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Neuronal MEK Is Important for Normal Fear Conditioning in Mice

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The extracellular signal-regulated kinase (ERK) cascade has received much attention for its possible role in neuronal synaptic plasticity. Although ERK activation has been linked to learning behaviors and activity-dependent neuronal function, much of the acquired data has relied upon pharmacological agents that suppress ERK function in both neurons and nonneuronal cells. To determine the function of neuronal ERK activity in learning, a new line of transgenic mice was generated wherein dominant-negative MEK1, the upstream obligate activator of ERK1/2, was expressed by using a neuronal-specific and pan-neuronal Tα1 α-tubulin promoter element. Mice expressing this construct exhibited decreased ERK1/2 activity in the hippocampus and thus were tested for learning impairments. In a battery of control tests, including open field, rotarod, and shock threshold, the transgenic mice displayed no deficits and performed as well as their wild-type littermate counterparts. However, the mice displayed a significant impairment in contextual fear conditioning compared with the wild-type littermates. These findings indicate that the MEK1/ERK1/2 cascade within neurons plays an important role in the processes of learning and memory.

Key words: ERK; MAP kinase; learning and memory

The extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase cascade was initially discovered for its role in cell cycle regulation, and, indeed, within the central nervous system, ERK activation has been linked to neuronal survival and differentiation (Meyer-Frank et al., 1995; Anderson and Tolkovsky, 1999; Grewal et al., 1999). More recently, emerging roles for this cascade have been proposed for processes of learning and memory. Specifically, ERK is activated in the hippocampus in models of rodent spatial learning (Selcher et al., 1999; Blum et al., 1999) and contextual fear conditioning (Atkins et al., 1998), two hippocampus-dependent learning paradigms. In addition to the hippocampus, the amygdala also exhibits ERK activation following fear conditioning (Schafe et al., 2000; Radwanska et al., 2002), and insular cortex displays ERK activation after training in a conditioned taste-aversion paradigm (Berman et al., 1998; Swank, 2000; Swank and Sweatt, 2001). ERK activation also occurs in a variety of in vitro preparations. Phosphorylation of ERK occurs following long-term potentiation (LTP)-inducing stimuli (English and Sweatt, 1997; Coogan et al., 1999; Watabe et al., 2000; Dudek and Fields, 2001) and can be activated pharmacologically by a wide variety of neurotransmitters, growth factors, and second messenger systems (English and Sweatt, 1997; Vossler et al., 1997; Impey et al., 1998; Roberson et al., 1999; Rosenblum et al., 2000).

Although the importance of ERK in models of learning has been investigated by numerous groups, work to date has relied largely upon pharmacological methods to block ERK signaling (Atkins et al., 1998; Selcher et al., 1999; Blum et al., 1999). Pharmacological approaches have two inherent disadvantages: they have nonspecific effects, and, more importantly, they lack cell type specificity. Although MEK inhibitors are some of the more selective kinase inhibitors that exist, they can affect phosphorylation and activation of ERK5 (Kamakura et al., 1999). Likewise, pharmacologic MEK inhibitors cannot be targeted to act in specific cell types. ERK activation is known to occur in glial cells within the nervous system following a wide variety of stimuli, many of them the same

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stimuli known to activate ERK in neurons (Bayatti and Engele, 2001; Kinkl et al., 2001; Ma and Quirion 2002; Berkeley and Levey, 2003). Therefore, the use of MEK inhibitors cannot distinguish between contributions made by neuronal and nonneuronal cells in processes such as learning.

Genetic manipulations of the cascade have provided limited data. An ERK1 null mouse was shown to be phenotypically normal, with no deficits in learning or LTP (Selcher et al., 2001), and Mazzuchelli and colleagues (2002) reported a limited hippocampal phenotype in a similar ERK1 knockout mouse. This group reported a diminution of a form of ERK-dependent LTP, accompanied by an enhancement of striatal-dependent learning. Interestingly, the viability and limited phenotype of the ERK1 knockout contrasts greatly with the lack of success in attempts to generate an ERK2 null mouse (Adams and Sweatt, 2002).

To assess better the roles of both ERK1 and ERK2 within the central nervous system, we generated a mouse that expressed specifically in neurons a dominant-negative (DN) form of MEK1, the obligate upstream activator of only ERK1/2. The DN-MEK contained a mutation in the ATP-binding domain (K97M) of human MEK1, rendering it kinase inactive (Mansour et al., 1994). Prior to generation of the mouse, we confirmed that this construct suppressed ERK1/2 activity in cultured neurons. We drove transgene expression in the mouse with the neuron-specific and pan-neuronal Tα1 tubulin promoter, which is activated when neurons are born (Gloster et al., 1994; Bamji and Miller, 1996). The mice expressing the transgene exhibited decreased ERK1/2 phosphorylation in the hippocampus, were viable, and appeared developmentally normal. Baseline behaviors were normal, and there were no overt central nervous system defects. However, we observed a significant contextual fear conditioning deficit, indicating that neuronal MEK/ERK1/2 activity is required either during nervous system development or in the adult central nervous system for fear conditioning and contextual learning.

MATERIALS AND METHODS

Generation of Recombinant Adenoviruses

Mitogen-activated protein kinase kinase (MEK1) cDNAs encoding a hemaglutinnin (HA) epitope tag (Gloster et al., 1994) were kindly provided by Natalie G. Ahn (Howard Hughes Medical Institute, University of Colorado). Wild-type or DN-MEK1 constructs were cloned into the BamHI site of pAdTR5F (Massie et al., 1995). The vector was modified to express enhanced green fluorescent protein (EGFP) in a bicistronic fashion (see Fig. 1B). Two hundred ninety-three cells were transiently infected to confirm expression of MEK and GFP. Western blot analysis was performed on the infected 293 cell lysates using polyclonal anti-HA (1:1,000; Babco), and anti-GFP (1:2,000; Clontech, Palo Alto, CA). Recombinant adenoviruses were generated, amplified, purified, and titered as described previously (Mazzoni et al., 1999).

Cell Culture and Infection

Sympathetic neurons of the superior cervical ganglion (SCG) were dissected from P1 Sprague-Dawley rats and dissociated as described previously (Ma et al., 1992). Neurons were trypsinized in 0.01% trypsin, triturated, and cultured on collagen-coated 96-well plates (5,000 cells/well) or six-well plates (80,000–100,000 cells/well). Cultures were maintained in Ultraculture medium (BioWhittaker) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 2% rat serum (Harlan), 7 μM cytosine arabinoside (CA), and 10 ng/ml nerve growth factor (NGF; Cedarlane Labs) for 5 days. At 5 days, medium was removed, and adenovirus diluted in DMEM (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) and 10 ng/ml NGF, was added to the cultures for 20–24 hr at the appropriate multiplicity of infection (MOI; MOI = plaque-forming units per cell). After 20–24 hr, medium was removed, and cells were again maintained in ultraculture medium containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (serum-free, CA-free) and 10 ng/ml NGF for 2 days prior to analysis.

Western Blot Analysis

Cells were cultured and infected as described above. Three days postinfection, NGF was washed out by three 1-hr washes in ultraculture media (BioWhittaker). Cells were stimulated with medium alone or NGF for the indicated times to assess the effect of DN-MEK. Cells were washed with PBS and lysed in ice-cold Tris-buffered saline (TBS) lysis buffer (Vaillant et al., 1999). Lysates (25 μg) were boiled with sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a 0.2-μm nitrocellulose membrane (Schleicher and Schuell, Keene, NH), and probed with rabbit polyclonal p44/p42 phospho ERK at 1:5,000 (NEB, Boston, MA), rabbit polyclonal anti-Protein Gene Product 9.5 at 1:2,000 (Chemicon, Temecula, CA), and mouse monoclonal anti-HA at 1:1,000 (Babco) overnight at 4°C. p44/p42 Phospho-ERK and PGP9.5 blots were incubated with goat anti-rabbit IgG (1:5,000; Roche Diagnostics, Indianapolis, IN) antibody, whereas HA blots were incubated with goat anti-mouse IgG (1:5,000; Roche Diagnostics) antibody, for 1 hr at room temperature. Detection was performed by using ECL (Amersham, Arlington Heights, IL).

Survival Assays

Cells were cultured in a 96-well dish and infected as described above. Three days postinfection, NGF was washed out by three 1-hr washes in medium. After washout, cells were fed Ultraculture with either 10 ng/ml NGF or no NGF. Two days after treatment, cell viability was assayed by 3[4,5-dimethylthio-zol-2-yl]2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) as described previously (Vaillant et al., 1999). In brief, 20 μl MTT reagent was added to each well of the 96-well plate. The plate was then incubated for 2.5 hr at 37°C. Cells were next lysed with 2 μl/ml concentrated HCl in isopropanol. After 20 min at room temperature, the absorbances of the lysates at 570 and 630 nm were measured with a Biotek model EL_X-800 UV plate reader (Mandel Scientific Inc.). *Percentage survival* refers to the survival relative to the survival

supported by 10 ng/ml NGF (100%) under experimental conditions (triplicates, with n = 3).

Generation of Tα1:DN-MEK Transgenic Mice

A 1.1-kb Tα1 α-tubulin promoter element that confers neuron-specific and pan-neuronal transgene expression (described in detail by Gloster et al., 1994; Bamji and Miller, 1996) was used to drive the expression of an HA-tagged DN form of MEK bearing a K97M mutation (Mansour et al., 1994). This mutation is within the ATP-binding site, yielding a kinase-inactive mutant. The mutant MEK competes with endogenous MEK1 and -2 for binding to the activating kinases Raf-1 and B-Raf but cannot in itself phosphorylate and activate ERK1 and -2. The transgene construct was generated, and the transgenic mice were derived as described by Majdan et al. (1997). Three transgenic mouse lines expressing DN-MEK were established in a C3H background strain, and the line that exhibited robust DN-MEK expression was selected for further analysis.

Genotyping of Transgenic Animals

Tα1:DN-MEK mice were genotyped by using Southern blot analysis and/or polymerase chain reaction (PCR) on tail DNA extracts. For Southern blot analysis, genomic DNA was digested with EcoRI, separated on 1% agarose gels, transferred to nitrocellulose, and probed with a ^{32}P-labelled riboprobe directed against 2 kb of the promoter-transgene sequence, using standard techniques. For PCR analysis, tail DNA was extracted using DNeasy Mini Spin columns (Qiagen, Chatsworth, CA) as described by the supplier. Primers for the PCR annealed to the tα1 promoter region and to the MEK cDNA. Primers to amplify the 484-bp tα-HA-DN-MEK transgene were sense 5′-CAC CCA CCC CCG TTT TCT TTC TTC, antisense 5′-TTC TTC TTC GGC TGC GGG TAG G.

Reverse Transcription-PCR

Total RNA extraction was performed using RNeasy Mini Spin Columns (Qiagen) as described by the supplier. cDNA synthesis and amplification of specific genes were performed using the Calypso reverse transcription (RT)-PCR system (DNAmp Ltd.). Primers for the GAPDH control (450 bp) were sense 5′-ACC ACA GTC CAT GCC ATC AC, antisense 5′-TCC ACC ACC CTG TTG CTG TA. Primers to amplify the HA-DN-MEK transgene (464 bp) were sense 5′-ATA CGA TGT TCC AGA TTA CG, antisense 5′-CGC ACC ATA GAA GCC CAC GAT G.

Histological Processing

For histological analysis, we followed the procedure described by Majdan and colleagues (2001). Briefly, Tα1:DN-MEK mice and wild-type littermates were deeply anesthetized with pentobarbitone and transcardially perfused with vascular rinse (0.8% NaCl, 0.025% KCl, 0.005% $CaCl_2$, 0.05% $NaHCO_3$, and 100 mM $NaHPO_4$), followed by fixation in 4% paraformaldehyde (PFA). Brains were subsequently removed and postfixed in the same fixative before cryoprotection at 4°C in 30% sucrose Tris buffer before freezing on dry ice and storage at −80°C. Frozen coronal sections 35–40 μm thick were collected in TBS-azide buffer for immunohistochemical staining. Neutral red staining and brightfield microscopy were performed on 35-μm sections to assess brain morphology.

Immunohistochemical Staining

For each brain, a series of sections through the hippocampal formation and cortical mantle was stained to reveal the distribution of neurons expressing the DN-MEK transgene. Immunoperoxidase staining was performed as described by Majdan et al. (2001). Briefly, 40-μm sections were immunostained with a primary polyclonal antibody against the HA tag (Babco). Sections were treated in 0.3% hydrogen peroxidase in phosphate-buffered saline (PBS), pH 7.4, for 30 min and incubated for 24 hr at room temperature with rabbit polyclonal anti-HA tag antibody (1:150). Sections were then rinsed three times for 5 min each in PBS and incubated for 1 hr at room temperature with biotinylated goat anti-rabbit IgG (1:200; Jackson Immunoresearch Laboratories, West Grove, PA). Primary and secondary antibodies were diltuted in PBS with 3% normal goat serum and 0.25% Triton X. After incubation in secondary antibody, sections were rinsed three times for 5 min each in PBS and incubated in avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. Sections were rinsed three times for 5 min each in PBS and treated with 0.05% diaminobenzidine (DAB) tetrachloride, 0.04% nickel chloride, and 0.015% hydrogen peroxide in 0.1 M PBS. After reaction with DAB, sections were rinsed, dehydrated through a series of ethanol gradients, coverslipped, and viewed under brightfield optics.

Animal Behavior

Mice were housed with littermates on a 12-hr light/dark schedule. Experiments were performed in accordance with the Baylor College of Medicine Institutional Animal Care and Use Committee and with national regulations and policies. Behavioral experiments were performed on 12-week-old male and female animals back-crossed two generations into C57/Bl6. The experimenter was blind to genotype in all behavioral experiments.

Open-field test. Each animal was placed in the center of a clear plexiglass box (43 × 42 × 30 cm) under standard room-lighting conditions. The field was divided into 225 equally sized squares by 15 photoreceptor beams on each side of the arena. Locomotor activity was quantitated using Opto-Varimex optical animal activity system and Auto Track System (Columbus Instruments). Activity measures represent the number of horizontal and vertical photoreceptor beam breaks collected over the 15-min testing period. Data are presented as total distance traveled (cm) and number of times and time spent in the center and periphery of the apparatus.

Rotarod test. Motor coordination, balance, and motor learning were assessed by using the accelerating rotarod task as described elsewhere (Selcher et al., 2001). Each animal was placed on a rotating bar, and the time for which the animal was able to maintain its balance was recorded. Over the 5-min trial, the rod accelerated from 4 to 40 rpm. Mice underwent four trials per day for 2 consecutive days, with 30–45 min between trials.

Fear conditioning. Fear conditioning was performed as previously described (Selcher et al., 2001). Briefly, on the

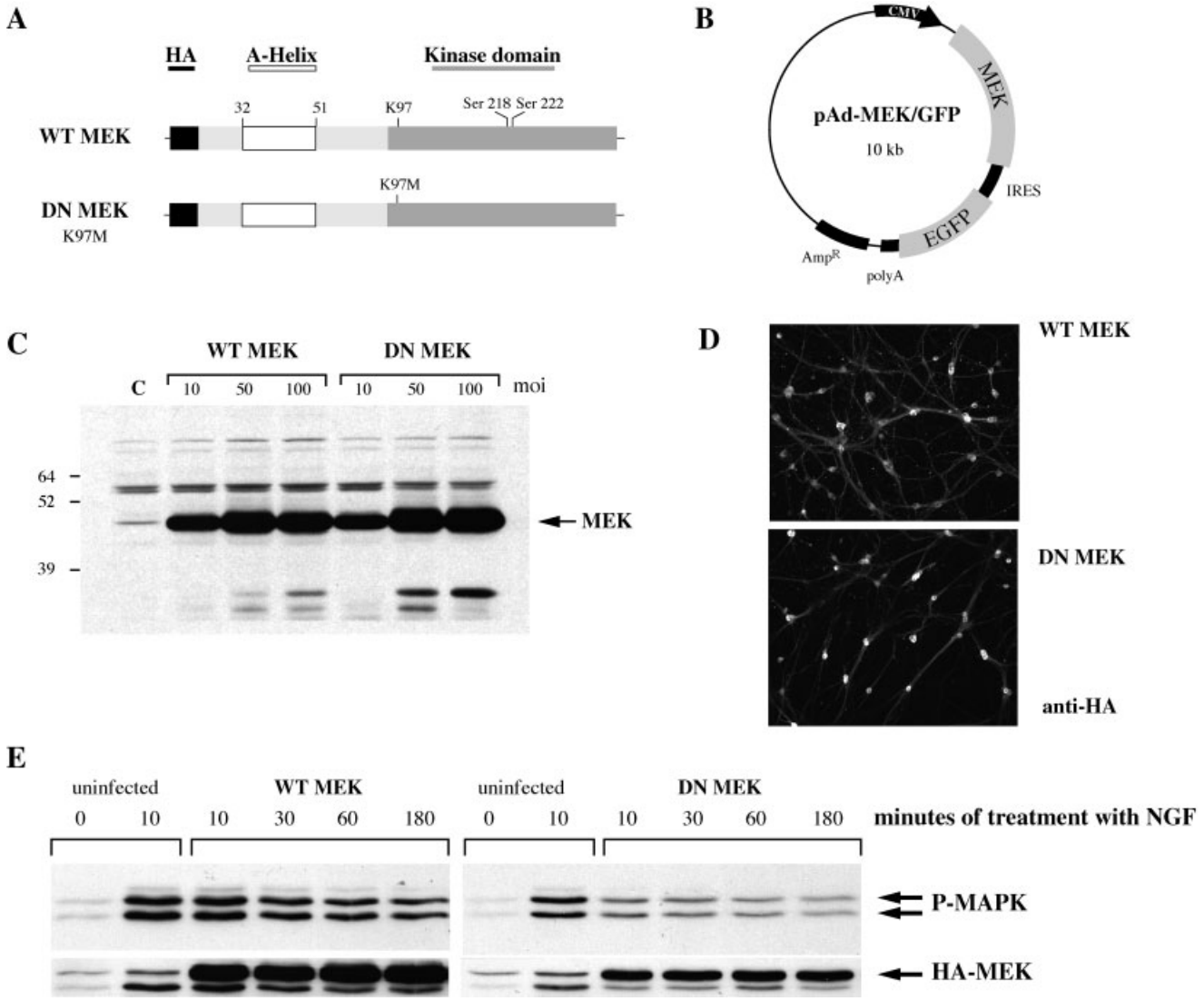


Fig. 1. **A:** N-terminally HA-tagged WT-MEK and the K97M DN mutant form of MEK. **B:** These MEK constructs were inserted into the adenoviral vector to be coexpressed with EGFP in a bicistronic fashion. **C:** Dose-dependent expression of exogenous protein in infected SCG neurons. **D:** Immunostaining against the HA-tag 3 days postinfection (MOI = 50) was performed to assess for transfection efficiency. Approximately 95% of neurons displayed cytosolic and neuritic transgene expression. **E:** Infected neurons were starved and subsequently treated with 50 ng/ml NGF to stimulate the ERK pathway. Cells were harvested after 10, 30, 60, or 180 min of treatment and probed for phospho-ERK levels. Cells expressing DN-MEK showed impaired p-ERK response.

training day, each animal was placed in the fear conditioning chamber and was allowed to explore for 3 min. A white noise (90 dB) was presented for 30 sec. During the last second of the cue, a mild foot shock (0.5 mA) was delivered for 1 sec to the metal floor grid. Two pairings of white noise and cue were administered, separated by 90 sec. Freezing behavior of the animals was measured during the 7-min training period by using a time sampling procedure whereby the animal was observed for approximately 1 sec every 5 sec. On the following day, learning was assessed by recording freezing behavior during a 5-min exposure to the fear conditioning chamber (context test) when no shock was administered. Cued fear conditioning was tested by placing the animal in a different test cage with altered cage dimensions, colors, and smells. The animal was allowed to explore the new cage for 3 min, followed by presentation of the auditory cue for 3 min, and freezing behavior was scored.

RESULTS

Expression of DN-MEK Decreases ERK Activation in Cultured Neurons

We first confirmed that the DN-MEK construct suppressed ERK/MAPK activity when expressed in cultured primary neurons. The HA-epitope-tagged K97M DN-MEK or wild-type MEK (WT-MEK; Fig. 1A) was

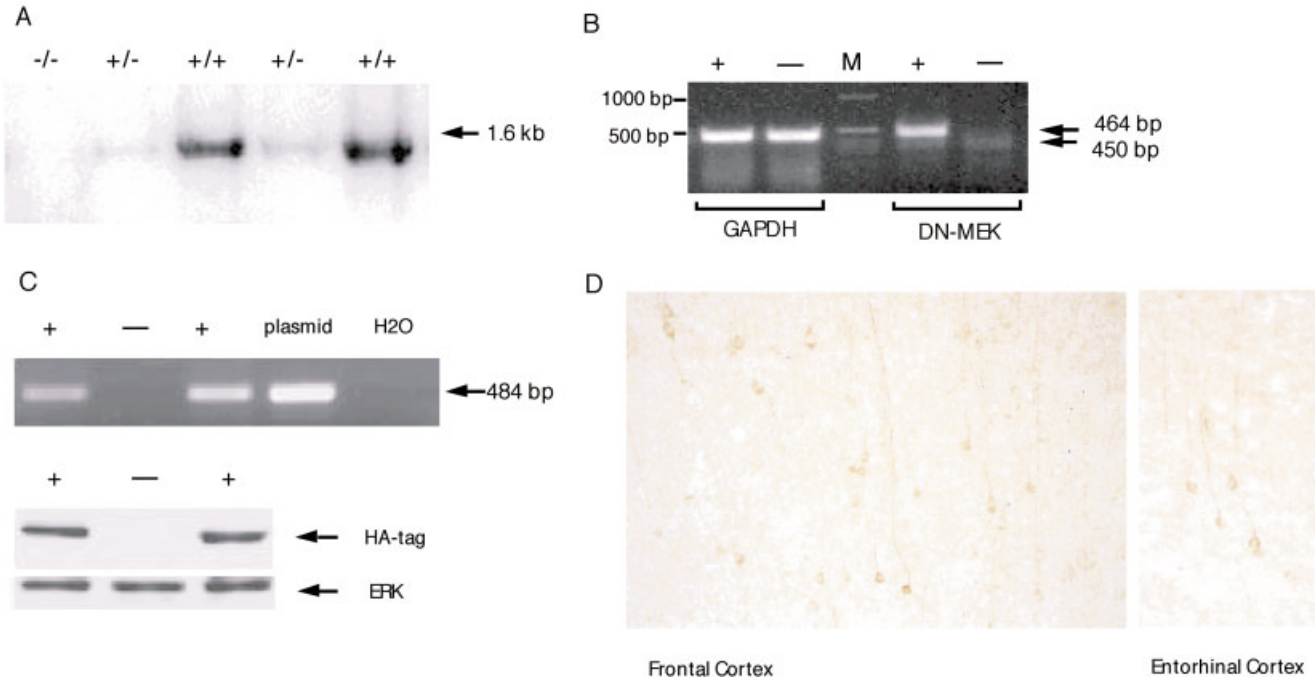
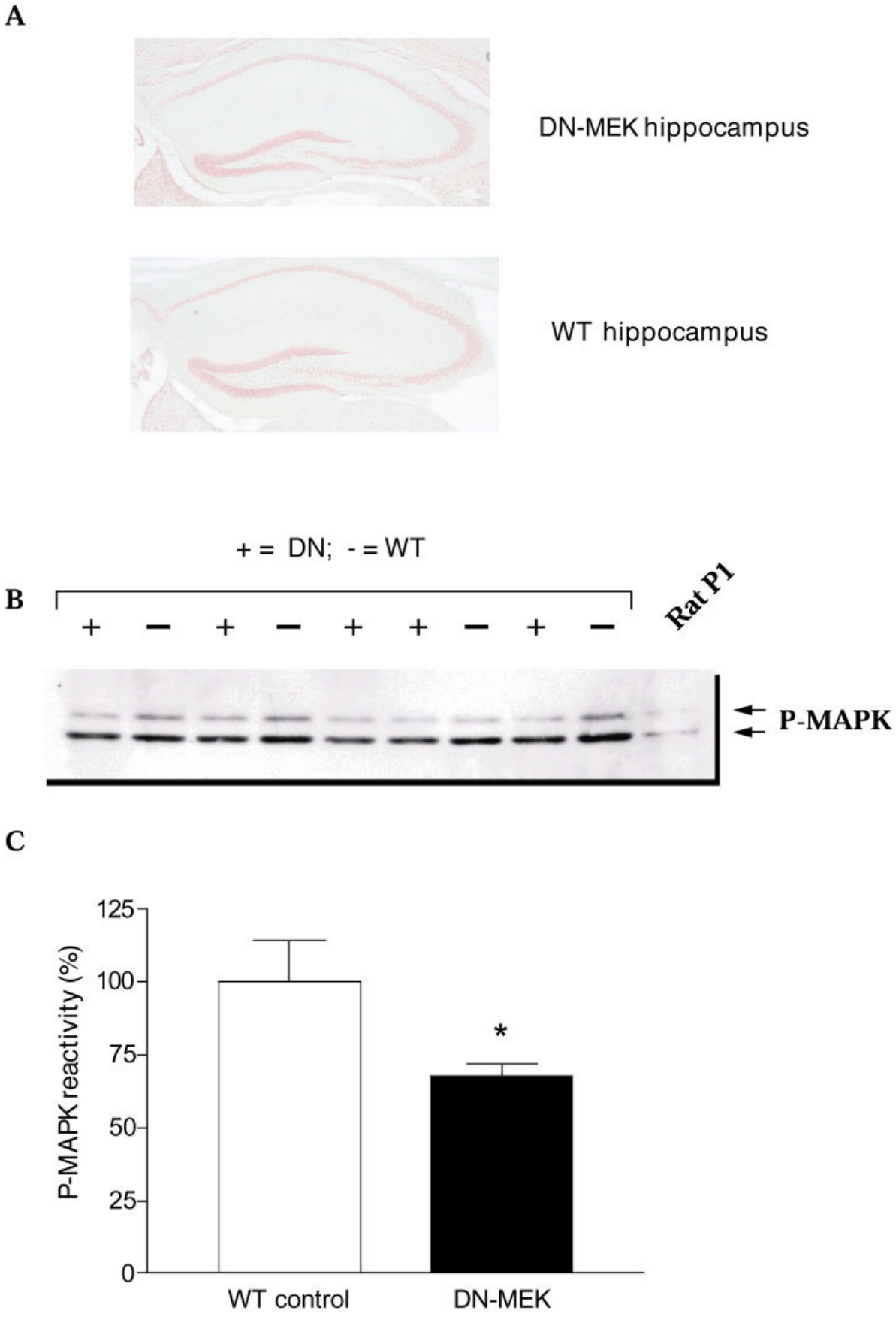


Fig. 2. Generation of Tα1:DN-MEK transgenic mice. **A:** Southern blot of tail DNA probed with a fragment spanning the promoter and DN-MEK region. **B:** Total RNA preparations from brain tissues analyzed by RT-PCR. GAPDH control primers detect the appropriate 450-bp product in both samples. Primers directed against DN-MEK show the expected product with RNA prepared from a transgenic (+) but not from a wild-type (–) animal. **C:** Genotyping of littermates via PCR amplification of tail DNA compared with the Western blot analysis of brain tissue taken from the same animals. A 1.5% agarose gel indicates a 484-bp PCR product with primers for Tα1 and MEK. A Western blot probed with antibodies against HA tag indicates the presence of the HA tag in transgenic (+) but not in wild-type (–) animals. **D:** Immunohistochemistry of brain sections localizes exogenous DN-MEK expression exclusively to neurons, with maximal expression in cytosolic compartments of frontal and entorhinal cortices. Figure can be viewed in color online via www.interscience.wiley.com.

inserted into an adenovirus vector (Fig. 1B) as described in Materials and Methods. Primary cultures of rat sympathetic neurons from the superior cervical ganglion (SCG) were infected with the recombinant adenoviruses. Both WT- and DN-MEK were expressed as assessed by Western blotting and immunofluorescence with anti-HA (Fig. 1C,D). The band recognized by the HA antibody in the uninfected cultures is a very faint background band that is always seen with this antibody in cultures. DN-MEK but not WT-MEK expression inhibited NGF-induced MAPK (ERK1/2) phosphorylation in the neurons (Fig. 1E). To assess whether DN-MEK suppressed neuronal survival, survival assays on the WT-MEK- and DN-MEK-infected neurons were performed with the MTT assay. DN-MEK did not alter the survival of the sympathetic neurons at 50 or 100 MOI (or virus particles per cell; data not shown), confirming results we have previously obtained in sympathetic neurons by using pharmacological MEK inhibitors (Mazzoni et al., 1999).

Expression of the Tα1:DN-MEK Construct in Transgenic Mice

After confirmation that the DN-MEK1 gene would inhibit MAPK activity when expressed in neurons, the gene was inserted into a vector that contained the Tα1 α-tubulin promoter, directing its expression exclusively to neurons. Transgenic mice were generated, founder animals were identified by Southern blotting (Fig. 2A) of tail DNA, and transmission of the transgene was verified in progeny of the founders by using the same technique. Confirmation that the DN-MEK transgene was expressed was obtained by RT-PCR analysis (Fig. 2B). Genotyping of the offspring was performed by using PCR analysis with specific primers for Tα1 and MEK. The expected 484-bp product was observed only with DNA prepared from transgenic animals and not from wild-type littermate controls (Fig. 2C). DN-MEK expression was also observed using Western blot analysis with anti-HA; DN-MEK was detected in transgenic mice with positive DN-MEK RT-PCR expression and not in wild-type littermate controls (Fig. 2C). To prove targeted expression of the transgene, we performed immunohistochemistry on brain sections with antibodies to the HA tag and localized exogenous protein in brain sections by using DAB immunostaining (Fig. 2D). The expression pattern in three independent lines was variable with respect to areas and expression levels, although in all lines transgene expression was observed only in neurons (Fig. 2D, and data not shown), as previously reported in studies with other transgenic mice



COLOR

Figure 3.

generated with this same vector and Tα1 α-tubulin promoter element (Gloster et al., 1994; Bamji and Miller, 1996; Majdan et al., 1997). For further analysis, we chose the line with the most robust neuron-specific expression of the Tα1:DN-MEK transgene.

DN-MEK-Expressing Animals Show Decreased Levels of Phosphorylated ERK1/2 in the Hippocampus

We focused on the hippocampal formation in the DN-MEK-expressing animals, because several studies using pharmacological MEK inhibitors have demonstrated a central role of the ERK pathway in the process of learning. Hippocampal morphology appeared normal in these animals as assessed by neutral red cell body staining (Fig. 3A). To determine whether ERK activity was suppressed in the hippocampus, we examined phospho-ERK1/2 levels. Lysates were prepared from the hippocampus from P4 animals, separated on SDS-PAGE, and probed with an activation-specific antibody to phosphorylated ERK1/2 (Fig. 3B). Animals expressing DN-MEK demonstrated significantly reduced phospho-ERK immunoreactivity compared with lysates prepared from the same region of wild-type littermates. The signal densities of phosphorylated ERK1 and ERK 2 were quantified (Fig. 3C). Animals expressing DN-MEK demonstrated approximately a 35–40% reduction in phospho-ERK1 and -2 immunoreactivity ($P < .05$ with unpaired Student's *t*-test).

Normal Behavioral Activity Levels in DN-MEK Mice

We next assessed whether the attenuated levels of phospho-ERK in the hippocampus of DN-MEK mice would result in impaired learning. Fear conditioning is routinely used as a paradigm for assessing learning, but it requires normal responses in both baseline sensory and motor functions. Behavioral characterization of the DN-MEK mice thus began with the open-field test, which monitors locomotor activity in both horizontal and vertical planes and provides an index of anxiety by calculating the ratio of distance traveled in the center of the open field to the distance traveled in the periphery.

DN-MEK mice displayed similar activity levels in the open-field test compared with their wild-type littermates. An unpaired Student's *t*-test revealed no difference between transgenic animals and wild-type littermate controls in the total average distance traveled or in the total average vertical activity in the open field ($P > .05$; Fig. 4A,B). These data suggest that the expression of DN-MEK does not cause hyper- or hypoactivity. Likewise, DN-MEK animals displayed similar center-to-total distance traveled ratios, suggesting that suppression of MEK activity has no effect on baseline anxiety levels ($P > .05$; Fig. 4C).

Motor Coordination and Learning Is Not Impaired in DN-MEK Mice

Motor coordination and motor learning were tested in the DN-MEK mice by using the accelerating rotarod task. The amount of time for which an animal can balance on the rotating rod was used as a measure of the animal's coordination, and improvements in the task over the course of eight trials was indicative of normal motor learning (Fig. 4D). The DN-MEK mice learned the rotarod task as demonstrated by their ability to balance on the rotating bar for longer periods with each successive trial (Fig. 4D). As would be expected, a two-way ANOVA was very significant for trial number [$F(7,296) = 5.45$, $P < .0001$]. More surprising was that the mice displayed a genotype effect [$F(1,296) = 5.74$, $P = .017$], indicating that the DN-MEK mice actually performed better than their wild-type littermate counterparts. However, this difference appeared to be due to the observation that the performance of the control mice plateaued (and worsened) on the second day of trials rather than continuing to improve, as is normally observed in wild-type mice (Selcher et al., 2001). There was no significant interaction between genotype and trial number observed in either DN-MEK or wild-type mice, suggesting that both sets of mice displayed similar improvements over time. Overall, these data suggest that the DN-MEK mice show normal motor learning and coordination.

Neuron-Specific DN-MEK Produces Specific Contextual Fear Conditioning Deficits

Prior studies have shown that infusion of pharmacologic agents that block MEK activity can cause impairments in the behavioral paradigm of fear conditioning. In fear conditioning, the animals learn to associate an unconditioned stimulus (a mild foot shock) with a conditioned stimulus (an auditory cue). If the fear conditioning occurs in a novel environment, animals also learn to associate the context of the new environment with the foot shock. Learning to fear both the context and the cue is manifested as increased freezing behavior in the animals when they are presented again with the cue or context. In the present experiments, 12-week-old mice were placed in a fear conditioning chamber and exposed to two pairings of white noise and a mild foot shock. Memory was assessed 24 hr later by measuring the amount of freezing that the mice displayed when placed in the fear conditioning chamber (contextual test) or when introduced to a new environment and presented again with the cue (cued test).

Fig. 3. Expression of the DN-MEK transgene impairs ERK activation in the hippocampus. **A:** Neutral red staining of fixed brain tissue from DN-MEK and wild-type littermates showed that hippocampal morphology was unaffected by the presence of the transgene. **B:** Western blot of hippocampal tissue samples from P4 littermates shows a decrease in phosphorylated-ERK1/2 (P-MAPK) in multiple DN-MEK-expressing transgenic animals (+, transgenic; –, wild-type littermates). Equivalent amounts of protein were loaded in each sample as determined by protein assay and by similar levels of Ponceau-S staining of protein bands in each lane of the Western blots. **C:** Quantified densitometry of the blot averaging the ERK1and ERK2 signals from the wild-type littermates (–) and DN-MEK (+) signals. There was a significant reduction in phospho-MAPK immunoreactivity in DN-MEK-expressing animals ($^{\star}P < .05$).

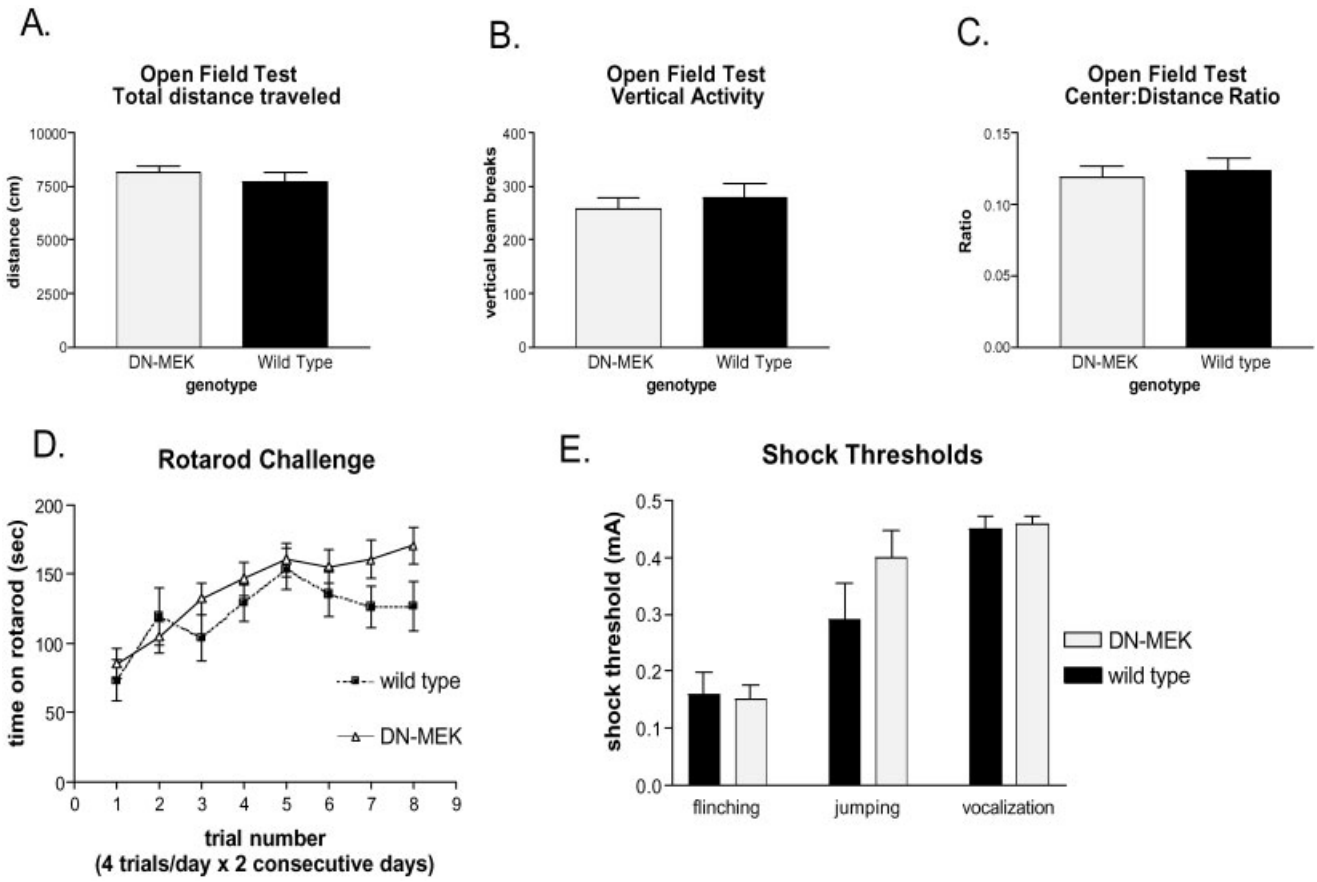


Fig. 4. Locomotor activity and anxiety are not affected by the DN-MEK transgene. **A:** Total distance traveled in an open field does not differ between DN-MEK mice (n = 28) and littermate controls (n = 11). **B:** Vertical activity is similar in both DN-MEK and wild-type animals. **C:** Center to distance ratio does not differ between DN-MEK and littermate controls. **D:** DN-MEK mice (triangles) showed better performance on the rotarod task than littermate controls (squares); however, this significant difference may be explained by the wild-type mice's failure to improve on the task on the second day of trials as is normally observed. Performance in all animals improved significantly over the course of the eight trials. **E:** DN-MEK animals had equivalent responses to different shock intensities. Amounts of flinching, jumping, and vocalization were similar in DN-MEK mice (open columns; n = 6) and littermate controls (solid columns; n = 6).

Mice expressing DN-MEK showed deficits in contextual fear conditioning relative to wild-type littermate controls (wild type 24.1% vs. DN-MEK 18.1%, $P < .05$; Fig. 5B). The DN-MEK mice and the wild-type controls were both able to sense the foot shock equally well, as demonstrated by equivalent levels of freezing during the training period (Fig. 5A). In addition, the DN-MEK and wild-type mice displayed equivalent responses of flinching, jumping, and vocalization in response to varying intensities of foot shock, indicating that there were no sensory deficits in the mice (Fig. 4E).

It is interesting to note that cued fear conditioning was normal in the DN-MEK mice. When placed in a new environment and presented with the cue, DN-MEK mice and the wild-type littermate controls showed equivalent levels of freezing (Fig. 5C). These data indicate that the observed contextual fear conditioning deficit is selective for hippocampus-dependent learning. The normal cued fear conditioning also serves as another internal control. By exhibiting normal cued fear conditioning, we could conclude that the animals did indeed sense the shock, that they have the capacity to freeze, and that they are capable of forming associations between the cue and the shock. Insofar as cued fear conditioning is widely considered to be strictly dependent on the amygdala, our observations suggest that DN-MEK does not affect amygdala-dependent learning.

DISCUSSION

In the current studies, we chose to address the role of MEK1 specifically in neurons by using a genetic approach. A transgenic mouse line was generated expressing a kinase-inactive mutant of MEK1, with expression driven selectively in neurons by using a Tα1 α-tubulin promoter element. This allowed us to investigate the role of neuronal MEK1, an important step forward in investigating the roles of the MEK1/ERK1/2 cascade in a cell-subtype-specific fashion.

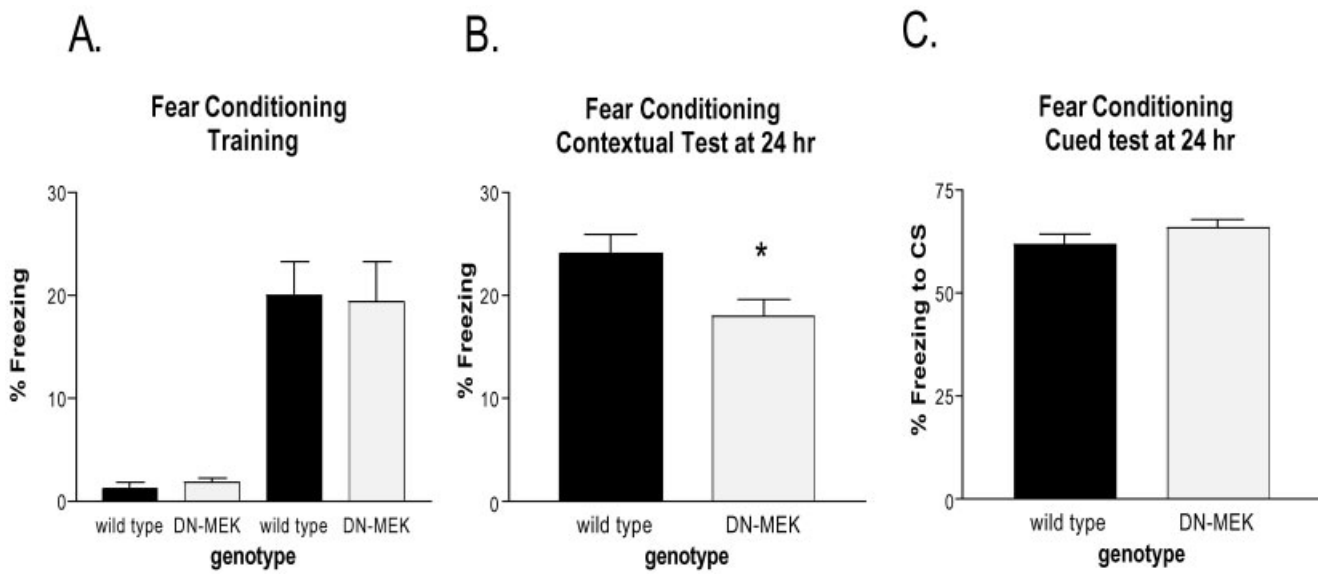


Fig. 5. Presence of DN-MEK transgene selectively affects contextual fear conditioning. **A:** Average freezing responses to two pairings of conditioned stimulus (tone) and unconditioned stimulus (foot shock) are shown as the training phase. Baseline behavior before the shock (first two columns) and freezing response after the shock (second two columns) were similar for both transgenic animals (n = 28) and littermate wild-type controls (n = 11). **B:** Compared with littermate controls, DN-MEK mice froze significantly less when reexposed to the fear conditioning context 24 hr after training ($*P < .05$). **C:** Twenty-four hours after training, DN-MEK animals and their littermate controls displayed equivalent amounts of freezing when reexposed to the conditioned stimulus.

In our limited characterization of the mice, we confirmed neuron-selective expression of DN-MEK1 in the forebrain. DN-MEK1 suppressed ERK1/2 activity in cultured neurons and in vivo, and mice expressing the transgene displayed apparently normal neuronal development. This observation suggests that the residual amount of ERK1/2 activity in the transgenic mice is sufficient to support normal growth and development of neuronal cells. We therefore proceeded to determine whether there were effects on neuronal function in vivo by undertaking a behavioral characterization of our mouse line. We observed fear conditioning deficits in mice expressing neuron-specific DN-MEK1. The expression of DN-MEK1 in neurons appeared to affect specifically contextual fear conditioning, a hippocampus-dependent learning task. Previous work has demonstrated a role for the MEK/ERK kinase cascade in hippocampus-dependent contextual fear conditioning (Atkins et al., 1998; Selcher et al., 1999; Blum et al., 1999). However, these studies relied on the use of the selective MEK inhibitors SL327 or U0126, which affect all cell types in the hippocampus and can suppress other MEK family members (Kamakura et al., 1999). Here we assessed the role of MEK1/ERK1/2 only, and selectively, in neurons. Our results showing impairments in contextual fear conditioning are consistent with the results obtained with the MEK inhibitors and extend these observations by demonstrating that the observed impairments likely are due to the suppression of ERK1/2 in neurons and not other ERK family members. Our data also suggest that the effects of isoform-nonspecific MEK inhibitors can be accounted for by inhibition of ERK1/2.

The fear conditioning deficit observed in the DN-MEK mice apparently was not due to sensory deficits, because the animals showed an ability to freeze as much as controls during training and during the cued fear conditioning test at 24 hr. In addition, the transgenic animals displayed activity levels and anxiety levels similar to those of controls, so their lack of freezing in the context cannot be explained by decreased overall activity or disinhibition of anxiety. The decrease in freezing also cannot be explained by an inability of the transgenic animals to feel the foot shock. Shock threshold testing, in which the amounts of flinching, jumping, and vocalization for various shock intensities were measured, showed no difference between DN animals and wild-type controls.

Other groups have observed behavioral learning deficits in mice in which genes regulating Ras activity, such as Ras-GRF and NF1, have been deleted by homologous recombination (Brambilla et al., 1997; Silva et al., 1997; Giese et al., 2001; Costa et al., 2001). These data have supported a critical role for Ras and its downstream signaling cascades in contextual learning. However, Ras regulates a number of signaling pathways besides MEK/ERK. Our studies, targeting MEK1 and therefore ERK1/2 specifically, greatly strengthen the conclusion that deregulation of upstream regulators of neuronal Ras cause disruption of learning and memory in part through derangements of MEK1 and ERK1/2 signaling.

Our studies do not distinguish which of the two neuronal targets of MEK1, ERK1 or ERK2, is/are involved in contextual fear conditioning. One approach to addressing this issue is the use of isoform-specific knock-

out mouse lines, although studies to date have not fully resolved this issue. Two groups have successfully generated an ERK1-selective knockout mouse. Selcher et al. (2001) reported mild hyperactivity and no hippocampus-dependent learning deficit in their studies and thus concluded that ERK2 was the predominantly important isoform for hippocampus-dependent learning. This conclusion was also reached independently by Mazzuchelli and colleagues (2002), who also generated an ERK1 knockout mouse in a different background strain. This group reported a minimal hippocampus-dependent learning phenotype, although they did discover an interesting enhancement of striatal learning in their studies. Overall, available results indicate that ERK1 and -2 have different functions and varying relative importance in different brain regions. Resolution of this question awaits further investigation. The most parsimonious interpretation of all the available data is that the impairment that we have observed is the result of an inhibition of neuronal ERK2 activity, in that neither of the ERK1 null animals generated by two separate groups displayed deficits in fear conditioning.

Previously described studies using genetically modified mice, such as the NF1 and Ras-GRF mutants (Brambilla et al., 1997; Silva et al., 1997; Giese et al., 2001; Costa et al., 2001); studies utilizing pharmacological MEK inhibitors (Atkins et al., 1998; Selcher et al., 1999; Blum et al., 1999); and our current observations all converge to provide a compelling case for a role of the neuronal MEK/ERK cascade in association and spatial learning in rodents. The various MEK inhibitor studies complement other previous work directly demonstrating activation of MEK with contextual and spatial learning (Atkins et al., 1998; Selcher et al., 1999; Blum et al., 1999). Given the complexities of MEK regulation in neurons and the wide variety of cellular targets of the cascade, future work will help to delineate the upstream regulators and downstream targets of the neuronal MEK cascade important for memory.

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