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Forebrain-Specific Knockout of B-raf Kinase Leads to Deficits in Hippocampal Long-Term Potentiation, Learning, and Memory

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Raf kinases are downstream effectors of Ras and upstream activators of the MEK-ERK cascade. Ras and MEK-ERK signaling play roles in learning and memory (L&M) and neural plasticity, but the roles of Raf kinases in L&M and plasticity are unclear. Among Raf isoforms, B-raf is preferentially expressed in the brain. To determine whether B-raf has a role in synaptic plasticity and L&M, we used the Cre-LoxP gene targeting system to derive forebrain excitatory neuron B-raf knockout mice. This conditional knockout resulted in deficits in ERK activation and hippocampal long-term potentiation (LTP) and impairments in hippocampus-dependent L&M, including spatial learning and contextual discrimination. Despite the widespread expression of B-raf, this mutation did not disrupt other forms of L&M, such as cued fear conditioning and conditioned taste aversion. Our findings demonstrate that B-raf plays a role in hippocampal ERK activation, synaptic plasticity, and L&M.

Key words: MEK-ERK; Ras; Cre-LoxP; conditional knockout water maze; contextual discrimination; fear conditioning; conditioned taste aversion

The Raf family of Ser/Thr kinases was originally identified as being protooncogenes. In mammalian cells, there are three Raf isoforms encoded by different genes: A-, B-, and C-raf (Bonner et al., 1985; Huleihel et al., 1986; Ikawa et al., 1988). Raf kinases are best characterized as the downstream effectors of Ras and upstream effectors of MEK-ERK (mitogen-activated protein kinase kinase-extracellular signal regulated kinase) signaling and are thought to mediate cell proliferation and differentiation. The three Raf kinases differ in their tissue distributions and biochemical properties, suggesting divergent physiological functions for each of these isoforms.

There are numerous studies implicating Ras and MEK-ERK signaling in learning and memory (L&M) and neural plasticity (Silva et al., 1997; Giese et al., 2001; Ohno et al., 2001; Adams and Sweatt, 2002; Dhaka et al., 2003). In recent years, a large body of evidence has demonstrated that Ras signaling pathways are involved in mechanisms of cognitive function, including transcriptional and translational controls of synaptic plasticity and memory (Atkins et al., 1998; Wu et al., 1999; Mazzucchelli et al., 2002; Kelly et al., 2003; Kelleher et al., 2004). However, it is still unknown whether Raf kinases in general, and B-raf in particular, play a role in bridging Ras and MEK-ERK signaling in plasticity and memory. Indeed, direct evidence for the involvement of Raf kinases in synaptic and behavioral memory is lacking. It is possible that different Raf isoforms may be involved in different processes of memory formation.

Among the three Raf isoforms, A-raf is mostly confined to urogenital organs (Storm et al., 1990). C-raf is ubiquitously expressed in both brain and peripheral tissues (Storm et al., 1990) and is the most extensively studied Raf with a defined role in the classic Ras-Raf-MEK-ERK pathway. B-raf is found predominantly in neural tissues, testis, and melanocytic and hematopoietic cells (Eychene et al., 1995). Compared with C-raf, B-raf is a stronger MEK-ERK activator. It has the higher MEK binding affinity and kinase activity and is necessary for long-lasting and sustained ERK activation (Wixler et al., 1996; Marais et al., 1997; Papin et al., 1998). In B-raf,

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but not in C-raf, null mutants, MEK activation is compromised in embryonic fibroblasts (Wojnowski et al., 2000; Huser et al., 2001; Mikula et al., 2001), suggesting that B-raf is the principal Raf kinase in the brain. Taken together, among the three Raf isoforms, B-raf is most likely to play an important role in L&M. Although C-raf is expressed in the soma of neurons, B-raf is present in both soma and neurites (Morice et al., 1999), supporting its potential involvement in plasticity. Indeed, B-raf is among a subset of hippocampal genes whose expression is triggered by the induction of long-term potentiation (LTP) and behavioral training (Morris water maze), suggesting a role for this gene in these processes (Thomas et al., 1994; Richter-Levin et al., 1998).

Furthermore, B-raf has been found to turn on ERK following cAMP activation (Vossler et al., 1997) and to modulate the nerve growth factor (NGF)-induced ERK pathway (Jaiswal et al., 1994). B-raf may be regulated by Rap1, another signaling molecule that, like Ras, has GTPase activity and mediates Rap1-dependent ERK activation invoked by cAMP and Ca^{2+} (Grewal et al., 2000; Morozov et al., 2003). These findings suggest that B-raf may be a critical nodal point for inputs mediated by Ras and other signaling molecules, such as cAMP. To determine the role of B-raf in plasticity and memory, we used a reverse-genetic approach. Because B-raf is essential for embryonic development (Wojnowski et al., 1997), we generated a conditional line of forebrain-restricted B-raf null mutant mice. Our studies demonstrate that B-raf kinase is involved in hippocampal synaptic plasticity and hippocampus-dependent L&M.

MATERIALS AND METHODS

Generation of *B-raf$^{f/f}$*, *Tg(αcre)*, and *Tg(αcre)B-raf$^{-/-}$* Mice

The floxed B-raf mice (*B-raf$^{f/f}$*) mice were generated by flanking a 1.2-kb targeting region including exon 12 with two LoxP sites. R1 embryonic stem cells (ES; 129/Sv background; Nagy et al., 1993) were transfected with the linearized targeting construct and screened by Southern blot analyses for correct homologous recombination. Mutant lines were established from male chimeras and bred into the 129/SvEms background. The *Tg(αcre)* mice (FVB/J background) were generated in the transgenic facility at the State University of New York at Stony Brook. The Cre gene with a nuclear localization signal (NLS) was driven by a 8.5-kb fragment of α-*CaMKII* promoter (Mayford et al., 1996a,b). The *Tg(αcre)B-raf$^{-/-}$* mice were F2 progeny from a cross between the *B-raf$^{f/f}$* and the *Tg(αcre)* mice. Genotypes of mice were determined by PCR analysis of tail DNA samples. It is important to note that, in all of the experiments mentioned here, we only used *Tg(αcre)B-raf$^{-/-}$* mice that were at least 6 months old to ensure the completeness of the Cre-mediated deletion. Expression data suggested increasing levels of deletion and Cre expression between 2 and 6 months. Consistently, we also found much milder behavioral and physiological phenotypes at 2 months in the knockout mice (data not shown).

Western Blot Analysis

Tissue samples were dissected rapidly from decapitated mice and homogenized. Samples containing equivalent amounts of protein, determined using a Pierce BCA protein assay kit (Pierce, Rockford, IL), were subjected to electrophoresis in 10% SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked before being incubated with the primary antibodies for 2 hr at room temperature. Membranes were washed and incubated with corresponding HRP-conjugated secondary antibodies (1:2,000; Bio-Rad) for 1 hr at room temperature. Signals were visualized by enhanced chemiluminescence (ECL Plus) as recommended by the manufacturer (Amersham, Arlington Heights, IL). The primary antibodies used were against: 1) B-raf C-terminal (C-19), 2) B-raf N-terminal (F-7), 3) C-raf (1:800; Santa Cruz Biotechnology, Santa Cruz, CA), 4) the dual phospho-Thr202/Tyr204 ERK p42/p44, 5) total ERK p42/p44 (1:1,000; New England Biolabs, Beverly, MA), and 6) β-actin (1:3,000; Sigma-Aldrich, St. Louis, MO). For the activity-induced ERK activation analysis, mice were placed into a conditioning chamber; 120 sec later, they received three foot shocks (0.75 mA, 2 sec) with a 58-sec intertrial interval (ITI). Then, mice were allowed to stay in the chamber for another 2 min before placed back in their home cages. Brain tissue was isolated 30 min after training.

Immunocytochemistry

Mice were anesthetized with chloral hydrate and intracardially perfused with saline (0.9%), followed by 4% paraformaldehyde in 0.1 M PBS. Brains were removed and postfixed with the same fixative overnight and cryoprotected with 30% sucrose in PBS until sectioning (40-μm coronal). For fluorescent immunocytochemistry, free-floating sections were blocked in PBST buffer [0.2% Triton X-100, 5% bovine serum albumin (BSA), 1% normal goat serum] for 2–4 hr at room temperature, then incubated with primary antibodies (β-galactosidase; 1:1,000; ICN Pharmaceuticals, Worthington, OH), or Cre recombinase (1:800; Covance, Berkeley, CA) in PBST (2.5% BSA) overnight at 4°C. Sections were then washed in PBST and incubated with fluorescent-conjugated secondary antibodies (Alexa 594 goat anti-rabbit or mouse IgG, 3 μg/ml; Molecular Probes, Eugene, OR) for 2 hr at room temperature. Finally, sections were washed and mounted with Vectashield mounting medium containing DAPI (Vector, Burlingame, CA). Immunoreaction was visualized using a fluorescence microscope. To determine whether the mutation produced gross morphological changes, parasagittal sections from adult mutant and WT brain were stained with cresyl violet. Regular light microscopic immunocytochemistry was performed with an antibody against Cre (1:10,000; Covance) with NiDAB staining, and an antibody against GAD-65/67 (1:3,500; Chemicon, Temecula, CA) with DAB staining.

Behavioral Analysis

In all behavioral experiments, nonknockout littermates of the mutants were used as the controls, and the experimenter was blind to the genotype of the mice studied. Mice were kept on a 12:12 hr light-dark cycle, and all behavioral experiments

were always conducted during the light phase of the cycle. All the behavioral procedures used were approved by the University of California at Los Angeles (UCLA) Animal Research Committee. The UCLA Franz Hall animal facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care, and the animals are maintained in accordance with the Animal Welfare Act and the Department of Health and Human Services (DHHS) guide. Statistical analyses of behavioral data were via one-way ANOVAs or two-way ANOVA with repeated measures.

Morris Water Maze

The water maze apparatus and procedures were previously described (Bourtchuladze et al., 1994). In the hidden-platform version of this task, the platform location is fixed for the training period and is submerged under opaque water. Mice were trained with 2 trials per day (1 min ITI) for 14 days. Spatial L&M was assessed in 60-sec probe tests conducted at the end of training on days 10 and 14, in which the platform was removed from the pool. The search pattern of the mice was recorded by a CCD camera and analyzed by using VHS software. The visible version of water maze was conducted after the hidden-platform test. Mice were trained to search for a marked platform for 2 days with three trials per day. The platform was placed at a different location in each trial.

Contextual Discrimination

The apparatus and procedures used were previously described (Frankland et al., 1998). The contextual discrimination experiment consisted of three stages: preexposure (1 day), training (1 day), and testing (2–3 days). On each day, mice were placed in chamber A in the morning and chamber B in the afternoon (or vice versa); order (chamber A vs. B) for each day was counterbalanced across groups (WT vs. mutant). On day 1, mice were preexposed to both chambers for a total of 10 min each, during which time no foot shocks were delivered. On day 2 and the following testing days, mice were placed in the chambers for 180 sec each day. In chamber A, a foot shock (2 sec, 0.75 mA) was delivered 150 sec after placement. No foot shock was delivered in chamber B. The percentage of time the mice spent freezing (absence of all but respiratory movement) during the first 150 sec (preceding the shock delivery in A) in both chamber A and chamber B was recorded by using automated procedures described previously (Anagnostaras et al., 2000).

Contextual and Cued Fear Conditioning

Separate groups of mice were placed in the conditioning chamber for 2 min before the onset of the 30-sec tone CS (2,800 Hz and 85 dB). In the last 2 sec of the CS, the US (a foot shock of 0.75 mA, 2 sec) was delivered. Mice were left in the conditioning chamber for an additional 2.5 min and were replaced back in the home cage. A test of contextual conditioning was performed 24 hr after training, in which the percentage of time mice spent freezing in the context in which they were previously shocked was assessed. Cued conditioning was tested 48 hr after training. Mice were placed in a novel context for 2 min (pre-CS test), after which they were exposed to the CS for 3 min (CS test). In all experiments described here, delivery of the CS and US and measurements of freezing were done with an automated procedure described elsewhere (Anagnostaras et al., 2000). In the slow-acquisition contextual conditioning paradigm, mice were given a foot shock (0.40 mA, 1 sec) 4 min after being placed into the chamber. Then, they were allowed to stay in the chamber for an additional 2 min before being placed back in their home cages. The same procedure was carried out once per day for 4 days. We measured freezing during the first 4 min before the onset of the shock. Extinction was tested on days 5–8. Each day, mice were placed in the same chamber for 30 min with no shock delivered.

Conditioned Taste Aversion

On days 1–4 of the experiment, mice were given limited access to water, and conditioning was carried out on day 5. Forty minutes following access to saccharin (0.1%, novel taste, for 15 min), mice received an IP injection of either lithium chloride (LiCl, 1.0 M, 2% of body weight, as malaise-inducing agent) or PBS as a control. Twenty-four hours later, mice were tested for conditioned taste aversion (CTA). They were presented with two bottles, one containing water and the other saccharin. The placement of water/saccharin bottles during conditioning and testing was pseudorandom. The bottles were weighed before and after the test, and the amount of liquid consumed was calculated. An aversion index (=amount of saccharin consumed/total amount of fluid consumed) was used to report CTA. A low score in the aversion index indicates learning.

Electrophysiology

All electrophysiological experiments were performed blind to genotype. Transverse hippocampal slices (400 μm thick) were maintained in a submerged recording chamber perfused with artificial cerebrospinal fluid (ACSF) equilibrated with 95% O_2 and 5% CO_2 at 30°C. The ACSF contained 120 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM D-glucose. Extracellular field excitatory postsynaptic potentials (EPSPs) were recorded with a metallic electrode from the stratum radiatum layer of the area CA1, and the Schaffer collaterals were stimulated with 100-μsec test pulses via a bipolar electrode. For synaptic input–output curves, different stimulation strengths (20–100 mA in steps of 10 mA) were applied. In the following experiments, the intensity of stimulation was adjusted to give field EPSP approximately 33% of maximum. Paired-pulse facilitation was tested by measuring the percentage increase in the slope of the second field EPSP in relation to the first one (20-, 50-, 100-, 200-, and 500-msec interpulse intervals). LTP was induced by a single tetanus delivered according to a high-frequency protocol (100 Hz, 1 sec) or a 2-theta-burst tetanus stimulation protocol (each burst consists of four pulses at 100 Hz with a 200-msec interburst interval). Before tetanization, the responses were monitored for at least for 20 min to ensure a stable baseline EPSP slope. To determine whether the magnitude of LTP differed significantly among the groups, responses from the last 10-min block of recordings (40–50 min for 2-theta-burst LTP; 80–90 min for 100-Hz LTP) were compared with a one-way ANOVA.

RESULTS

Generation of Conditional B-raf Knockout Mice

Developmental deficits can confound the interpretation of knockout studies of adult brain function. B-raf is known to be involved in cell survival during development and mice with a homozygous global B-raf disruption are not viable (Wojnowski et al., 1997). To circumvent these and other related concerns, we used the Cre-LoxP system to generate a conditional B-raf knockout. Two mouse lines were generated for this strategy: a *B-raf*$^{f/f}$ "floxed" line and a *Tg(αcre)* transgenic "deleter" line expressing Cre recombinase specifically in postnatal forebrain (Fig. 1A,B). By crossing these two lines of mice, we were able to derive a homozygous brain-specific B-raf knockout restricted to excitatory neurons of the forebrain.

We floxed exon 12, which is the first exon that encodes the B-raf kinase domain. It also contains a critical GXGXXG motif that is common to all Ser/Thr kinases (Bossemeyer, 1994). The deletion of exon 12 led to a shift in the open reading frame (ORF) and resulted in a null mutation of B-raf, in that no protein was detected by antibodies against either the N-terminal or the C-terminal portion of the protein in Western blot analysis (data not shown). Western blot analysis also demonstrated that the insertion of loxP sites per se did not affect B-raf expression, insofar as the B-raf protein level was normal in *B-raf*$^{f/f}$ mice (data not shown).

To achieve regional and cell-type specificity, we derived *Tg(αcre)B-raf*$^{-/-}$ mice with the Cre recombinase gene under the regulation of a postnatally active α-*CaMKII* promoter (Mayford et al., 1996a,b). As previously reported by others (Tsien et al., 1996), the Cre transgene driven by the α-*CaMKII* promoter is expressed specifically in forebrain postnatal excitatory neurons. Immunocytochemical analysis of line *Tg(αcre)1557* brain slices with an anti-Cre antibody showed that Cre expression is confined to forebrain structures, i.e., cortex, striatum, hippocampus, amygdala, but absent in other brain regions, such as midbrain and cerebellum (Fig. 2A, and data not shown). Within hippocampal structures, Cre is highly expressed in the CA1 region and less so in CA3 and dentate gyrus of line *Tg(αcre)1557*. Furthermore, immunohistochemical staining showed that Cre is expressed in cells negative for both glutamic acid decarboxylase (GAD) and glial fibrillary acidic protein (GFAP), which suggests that Cre is specifically expressed in excitatory neurons (Fig. 2C–F). This deleter line was used in subsequent experiments.

To examine Cre-mediated gene deletion in brain, *Tg(αcre)1557* mice were crossed to a *lacZ* reporter mouse line (*cAct-XstopXlacZ*; Tsien et al., 1996; Zinyk et al., 1998). This reporter line carries a transgene in which the chicken actin promoter drives the expression of a *lacZ* gene, whose transcription and translation are prevented by a stop site floxed by LoxP sites. Cre-mediated recombination deletes the stop site and activates *lacZ* gene expression. In mice that possess both the Cre transgene and the *lacZ* reporter, the *lacZ* gene is activated in forebrain as a result of Cre-loxP recombination. In agreement with the Cre expression pattern, immunocytochemical analysis with a β-galactosidase antibody showed that Cre-dependent *lacZ* expression occurred in forebrain, but not in other brain structures, such as cerebellum and midbrain (Fig. 2B, and data not shown).

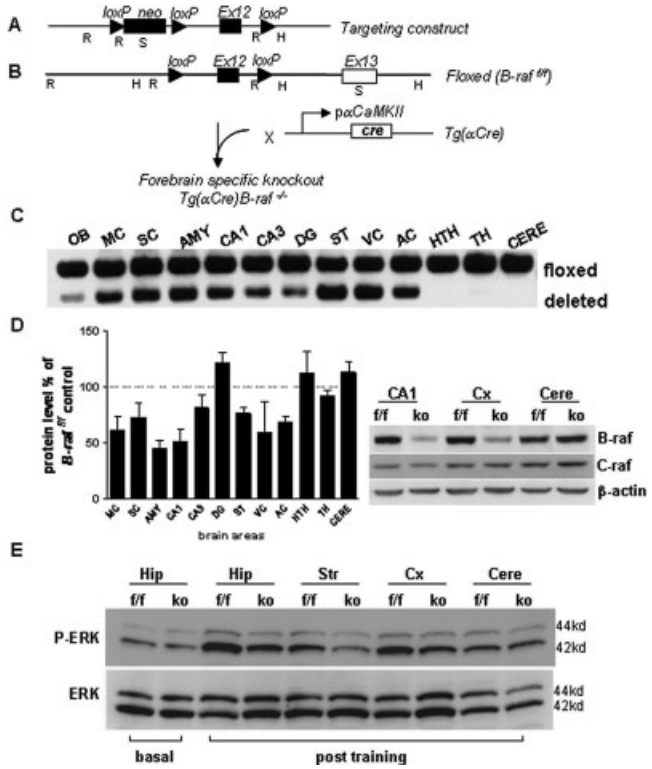


Fig. 1. Generation of the *Tg(αcre)B-raf*$^{-/-}$ knockout (ko) mice. **A:** Scheme of the targeting construct. A 1.2-kb fragment containing exon 12 of B-raf was floxed by loxP sites. A *neo* gene was used as an ES cell selection marker and deleted in a late stage of ES cell culture. H, HindIII; R, EcoRI; S, StuI. **B:** Scheme of the cross between the floxed mice (*B-raf*$^{f/f}$) and *Tg(αcre)* transgenic mice to produce the forebrain specific B-raf knockout [F2 homozygous *Tg(αcre)B-raf*$^{-/-}$]. **C:** PCR analysis of B-raf deletion in microdissected tissues of the *Tg(αcre)B-raf*$^{-/-}$ knockout mouse. The upper band (413 bp) results from the floxed B-raf gene and the lower band (282 bp) from Cre-mediated deletion. OB, olfactory bulb; MC, motor cortex; SC, sensory cortex; AMY, amygdala; DG, dentate gyrus; ST, striatum; VC, visual cortex; AC, auditory cortex; HTH, hypothalamus; TH, thalamus; CERE, cerebellum. **D:** Western blot analysis of microdissected brain areas from the *Tg(αcre)B-raf*$^{-/-}$ knockouts with antibodies against B-raf, compared with that from the their non-ko littermates (*B-raf*$^{f/f}$). The percentage of B-raf protein level of their non-ko counterparts is plotted and shown at left. The right panel shows a Western blot of different brain regions from the ko and control mice (f/f) with antibodies against B-raf (95 kd) and C-raf (74 kd). β-Actin signal was used as loading control. **E:** Western blot analyses of ERK1/2 phosphorylation before and after training (conditioning). Mice were trained in a conditioning chamber with three foot shocks (0.75 mA, 2 sec, 1 min ITI). Samples from different brain regions were extracted 30 min after training and subjected to Western blot analysis by using an antibody against dual phospho-ERK p44/p42. The same membrane was also blotted with an antibody for total ERK1/2 proteins (lower panel). f/f, *B-raf*$^{f/f}$; ko, *Tg(αcre)B-raf*$^{-/-}$; Hip, hippocampus; Str, striatum; Cx, cortex; Cere, cerebellum.

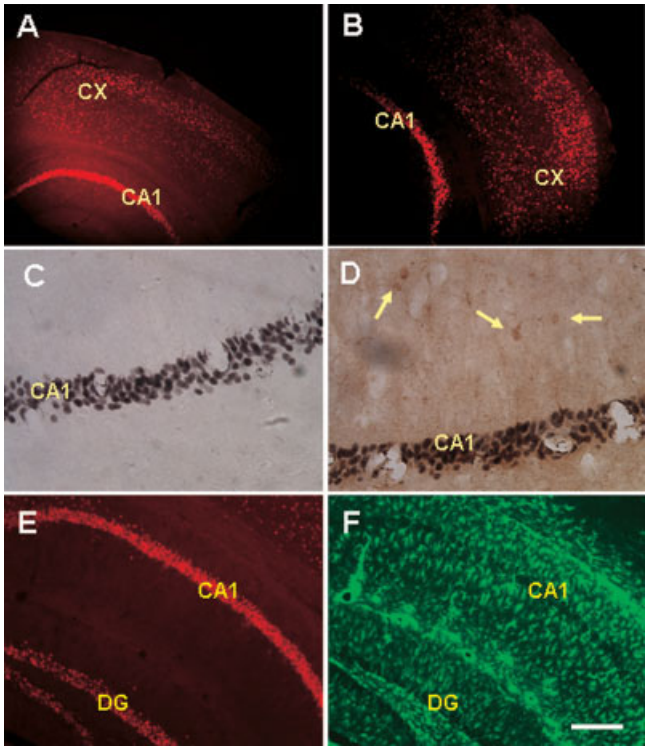


Fig. 2. Cre recombinase expression and activity in forebrain excitatory neurons. **A,B:** Fluorescent microscopy of Cre expression in *Tg(αcre)* mice (A, immunostained with an antibody against Cre) and Cre-mediated gene deletion in *Tg(αcre)LacZ* mice (B, immunostained with an anti-β-galactosidase antibody). CA1 and cortex regions are shown. LacZ expression indicated Cre activity. **C,D:** Cre expression was not colocalized with GAD expression. C: NiDAB staining of Cre expression in CA1. D: Double staining of Cre (black nuclear staining) and GAD (brown cell body and fiber staining; arrows). **E,F:** Cre expression was not colocalized with GFAP expression. Brain slices were immunostained with antibodies against Cre and GFAP that were visualized with different fluorescent-conjugated secondary antibodies. Cre is shown as red and GFAP as green; the same field is shown in E and F. Sacle bar = 250 μm in A,B; 40 μM in C,D; 125 μM in E,F.

Homozygous *Tg(αcre)B-raf*$^{-/-}$ knockout mice were obtained from a F2 cross between the *B-raf*$^{f/f}$ mice and *Tg(αcre)1557* mice. A PCR analysis of DNA from microdissected different brain subregions revealed that the deletion took place exclusively in the forebrain, including cortical, hippocampal, and amygdala regions, but not in cerebellum, thalamus, and hypothalamus (Fig. 1C). Western blot analysis on microdissected brain regions also showed that B-raf protein level is decreased in forebrain structures but not in midbrain and cerebellum areas. If anything, it appears that B-raf protein levels may be slightly increased in these other areas (Fig. 1D). Among hippocampal areas, B-raf is largely deleted in CA1 region and less so in other regions. It is important to note that, in all of the behavioral and electrophysiological experiments in this report, we used only mice that were at least 6 months old to ensure the completeness and stability of the Cre-mediated deletion of mice used in the experiments described here.

Disruption of B-raf Led to a Down-Regulation of the ERK Pathway

To examine whether the deletion of B-raf results in intracellular signaling deficits, we analyzed the activation of signaling proteins. Western blot analysis with antibodies against the phosphorylated ERK p42/p44 and total ERK protein was used to analyze ERK activity. Our results showed that the expression of total ERK protein was not changed in the *Tg(αcre)B-raf*$^{-/-}$ mutants compared with the control animals (β-actin blotting as loading control; data not shown). Interestingly, the basal levels of ERK phosphorylation were also not detectably altered in the mutants (Fig. 1E basal, and data not shown), perhaps because B-raf was deleted only in excitatory neurons, which constitute a small portion of the tissue sample examined. We observed that the basal level of ERK phosphorylation in hippocampus is much higher than in most other brain regions and organs (data not shown), which suggests that this kinase is normally engaged in hippocampal function.

Although ERK activity is low under basal physiological conditions, behavior training can elevate ERK phosphorylation (Atkins et al., 1998; Blum et al., 1999). Hence, contextual conditioning (with three mild foot shocks) resulted in an increase in ERK phosphorylation levels in several brain regions 30 min after training. Importantly, in forebrain structures, but not in cerebellum, this increase in ERK phosphorylation was clearly suppressed in *Tg(αcre)B-raf*$^{-/-}$ mutants but not in controls (Fig. 1E, P-ERK, posttraining). This result confirms that ERK signaling is activated after behavioral training and indicates that this activation is mediated by B-raf.

Deletion of B-raf Severely Disrupted Spatial Learning

To examine whether B-raf is involved in learning and memory, we studied the impact of our conditional B-raf mutation on several learning tasks, some of which are known to be dependent on hippocampal function. In our behavioral tests, *B-raf*$^{f/f}$ mice were used as the control for the *Tg(αcre)B-raf*$^{-/-}$ mice. To exclude the possible impact of each of the single transgenes on learning, we tested the *Tg(αcre)1557* mice, the *B-raf*$^{f/f}$ mice, and their respective wild-type littermates in the tasks described below. Our results demonstrated that each of the transgenes alone does not affect learning and memory (data not shown).

The Morris water maze is a sensitive and reliable measure of hippocampus-dependent learning (Brandeis et al., 1989). In this test, mice are placed in a pool with opaque water and must swim to a submerged platform to escape the water. In the hidden-platform version of the water maze test, the escape platform is located in a fixed location in the pool, and there are no objects directly indicating the platform location. Mice were trained on this task for 14 days with 2 trials per day. The results show that *Tg(αcre)B-raf*$^{-/-}$ mutant mice performed poorly compared with their *B-raf*$^{f/f}$ control littermates. Whereas the controls (n = 14) improved during training ($F_{13,169}$ =

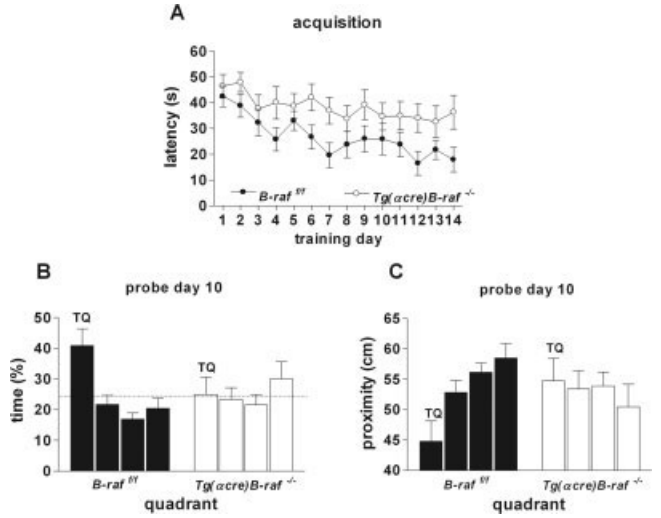
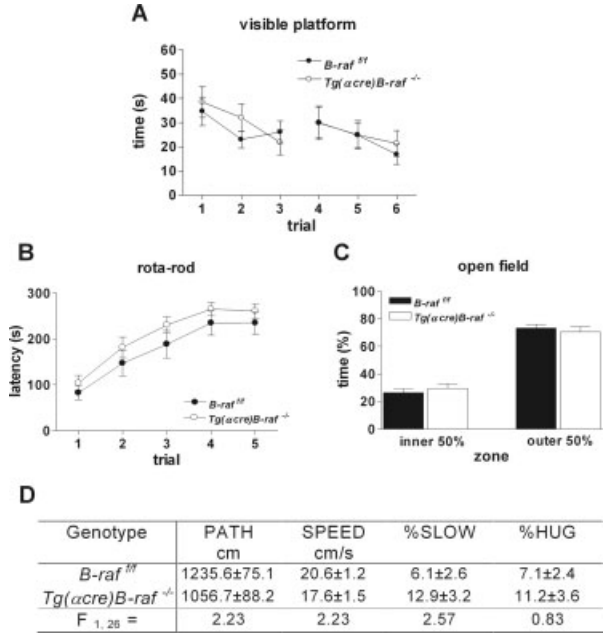


Fig. 3. Impaired spatial learning in conditional B-raf knockouts. The *Tg(αcre)B-raf*$^{-/-}$ knockouts and *B-raf*$^{f/f}$ littermate controls were trained for 14 days with two trials per day in the Morris water maze. The average latency to reach the hidden platform is plotted for each day in **A**. Results from the probe trial at day 10 are shown in **B** (% time spent in each quadrant) and **C** (proximity to the platform). TQ, target quadrant. The other three bars represent the results for the adjacent left, right, and opposite quadrants (in that order).



Genotype	PATH cm	SPEED cm/s	%SLOW	%HUG
B-raf $^{f/f}$	1235.6±75.1	20.6±1.2	6.1±2.6	7.1±2.4
Tg(αcre)B-raf $^{-/-}$	1056.7±88.2	17.6±1.5	12.9±3.2	11.2±3.6
$F_{1,26}$ =	2.23	2.23	2.57	0.83

Fig. 4. Normal performance in the visible water maze, rota-rod, and open-field tests in conditional B-raf knockouts. **A:** After the hidden-platform water maze, mice were tested in the visible-platform version of this test for 2 days, with three trials per day. Average latencies to reach the platform are plotted. **B:** Rota-rod test for motor coordination. Mice were tested with 4–40 rpm accelerating speeds (maximal duration 300 sec) for five trials with 30-min intertrial interval. Average duration for each group is presented. **C:** Open-field test for exploratory activity. Mice were allowed to explore an open field for 5 min (diameter 1 m). Percentage time animals spent exploring the innermost 50% vs. outmost 50% areas of the field is shown. **D:** Performance measures from the water maze day 10 probe trial are presented: path length (path; cm), swimming speed (speed; cm/sec), percentage time spent swimming slower than 5 cm/sec (%slow), and percentage time spent hugging the outmost 10% area of the pool (%hug). The F values of one-way ANOVA analysis for each group are shown. None of the measures above are significantly different between mutants and controls ($P > 0.05$).

3.64, $P < 0.05$), mutants (n = 14) showed poor acquisition of the task ($F_{13,169} = 1.53$, $P > 0.05$). The acquisition for mutants is significantly slower than that for controls ($F_{1,26} = 5.80$, $P < 0.05$; Fig. 3A). Because the time to find the platform during training is known to be a poor measure of spatial learning (Brandeis et al., 1989), we used probe trials to evaluate the performance of the mice. In these probe tests, the platform was removed and the mice were given 60 sec to search for it. Probe tests were given at days 10 and 14 of training. In the probe trial at day 10, *B-raf*$^{f/f}$ controls spent a significantly higher percentage of time searching within the target quadrant compared with other quadrants ($F_{3,39} = 7.12$, $P < 0.05$), but the *Tg(αcre)B-raf*$^{-/-}$ mutants did not ($F_{3,39} = 0.63$, $P > 0.05$; Fig. 3B). Similarly, analysis of the average proximity to the exact platform location during the probe trial (Gallagher et al., 1993) showed that, whereas control mice searched significantly closer to the target platform position than to other, nontarget positions ($F_{3,39} = 13.0$, $P < 0.05$), the mutant mice showed no preference for the target location ($F_{3,39} = 1.13$, $P > 0.05$; Fig. 3C), confirming the deficit of the mutants.

To determine whether abnormalities in motivation, motor coordination, or vision account for the deficits in spatial learning, the same animals were tested in the visible platform of the water maze, a hippocampus-independent task in which mice have to locate a platform marked with a visible cue (Cho et al., 1999). The time taken to reach the visible platform was not different between the groups ($F_{1,19} = 1.40$, $P > 0.05$; Fig. 4A), and no differences in swimming speed, floating, or thigmotaxic behavior were observed between the *Tg(αcre)B-raf*$^{-/-}$ mutants and the *B-raf*$^{f/f}$ controls (day 10 probe trial shown in Fig. 4D). Additionally, knockout animals also did not show deficits in either motor coordination in a rota-rod test ($F_{4,80} = 0.14$, $P > 0.05$; Fig. 4B) or exploratory activity in an open-field test ($F_{1,21} = 0.41$, $P > 0.05$; Fig. 4C). These data suggest that the poor performance of the conditional knockouts in the hidden-platform version of the water maze was due to deficient spatial L&M, rather than impaired motivation, motor coordination, or vision.

B-raf Deletion Impaired Contextual Discrimination

We used the contextual discrimination task to address the possibility that the water maze deficits of the mutants are due to abnormal hippocampal function. Contextual discrimination tests a mouse's ability to distinguish between two similar contexts, and, as with the water maze, it is very sensitive to hippocampal lesions (Frankland et al.,

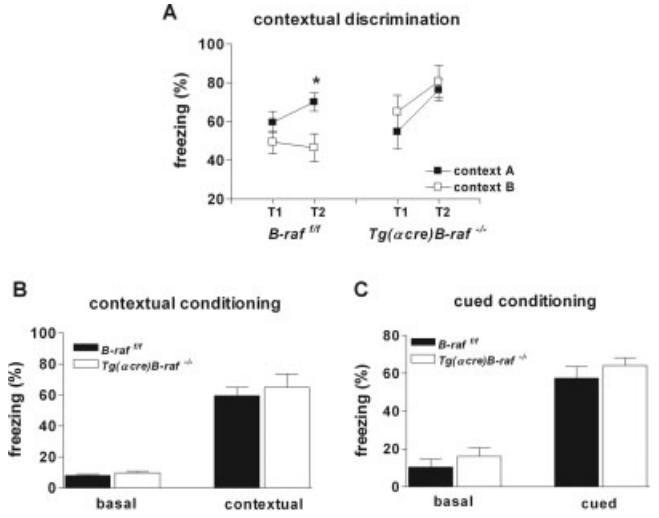


Fig. 5. Impaired contextual discrimination in conditional B-raf knockouts. **A:** Contextual discrimination. Percentage time the mice spent freezing in chamber A (shock context, solid squares) vs. chamber B (no-shock context, open squares) is shown for each of the 3 test days. *Significant difference, $P < 0.05$; T1–3, test days 1–3. **B:** Contextual conditioning in chamber A measured 24 hr after the mice were initially shocked. **C:** Cued conditioning test with naïve mice. Mice were trained with a shock-tone pairing (0.5 mA, 2 sec) and tested for cued freezing 24 hr later.

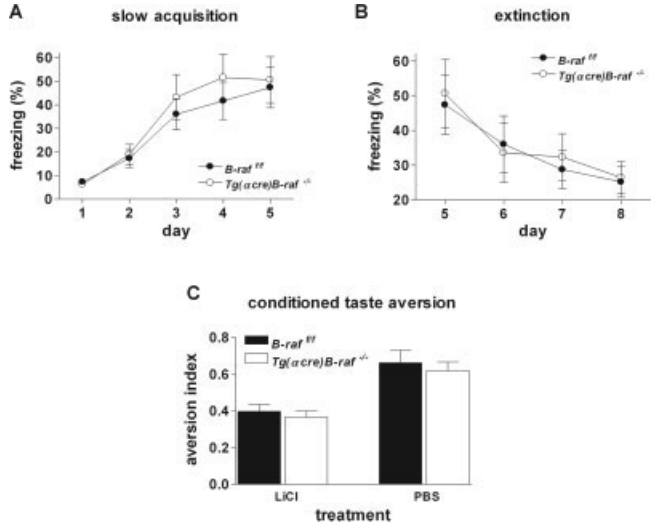


Fig. 6. Normal acquisition and extinction of contextual conditioning as well as intact conditioned taste aversion in conditional B-raf knockouts. **A:** Slow acquisition of contextual conditioning. Mice were trained with a milder (0.4 mA, 1 sec) foot shock each day for 4 days. Average freezing during the first 4 min before the onset of shock is presented (no shock on day 5). **B:** Extinction of contextual conditioning. Starting upon completion of the fifth day of acquisition, mice were allowed to stay in the conditioning context for 30 min (no shock was delivered). Extinction was continued on days 5–8. Average freezing during the first 4 min in each day is presented. **C:** Conditioned taste aversion. Aversion index is shown for the B-raf mutants and their littermate controls (*B-raf*$^{f/f}$) treated with malaise-inducing LiCl. PBS was used as a treatment control. Aversion index = saccharin consumed/total water and saccharin consumed.

1998). In this test, animals are required to discriminate between two similar chambers, one in which they receive mild foot shocks (chamber A) and the other in which they do not (chamber B). The two contexts consisted of both unique and common cues. Contextual discrimination is assessed by measuring the time mice spent freezing (i.e., the absence of bodily movements aside from respiration) in each chamber. The data in Figure 5A demonstrate a clear impairment of the knockout mice in the contextual discrimination task. The *B-raf*$^{f/f}$ control mice (n = 19) learned to discriminate between the two contexts. On test day 2, they showed significantly less freezing in the no-shock context, B (46.5% ± 7.2%), than in the shock context, A (70.1% ± 4.7%; $F_{1,18} = 20.3$, $P < 0.05$). In contrast, *Tg(*α*cre)B-raf*$^{-/-}$ mutants (n = 13) responded similarly to both contexts (i.e., 80.7% ± 8.3% freezing in context A vs. 76.3% ± 5.7% in context B; $F_{1,12} = 0.44$, $P > 0.05$; test day 2).

It is noteworthy that, although the *Tg(*α*cre)B-raf*$^{-/-}$ mice could not discriminate between contexts, shock reactivity and freezing responses were normal in these mutants. For example, when the mice were brought back to context A 1 day after being given the foot shock (test day 1 in context A), knockout mice and their control littermates exhibited comparable levels of contextual conditioning ($F_{1,30} = 0.02$, $P > 0.05$; Fig. 5B). Additionally, in a cued conditioning test with naïve mice (one tone-shock pairing; 0.5 mA, 2 sec), *Tg(*α*cre)B-raf*$^{-/-}$ mice showed normal levels of response compared with wild-type controls ($F_{1,17} = 0.45$, $P > 0.05$; Fig. 5C). This result is consistent with previous findings indicating that the hippocampus is not always essential for contextual conditioning (Frankland et al., 1998), and it also suggests that amygdala function is intact in the knockouts. Importantly, contextual discrimination is more sensitive to hippocampal lesions than contextual conditioning (Frankland et al., 1998), a result consistent with deficient hippocampal function in the mutant mice.

B-raf Deletion Did Not Disrupt the Formation and Extinction of Contextual Conditioning

To test whether learning is generally affected in the *Tg(*α*cre)B-raf*$^{-/-}$ knockouts, we tested them in a sensitive fear-conditioning protocol. In this procedure, animals received a very mild foot shock (0.4 mA, 1 sec) every day for 4 days. The mice exhibited gradually increasing freezing responses. As shown in Figure 6A, both the *Tg(*α*cre) B-raf*$^{-/-}$ knockouts (n = 12) and the controls (n = 11) showed low baseline freezing and gradually higher freezing responses over the 4 training/test days. Consistently with the results presented above, we found no differences between the freezing responses of the mutants and controls ($F_{1,21} = 0.25$, $P > 0.05$). This gradual increase in freezing responses over the 4 days of training confirmed that the lack of differences between mutants and controls was not due to a ceiling effect. It is important to note that, although acquisition of contextual discrimination is

always dependent on hippocampal strategies, contextual conditioning is not, insofar as mice can use nonhippocampal strategies in this task (Frankland et al., 1998).

To test whether the B-raf disruption affected extinction, the same group of contextually conditioned mice was reexposed to the conditioning context for 30 min each day for 4 days. The results show that the rate of extinction of freezing responses was also normal in the knockouts ($F_{1,21} = 0.02$, $P > 0.05$; Fig. 6B).

B-raf Knockouts Showed Normal CTA Task

Since tone and contextual conditioning was unaltered by the deletion of B-raf, it is possible that the forebrain excitatory neuron-specific B-raf knockout that we derived does not affect either amygdala- or cortex-dependent learning. To test this possibility further, we examined the mutant mice in the CTA test, a paradigm known to be dependent on amygdalar and cortical function, but not on the hippocampus (Welzl et al., 2001). Previous studies in rats suggest that novel tastes activate ERK activity and that MEK inhibition can block CTA (Berman et al., 1998). In the CTA task, mice were trained to associate saccharine flavored water with an IP injection of malaise-inducing LiCl. The aversion index (I = saccharin consumed/total fluid consumed) reflects a mouse's choice between saccharin and water after conditioning. A low index indicates good learning, in that it reflects a strong association between saccharin and LiCl. Consistently with a specific role for B-raf in hippocampal-dependent learning, we found that B-raf conditional knockouts did not disrupt the acquisition of CTA (Fig. 6C).

As expected, both knockout (n = 3, I = 0.67 ± 0.08) and control mice (n = 4, I = 0.71 ± 0.09) injected with PBS (instead of LiCl) showed no avoidance of saccharin (Fig. 6C, PBS treatment). In contrast, both *Tg(αcre) B-raf*$^{-/-}$ (n = 8, I = 0.37 ± 0.07) and control mice (n = 13, I = 0.39 ± 0.03) injected with LiCl exhibited an equally strong avoidance of saccharin ($F_{1,19} = 0.40$, $P > 0.05$; Fig. 6C, LiCl treatment). Taken together, the behavioral data presented above indicate that our B-raf mutation specifically disrupts hippocampus-dependent learning.

Hippocampal CA1 LTP Is Impaired in B-raf Conditional Knockouts

Because the B-raf mutants showed clear hippocampus-dependent learning deficits, we determined whether these mutations also disrupted hippocampal LTP, an experimental model of the synaptic changes thought to underlie L&M. Studies in aplysia demonstrated the involvement of MAPK signaling in later stages of long-term synaptic facilitation (Martin et al., 1997), and a number of mammalian studies implicated MAPK signaling in early stages of LTP. For example, previous studies reported ERK activation during LTP induction in various brain structures, including the hippocampal CA1 region, and inhibition of the ERK pathway results in impairments in the early phase of LTP (e-LTP; English and Sweatt, 1996, 1997). Thus, we focused our LTP studies on e-LTP.

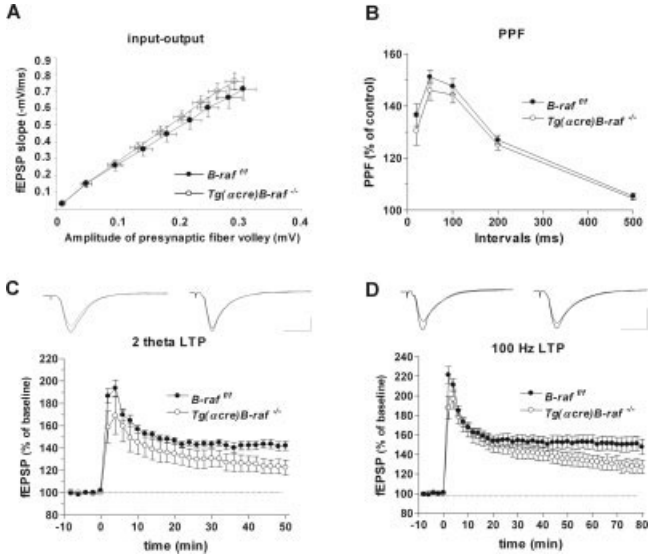


Fig. 7. Impaired hippocampal CA1 LTP in conditional B-raf knockouts. **A:** The B-raf mutants showed normal input–output curves. Field EPSP slopes are plotted with respect to their presynaptic fiber volleys induced by different strengths of stimulation (20–100 μA in steps of 10 μA). **B:** The mutants also showed normal paired-pulse facilitation. Percentage facilitations, calculated as the ratio of the slopes of the second vs. the first field EPSPs, are plotted for interpulse intervals of 20–500 msec. **C,D:** LTP induced by either a 2-theta-bursts (C) or by a 100-Hz (D) tetanus. Each point indicates the field EPSP slope (mean ± se) normalized to the average baseline response before the tetanus delivered at time 0; traces (control, left; mutant, right) are the average of fEPSPs recorded during baseline and 40–50 min (for 2-theta-burst LTP) or 70–80 min (for 100-Hz LTP) after tetanization. Calibration bars = 10 msec, 0.5 mV.

The relationship between evoked fiber volleys and field EPSPs (fEPSPs) was indistinguishable between the *Tg(αcre)B-raf*$^{-/-}$ mutants (n = 12) and their *B-raf*$^{f/f}$ controls (n = 13; Fig. 7A). Additionally, paired-pulse facilitation was also indistinguishable between the knockouts (n = 11) and the control mice (n = 12), suggesting that this forebrain- and neuron-specific knockout did not disrupt either synaptic transmission or presynaptic plasticity (Fig. 7B). Altogether, these results indicate that the conditional mutation of the B-raf gene does not compromise the general health of hippocampal slices, synaptic transmission, or certain measures of presynaptic plasticity.

e-LTP was tested with extracellular field recordings in stratum radiatum of hippocampal slices and was studied with a 2-theta-burst tetanus, because this pattern of stimulation is thought to reflect normal hippocampal patterns of activation during learning (Ranck, 1973; Otto et al., 1991). Our results demonstrate that e-LTP was deficient in the mutants. Between 40 and 50 min after the tetanus, the mutants (n = 6 mice) showed 123.8% ± 6.5% potentiation, whereas control mice (n = 6 mice) showed 143.6% ± 3.8% ($F_{1,10} = 6.21$, $P < 0.05$; Fig. 7C). Furthermore, LTP induced with a 100-Hz (1-sec) tetanus was also impaired in the mutants. Between 70 and 80 min after the tetanus, the knockouts (n = 9 mice) showed 128.1% ±

6.7% potentiation, and the control mice (n = 10 mice) showed 150.1% ± 7.5% ($F_{1,17} = 4.6$, $P < 0.05$; Fig. 7D).

DISCUSSION

In this study, we used forebrain conditional gene targeting to investigate the involvement of the B-raf kinase in synaptic plasticity and L&M. Our results demonstrate that, in the adult brain, B-raf is required for hippocampal synaptic plasticity and for hippocampus-dependent learning. Our findings also suggest that B-raf dependent activation of the ERK pathway in excitatory neurons is required for LTP and learning.

Our biochemical studies show that the region-restricted disruption of B-raf affects the levels of ERK activation in hippocampal neurons. Accordingly, just as pharmacological disruptions of ERK activation interfere with LTP (English and Sweatt, 1997), the B-raf null mutation in excitatory CA1 pyramidal neurons also impairs LTP. These results are consistent with the hypothesis that B-raf kinase is one of the upstream regulators of ERK activation and that this is important for synaptic plasticity. Basal synaptic transmission and paired-pulse facilitation, however, were normal in the B-raf mutants, suggesting that the loss of B-raf affects only certain aspects of synaptic function. Furthermore, LTP induced with different stimulation protocols were all affected in the conditional mutants, suggesting that B-raf is an universal component of LTP in the CA1 region of the hippocampus.

Consistently with a role for B-raf in hippocampal function, we found that B-raf knockout mice were impaired in contextual discrimination. In contrast, contextual and cued conditionings were normal in these mutants. Previous studies suggest that the contextual discrimination task is a more faithful measure of hippocampal function than contextual fear conditioning (Frankland et al., 1998), in that animals with hippocampal deficits can use nonhippocampal strategies to recognize the conditioning context. Instead of creating an integrated polymodal representation of context, they are thought to make several independent associations between shock and different cues in the context. The B-raf mutants showed seemingly normal freezing responses in the context in which they were trained, but, unlike their controls, they were unable to distinguish between similar contexts. These results strongly suggest that the disruption of B-raf specifically affects a hippocampal component of fear memory formation.

Cre-mediated disruption of B-raf protein was not restricted to the hippocampus. Previous studies have suggested that the inhibition of ERK activation affects neuronal function not only in the hippocampus but also in the cortex, striatum, and amygdala. For example, fear conditioning activates ERK in the amygdala, and amygdala-specific inhibition of ERK activation also blocks fear conditioning (Schafe et al., 2000). Loss of ERK1 leads to enhanced locomotor activity and enhanced active-avoidance L&M (Mazzucchelli et al., 2002) but does not affect fear conditioning or passive avoidance (Selcher et al., 2001). Microinjection of a MEK inhibitor into the insular cortex decreases ERK phosphorylation and impairs CTA (Berman et al., 1998), and infusion of a MEK inhibitor into medial prefrontal cortex no later than 4 hr after extinction training resulted in the return of conditioned fear response (Hugues et al., 2004). However, our studies show that the forebrain-specific B-raf disruption does not affect either acquisition or extinction of fear conditioning, nor does it disrupt CTA. These learning tasks are known to be sensitive to functional lesions of the amygdala and cortex. Therefore, these data demonstrate that B-raf is critical to L&M processes in the hippocampus but not amygdala or cortex. However, our findings do not contradict previous findings, insofar as these other manipulations targeted different subsets of cells and pathways. The apparent lack of an effect of the B-raf disruption on cortex and amygdala does not necessarily indicate that B-raf is not involved in amygdala and cortical function. For example, it is possible that B-raf activity in inhibitory neurons (but not in excitatory neurons, where the kinase was deleted in the experiments described here) is crucial for cortical and amygdala L&M. It is also possible that other pathways compensate for the loss of B-raf in the amygdala and cortex, but not in the hippocampus. Furthermore, previous studies demonstrated a specific activation of phosphatidylinositol 3-kinase (PI-3 kinase) in the amygdala after LTP induction and after fear conditioning (Lin et al., 2001). Importantly, PI-3 kinase inhibitors blocked ERK activation, LTP, and fear conditioning, suggesting that PI-3 kinase-dependent activation of the ERK pathway is critical for amygdalar synaptic plasticity and learning. Nevertheless, it is also possible that the specificity of our behavioral results reflects evolutionary specializations, with different variants of signaling pathways subserving unique functional requirements of individual brain regions.

What signaling processes activate the B-raf/ERK cascade during LTP and learning in the hippocampus? Ras is likely a strong upstream regulator, in that it has been shown that defective Ras-ERK signaling leads to learning deficits (Brambilla et al., 1997; Silva et al., 1997; Giese et al., 2001; Ohno et al., 2001). However, it is unknown whether Ras signals through B-raf during learning. Interestingly, the loss of neurofibromin (the product of the *Nf1* gene), a negative Ras regulator, causes synaptic plasticity and learning deficits, perhaps because of an increase in γ-aminobutyric acid (GABA)-mediated inhibition (Costa and Silva, 2002). Interestingly, although *K-* and *N-Ras* mutations were able to rescue the synaptic dysfunction and learning deficits of the Nf1 mutation (Costa and Silva, 2002), the B-raf mutation was not (Chen and Silva, unpublished results). This raises the possibility that neurofibromin and B-raf may be important for regulating Ras signaling in inhibitory and excitatory neurons, respectively.

Previous studies of L&M have demonstrated a universal role for cAMP-dependent signaling (Bailey et al., 1996). It is important to note that Rap1 couples cAMP signaling to ERK in synaptic plasticity and L&M (Morozov et al., 2003) and that Rap1 can regulate B-raf activity. It is, therefore, possible that B-raf contributes to LTP and

learning by mediating the activation of ERK in a cAMP/Rap1-dependent manner (Vossler et al., 1997; Dugan et al., 1999; Sweatt, 2001). Although further investigation is required, it is also possible that B-raf is a critical nodal point between Ras- and cAMP-dependent signaling in the ERK cascade during synaptic plasticity and learning. Our results demonstrate an important role for the B-raf kinase in hippocampal synaptic plasticity and L&M. They also suggest that, despite the widespread conservation of mechanisms of L&M among brain structures and species, there are molecular specializations that reflect the unique functional requirements of each memory system.

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