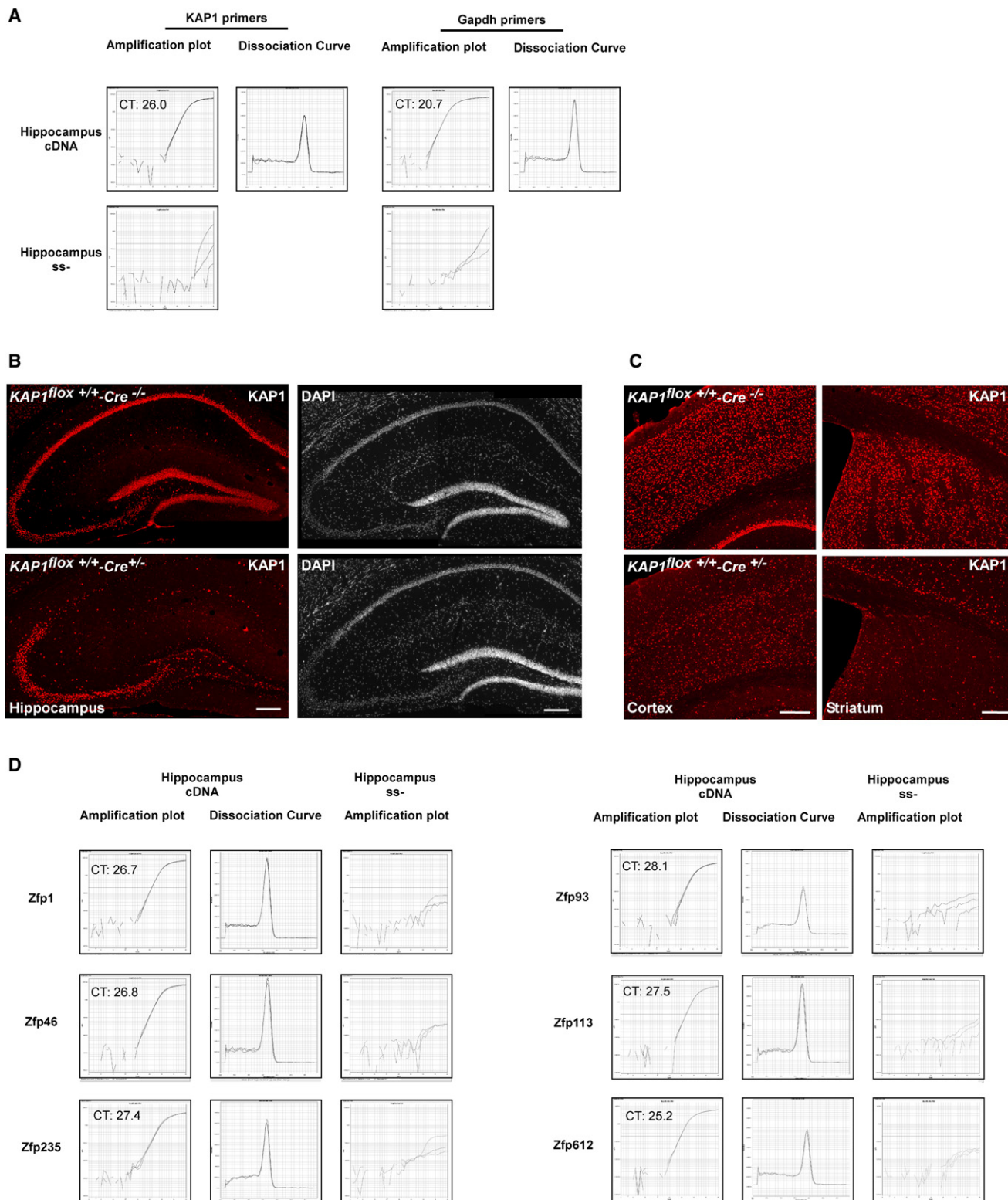


Figure 1. Conditional Deletion of KAP1 in the Forebrain

(A) Real-time PCR data demonstrating high levels of KAP1 mRNA in the mouse hippocampus.

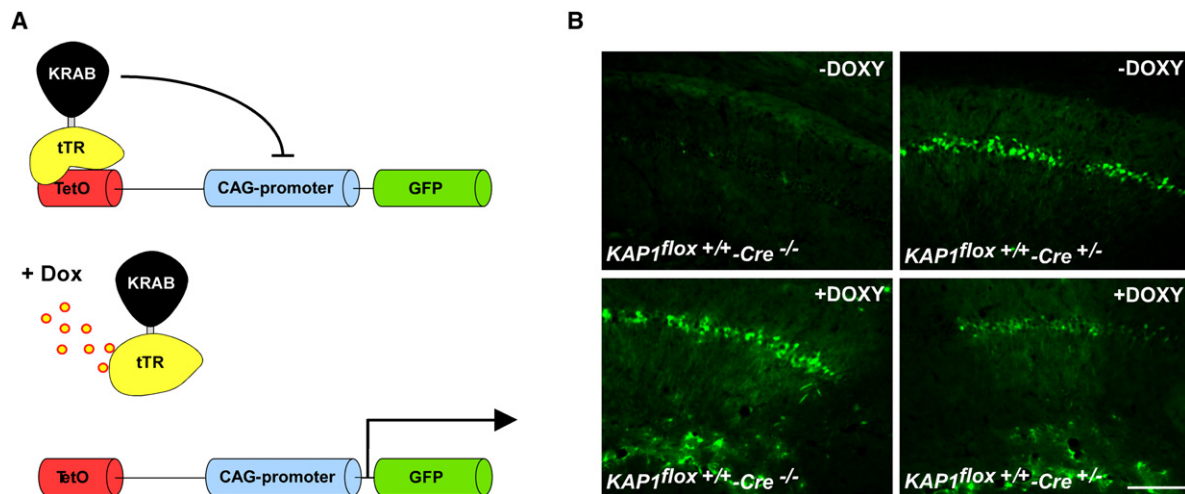


Figure 2. KAP1-Dependent KRAB-Mediated Gene Regulation in Neurons Demonstrated with a Lentiviral Reporter Vector

(A) In the absence of doxycycline, the tTR-KRAB fusion protein binds to tetO sites in the lentivector and represses expression (top left). When doxycycline is present, tTR-KRAB binding is relieved and GFP expression initiated (bottom left).

(B) Stereotaxic injection of the lentiviral vector into the hippocampus of wild-type mice (left) reveals doxycycline-dependent GFP expression. In *KAP1* mutant mice GFP expression can be detected in neurons irrespective of prior doxycycline administration, demonstrating that KAP1 is essential for KRAB-mediated repression in neurons in vivo. Scale bar, 100 μ m.

in the central area ($t = 2.06$, $p < 0.05$; Figure 3G) than control *KAP1^{flox+/+}-CamKII α Cre^{-/-}* mice (but note that differences in the percent time spent in the central area did not reach significance; Figure 3F), while total locomotor activity in the arena was equal for both genotypes ($t = 1.19$, n.s.; Figure 3H). Therefore, the behavioral pattern displayed by *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice (i.e., reduced percent time in open arms of the EPM and reduced movement in the central area of the OF, while no differences in total locomotion) is indicative of an increased anxiety-like phenotype. To assess directed exploratory behaviors, at the end of the OF testing and while kept in the same arena, mice were exposed to a novel object (NO) placed in the center of the OF while their exploration of this object was monitored and to the hole board (HB) test, in which head-dipping responses in a 16-hole board are recorded. In both tests, the *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice displayed higher exploratory behaviors. Indeed, in the NO test (Figures 3I–3L), mutant mice spent more time in close proximity to the object, as indicated by a higher frequency of entries to the center ($t = 2.29$, $p < 0.03$), a higher percentage of time spent in the central area ($t = 3.22$, $p < 0.003$; Figure 3J) and a longer distance traveled in this area ($t = 3.12$, $p < 0.004$; Figure 3K). Of note, total locomotor activity in the arena was equal between wild-type and KAP1-deficient mice ($t = 0.18$, n.s.; Figure 3L). When the HB test was initially run for 8 min (Figures 4A–4C), we did not observe significant differences between the two genotypes in the total number of nose pokes produced [Figure 4A, repeated-measures ANOVA: $F(1,32) = 0.67$, n.s.; Figure 4B: $t = 0.82$, n.s.]. However,

analyzing data in blocks of 2 min suggested a potential differential behavior between the two genotypes toward the end of the testing time (Figure 4A, last 2 min block). A second experiment was performed in a new set of mice with a prolonged testing time. Confirming the initial results, when mice were examined for 20 min (Figures 4D–4F), significant differences were found between the two genotypes, with *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice displaying more frequent sniffing at holes than control *KAP1^{flox+/+}-CamKII α Cre^{-/-}* mice [Figure 4D, repeated-measures ANOVA: $F(1,16) = 4.81$, $p < 0.05$; Figure 4E: $t = 2.19$, $p < 0.05$], with post hoc analyses indicating a significant difference during the last 2 min block; Table S3). As in all previous tests, the general locomotion patterns of the two groups of mice in the first replication experiment (Figure 4C) were indistinguishable ($t = 0.39$, n.s.), although *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice moved significantly more than controls when the test was given for 20 min (Figure 4F; $t = 3.18$, $p < 0.006$), owing to the persistence of their exploratory behavior during the second part of the test (Figure S3). To investigate further the anxiety-like phenotype of the KAP1 knockout mice, this set of animals was also evaluated through a conflict-based test, the novelty suppressed feeding (NSF) (Gordon and Hen, 2004; Weisstaub et al., 2006). This task is based on the conflict between hunger and the suppression of feeding induced by a novel environment. Mice are food-deprived for 24 hr before measuring their latency to approach a food pellet placed in the center of a novel cage. In agreement with the anxiety-like phenotype observed in the EPM and OF, *KAP1^{flox+/+}-CamKII α Cre^{+/-}* mice showed a longer latency to

(B) Immunostaining for KAP1 (red) and DAPI staining labeling the nuclei of all cells (white). KAP1 is expressed in all neuronal populations in the hippocampus of wild-type animals (top left). *KAP1* mutant mice completely lack expression in the dentate gyrus and CA1 area of the hippocampus (bottom left). DAPI staining revealed that there was no loss of neurons when KAP1 was not expressed (bottom right).

(C) Same analysis on motor cortex and striatum, showing partial loss of KAP1 in mutant mice.

(D) Real-time PCR analysis of KRAB-ZFP expression. Analysis of several other KRAB-ZFP transcripts are found in the Supplemental Data. Scale bar, 200 μ m.

Elevated Plus Maze

Open Field

Novel Object

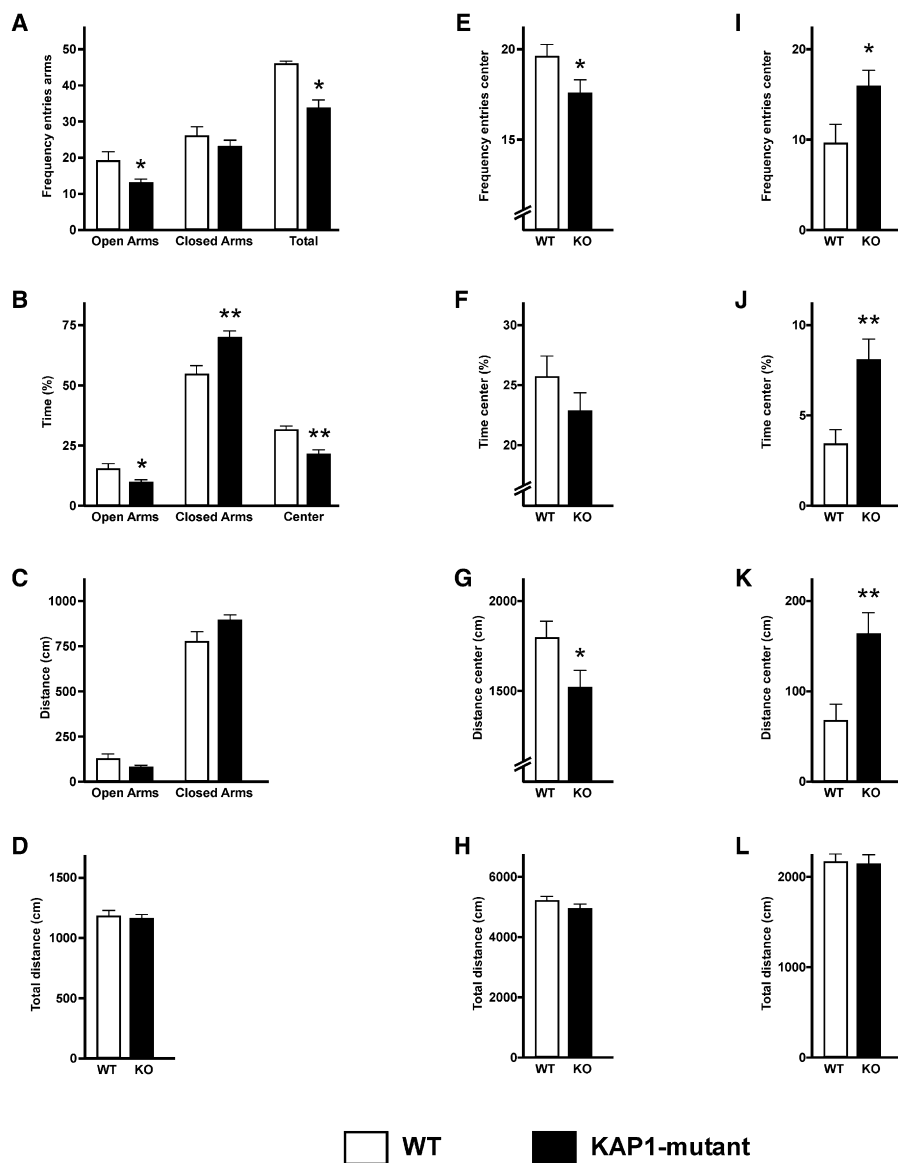


Figure 3. Behavioral Analysis of KAP1 Mutant Mice through the Elevated Plus Maze, Open Field, and Novel Object Tests

Behavioral measurements in the elevated plus maze are represented as frequency of entries in the different arms and total (A), percent time spent in the different EPM areas (B), distance (cm) moved in the different arms (C), and total distance (cm) moved in the maze (D). Behavioral measurements in the open field are represented as frequency of entries in the central area (E), percent time spent in center (F), distance (cm) moved in center (G), and total distance (cm) moved in the arena (H). Directed exploration was evaluated in the novel object test and is represented as frequency of entries in the central area including object (I), percent of time spent in the center (J), distance (cm) moved in center (K), and total distance (cm) moved during the test (L). Results are the mean ± SEM, * $p < 0.05$ versus WT, ** $p < 0.01$ versus WT. WT $n = 16$, KO $n = 18$.

approach the food pellet than controls (Figure 5A; normality rejected: Mann-Whitney test $U = 19$, $z = 2.12$, $p < 0.05$), while no difference between the two genotypes was observed in body weight loss (Figure 5B; $t = 1.68$, n.s.) or feeding activity in the home cage (Figure 5C; normality rejected: Mann-Whitney test $U = 38$, $z = 0.22$, n.s.). In sum, the behavioral analyses revealed a combination of hyperanxiety-like behavior and increased

directed exploration phenotype in the KAP1 forebrain knockout mice, without alteration in locomotion.

We then examined the impact of KAP1 deficiency on cognitive abilities known to depend on hippocampal function, such as spatial learning and memory. In the Morris water maze task, animals learn to locate a submerged platform in a tank of opaque water by orienting themselves using distal visual cues. No

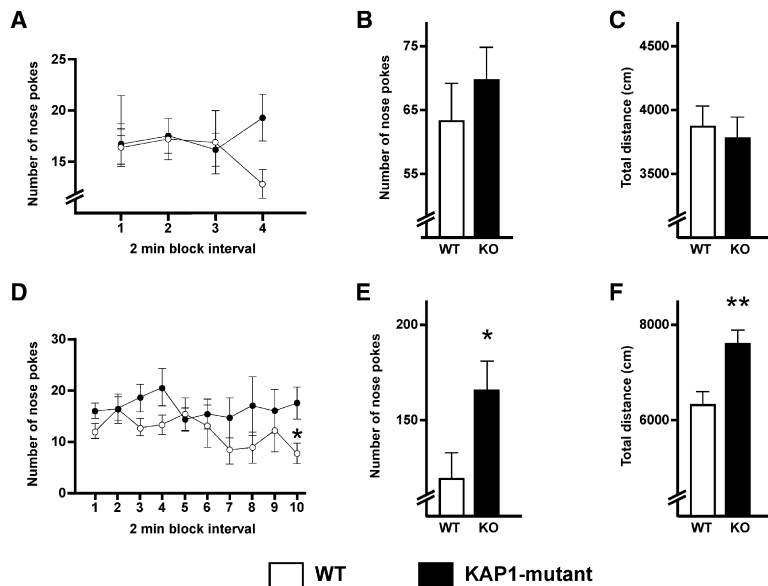


Figure 4. Directed Exploration in the 16 Hole Board Test of KAP1 Mutant Mice

Two replication experiments were performed in the 16 hole board test, a first one involving the same animals as in Figure 3 and run for a total of 8 min (A–C), and a second one addressed to explore potential differences between the genotypes when the testing time is extended to 20 min (D–F). Results are the mean \pm SEM. * $p < 0.05$ versus WT, ** $p < 0.01$ versus WT. WT $n = 16$ (A–C) and $n = 9$ (D–F); KO $n = 18$ (A–C) and $n = 9$ (D–F).

following exposure to stress. Chronic stress schedules involving exposure of mice to daily stressors have been repeatedly shown to impair hippocampus-dependent learning (Sandi, 2004). We selected a mild subchronic stress procedure that does not affect performance in the water maze in wild-type mice and asked whether it would impact the performance of KAP1-deficient mice. A new set of control and mutant mice was subjected to 2 week sporadic exposure to different stressors, including mild foot

differences in spatial learning and memory were observed between $KAP1^{flox/+}-CamKII\alpha Cre^{+/-}$ and their control $KAP1^{flox/+}-CamKII\alpha Cre^{-/-}$ littermates during spatial learning [Figure 6A; repeated-measures ANOVA trials 2–12: $F(1,17) = 0.351$, n.s.] or during the probe test [Figure 6B: ANOVAs on percent time in quadrant for each genotype indicated focused navigation toward the target quadrant; WT: $F(3,32) = 9.85$, $p < 0.001$; post hoc analyses were $p < 0.001$ for percent time swimming in quadrant NW (target) versus quadrants NE and SE –opposite–, and n.s. versus SW –releasing–; KAP1 mutant: $F(3,36) = 24.23$, $p < 0.0001$; post hoc analyses were $p < 0.001$ for percent time swimming in quadrant NW (target) versus each of the other quadrants]. No differences were found in the ratio for the percent time spent in the target versus the opposite quadrant between the two genotypes (Figure 6C, $t = 0.62$, n.s., Table S4). Since anxiety is a risk factor for the development of stress-related cognitive impairments, we reasoned that KAP1-mutant mice might have a greater vulnerability to develop cognitive deficits

shocks and reminders of the aversive experience, and 1 week later they were tested for spatial learning. As compared to $KAP1^{flox/+}-CamKII\alpha Cre^{-/-}$ control mice, $KAP1^{flox/+}-CamKII\alpha Cre^{+/-}$ mice showed impaired learning and memory. A repeated-measures ANOVA on escape latencies (trials 2–12) indicated a significant genotype effect [Figure 6D; $F(1,21) = 5.23$, $p < 0.033$], with mutant mice displaying higher latencies to find the platform. Post hoc analyses further confirmed significant differences in latency to find the platform in individual trials on day 2 (trial 7, $p < 0.001$) and day 3 (trial 9, $p < 0.04$). Moreover, when tested for long-term spatial memory, control animals spent more time searching for the platform in the quadrant in which it resided during the training period than in the other quadrants [$F(3,40) = 13.72$, $p < 0.0001$; post hoc analyses were $p < 0.001$ for percent time swimming in quadrant NW (target) versus all other quadrants], indicating a focused navigation strategy toward the target quadrant. Conversely, $KAP1^{flox/+}-CamKII\alpha Cre^{+/-}$ mice performed at chance level and spent similar time swimming in each quadrant [Figure 6E, and Table 4; $F(3,44) = 1.6$, n.s.]. This impaired long-term memory following a mild stress period in the KAP1-deficient mice was further supported by significant differences in the time spent in the target quadrant between the two genotypes (Figure 6E, $t = 2.11$, $p < 0.05$) and in the ratio between the time spent in the target versus opposite quadrant, with mutant mice displaying at chance level while controls showed a tendency to swim in the target quadrant (Figure 6F, $t = 2.91$, $p < 0.009$). These results were confirmed independently in two experiments performed on groups of mice generated close to a year apart. Taken together, these data indicate that spatial learning and memory capabilities are preserved in KAP1-deficient mice under non-stress conditions, but these animals show an increased vulnerability to develop hippocampus-related cognitive deficits after a period of mild stress. Thus, KAP1 expression in the fore-brain modulates behavioral traits such as anxiety and directed exploration and affects the vulnerability to develop stress-induced impairment in cognitive performance.

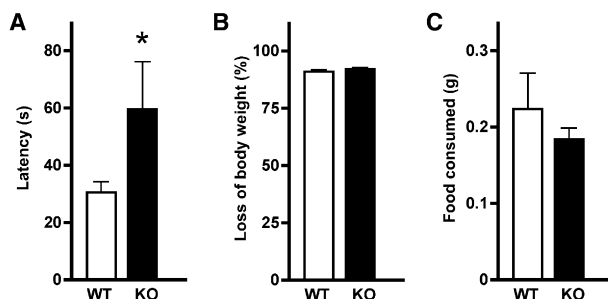
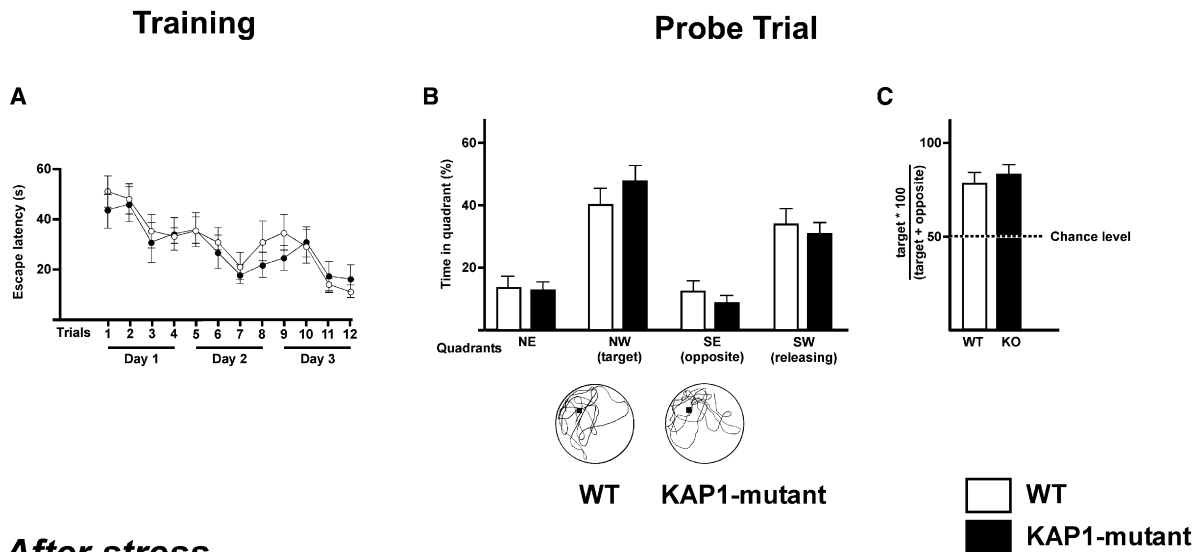
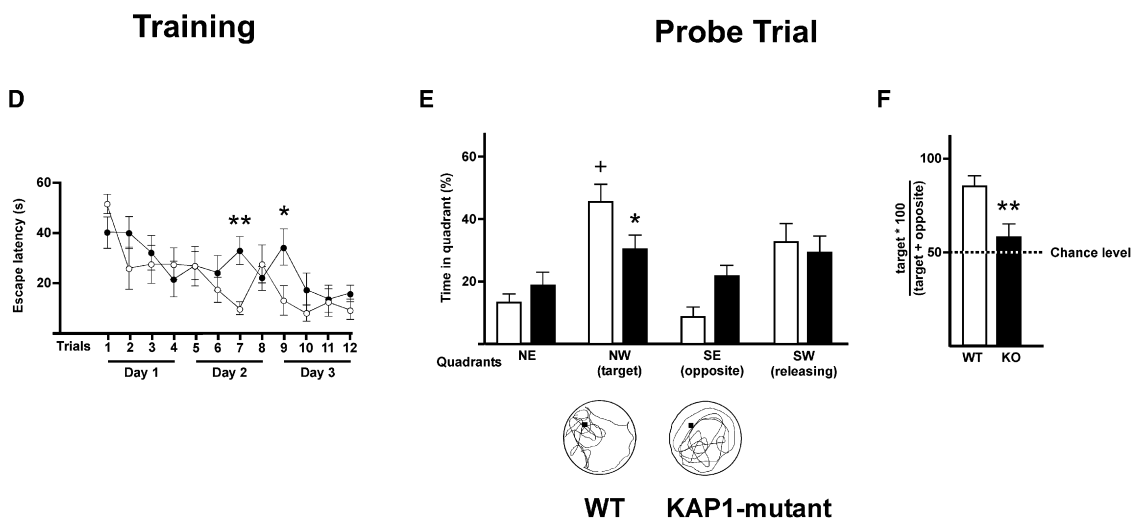


Figure 5. Conflict-Based Anxiety-like Behavior of KAP1 Mutant Mice in the Novelty Suppressed Feeding Test

Latency to approach the food pellet(s) (A), percentage of body weight lost after 24 hr food deprivation (B), amount of food consumed in the home cage during the 5 min post-NSF period (C). Results are the mean \pm SEM. * $p < 0.05$ versus WT. WT $n = 9$, KO $n = 9$.

- No prior stress -**- After stress -****Figure 6. Cognitive Abilities of KAP1 Mutant Mice Pre- and Poststress**

Learning in the Morris water maze task was evaluated by the latency to find a hidden platform during 3 days (12 trials in total), and spatial memory was assessed by the percent of time spent in the target quadrant, under naive “no prior stress” conditions (A–C) and after a period of “stress” (D–F). Representative swimming paths of $KAP1^{flox/+}-CamKII\alpha^{Cre-/-}$ (WT) and $KAP1^{flox/+}-CamKII\alpha^{Cre+/-}$ (KAP1 mutant) mice during the probe test are included under (B) and (E); note that, in contrast to the nonstressed KAP1 mutant or WT groups, which swim preferentially in the area where the platform was previously located, the swim path of pre-stressed KAP1 mutant mice is more erratic and distributed all over the pool (E). Results are the mean \pm SEM. * $p < 0.05$ versus WT, ** $p < 0.01$ versus WT, + $p < 0.01$ versus all other quadrants same genotype. No prior stress groups, WT $n = 9$, KO $n = 10$; after stress groups, WT $n = 11$, KO $n = 12$.

Transcriptional Dysregulations in the Hippocampus of KAP1 KO Mice

To explore the molecular bases of the behavioral impairment of KAP1 mutant mice, we extracted total RNA from the hippocampus of animals a few weeks after testing and performed transcriptome analysis using genome-wide microarrays. Initial cluster analyses of dysregulated transcripts revealed that samples from $KAP1^{flox/+}-CamKII\alpha^{Cre+/-}$ mice were distinct from samples from the three control groups ($KAP1^{+/-}$ heterozygous mice and

two wild-type KAP1 control groups) (Figure 7A). Further analyses of candidate genes that were either up- or downregulated at least 1.5-fold ($p < 0.05$, t test) in KAP1 mutant mice led to the identification of about 30 transcripts (Figure S4), which were confirmed by quantitative PCR (Figure 7B). Most notable was the markedly elevated expression of transcripts encoding the cell adhesion molecule Protocadherin beta 6 (*Pcdh6*), Bgalp-RS, the Mkn3 imprinted gene product, the Tcf5 transcription factor, as well as of a transcript predicted to encode an approximately one

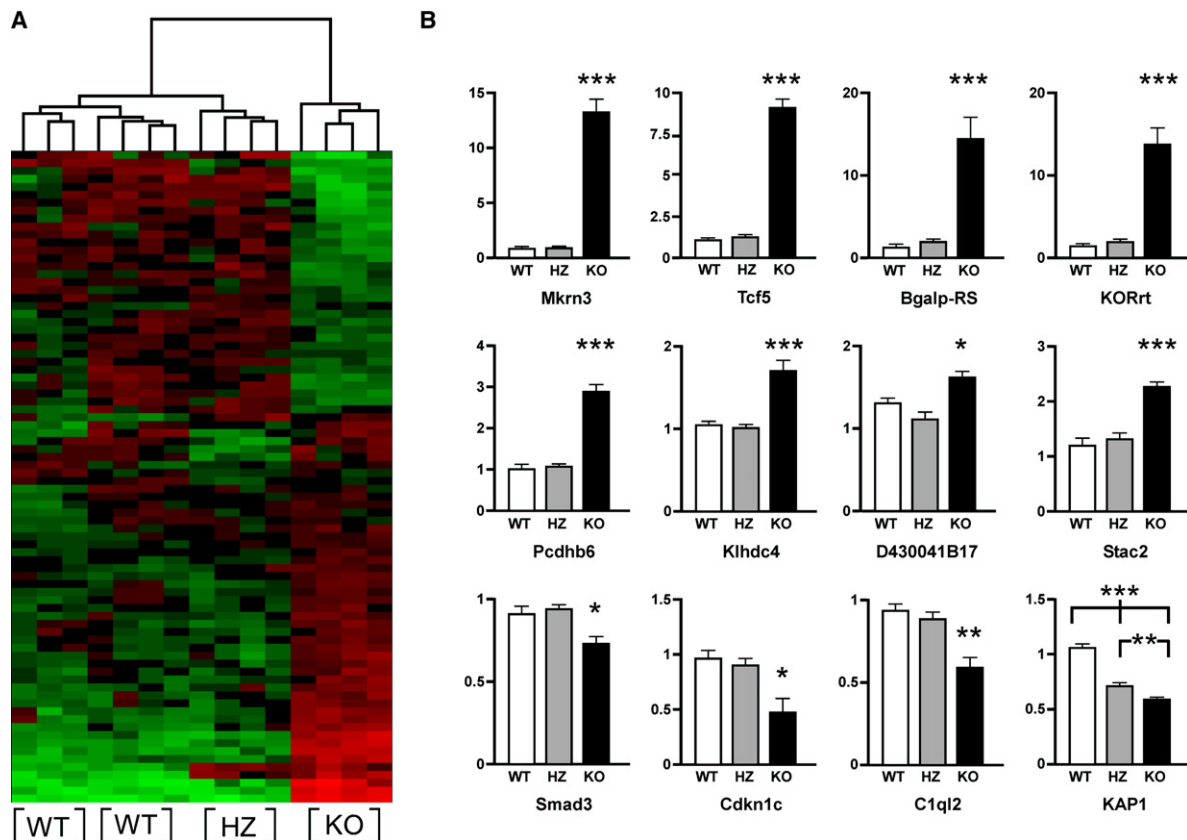


Figure 7. Microarray Analysis of Hippocampus RNA from KAP1 Mutant Mice

(A) Cluster analysis of dysregulated transcripts ($p < 0.005$, ANOVA) from an Illumina whole-genome array. The list of genes for cluster analysis were selected on the basis of differences in expression between any of the groups (KAP1 mutant mice: $KAP1^{flox+/+} CamKII\alpha Cre^{+/-}$, heterozygous: $KAP1^{flox+/+} CamKII\alpha Cre^{+/-}$ and wild-type: $KAP1^{flox+/+} CamKII\alpha Cre^{-/-}$ and $KAP1^{flox+/+} CamKII\alpha Cre^{-/-}$). A list of the genes used for the cluster analysis is found in Table S2.

(B) Real-time PCR validation of dysregulated transcripts. Results are the mean \pm SEM. y axis represents relative mRNA levels. One (randomly chosen) of the WT samples was used as a reference sample (value = 1). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Heat map: red, upregulated; green, downregulated.

hundred amino acids-long protein corresponding to the N-terminal domain of the kappa opioid receptor (KORrt).

Chromatin Modifications at Promoters of KAP1 Target Genes

To investigate the mechanism of KAP1 transcriptional repression, we performed chromatin immunoprecipitation assays (ChIP) on hippocampal DNA, using antibodies for different types of modified histones including acetylated histone 3 (H3ac), acetylated histone 4 (H4ac), and histone 3 trimethylated on lysine 9 (H3K9trimet) or lysine 27 (H3K27trimet). DNA was amplified with primers specific for the Gapdh promoter, as a reference for a highly transcribed gene, or the developmentally silenced globin promoter, as a reference for a repressed gene. Importantly, expression levels of these two genes were identical in control and $KAP1^{flox+/+} CamKII\alpha Cre^{+/-}$ mice. As expected, we found high levels of the active histone marks H3ac and H4ac on the Gapdh promoter while they were very low on the globin promoter (Figures 8A and S5). On the contrary, the globin promoter was enriched for the repressive marks H3K9trimet and H3K27trimet, while these were virtually undetectable on the Gapdh promoter (Figures 8A and S5). These results validated our ChIP protocol

and were in line with data previously reported for hippocampal tissue (Tsankova et al., 2004, 2006). We thus turned to the analysis of the Mkx3 and Pcdhb6 promoters, since both drove transcripts strongly upregulated in $KAP1^{flox+/+} CamKII\alpha Cre^{+/-}$ mice (Figure 7B), suggesting that they were direct targets of KAP1 repression. In the hippocampus of KAP1-competent mice, levels of H3ac and H4ac were low on these two promoters, while in striking contrast those of H3K9trimet were even higher than measured on the globin promoter (Figures 8A and S5). Such pattern of chromatin modification was highly suggestive of KAP1-induced repression, since this transcriptional regulator is known to recruit the H3K9 histone methyltransferase SETDB1 and the histone deacetylase-containing complex NuRD (Sripathy et al., 2006; Urrutia, 2003). Accordingly, in the hippocampus of $KAP1^{flox+/+} CamKII\alpha Cre^{+/-}$ mice, the Mkx3 and Pcdhb6 promoters exhibited a 2-fold enrichment in H3ac and H4ac and 2-fold reduction in H3K9trimet compared with $KAP1^{flox+/+} CamKII\alpha Cre^{-/-}$ controls (Figures 8B and S5). Considering that these samples included both KAP1-negative and KAP1-positive cells (see Figure 1B), these changes are indicative of robust KAP1-mediated chromatin modulation. Consistently, the Mkx3 and Pcdhb6 promoter-association of H3K27trimet, a chromatin mark suggested

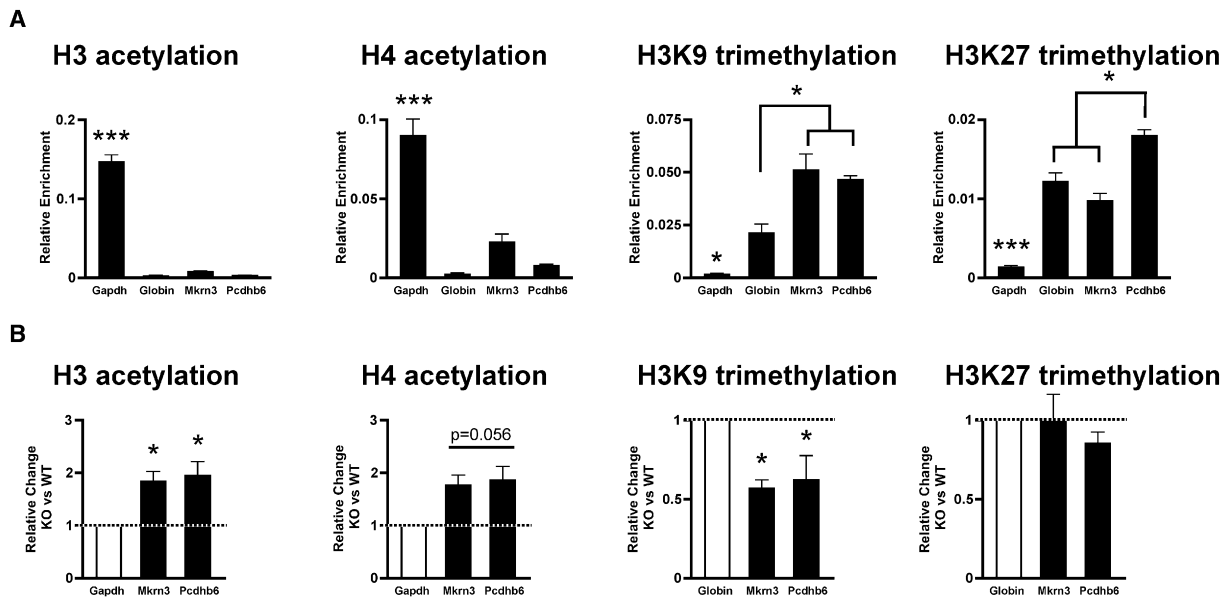


Figure 8. Chromatin Immunoprecipitation

(A) Quantification of the abundance of H3 acetylation, H4 acetylation, H3 lysine-9-trimethylation, and H3 lysine-27-trimethylation on KAP1 target genes Mkrn3 and Pcdhb6 in whole hippocampus of WT animals, showing a strong enrichment of H3K9trimet.

(B) Relative ratios of indicated modified histones on these promoters in whole hippocampus of KO versus WT animals, demonstrating that loss of KAP1 leads to a pattern of chromatin modification typical of gene activation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

to be KAP1 independent (O'Geen et al., 2007), was similar in control and knockout mice (Figures 8B and S5). Altogether, these data establish a correlation between KAP1-mediated chromatin modulation of the promoter of Mkrn3 and Pcdhb6 and levels of expression of these genes in the mouse hippocampus.

DISCUSSION

This work demonstrates that KAP1 acts as a transcriptional regulator in adult neurons in vivo and that loss of KAP1 in the mouse forebrain leads to alterations in well defined behavioral traits, including increased anxiety-like and directed exploration behaviors. In rodents, these phenotypic characteristics have been found to be associated in some situations and dissociated in others (Bouwknicht et al., 2004; Fernandes et al., 2004; Holmes et al., 2003; van Gaalen and Steckler, 2000). In humans, the combination of enhanced anxiety and directed exploration is a phenotype observed in some behavioral disorders such as borderline personality (Joyce et al., 2003) or bipolarity (Kazlauckas et al., 2005; Leahy, 2007), suggesting that KAP1 knockout mice may represent a model of increased vulnerability to psychopathology. Moreover, although under non-stressful conditions the performance of KAP1-deficient mice in a hippocampus-dependent spatial learning task was equivalent to that of their wild-type littermates, they exhibited cognitive deficits following a mild sub-chronic stress period that did not affect performance in control animals, suggesting an increased vulnerability to stress-related disorders.

Deletion of KAP1 in $KAP1^{flox/+/-CamKII\alpha Cre^{+/-}}$ mice was maximal in the hippocampus, with complete deletion in the dentate gyrus and CA1 hippocampal subregions. The hippocampus

is known to play key roles in both memory formation and the genesis of emotions such as anxiety (Morris, 2006; Squire et al., 2004). Furthermore, personality traits are the result of genetic and environmental factors, the latter acting at least partly through epigenetic regulation (Gross and Hen, 2004; Szyf et al., 2007). The increased anxiety-like behavior of KAP1 mutant mice in some tasks suggests a model in which KAP1 and KRAB-ZFPs may modulate the generation of anxiety in the hippocampus.

Individual differences exist in both resilience to stress and stress-induced psychopathological alterations (Levinson, 2006; Southwick et al., 2005). In humans, the neuroticism-anxiety trait is a particularly important risk factor for the development of diverse psychiatric conditions, such as generalized anxiety, posttraumatic stress disorder and major depression (Ball and Schottenfeld, 1997; Wang et al., 2004), and has recently been implicated in stress-induced cognitive impairments in both animals and humans (Burriss et al., 2007; Herrero et al., 2006). The hippocampus seems to be particularly vulnerable to the effects of repeated stress exposure on functions such as activity-dependent synaptic plasticity or spatial learning and memory (de Kloet et al., 2005; McEwen, 1999; Sandi, 2004). Here, KAP1 mutant mice exhibited a marked vulnerability to stress, as revealed by the development of learning and memory impairments in a hippocampus-dependent task following exposure to a sub-chronic mild stress period, in a form reminiscent of the altered performance classically found in rodents after exposure to heavier and longer-lasting stressors (Sandi, 2004).

Although different cellular and molecular mechanisms have been implicated in the adverse effects of chronic stress on brain function and behavior (de Kloet et al., 2005; McEwen, 1999; Sandi, 2004), the interactions between genetic, epigenetic, and

environmental factors are not well understood. While the role of epigenetics in mediating the long-term consequences of stress is indicated by the finding of chromatin changes at particular genetic loci and altered activities of chromatin modifiers in animal models of stress, how epigenetic modulation is targeted to specific genes has remained largely unknown. Our results suggest that KRAB-ZFPs may play a role in this process by tethering KAP1 and associated transcriptional repressors to specific genes in forebrain neurons in response to stress-related inputs.

Differences detected between the hippocampal transcriptomes of *KAP1* mutant and control mice were subtle, with only 30 genes exhibiting significantly altered levels of expression in the microarray analysis. The heterogeneity of the samples under analysis, harvested through dissection of whole hippocampi containing both *KAP1*-deleted and *KAP1*-positive cells (as reflected by the only 2-fold decrease recorded for the transcripts levels of *KAP1* itself, Figure 7B), may have blurred some of the difference. Nevertheless, we believe that the likelihood of a high rate of false positives and false negatives is small owing to the filtering method used here. At least in neurons of the adult forebrain, *KAP1* thus appears to play the role of a fine-tuner of expression, rather than that of a master transcriptional regulator. This may appear surprising in view of the recent identification of more than 7000 *KAP1*-binding sites through a genome-wide chip-on-ChIP analysis performed in a human tumor cell line (O'Geen et al., 2007). While the cellular context and cut-off of this latter study clearly differed, it is interesting to note that it identified *KAP1*-binding sites in the transcribed region of many KRAB-ZFPs, suggesting auto-regulatory feedback loops. Consistent with this model, 4 out of 14 KRAB-ZFPs analyzed by real-time PCR in *KAP1* mutant mice were upregulated, albeit modestly (Figure S6).

Chromatin immunoprecipitation studies performed on two genes markedly upregulated in the hippocampus of knockout animals, *Mkx3* and *Pcdhb6*, revealed a signature consistent with *KAP1*-mediated control. Indeed, in control animals, high levels of the silencing marker H3K9trimet and low levels of the activation markers H3ac and H4ac were detected on the promoters of these genes, whereas their transcriptional activation in knockout animals correlated with a drop in H3K9trimet and an increase in H3ac and H4ac. This result not only provides the molecular bases of the observed gene dysregulation, but also demonstrates *KAP1*-mediated chromatin modulation of endogenous promoters *in vivo*.

The nature of the genes dysregulated in the hippocampus of *KAP1* knockout mice does not provide a straightforward explanation for their phenotype. Two of these genes, *Mkx3* and *Cdkn1c*, are normally imprinted, i.e., expressed from only one allele, while the other is epigenetically inactivated by DNA methylation (Wood and Oakey, 2006). Imprinted genes have previously been implicated in neural disorders (Wilkinson et al., 2007). In particular, *Mkx3* resides in the Prader-Willi/Angelman syndromes locus, deletions of which result in genetic diseases characterized by behavioral and neurodevelopmental abnormalities (Hershko et al., 1999). While the more than 10-fold increase in *Mkx3* expression in the hippocampus of *KAP1* knockout mice suggests more than the sole activation of the imprinted allele, it is tempting to speculate that *KAP1* may be involved in maintaining the imprinting of specific genes in neurons or other adult tissues.

Consistent with a role for *KAP1* in imprinting, the KRAB-containing ZFP57 was recently demonstrated to participate in the post-fertilization maintenance of imprinted loci in the mouse (Li et al., 2008), and mutations in the corresponding gene are associated with hypomethylation in multiple imprinted loci in patients with transient neonatal diabetes (Mackay et al., 2008). Interestingly, one of the other upregulated genes, *Bgalp-RS* (also known as mouse osteocalcin-related gene), contains an endogenous IAP retroelement (Tabernero et al., 1997). IAP-elements are known to be able to influence the expression of neighboring genes by attracting epigenetic modifications including DNA methylation (Morgan et al., 1999). Another of the transcripts upregulated in the hippocampus of *KAP1* knockout mice, protocadherin beta 6 (*Pcdhb6*), encodes a calcium dependent cell-adhesion protein. Cell-adhesion proteins establish and maintain specific neuronal synaptic connections and have been implicated in stress-related mood disorders (Sandi, 2004; Sandi and Bisaz, 2007). It could be that disturbances in *Pcdhb6* induce the formation of improper synaptic connections following stress in *KAP1* knockout mice. Several of the other genes altered in the hippocampus of these animals are transcription factors with functions identified in some peripheral tissues but not in the central nervous system. *Smad3* is downstream target of TGF β and partakes in phenomena such as inflammation, hematopoiesis and tumorigenesis (Brown et al., 2007), and *Tcf5* (also known as C/EBP- β) is involved in interferon gene expression (Kalvakolanu and Roy, 2005). Targets of these two factors, however, have yet to be identified in neurons. Finally, one markedly increased transcript (*KORrt*), is predicted to encode an approximately 100 amino acids long protein completely identical to the N-terminal domain of the kappa opioid receptor. The function of this peptide, which in the full-length receptor sits on the outside of the membrane, is completely unknown. Nevertheless, it is interesting to note that the stimulation of the kappa opioid receptor by the endogenous opioid peptide dysphorin has recently been implicated in the dysphoric properties of chronic stress in the mouse (Land et al., 2008). It could be that functionally significant changes in gene expression also occurred at the level of single cells in the hippocampus of the knockout mice, rather than at that of the whole structure. Such changes would have escaped our analyses.

Although the results of behavioral tests in rodents should not be interpreted in an overly anthropocentric fashion, the stress vulnerability of *KAP1*-mutant mice suggests that *KAP1* and KRAB-ZFPs may modulate responses to stress in humans. Most individuals maintain largely normal psychological functions in face of stressful events, yet exposure to acute or chronic stress can have psychopathological consequences, for instance post-traumatic stress syndrome and depression. The biological bases for inter-individual differences in susceptibility to stress are poorly understood, but it is clear that complex interactions between genetic and environmental factors are at play (Krishnan et al., 2007). The identification of *KAP1* as a key player in stress vulnerability opens up new areas for investigation. Considering its large size and recent expansion, the human KRAB-ZFP gene family is indeed likely to exhibit significant levels of polymorphism, which could influence behavioral responses. Furthermore, elucidating the mechanisms by which *KAP1* modulates behavior may point to novel approaches for the treatment of psychiatric disorders.

EXPERIMENTAL PROCEDURES

Transgenic Mice

Generation and genotyping of mice with a floxed KAP1 allele have been described previously (Weber et al., 2002). The *CamKII α -CRE* (Line 1950) mouse was generated as previously described (Schweizer et al., 2003). The *CamKII α -CRE* (Line 1950) gives a broad pattern of recombination in forebrain neurons in the adult brain including the olfactory bulb, cortex, striatum and the hippocampal formation as visualized by breeding with the *Rosa26R-lacZ* reporter mouse (Soriano, 1999) (Figure S1C). The recombination pattern in the floxed KAP1 mice was similar to the R26R mice. The CRE-allele was genotyped with quantitative real-time PCR using SYBR green according to standard procedures. Transgenic mice were backcrossed to a C57/BL6-background for at least eight generations. To obtain mutant KAP1-mice, we bred *KAP1^{fllox/+}-CamKII α Cre^{+/+}* males with *KAP1^{fllox/+}-CamKII α Cre^{-/-}* females. The resulting offspring were born at the expected rate of the four different genotypes. For subsequent experiments, we also bred *KAP1^{fllox/+}-CamKII α Cre^{+/+}* with *KAP1^{fllox/+}-CamKII α Cre^{-/-}* females. *KAP1^{fllox/+}-CamKII α Cre^{+/+}* males are fertile but stop producing offspring earlier (after about three broods) compared to wild-type mice. All animal experiments were approved by the local veterinary office and carried out in accordance with the European Community Council Directive (86/609/EEC) for care and use of laboratory animals.

Histology

Mice were perfused with cold PBS (Gibco) followed by 50 ml of 4% paraformaldehyde (Sigma). The brains were removed and postfixed for 2 hr and then transferred to 25% sucrose solution. After calibration, brains were sectioned on a freezing-stage microtome at 30 μ m. For immunohistochemical staining, sections were first rinsed three times in PBS and were then preincubated in PBS containing 2.5% goat-serum and 0.25% Triton X-100. Following preincubation, sections were incubated with a monoclonal mouse anti-KAP1 antibody (Chemicon, MAB3662, diluted 1:1000) overnight at room temperature. The next day sections were rinsed three times in PBS and then incubated 2 hr with a fluorochrome secondary antibody (Cy3-goat anti-mouse, Molecular probes, 1:500). Sections were then rinsed 3 times and then mounted on gelatinized slides. DAPI (1:1000) was added at one of the last washes. Sections were coverslipped with DABCO (Sigma) and then analyzed with a Zeiss Axiovert 200M microscope.

In Vivo Injection of Lentiviral Vectors

KAP1^{fllox/+}-CamKII α Cre^{+/+} males were bred with *KAP1^{fllox/+}-CamKII α Cre^{-/-}* females. A total of 12 age-matched male offspring were used for viral injections (six of each genotype). Half of the mice (three of each genotype) received doxycycline (2 g/l) in their drinking water starting the week before surgery and kept until the end of the experiment. LVCT-tTRKAB lentiviral vectors were produced and titered as previously described (Szulc et al., 2006). The titer of the vector used in this experiment was estimated to 1 \times 10⁹ TU/ml as titered on 239T cells. The vector was diluted 1:2 in PBS before injection. Animals were anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg) before being placed in a stereotaxic frame. Injections were done with a 5 μ l Hamilton syringe. 1 μ l of lentiviral vector was unilaterally injected at a single site in the hippocampus. Coordinates were as follows: AP: -2 mm, L: -1.5 mm, V: -1.7 mm, TB: -2 mm. Four weeks after vector injection, mice were perfused and sectioned as described above. Following mounting of sections on gelatinized slides, GFP expression was analyzed using a Zeiss Axiovert 200M microscope. We found very similar results within the groups. In *KAP1^{fllox/+}-CamKII α Cre^{-/-}* that did not receive doxycycline, we never detected more than few (<10) GFP-expressing cells, while in all *KAP1^{fllox/+}-CamKII α Cre^{+/+}* animals we detected a large number of GFP-expressing cells.

Behavioral Testing

For the behavioral tests presented in Figures 3–5, *KAP1^{fllox/+}-CamKII α Cre^{+/+}* males were bred with *KAP1^{fllox/+}-CamKII α Cre^{-/-}* females. A total of 34 age-matched male offspring were used (18 mutant and 16 wild-type). All the behavioral results were confirmed in a second experiment where we bred

KAP1^{fllox/+}-CamKII α Cre^{+/+} males with *KAP1^{fllox/+}-CamKII α Cre^{-/-}* females. Heterozygous offspring had a similar phenotype as wild-type animals. This experiment also confirmed that the behavioral phenotype is not due to flanking sequences surrounding the CRE allele.

All animals, starting at 3 months of age, were exposed to the EPM, open field, novel object and 16 hole board, in this order with 1 week in between the tests. All behavioral testing took place during the light cycle (9 am to 2 pm). To eliminate odor cues, each apparatus was thoroughly cleaned with 4% acetic acid in tap water and dried after each animal. During all trials and tests, the trajectories of the mice were recorded with a vertically mounted camera and analyzed with a video tracking software (Ethovision 3.1.16, Noldus).

Anxiety-like behavior was assessed using the elevated plus maze (EPM) task, a standardized test for anxiety-like behavior in rodents. The maze consists of two opposite open arms and two opposite closed arms (30 \times 5 \times 14 cm) arranged at right angles. The arms extended from a common central platform (5 \times 5 cm) that gave access to all arms. The maze was elevated 70 cm above the floor under dim and dispersed light conditions. Mice were placed on the central platform facing a closed arm and allowed to explore the maze for 5 min. The total distance moved, time spent in the center, open and closed arms, number and latency of entries to the open and closed arms were analyzed. The total distance the mice moved served as indicator of spontaneous locomotor activity, while differences in the proportions of time spent in the open arms and in the center are used as measure of anxiety-like behavior.

Moreover, anxiety-like responses were also evaluated in a conflict paradigm, the novelty suppressed feeding (NSF), previously validated as a test for anxiety-like behaviors in mice (Gordon and Hen, 2004). This task was performed as described (Weisstaub et al., 2006). Briefly, mice were food deprived for 24 hr prior to the test. At the time of testing, a food pellet (familiar laboratory mouse chow) was placed on a white filter paper (12 cm diameter) located in the middle of the testing cage (50 \times 50 \times 30 cm, 450 luxes) covered with approximately 2 mm of sawdust bedding. At the beginning of the test, the mouse was positioned in one corner and allowed to explore for a maximum of 10 min. Latency to approach the pellet (touching the filter and pellet) was recorded. As soon as the mouse started eating, it was transferred to its home cage and allowed to free-feed for 5 min. The amount of home cage food consumption was recorded. Each mouse was weighed prior to and following food deprivation to establish whether any genotype effects on weight loss were present.

Locomotion and reactivity to an open field (OF) was assessed in a white quadratic box (50 \times 50 \times 37 cm) under dim and dispersed light conditions. The mouse is placed into the center of the field and allowed to move freely during 10 min. The total distance moved, frequency of entries to the center, time and percent time in the center of the OF were analyzed. Avoidance of the interior or "unprotected" area of the field is interpreted as an anxiety-like behavior. Measures of total distance are used as an index of activity.

Exploratory behavior was assessed using two behavioral paradigms, the novel object (NO) test and the 16 hole board (HB). The NO was performed immediately after the OF test. A small, metallic object (3 \times 1.5 \times 5 cm) was placed into the center of the open field while the mouse was inside. Mice were given then 5 min to freely explore the novel object. The time spent in the center and the periphery of the compartment, number and latency of entries to the center, total distance moved in the center and the whole compartment were analyzed. Percent time and distance the mice spent in the center, exploring the novel object were considered as indicators of "focused" exploratory activity.

The hole board HB (TSE, Bad Homburg, Germany) was used to further characterize the exploratory activity of the mice. It consisted of a light gray quadratic box (44 \times 44 \times 17.5 cm), which contained 16 holes (diameter 12.5 mm) evenly distributed over the floor (4 \times 4 holes, 9.5 cm between two holes). Each hole is fitted with a light barrier sensor to record visits to the hole (nose poking). Total distance traveled was analyzed. Animals were placed at the center of the board and allowed to freely explore it during 8 min. Total hole visits are considered as a measure for exploratory activity.

Cognitive abilities were studied either under normal conditions (i.e., "no prior stress condition": test given immediately after the battery of behavioral characterization described above) or after a 2 week period of subchronic mild stress ("stress condition"). Stress started 1 week after the behavioral characterization (i.e., HB test) and consisted of one stress session in which animals

were submitted to one electric shock (0.7 mA, 2 s) given 2 min after they were introduced in context A (a shocking chamber [24 × 20 × 20 cm, grill floor connected to a shock generator, Panlab, Spain]). On the subsequent day, all mice were re-exposed to Context A (stress reminder) for 5 min. On the second week, mice were again submitted to three electric shocks (0.7 mA, 2 s, 1 min inter-shock interval) in Context B (20 × 20 × 27.5 cm, SD. Instruments, USA) and then re-exposed three times over the following 2 days (two times on the first day and once on the second) for 8 min to context B without any further shock being delivered. Morris water maze training was performed 9 days after the last recalled session.

To evaluate spatial learning (hippocampus-dependent task) we used the Morris water maze test. The water maze apparatus consists of a large white circular pool (140 cm diameter) filled with opaque colored water (26°C ± 1°C). A platform (10 cm diameter) is submerged 1 cm under the water surface. The water maze is surrounded by gray curtains (25 cm from the pool periphery) containing several prominent visual cues. One day before training, all mice were habituated to the room, apparatus, and water by giving them a 2 min free swim trial without platform. Performance on this session was later evaluated for locomotion and swimming abilities. Spatial learning sessions were conducted on 3 consecutive days (days 1–3) with four trials per day and an intertrial interval of 15 min. Each trial started by introducing the mouse, facing the pool wall and with the aid of a cup, at one of five starting points, pseudorandomly counterbalanced between trials and days. The latency to find the platform is measured as an index of the learning and memory abilities of the animals, since no differences in swim speed were found between mice from the two genotypes. If a mouse did not find the platform within 60 s, it was gently guided toward it. Each mouse remained on the platform for 15 s before it was taken out with a grid and put back into a waiting cage. During all learning trials, the platform remained in the same position. On day 4, a 30 s probe test was conducted in order to test the spatial memory of the animals. For this probe test the platform was removed from the pool. Percent time the mice swam in the target and opposite quadrant were measured and served as an indication of spatial memory.

Statistical Analyses of the Behavioral Data

The Kolmogorov-Smirnov and Shapiro-Wilk test was used to test for normality. When normality was accepted, parametric tests applied included Student's *t* tests (for mean comparisons of two groups and one single dependent variable measure) and analysis of variance (ANOVA) with (for HB data on 2 min blocks and training in the water maze for trials 2–12 representing learning and memory, with trial 1 excluded since it has none of these components) or without (probe test data for navigation time in the different quadrants for each genotype) repeated-measures, as appropriate. When significant differences were observed, post hoc analyses were performed with the PLSD Fisher test. Genotype was set as independent variable. When normality was rejected, the nonparametric Mann-Whitney U test was applied. The criterion for significance for all analyses was *p* < 0.05. All data are expressed as mean ± SEM.

RNA Studies

Whole hippocampi were dissected and snap-frozen on dry ice. Tissue was homogenized by sonication. Total RNA was isolated using Trizol (Invitrogen) according to supplier's recommendations. For real-time PCR analysis RNA was treated twice with DNase (DNAfreeTurbo, Ambion). 0.5 µg of RNA was used for the reverse transcription performed with random primers (Invitrogen) and SuperscriptII (Invitrogen) according to supplier's recommendations. SYBR green quantitative real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) using standard procedures. Data was quantified using the $\Delta\Delta C_t$ -method and were normalized to Gapdh and β -actin expression. All data shown is normalized to Gapdh. Actin-normalized data were very similar to Gapdh data. Primers were designed using PrimerExpress software (Applied Biosystems). The efficiency of all primer pairs were confirmed by performing reactions with serially diluted samples. The specificity of all primer pairs were confirmed by analyzing the dissociation curve. Primer sequences are found in Table S5.

For the microarrays *KAP1^{fllox/+}-P-CamKII α CreP^{+/-}* males were bred with *KAP1^{fllox/+}-P-CamKII α CreP^{-/-}* females. Four male offspring of each of the four resulting genotypes were used for the experiment. All mice had been

through the entire behavioral testing. Total RNA was isolated as described above. RNA quality was verified by an Agilent Bioanalyser prior to labeling and hybridization onto Illumina-Mouse full genome arrays (Illumina). One sample (a *KAP1^{fllox/+}-P-CamKII α CreP^{-/-}* sample) was removed due to poor RNA quality. Analysis and normalization of expression data from the 46,628 transcripts was carried out using BeadStudio 3.1 (Illumina, San Diego, CA, USA). Cubic spline normalization was used for all samples. ANOVA analysis on the log₂ of the signal was applied to all samples using Matlab (Mathworks, MA, USA) and a selection base on the Beadstudio Detection (*p* value < 0.005) where used in order to select dysregulated transcripts. For the cluster analysis the expression of the selected genes were standardized using the *z* transformation (subtraction of the mean and divided by the standard deviation). The standard parameters in Matlab bioinformatic toolbox were used to create a dendrograms based on the expression levels of genes across the samples.

The design of the microarray allowed the use of three transcripts to validate and control the results. The *KAP1* probe were downregulated in *KAP1^{fllox/+}-P-CamKII α CreP^{+/-}*-samples and partially downregulated in *KAP1^{fllox/+}-P-CamKII α CreP^{-/-}*-samples (Table S1). However, the loss of *KAP1* signal in mutant mice are only 50% (confirmed by PCR analysis, Figure 6B) reflecting the heterogeneity of the samples. There were also two CRE-probes present in the array (5' and 3'). These two probes were upregulated in *KAP1^{fllox/+}-P-CamKII α CreP^{+/-}*-samples and *KAP1^{fllox/+}-P-CamKII α CreP^{-/-}*-samples (Table S1). The list of genes used for the cluster analysis is generated using an ANOVA (*p* < 0.005) by looking for differences in transcription levels among groups. In this list both *KAP1* and the two CRE probes are detected. After manual inspection of the list (Table S1), it was obvious to us that several of these genes were to be considered as false positives. To avoid this problem we filtered the data based on differences in the fold of expression level (1.5-fold) and significant differences among groups (*p* < 0.05) and generated volcano plots to visualize differences (Figure S4). Only when comparing *KAP1^{fllox/+}-P-CamKII α CreP^{+/-}*-samples to any of the other groups were we able to detect dysregulated transcripts (Figure S4), suggesting that the rate of false positives was likely to be very low. A potential problem is that several dysregulated genes are potentially missed and it is difficult for us to estimate the proportion of false negatives in our analysis. However, we have analyzed the expression of about 20 genes expressed in the hippocampus using real-time PCR. The PCR data of these genes were all in accordance with the microarray data suggesting that the rate of false negatives is not extremely high (data not shown).

For analysis of the real-time PCR data one-way ANOVA followed by Tukey's post hoc test was used. The criterion for significance for all analyses was *p* < 0.05. All data are expressed as mean ± SEM.

Chromatin Immunoprecipitation

ChIP of brain tissue was performed as previously described (Tsankova et al., 2004; Wells et al., 2002) with minor modifications. In brief, whole hippocampus was quickly dissected from decapitated mice and minced into about 1 mm pieces that were immediately frozen on dry ice and stored at -80°C until further use.

At the beginning of the procedure the tissue was fixed in 10 ml of 1% formaldehyde for 15 min at room temperature. Fixation was quenched by adding glycine at a final concentration of 0.125 M. The tissue was then rinsed three times with cold PBS containing protease inhibitors and was then twice homogenized for 10 s in 10 mM Tris, 10 mM NaCl, 0.2% NP40. The homogenate was centrifuged at 4500 × *g* for 5 min. The supernatant was removed and the cell pellet was then homogenized two more times using nuclear lysis buffer (ChIP kit number 17-295, Upstate Biotechnology). The cell mixture was diluted into 1 ml nuclear lysis buffer and sheared into about 300–700 bp pieces using a Branson digital sonicator 250. Each sample was sonicated on ice for four times, 20 s each, at 30% of maximum power.

Two hundred microliters of sonicated lysate were used for each ChIP reaction. One percent of the sample was saved as input-sample for normalization. The lysate was precleared with 80 µl salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate) and immunoprecipitated overnight at 4°C with 5 µg of antibody directed against acetylated H3 (Upstate, 06-599), acetylated H4 (Upstate, 06-866), trimethylated H3K9 (Abcam, ab8898), and trimethylated H3K27 (Upstate, 07-449). Chromatin-antibody complexes were collected with 60 µl of salmon sperm DNA/protein A-agarose beads for 1 hr. Beads

were sequentially washed with low salt, high salt, LiCl, and TE (twice) buffers (Upstate Kit). Chromatin was eluted with 500 μ l NaHCO₃/SDS buffer (Upstate Kit). The crosslink between DNA and histones was reversed in high salt conditions at 65°C overnight. Samples were then treated with Proteinase K for 1.5 hr at 55°C, extracted with phenol/chloroform, ethanol precipitated, and resuspended in 60 μ l H₂O. Levels of the various modified histones were determined by using quantitative real-time PCR (ABI Prism 7700). Primers were designed to amplify about 80 bp amplicons targeted to about 200 bp upstream of the transcription start site. Primer sequences are found in Table S5. PCR SYBR green quantitative real-time PCR reactions were run in triplicates with Power SYBR Green PCR Master Mix (Applied Biosystems) using standard procedures. Negative control reactions without antibody were run for each sample. To quantify the relative enrichment of each promoter sequence a Δ Ct for each sample was determined ($Ct_{Input} - Ct_{Sample}$). The relative enrichment was then calculated by raising 2 to the Δ Ct power and multiplying by 0.01. Relative quantification between KO and WT samples was performed by calculating a $\Delta\Delta$ Ct value for each pair of samples that were run in parallel ($KO\Delta Ct - WT\Delta Ct$). The fold difference was then determined by raising 2 to the $\Delta\Delta$ Ct power. The fold difference among pairs was then normalized to the Gapdh (H3ac and H4ac) or Globin values (H3K9 and H3K27). For statistical analysis one-way ANOVA followed by Tukey's post hoc test was used. The criterion for significance for all analyses was $p < 0.05$. All data are expressed as mean \pm SEM, based on the results of three complete independent experiments.

SUPPLEMENTAL DATA

The Supplemental Data include six figures and five tables and can be found with this article online at [http://www.neuron.org/supplemental/S0896-6273\(08\)00839-8](http://www.neuron.org/supplemental/S0896-6273(08)00839-8).

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