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The immediate early gene Egr3 mediates adaptation to stress and novelty

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Abstract

Stress and exploration of novel environments induce neural expression of immediate early gene transcription factors (IEG-TFs). However, as yet no IEG-TF has been shown to be required for the normal biological or behavioral responses to these stimuli. Here we show that mice deficient for the IEG-TF early growth response gene (Egr) 3, display accentuated behavioral responses to the mild stress of handling paralleled by increased release of the stress hormone corticosterone. Egr3−/− mice also display abnormal responses to novelty, including heightened reactivity to novel environments and failure to habituate to social cues or startling acoustic stimuli. In a Y-maze spontaneous alternation task, they perform fewer sequential arm entries than controls, suggesting defects in immediate memory. Because stress and novelty stimulate hippocampal long-term depression (LTD), and because abnormalities in habituation to novelty and Y-maze performance have been associated with LTD deficits, we examined this form of synaptic plasticity in Egr3−/− mice. We found that Egr3 −/− mice fail to establish hippocampal LTD in response to low frequency stimulation and exhibit dysfunction of an ifenprodil-sensitive (NR1/NR2B) NMDA receptor subclass. LTP induction was not altered. The NR2B-dependent dysfunction does not result from transcriptional regulation of this subunit by Egr3, because NR2B mRNA levels did not differ in the hippocampi of Egr3−/− and control mice. These findings are the first demonstration of the requirement for an IEG-TF in mediating the response to stress and novelty, and in the establishment of LTD.

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Keywords

immediate early gene; LTP; LTD; corticosterone; stress

Immediate early genes (IEGs) are rapidly activated in the brain in response to changes in the environment including novelty and stress (Senba and Ueyama, 1997, Martinez et al., 2002, Ramanan et al., 2005, Ramirez-Amaya et al., 2005). However, little is known about the role IEGs play in the biological or behavioral response to these stimuli. The early growth response (Egr) genes are a family of IEG transcription factors (IEG-TFs) expressed at basal levels throughout the brain, including in the cortex, hippocampus and other limbic areas, and the basal ganglia. Egr expression is induced at high levels in these regions in response to changes in the environment. Stressful stimuli across a range of intensities activate Egrs, including handling, exposure to novel environments, restraint, and pain (Olsson et al., 1994, Herdegen and Leah, 1998, Rosen et al., 1998, Honkaniemi et al., 2000, Meaney et al., 2000, Staiger et al., 2000, Sabban et al., 2004, Ko et al., 2005, Ramanan et al., 2005). Although much is known about stimuli that activate IEGs, as yet no IEG-TF has been demonstrated to have an essential role in the biological or behavioral response to stressful events or novel stimuli.

Recent studies indicate that exposure to novelty or stress is associated with generation of hippocampal long-term depression (LTD) (Kim et al., 1996, Xu et al., 1997, Manahan-Vaughan and Braunewell, 1999, Kemp and Manahan-Vaughan, 2004, Artola et al., 2006). LTD occurs in the hippocampus of freely moving animals as they explore a novel environment, but does not occur when animals explore a familiar space (Manahan-Vaughan and Braunewell, 1999). Stressful conditions likewise facilitate hippocampal LTD, while having the opposite effect on long term potentiation (LTP), a form of plasticity thought to be involved in spatial reference learning (Kim et al., 1996, Xu et al., 1997, Xiong et al., 2003, Artola et al., 2006). LTD appears to be important for the formation of immediate spatial memory, as performance of rodents in tests of spatial cognitive ability, such as spontaneous alternation in a Y maze, correlates with the magnitude of hippocampal LTD (Nakao et al., 2002). Similarly, deficits in LTD are associated with impairment in immediate memory of novel contexts (Etkin et al., 2006) and one-trial forms of spatial learning (Zeng et al., 2001). Although Egr1 is required for retention of long-term memories and late phase hippocampal LTP (Jones et al., 2001, Bozon et al., 2002, Bozon et al., 2003), as yet no IEG-TF has been demonstrated to play a role in LTD.

To evaluate whether a member of the Egr family plays a role in novelty and stress responsivity, as well as in the specific form of synaptic plasticity associated with these responses, we performed behavioral tests as well as electrophysiological studies of mice deficient for Egr3.

Experimental procedures

Animals

Previously-generated Egr3−/− mice (Tourtellotte and Milbrandt, 1998) created from S129/SvJ embryonic stem cells injected into C57BL/6 pseudopregnant females, were back-crossed to C57BL/6 mice for 13 generations. Animals were housed on a 12 hour light/dark schedule with *ad libidum* access to food and water except where otherwise specified. Studies were conducted on homozygous adult male littermate progeny of heterozygote matings except where indicated otherwise. The term wildtype (WT) refers to the +/+ littermates of Egr3−/− mice generated in these crossings. Investigators were blind to the genotype of animals in all studies.

Behavioral Testing

Behavioral testing was performed during daytime hours under ambient light. Cohorts of animals were tested in multiple behavioral studies with several intervening test-free days. Tests

were ordered to proceed from less invasive (i.e. less stress-producing) to more invasive, and to defer tests requiring that animals be individually-housed until the end of the battery to minimize cage costs. Tests were performed in the following order: reactivity to handling, locomotor activity, social investigatory behavior, hidden cookie, and corticosterone assay. Acoustic startle response/pre-pulse inhibition (PPI) was performed on a separate, larger cohort of animals. Y-maze was performed on a later cohort of animals selected for electrophysiologic studies.

Breeding of Egr3−/− mice to a pure genetic background resulted in a diminution of the motor phenotype described in an SvJ/129 × C57BL/6 mixed background (Tourtellotte and Milbrandt, 1998). This included improvement in ataxia and motor coordination and the resolution of ptosis. However, because of persistent systemic defects in Egr3−/− mice such as scoliosis (Tourtellotte and Milbrandt, 1998) and a sensitivity to repeated food-deprivation (see Methods, Hidden Cookie Test), some behavioral tests requiring swimming or repeated food-deprivation were precluded. In this study, we employed tests involving mild stress after careful evaluation for possible confounding based on motor abnormalities.

Reactivity to Handling

Reactivity of mice to handling was simultaneously evaluated by three experienced technicians who were unaware of the genotype and each others' ratings during three consecutive days of assessment. Each mouse rated for wildness and ease of handling and was assigned a score between 1 and 5 for each evaluation according to a modified version of a rating scale for rats (Wozniak et al., 1989) that was adapted to include a rating scale for mice (Wahlsten et al., 2003). In our procedure one evaluator picking up the test mouse by the tail and placed it on a wire grid. While continuously holding its tail, the evaluator marked the tail with an indelible marker (a standard laboratory procedure). This process was observed and rated by the evaluator and two other observers according to a five point scale: 1 -- the mouse offers minimal resistance to being picked up by the tail and is relatively inactive on the grid during tail marking; 2 -- the mouse offers minimal resistance but is active on the grid for a relatively brief portion of the time; 3 -- the mouse offers some resistance but is captured relatively quickly or is active on the grid for the majority of time ; 4 -- the mouse offers resistance and requires an extended effort to be captured or jumps around for a relatively brief portion of time on the grid ; 5 -- the mouse offers resistance and requires an extended effort to be captured *and* jumps around on the grid for the majority of time during marking *or* successfully escapes the handlers' grasp *or* bites the handler. Means were computed from the scores of each of the three evaluators and one score was assigned for each of the three assessments.

1-Hour Activity

Activity was evaluated for a one-hour period in transparent (47.6 × 25.4 × 20.6 cm high) polystyrene enclosures using a computerized photobeam system (MotorMonitor, Hamilton Kinder, Poway, CA) as previously described (Schaefer et al., 2000). Activity was measured using number of movements (total photobeam breaks) as the dependent variable for total activity.

Acoustic startle response

The startle response of male and female mice to a 120 dBA auditory stimulus (40-ms broadband burst) was tested using a computerized apparatus designed for mice (StartleMonitor, Hamilton-Kinder LLC, Poway, CA). Beginning at stimulus onset, 65 1-ms force readings were averaged to obtain an animal's startle amplitude. The protocol used involved measuring the startle response and PPI contemporaneously (Hartman et al., 2001). A total of twenty startle trials were presented over a twenty minute test period in which the first 5 minutes was an acclimation period during which no stimuli above the 65 dBA white noise background was presented. The

session began and ended by presenting 5 consecutive startle trials (120 db pulse alone) unaccompanied by other trial types. The middle 10 startle trials were interspersed with PPI trials (consisting of an additional 30 presentations of 120 dB startle stimuli preceded by pre-pulse stimuli of either 4, 12, or 20 dB above background) that were not included in the analysis of startle. Animals that failed to demonstrate a significant difference in response to 10 startle versus 10 "non-startle" middle trials were presumed to have auditory deficits and were eliminated from analyses. No statistical difference was detected between male and female mice so data from both sexes were combined.

Social Investigatory Behavior

Littermate mice were sorted into groups of 3 to 5 mice at the time of weaning to permit consistent test groups. Prior to testing, animals' tails were marked with indelible ink. A key linking these markings to each animal's identification number was placed adjacent to the cages in the view of the videocamera to allow subsequent identification. Animals were transferred to a test cage and videotaped in groups of 3 (either 1 −/− and 2 +/+, or 1 each of −/−, +/−, +/+) for 30 minutes. Tapes were subsequently scored for total duration of social interactions including sniffing in the head and anogenital regions in 3 consecutive 10 minute blocks using Stopwatch+ software (Brown). Our studies have shown that Egr3−/− mice are highly reactive to novel environments. To focus on the reaction of Egr3−/− mice to a familiar social stimulus we attempted to minimize the novelty of the environment in which this test was performed, and therefore avoided employing a test in which mice are presented with familiar and unfamiliar animals in a separate testing arena.

Hidden Cookie Test of Olfaction and Reaction to Environmental Novelty

Olfactory function was evaluated using a modified hidden cookie test (Ferguson et al., 2000). Testing was initially performed in a novel test cage, then repeated in the home cage on a second cohort of individually-housed animals to control for environmental novelty. Animals were acclimated to the test food (graham cracker) for 2 days and then tested for the latency to find and eat the piece of cookie hidden under 1 cm of cage bedding following 16–18 hours of food-deprivation. Several Egr3−/− mice appeared ill following overnight food deprivation, displaying a hunched stance, immobility and stereotypic behavior, and were not included in the analyses. This phenomenon was never seen in WT littermates.

Spontaneous alternation in a Y-maze

Testing was conducted using previously published methods (Nakao et al., 2002, Izumi et al., 2005a) in a black metal Y-maze in which the three trough-shaped arms (8 cm in depth, 3cm bottom width, 6 cm open surface width, 45 cm length) are separated an angle of 120°. A mouse was placed in the center of a maze where three arms intersected and was allowed to explore the apparatus for up to 10 min or until 12 arm-entries were made. A successful alternation was defined as any three consecutive choices of different arms without re-exploration of a previously-visited arm. Each successful alternation was given a score of "1" for a maximum possible score of "10". For studies with ifenprodil (Sigma-Aldrich Co., St. Louis, MO), the drug was dissolved in water and then diluted to a final concentration of 0.3 mg/ml in sterile physiologic saline. Ifenprodil or vehicle was administered to WT mice in a volume of 10 ml/kg via intraperitoneal injection 40 minutes prior to testing.

Corticosterone levels

Serum samples for corticosterone were obtained via retro-orbital bleed (completed within 90 seconds of touching the animals' cage) from adult males at circadian nadir (8:00 a.m.) for baseline levels. One week later animals were handled for 90 seconds (held in open palms), then replaced in their home cages. 25 minutes later blood was obtained for post-stress corticosterone

levels. Samples were placed on ice until centrifugation (10,000 r.p.m. at 4 °C × 5 minutes), and serum was transferred and stored at −20 °C. Hormone concentrations were determined by radio-immunoassay using a kit from MP Biomedicals (Diagnostics Division, New York, catalog # 07120102) according to the instructions included, as previously described (Brewer et al., 2003).

Electrophysiology

60-day old mice were anesthetized with isoflurane and decapitated. Hippocampi were rapidly dissected, placed in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 22 $NaHCO_3$, 10 glucose, gassed with 95% O_2 - 5% CO_2 at 4–6 °C, and sliced transversely into 400 m slices using a vibratome. Slices were prepared from the septal half of the hippocampus and were placed in an incubation chamber containing gassed ACSF for 1 hr at 30 °C. At the time of study, slices were transferred individually to a submersion recording chamber. ACSF (30 °C) was perfused at 2 ml/min.

Extracellular recordings were obtained from the CA1 apical dendritic region for analysis of EPSPs using 2 M NaCl glass electrodes with resistances of 5–10 MΩ. Evoked synaptic responses were elicited with 0.2 msec constant current pulses through a bipolar electrode in the Schaffer collateral pathway. Synaptic responses were monitored by applying single stimuli every 60 sec at an intensity sufficient to elicit 50% maximal excitatory postsynaptic potentials (EPSPs). After establishing a stable baseline, LTP was induced by applying high frequency stimulation consisting of a single 100 Hz × 1 sec stimulus train using pulses of the same amplitude. Following the tetanus, responses were monitored every 60 sec. LTD was induced using a low frequency stimulus train consisting of 900 single pulses at 1 Hz.

Isolated N-methyl d-aspartate receptor (NMDAR) synaptic responses were studied in extracellular solution containing 2 mM calcium and 0.1 mM magnesium. CNQX (30 μM) was added to inhibit non-NMDAR EPSPs and responses were evoked once per minute.

Quantitative RT-PCR

Mice were anesthetized with halothane and decapitated. Hippocampi were rapidly dissected and placed in RNA-Later (Ambion Inc., Austin, TX) for several minutes, frozen on dry ice and stored at −80 °C. Total RNA was isolated with Trizol Reagent (Invitrogen Corporation, Carlsbad, CA) using the protocol provided with the product. Quantitative RT-PCR was performed in triplicate on reverse transcribed cDNAs from 6 Egr3−/− and 6 WT littermate hippocampi, according to a previously published protocol (Nagarajan et al., 2001). Primer sequences are available upon request.

Data analysis

For behavioral studies (excluding the Y-maze) data were analyzed using SPSS (Chicago, IL) and Systat (Point Richmond, CA) programs to conduct T-tests, analysis of variance (ANOVA), and repeated measures ANOVA as appropriate. For ANOVAs with repeated measures, the Huynh-Feldt adjustment of degrees of freedom was used for all within-subjects effects containing more than two levels to generate corrected “p” values to help protect against violations of the sphericity/compound symmetry assumptions. Bonferroni corrected “p” values were computed when appropriate following multiple comparisons. For electrophysiologic and Y-maze studies data were analyzed using SigmaStat (Jandel Scientific Software, San Rafael, CA). Statistical significance was determined using Student t-test where appropriate or Mann-Whitney U test with $p < 0.05$.

Results

Egr3−/− mice are hypersensitive to stress

To evaluate whether Egr3 plays a role in the behavioral reaction to stress, we examined the response of Egr3−/− mice to a range of mildly stressful stimuli. To minimize the effect of mouse strain variability on behavior we back-crossed Egr3−/− mice to a C57BL/6 background for 13 generations prior to these studies.

We began by evaluating the response of Egr3−/− mice to handling, a mild stressor that is known to activate Egr-family genes (Meaney et al., 2000). We found that Egr3−/− mice were consistently hyper-reactive to handling, while WT littermate mice scored in the normal range in this test (Fig. 1a). Repeated measures ANOVA revealed significant main effects of genotype [$F(1,18) = 32.10$, $p < 0.001$] and day [$F(2,36) = 14.33$, $p < 0.001$] and significant genotype × day interaction [$F(2,36) = 6.93$, $p = 0.011$]. Although Egr3−/− mice demonstrated diminished reactivity over three days of testing, they remained more reactive than WT controls on each test day. Significant differences were found on each of the 3 testing days ($p < 0.001$, $p = 0.006$, and $p = 0.002$, respectively).

To evaluate whether Egr3 is also essential for modulating the physiologic response to stress, we measured serum corticosterone levels. Egr3−/− mice showed no difference in baseline, unstressed levels of corticosterone (Fig. 1b). Following a one-week recovery, the mice were handled for 90 seconds 25 minutes prior to measuring corticosterone levels. This mild stressor augmented the level of corticosterone in both Egr3−/− and WT mice. However, in Egr3−/− mice the increase in corticosterone release was more than twice that of controls ($p = 0.002$; Fig. 1b). Repeated measures ANOVA revealed significant effects of genotype [$F(1,12) = 14.81$, $p = 0.002$] and condition (baseline vs. handling) [$F(1,12) = 87.82$, $p < 0.001$] as well as a significant genotype × condition interaction [$F(1,12) = 13.74$, $p = 0.003$].

We also evaluated responses of Egr3−/− mice to the mild stress of being placed in a novel “open field” by measuring their locomotor activity over a 1-h test period (Fig. 2). We found that Egr3−/− mice were significantly more active than WT littermates in a novel environment (significant main effect of genotype, [$F(1,34) = 13.08$, $p = 0.001$]). The activity of both groups decreased over time (significant effect of time, [$F(5,170) = 56.22$, $p < 0.001$]) but the pattern of this habituation to the novel environment differed between Egr3−/− mice and WT controls (indicated by a significant genotype by time interaction, [$F(5,170) = 3.49$, $p = 0.028$]) due to significantly greater activity of the Egr3−/− mice in the early part of the testing period (Fig. 2).

The hyperactivity of the Egr3−/− mice in the present study, which are on a C57BL/6 background, is notable given our earlier findings of motor impairments in Egr3−/− mice on a mixed background (Tourtellotte and Milbrandt, 1998). The present results indicate that Egr3 −/− mice show exaggerated locomotor activity responses to a novel environment, suggesting that Egr3 may be involved in dampening behavioral as well as physiologic responses to stress.

Immediate memory deficits in Egr3−/− mice

The finding of heightened responses to novelty in Egr3−/− mice prompted us to examine the response of these mice toward a familiar stimulus in the context of a novel environment by observing social interactions between Egr3−/− mice and WT cagemates, with whom they had been housed since weaning.

Social behavior in rodents is mediated primarily through the olfactory system. Mice sniff and lick to investigate and identify one another. We videotaped and scored the interaction among groups of three cagemate mice for a period of 30 minutes after being transferred to a test cage.

We found that Egr3−/− mice showed markedly elevated levels of social investigation in the anogenital region of familiar animals (5–6 fold increase) compared to WT controls (Fig. 3a; significant main effect of genotype [$F(1,13) = 37.37$, $p = 0.001$]). This hyper-investigation decreased over the 30 minute period (indicated by a significant genotype by time interaction [$F(2,26) = 4.06$, $p = 0.029$]), suggesting that this behavior may have been accentuated by the novel environment. Comparison between genotypes at the end of the evaluation period indicated that Egr3−/− mice showed persistent increases in anogenital investigatory behavior ($p < 0.001$). Olfactory investigation of the head region was more modestly increased in Egr3 −/− mice (58% increase over that of WT mice; Fig. 3b, [$F(1,13) = 7.87$, $p = 0.015$]), and did not habituate over the testing period. This effect appears to be specific for social investigatory behavior, as Egr3−/− mice did not differ from controls in the total duration of physical contact with cagemates or in the duration of non-social behaviors such as grooming (not shown). Although group-housing necessitated transfer of mice for testing (see methods), the increased social investigatory behavior of Egr3−/− mice is seen upon opening the home cage in otherwise undisturbed conditions (A. Gallitano-Mendel, unpublished observation). Notably, the test we have employed cannot distinguish whether the increased social investigatory behavior displayed by Egr3−/− mice is due to a failure to form a social memory or due to an overall increased social interest on the part of the Egr3−/− mice. However, increased social investigation of familiar mice suggests a possible defect in habituation to social stimuli (Ferguson et al., 2000).

To rule out a deficit in olfaction as a reason for the sustained olfactory investigatory behavior, we tested the mice for their ability to locate a food source using the “hidden cookie” test (Ferguson et al., 2000). We found that Egr3−/− mice took significantly longer to locate the hidden food than did WT mice when tested in a novel testing cage (Fig. 3c, [$t(12) = -2.505$, $p = 0.028$]). However, when testing was conducted in the home cage using a new cohort of mice, Egr3−/− mice did not differ in their latencies to find the hidden cookie (Fig. 3c, $p = 0.229$). These results demonstrate that Egr3−/− mice have intact olfactory detection capabilities, and confirmed that they display an accentuated response to a novel environment.

To examine adaptation of Egr3−/− mice in another stressful context we measured acoustic startle responses (ASR). Egr3−/− mice did not differ from WT controls in their initial ASR (i.e., block 1) to the 120 dB pulse (Fig. 4a). However, comparison across test sessions demonstrated that Egr3−/− mice differed from WT mice in their response to the repeated sound bursts (repeated measures ANOVA revealed a significant main effect of genotype [$F(1,59) = 8.48$, $p = 0.005$] and a startle block × genotype interaction [$F(3,177) = 3.72$, $p = 0.013$]). Whereas WT mice habituated to the startle stimulus (demonstrated by a significant decrease in startle amplitude between stimulus blocks 1 and 4 ($p < 0.001$)) Egr3−/− mice failed to habituate to repeated stimulus presentations (difference between startle blocks 1 and 4 was not statistically significant). No significant differences were observed with regard to PPI between the Egr3−/− and WT mice (data not shown).

The abnormal habituation response in Egr3−/− mice across a range of stimuli suggests a possible defect in immediate memory formation (File and Wardill, 1975, Platel and Porsolt, 1982, Etkin et al., 2006). To evaluate this possibility, we tested Egr3−/− mice and WT littermates on a spontaneous alternation task in a Y-maze (Fig. 4b). This test is correlated with working memory, and impaired performance is seen following administration of amnestic drugs or hippocampal lesions (Lalonde, 2002). We found that Egr3−/− mice displayed significantly fewer spontaneous alternations than did WT controls in the Y-maze (Fig. 4b), a result suggestive of hippocampusbased memory deficits.

Egr3−/− mice have deficits in LTD

Our findings that Egr3−/− mice display abnormalities in response to these classes of stimuli suggested that Egr3 may be required for induction of hippocampal LTD, a form of synaptic plasticity correlated with Y-maze performance and stress and novelty response (Kim et al., 1996, Xu et al., 1997, Manahan-Vaughan and Braunewell, 1999, Nakao et al., 2002, Kemp and Manahan-Vaughan, 2004, Izumi et al., 2005a, Artola et al., 2006). In hippocampal slices from WT mice, 1 Hz × 900 pulse low frequency stimulation (LFS) of the Schaffer collaterals resulted in a persistent depression of EPSPs in the CA1 region (Fig. 5a; EPSP change 60 min after LFS = −24.3 +/− 6.5 %, n = 5). In contrast, LTD was not induced in slices from Egr3−/− mice (Fig. 5a; − 1.9 +/− 4.3 %, n = 5, p = 0.02).

LTP, a form of synaptic plasticity correlated with spatial reference learning, was not altered in slices from Egr3−/− mice. No significant differences were found in the magnitude of LTP induced by a single 100 Hz × 1 sec high frequency stimulation of the Schaffer collateral pathway in Egr3−/− mice compared with controls (Fig. 5b; EPSP change 60 min after HFS: 22.9 +/− 4.2 %, n=6, in WT mice; 21.9 +/− 6.9%, n = 4, in Egr3−/− mice).

NMDA receptor properties are disrupted in Egr3−/− mice

The induction of LTD in the CA1 region is dependent upon activation of NMDARs, and CA1 synapses express several NMDAR subunits including NR1, NR2A and NR2B. To determine whether NMDAR dysfunction contributes to the LTD deficits seen in Egr3−/− mice, we isolated NMDAR components of synaptic responses and examined the effects of ifenprodil, a noncompetitive NMDAR inhibitor that is selective for NMDARs expressing NR1 and NR2B subunits (Williams, 1993, Priestley et al., 1995). In slices from WT mice, 10 μM ifenprodil blocked synaptic NMDARs by about 40–50% (Fig. 6a; −43.3 ± 2.8% change, n = 5). The effects of 10 μM ifenprodil were markedly diminished in slices from Egr3−/− mice (Fig. 6a; −5.5 ± 6.8% change, n = 5, $p < 0.001$), suggesting that abnormalities in the function of NR1/NR2B receptors may contribute to the defect in LTD.

To evaluate whether the defect in Y-maze performance in Egr3−/− mice might result from dysfunction of NR2B-containing NMDA receptors, we tested the effect of ifenprodil on spontaneous alternations of WT in the Y-maze (Fig. 6b). We found that administration of 3 mg/kg ifenprodil, a non-sedating dose, significantly impaired performance of WT mice in this test of immediate memory in a novel environment compared to age-matched vehicle-treated WT mice. This suggests that the poor performance of Egr3−/− mice in the Y maze may result, at least in part, from their deficient function of NR2B-containing NMDARs.

To examine whether dysfunction of NR2B-containing NMDARs results from decreased levels of NR2B mRNA, implying that Egr3 may normally regulate expression of this gene in the hippocampus, we compared expression of NR2B in the hippocampi isolated from Egr3−/− and WT control mice using quantitative Reverse Transcription Polymerase Chain Reaction (RTPCR). Our results demonstrated no significant difference in the levels of NR2B mRNA in Egr3−/− and WT mice (data not shown). This suggests that the dysfunction of NR2B-containing NMDARs does not result from deficient levels of NR2B gene expression in Egr3−/− mice.

Discussion

Egr3 mediates adaptation to stress and novelty

Stress and novelty are two major classes of stimuli that activate IEGs, including Egr-family genes (Senba and Ueyama, 1997, Ramanan et al., 2005). However, as yet, no IEG-TF has been demonstrated to play an essential role in the biological or behavioral response to these stimuli.

We show that Egr3 is required for the normal behavioral response of mice to a range of stressful stimuli, including handling, novel environments, and startling sounds. Egr3−/− mice showed heightened reactivity to the mild stress of handling which diminished only slightly across the three test days such that the Egr3−/− mice still showed robust differences relative to littermate controls even on the third day (Fig. 1a). Interestingly, Egr3−/− mice do not appear to have higher levels of stress at baseline, as corticosterone levels of unstressed Egr3−/− mice do not differ from those of littermate controls. Rather, they differ only in their response to stress, demonstrated by accentuated release of corticosterone in response to handling (Fig. 1b).

Egr3−/− mice also displayed heightened reactivity to novel environments (Fig 2 and Fig. 3c). Exploration is an essential step in the cognitive processing of novel stimuli and may be indicative of information acquisition (Eichenbaum, 1996). Conversely, habituation represents a decrease in response to a stimulus as it becomes familiar. Thus a delay in habituation suggests a deficit in information acquisition (File and Wardill, 1975,Platel and Porsolt, 1982). The increased activity is more prominent in the early phases of the one-hour testing period, suggesting that this is a response to the novelty of the environment. The effect of a novel environment on the behavior of Egr3−/− mice was further demonstrated by a significant increase in the time spent by the mutant mice to find and eat hidden food compared to controls, a difference that disappeared when mice were tested in a familiar environment (Fig. 3c).

The heightened response to stress displayed by Egr3−/− mice may result from abnormalities in cognitive processing or memory, as suggested by our findings of abnormalities in habituation to acoustic-startle stimuli and spontaneous alternations in the Y-maze, and increased social investigatory behavior that may indicate a failure to habituate to social stimuli. If Egr3−/− mice fail to recall a prior stimulus or event, then their response to the stimulus would be similar to that following a first experience. Repeated “novel-seeming” experiences would create a scenario of frequent stress, which could have damaging effects on the brain and other tissues over time. Development of Egr3 conditional knockout mice will permit regionally and temporally specific inactivation of Egr3 in the brain, thus allowing a more detailed characterization of the role of Egr3 in learning and memory.

Egr3 is required for LTD

Egr3 is activated in the hippocampus in response to either electroconvulsive seizures or high frequency stimulation of perforant pathway neurons, the latter of which induces synaptic plasticity (Yamagata et al., 1994). Notably, this activation is NMDAR-dependent, as application of the NMDAR antagonist MK-801 blocks both expression of Egr3 and induction of plasticity (Yamagata et al., 1994). However, prior to our current study no role for Egr3 in hippocampal synaptic plasticity has been reported.

Several studies have reported a relationship between stress, facilitation of LTD and concomitant inhibition of LTP (Kim et al., 1996, Xu et al., 1997, Xiong et al., 2003, Artola et al., 2006). Stress represses induction of LTP in the CA1 hippocampal region, but facilitates LTD (Kim et al., 1996). Stress influences LTD through the glucocorticoid receptor (GR) and the mitogen activated protein kinase (MAPK) – signaling pathway leading to ERK1/2 activation (Yang et al., 2004, Boyle et al., 2005). This pathway also activates Egr-family IEG-TFs (Revest et al., 2005). Furthermore, LFS that induces the longest-lasting LTD induces the highest expression of IEGs (Abraham et al., 1994). In contrast to Egr1, which is involved in spatial learning and late phase LTP (Jones et al., 2001), our results indicate that Egr3 is pivotal for induction of LTD, but not LTP.

Recent studies have also shown an association between acquisition and retention of novel information and induction of LTD (Manahan-Vaughan and Braunewell, 1999, Kemp and Manahan-Vaughan, 2004, Artola et al., 2006, Etkin et al., 2006, Plath et al., 2006). LFS not

only induces LTD but it also facilitates exploratory behavior in rats (Manahan-Vaughan and Braunewell, 1999). Similarly, *in vivo* LTD is enhanced by exposure to a novel environment, indicating the role of novelty in LTD induction (Manahan-Vaughan and Braunewell, 1999). Performance of animals on a spontaneous alternation task is an additional indicator of abnormalities in exploration of a novel environment and is correlated with the degree of LTD (Nakao et al., 2002). We recently reported poor spontaneous alternation performance and deficits in LTD induction in 30-day old rats exposed to ethanol at postnatal day 7, a model of fetal alcohol syndrome. Defects in LTD in these animals correlated with diminished contribution of an ifenprodil-sensitive NMDAR subclass (Izumi et al., 2005a).

In the present study, we observed that in Egr3−/− mice synaptic NMDARs showed altered sensitivity to ifenprodil, indicating dysfunction of the NR1/NR2B receptor subclass in these animals. Thus the insensitivity to ifenprodil correlates with the LTD-induction deficit in these mice. To further support a potential link between NR1/NR2B-type NMDAR abnormalities and defects in immediate memory function and novelty response, we examined whether ifenprodil impairs performance of WT mice in the Y maze. We found that ifenprodil administration did, in fact, disrupt spontaneous alternations in the Y maze. This suggests that the defects in immediate memory and in Y maze performance displayed by Egr3−/− mice may be, at least in part, explained by the dysfunction of NR1/NR2B-type NMDARs. While the role of specific NMDAR subtypes in various forms of synaptic plasticity is controversial (Liu et al., 2004, Berberich et al., 2005, Izumi et al., 2005b, Weitlauf et al., 2005, Izumi et al., 2006, Bartlett et al., 2007, Morishita et al., 2007)), our results suggest that NMDAR dysfunction plays at least some role in the altered synaptic function exhibited by Egr3−/− mice.

It is important to note that the activation of the Egr3 gene in response to an acute stimulus such as the Y-maze (a test which is completed in less than 10 minutes), is unlikely to account for the abnormalities we saw in immediate responses to acute stimuli. Based on reports of the timing of activation of IEGs in response to a stimulus, and subsequent translation of Egr3 protein, binding of that protein to DNA, and activation of target genes, it is highly unlikely that a deficit in the acute expression of Egr3 accounts for the abnormal behavior of Egr3−/− mice in this test. Instead, we would assume that Egr3 is required for the basal expression of genes encoding proteins that are themselves essential for such an acute response to an environmental stimulus. One such example would be a cell-surface receptor, such as one of the NMDA receptors.

To further examine the mechanism by which loss of Egr3 leads to NR1/NR2B NMDA receptor dysfunction we tested the most parsimonious explanation, that Egr3 regulates expression of the NR2B gene. However, this does not appear to be the mechanism, since we detected no difference in the levels of NR2B mRNA measured by qRT-PCR in the hippocampi of Egr3−/− versus WT mice. Alternative possible explanations for dysfunction of this receptor subtype in Egr3−/− mice include that Egr3 may regulate genes that are required for the normal processing, trafficking, localization, or function of NR2B-containing receptors, or genes involved in some step in the subsequent signal transduction pathway downstream of the receptors activation. Notably Arc/Agr3.1, an Egr3-regulated gene (Li et al., 2005), affects internalization of AMPA receptors which in turn affects LTD (Chowdhury et al., 2006, Plath et al., 2006, Rial Verde et al., 2006, Tzingounis and Nicoll, 2006). Thus this, or a parallel effect on NR2B-type NMDA receptors, could provide an explanation for the LTD abnormalities in Egr3-deficent mice. Alternatively, Egr3 may be required for normal hippocampal development, and the abnormal response to acute stimuli may result from such a developmental defect. Further study using a broad approach such as gene expression profiling will be essential to elucidate how Egr3 influences the function of ifenprodil-sensitive NMDARs.

Putative biological pathway for LTD and the response to novelty

Recently, mice deficient for forebrain expression of Serum Response Factor (SRF) were found to have deficits in both LTD and novelty memory (Etkin et al., 2006). In a preceding study, SRF was found to regulate forebrain expression of both Egr1 and Egr2 in response to exposure to a novel environment (Ramanan et al., 2005). Although the activity of Egr3 in SRF-deficient mice was not reported in the prior studies, the Egr3 promoter contains multiple Serum Response Elements, the binding sequence for SRF (AGM, unpublished observation). This suggests that the regulation of Egr3 may be the mechanism through which SRF regulates LTD.

In addition, Egr3 is regulated by the calcium-responsive protein phosphatase calcineurin (CN) (Mittelstadt and Ashwell, 1998), which is triggered by calcium influx through NMDARs (Sheng and Kim, 2002). CN-deficient mice also display deficits in LTD and one-trial spatial learning (Zeng et al., 2001). Furthermore, CN-deficient mice were reported to display a heightened responsiveness to handling (suppl. online data in (Miyakawa et al., 2003)) similar to that which we report in Egr3−/− mice, suggesting that CN−/− mice may also have a heightened sensitivity to stress. In turn, Egr3 regulates expression of the effector IEG Arc/Arg3.1 (activity-regulated cytoskeleton-associated gene) (Li et al., 2005). Arc/Arg3.1 was recently demonstrated to be required for LTD as well as memory retention (Plath et al., 2006, Rial Verde et al., 2006, Tzingounis and Nicoll, 2006).

These findings suggest a possible pathway for induction of LTD in response to stimuli such as novelty and stress. This pathway would include stimulation of NMDARs, increased intracellular calcium, and activation of CN. CN, together with other factors, activates SRF (Hao et al., 2003). SRF may then regulate Egr3 which activates expression of Arc/Arg3.1 (Li et al., 2005), leading to induction of LTD and establishment of enduring memory of a stressful or novel stimulus.

Although a defect in LTD and altered response to stress and novelty implicate CN, SRF and Egr3 in a biological pathway, the results of memory tests in mice deficient for these genes differ. While Egr3−/− show hyperactivity in a novel environment, as seen in both CN and SRF-deficient mice, Egr3−/− mice do eventually habituate to this stimulus whereas SRF−/− mice and CN−/− mice do not (Miyakawa et al., 2003, Etkin et al., 2006). In addition, Egr3−/− mice fail to habituate to a startling auditory stimulus, while SRF−/− mice do not (Etkin et al., 2006). One notable difference is that Egr3−/− mice are conventional knockout mice, lacking function of the gene in all cells throughout development and life, whereas CN−/− and SRF−/− mice were created by selectively knocking out these genes in αCAMKII-expressing cells, an adult-forebrain selective pattern (Zeng et al., 2001, Etkin et al., 2006). Thus some of these differences may be developmental or regional in nature. Generation of conditional Egr3−/− mice will permit extensive evaluation of the role of Egr3 in learning and memory in response to stress and novelty, and comparison with the roles of SRF and CN in a comparable scenario.

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Glossary

List of Abbreviations, in order of appearance in the text

IEG
: Immediate early gene

IEG-TF
: immediate early gene transcription factor

Egr3
: early growth response gene 3

LTD
: long-term depression

LTP
: long term potentiation

PPI
: pre-pulse inhibition

NMDA
: N-methyl-D-aspartate

ANOVA
: analysis of variance

WT
: wildtype

ASR
: acoustic startle responses

GR
: glucocorticoid receptor

MAPK
: mitogen activated protein kinase

SRF
: Serum Response Factor

CN
: calcineurin

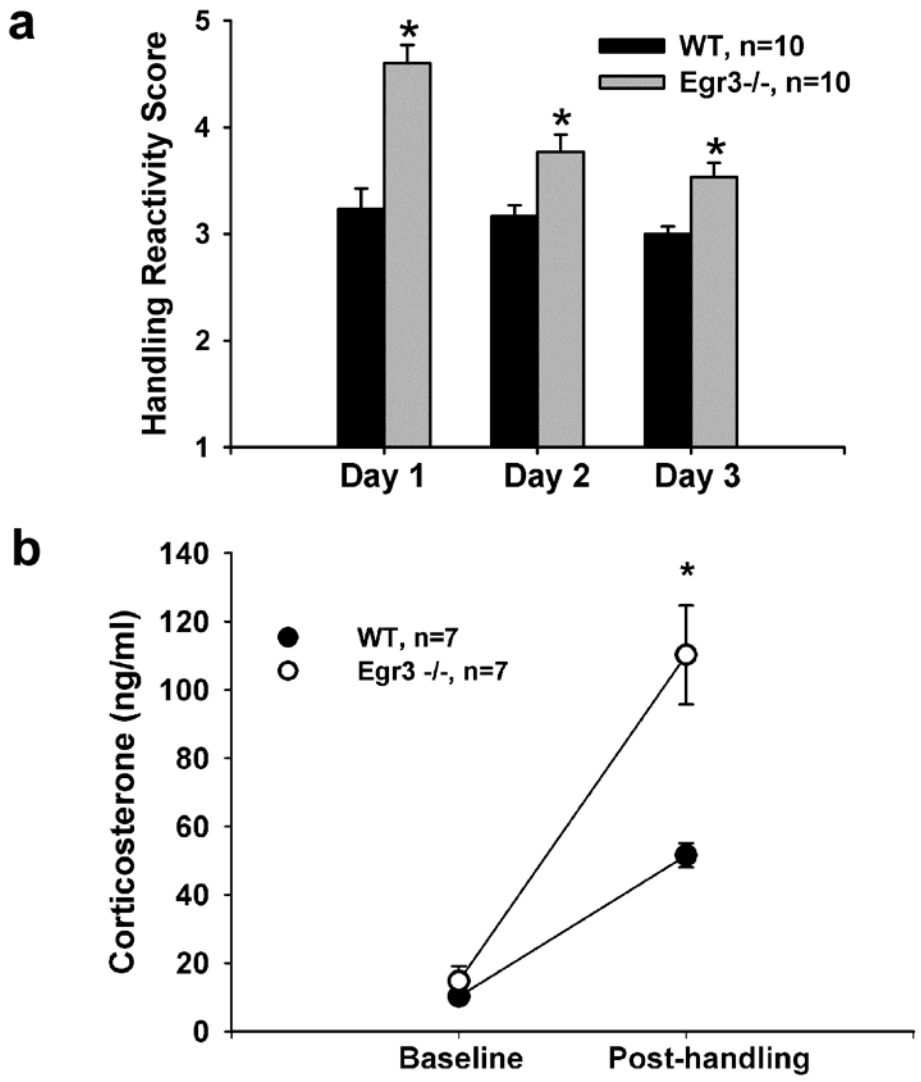

Fig. 1. Egr3−/− mice are hypersensitive to handling stress

a, Egr3−/− mice were significantly (*) more reactive to handling on each of 3 consecutive test days. **b**, Baseline corticosterone levels in Egr3−/− mice did not differ from controls, but handling induced significantly greater corticosterone release in Egr3−/− mice (*p = 0.002), indicating heightened physiologic response to mild stress.

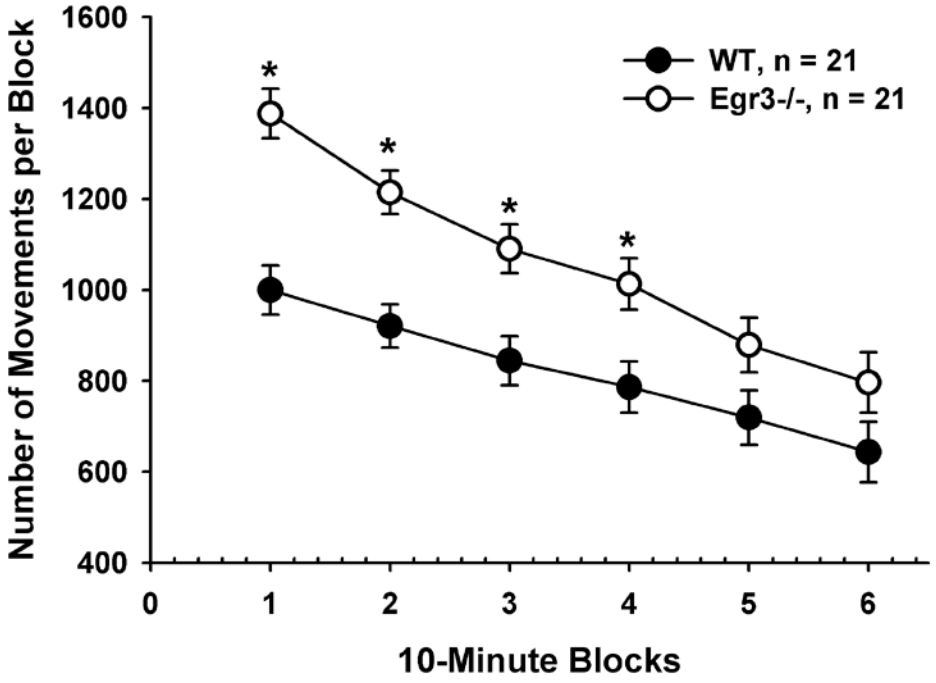

Fig. 2. Egr3−/− mice display heightened reactivity to novelty stress

Egr3−/− mice were more active in a 1-hour open-field activity test compared to WT control mice ($p = 0.001$). This effect was greatest early in the test and decreased to control levels over time, indicating that the heightened activity was a response to the novel environment (p values for blocks 1–6 respectively: $p < 0.00005$; $p = 0.0001$; $p = 0.003$; $p < 0.008$; $p = 0.067$; $p = 0.11$).

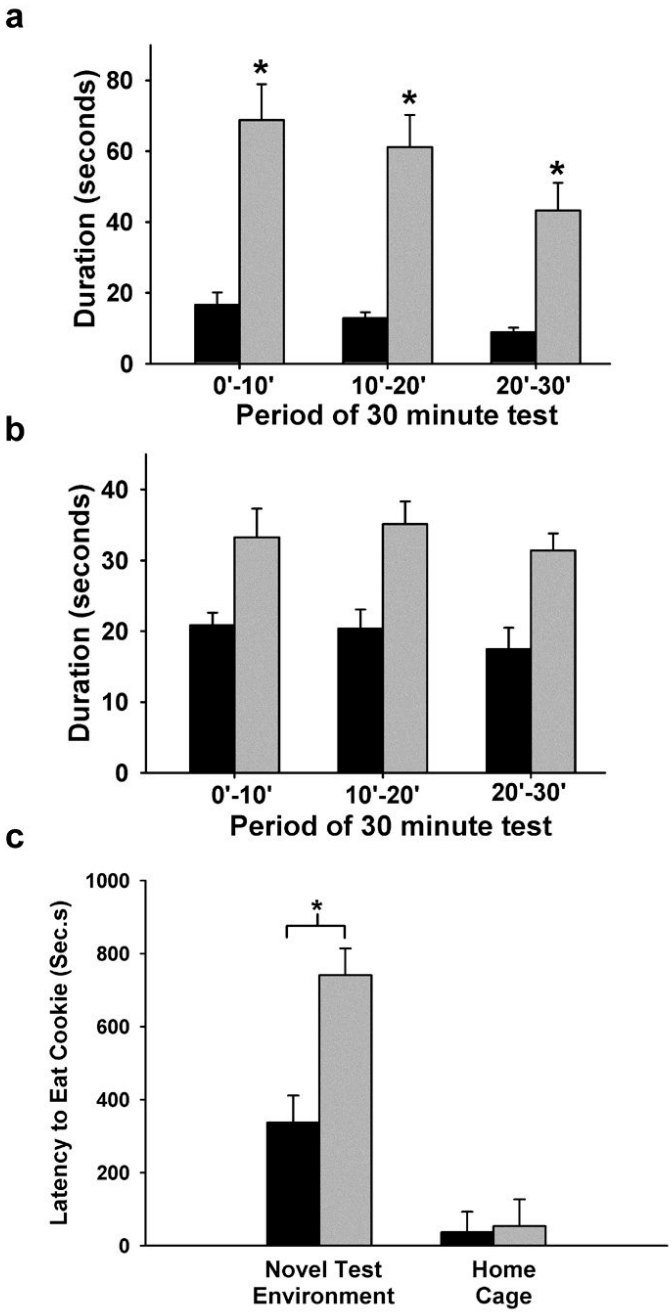

Fig. 3. Egr3−/− mice show increased social investigation of familiar mice suggesting failure to habituate to a social stimulus

a, **b**, Egr3−/− mice showed significantly more social investigation of familiar cagemates compared to that of WT mice (n = 8 WT, n = 7 Egr3−/−). **a**, This effect was greatest for investigation of the anogenital region ($p < 0.001$) but **b**, was also significantly different in the head region ($p = 0.015$). **c**, the hidden cookie test of olfactory function revealed that Egr3−/− mice took significantly longer to find and eat hidden food when tested in a novel cage (*, $p = 0.028$; n = 5 WT, n = 5 Egr3−/−), but showed no difference when tested in a familiar cage (n = 12 WT, n = 7 Egr3−/−; see methods for explanation of variance in n), indicating that Egr3 −/− mice are able to smell but their behavior is highly affected by novelty stress.

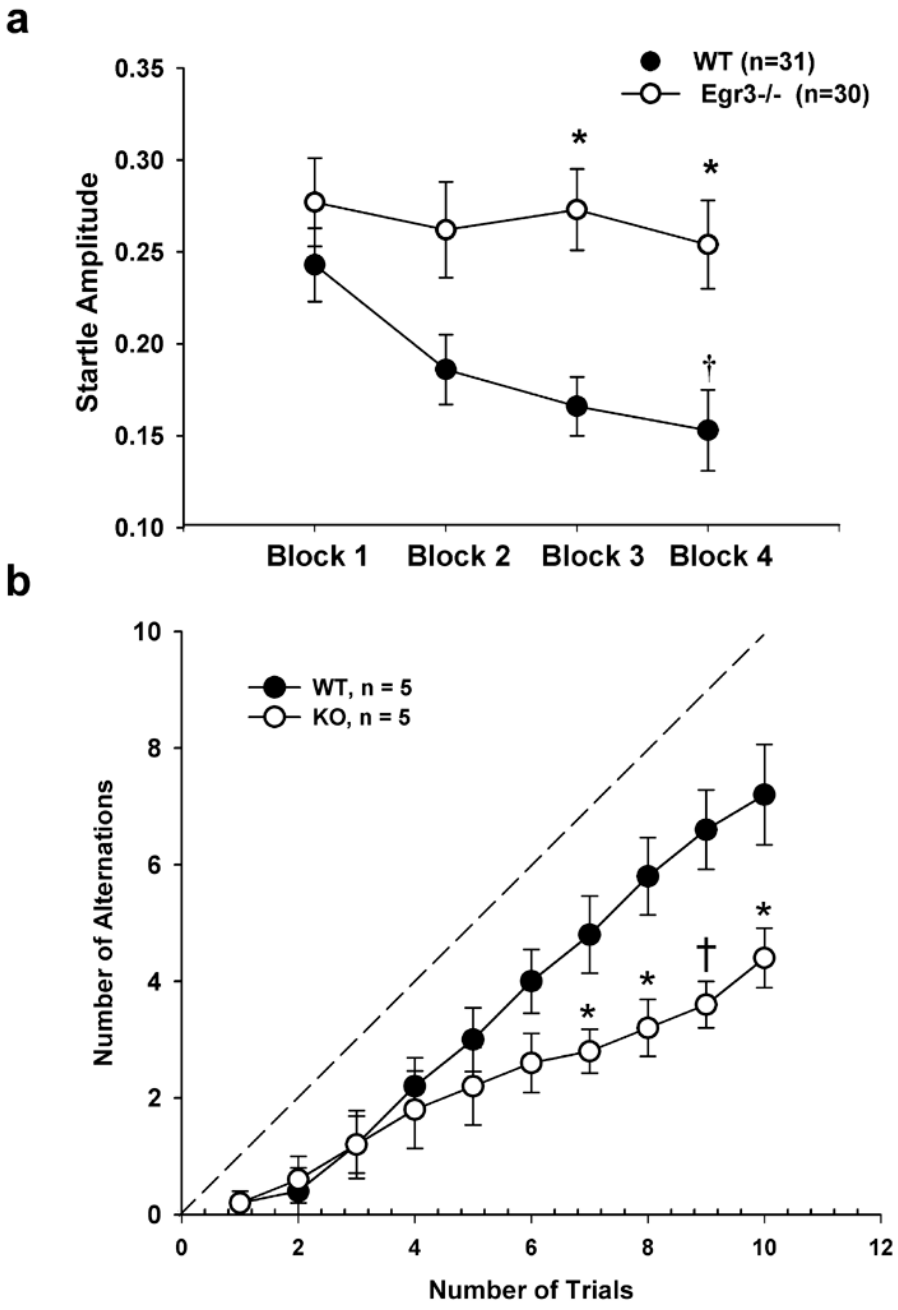

Fig. 4. Egr3−/− mice fail to habituate to a startling sound and show deficits in spontaneous alternation in a Y-maze

a, Initial startle response, displayed as means of blocks of 5 startle trials, did not differ between Egr3−/− and WT mice. However, while the response of WT mice decreased significantly over time (†), Egr3−/− mice failed to habituate to the stimulus, resulting in significantly (*) increased startle response compared to WT in blocks 3 and 4 ($p < 0.001$, and $p = 0.004$ respectively). **b**, Egr3−/− mice made significantly fewer successful alternations in the Y-maze (* $p < 0.05$, students t-test; † $p < 0.05$, Mann-Whitney U test).

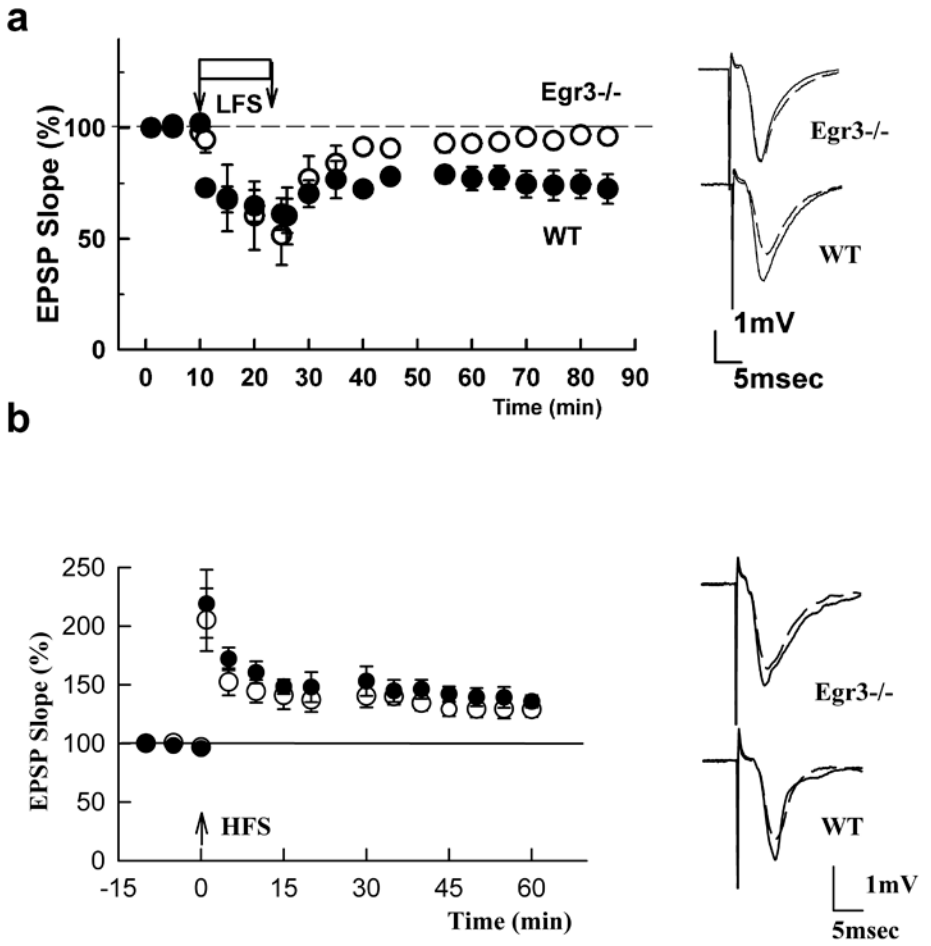

Fig. 5. Impaired induction of hippocampal LTD, but not LTP, in Egr3−/− mice

a, Time course of change in EPSPs in the CA1 region following LFS in slices from WT (filled circles; n = 5) and Egr3−/− mice (open circles; n = 5) demonstrate a deficit in LTD in Egr3−/− mice. Traces to the right depict EPSPs obtained before (solid line) and 60 min after (dotted line) LFS in slices from an Erg3−/− mouse (upper) and a WT mouse (lower). **b**, Change in CA1 region EPSPs following 100 Hz × 1 s HFS of the Shaffer Collaterals in slices from WT (filled circles; n = 5) and Egr3−/− mice (open circles; n = 5) demonstrate no difference in LTP based on Egr3 genotype. Traces to the right depict EPSPs obtained before (solid line) and 60 min after (dotted line) HFS in slices from an Erg3−/− mouse (upper) and a WT mouse (lower).

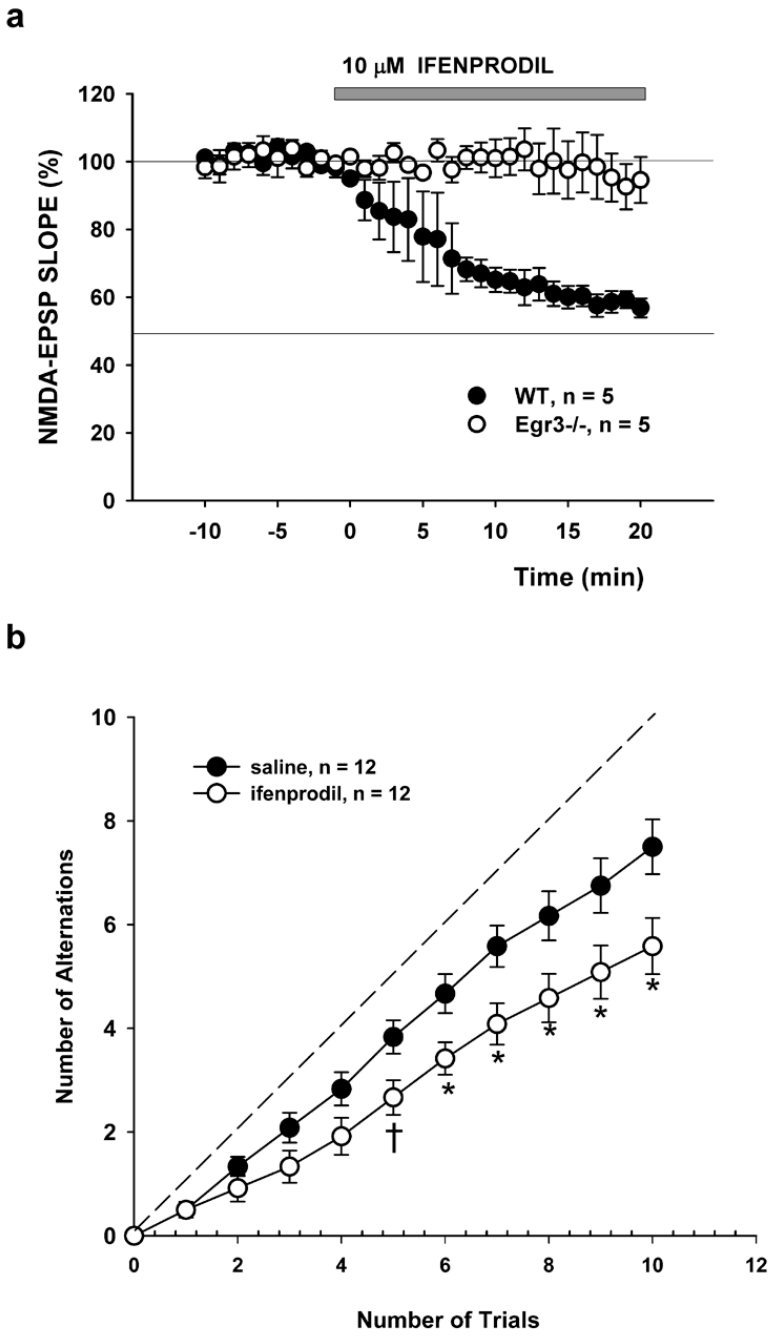

Fig. 6. Dysfunction of NMDARs in Egr3−/− mice

a, Loss of ifenprodil sensitivity of synaptic NMDARs in the CA1 region of slices from Egr3 −/− mice. The time course of change in isolated NMDAR-mediated EPSPs in slices from WT mice (filled circles) and Egr3 −/− mice (open circle) following treatment with 10 μm ifenprodil. **b**, WT mice treated with 3mg/kg ifenprodil made significantly fewer successful alternations in the Y-maze than vehicle-treated WT mice (* $p < 0.05$, students t-test; † $p < 0.05$, Mann-Whitney U test).