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NF-κB activity affects learning in aversive tasks: possible actions via modulation of the stress axis

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Abstract

The role of altered activity of nuclear factor κB (NF-κB) in specific aspects of motivated behavior and learning and memory was examined in mice lacking the p50 subunit of the NF-κB/rel transcription factor family. Nfkb1-deficient mice are unable to produce p50 and show specific susceptibilities to infections and inflammatory challenges, but the behavioral phenotype of such mice has been largely unexamined, owing in large part to the lack of understanding of the role of NF-κB in nervous system function. Here we show that Nfkb1 (p50) knockout mice more rapidly learned to find the hidden platform in the Morris water maze than did wildtype mice. The rise in plasma corticosterone levels after the maze test was greater in p50 knockout than in wildtype mice. In the less stressful Barnes maze, which tests similar kinds of spatial learning, the p50 knockout mice performed similarly to control mice. Adrenalectomy with corticosterone replacement eliminated the differences between p50 knockout and wildtype mice in the water maze. Knockout mice showed increased levels of basal anxiety in the open-field and light/dark box tests, suggesting that their enhanced escape latency in the water maze was due to activation of the stress (hypothalamic-pituitary-adrenal) axis leading to elevated corticosterone production by strongly but not mildly anxiogenic stimuli. The results suggest that, as in the immune system, p50 in the nervous system normally serves to dampen NF-κB -mediated intracellular activities, which are manifested physiologically through elevated stress responses to aversive stimuli and behaviorally in the facilitated escape performance in learning tasks.

Keywords

NF-κB; Spatial learning and memory; Hypothalamic-pituitary-adrenal axis; Corticosterone; Anxiety; Hippocampus; Barnes maze; Morris water maze

1. Introduction

The nuclear factor κB (NF-κB) family of transcription factors (p65, c-ReL, RelB, p50, and p52) is classically associated with the control of biological processes active in pathological settings such as inflammatory and innate immune responses (Ghosh et al., 1998), immune dysfunction (Hayden and Ghosh, 2004), and cancer (Aggarwal, 2004). Recent studies have also implicated NF-κB in a broad spectrum of CNS functions ranging from development and

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synaptic plasticity to emotional learning and memory (O'Neill and Kaltschmidt, 1997; Albensi and Mattson, 2000; Yeh et al., 2002; Meffert et al., 2003; Wang et al., 2004; Meffert and Baltimore, 2005). The NF-κB family consists of five different subunit proteins that pair to form homo- and heterodimers that recognize specific DNA sequences in *cis*-acting elements of target genes (Hayden and Ghosh, 2004). Each monomer contains a Rel region through which it can bind to DNA and dimerize. Only three family members—p65 (RelA), cRel, and RelB—contain transcriptional activation domains (TAD) enabling them to induce transcriptional activity. In contrast, p50 and p52 lack a TAD and therefore form homodimers with no intrinsic ability to activate transcription, although they form transcriptionally active heterodimers in association with p65, cRel, and RelB

The prototypical NF-κB heterodimer p65-p50 is sequestered in the cytoplasm through interaction with inhibitory IκB proteins. However, the constitutive presence of p50 has been detected in the nucleus of unstimulated monocytes and other cell types (Kang et al., 1992; Kaufman et al., 1992; Kaltschmidt et al., 1994). Because p50 lacks a TAD, p50-p50 homodimers are thought to act predominately as repressors of gene expression in resting conditions (Ghosh and Karin, 2002; Zhong et al., 2002). These transcription-repressive functions may serve to fine-tune NF-κB -directed immune and other responses. Indeed, the loss of this repressor function can be illustrated by the exaggerated cytokine response to LPS (Sha et al., 1995) and to IL-1β (Campbell et al., 2008), relative to wildtype mice, observed in mice lacking the p50 subunit by virtue of genetic deletion of the Nfkb1 gene (Sha, 1995), which encodes the precursor p105 and p50.

Although its function in the immune system is well characterized, comparatively little is known of the consequences of NF-κB activation, and the involvement of p50, in CNS processes. Besides immune dysregulation, loss of the p50 subunit affects emotional learning (Kassed, 2002), motivated behavior (Kassed and Herkenham, 2004), and adult hippocampal neurogenesis (Denis-Donini et al., 2008), indicating that p50 and its action on genes influenced by NF-κB are clearly important for some aspects of normal CNS function. The current study examines the relationship between stress and learning in Nfkb1−/− (p50−/−) mice, using the Morris water maze (MWM) and the Barnes maze. These spatial learning tasks provide different degrees of aversive stimulation to measure the performance of hippocampal dependent learning and memory. In the first experiment, we show that mice lacking p50 show significant enhancement of learning in the MWM, a highly anxiogenic task (Harrison et al., 2009). However, this enhanced performance is not seen in another task of spatial learning—the Barnes maze, a less aversive/anxiogenic task (Holmes et al., 2002; Harrison et al., 2009). We hypothesized that the p50−/− mice have an altered stress response, which accounts for their improved spatial learning ability under aversive conditions. To test this hypothesis, a second experiment assayed corticosterone (CORT) response during the MWM task and examined the effect of clamping hypothalamus-pituitary-adrenal (HPA)-axis activity during spatial learning. If the p50−/− mice perceive the MWM task as more stressful than their wildtype littermates, increased circulating levels of corticosterone—which can facilitate learning—could account for the performance differences between groups. If glucocorticoids are playing a key role, clamping the HPA-axis via adrenalectomy should eliminate the superior learning ability displayed by p50−/− mice in the MWM task. We report that enhanced learning ability displayed by p50−/− mice in the MWM task is dependent on elevated CORT response. These findings suggest that p50−/− mice are resistant to the adverse effects of anxiety on memory and that elevated CORT release from MWM exposure is, in part, responsible for the enhanced spatial learning displayed in p50−/− mice.

2. Methods and Materials

2.1 Animals and surgery

The current experiment used adult (10–12 week-old) male wildtype (WT p50+/+) and Nfkb1-deficient (p50−/−) mice (Sha, 1995) obtained from Phillip Scott, University of Pennsylvania, PA where they had been backcrossed onto a C57BL/6 background. Animals were bred and genotyped in-house. WT p50+/+ mice were littermates of p50 knockout (p50−/−) mice. All were group-housed with food and water provided ad libitum. Lighting was maintained on a 12-h light-dark cycle with lights on at 0600, and all behavioral testing was performed during the light phase of the cycle. The NIMH Intramural Research Program Animal Care and Use Committee approved all procedures, which conformed to the NIH Guide for the Care and Use of laboratory animals.

Mice were anesthetized with isoflurane (5% induction, 2% maintenance). A 2-cm midline incision was made on the dorsal hump, and using the kidney as a landmark, both adrenal glands were exposed and removed with looped forceps. Ketoprofen (.01 mg/kg) was administered directly into the intraperitoneal cavity and the skin was sutured shut. Adrenalectomized mice were provided normal 0.9% saline with 25 μg/ml corticosterone (C2505, Sigma Chemical Company) ad libitum in lieu of drinking water to emulate basal CORT levels. Corticosterone was prepared by dissolving in EtOH (10 mg/ml) that was then added to the water. In sham-operated mice, all surgical procedures were identical, but adrenal glands were not removed.

2.2 Experimental procedures

2.2.1 Open Field Test—Exploratory locomotor activity was examined in an automated open-field test (Accuscan, Columbus, OH). Mice were placed in the center of the open field (40 × 40 × 30.5 cm) and allowed to explore for 15 min. Rearing and exploratory activity were collected using the Versamax activity monitor and analyzer software system (Accuscan).

2.2.2 Light/dark box test—The light/dark box test was conducted using a Plexiglas box (50 cm × 25 cm with 30 cm walls) consisting of a dark (one-third of the box) and a transparent (two-thirds of the box) compartment that was illuminated to approximately 40 lux. An open door divided the compartments. Each mouse was placed in the light compartment and allowed to freely move within the compartments for 10 min. The time spent in the dark compartment was measured as an indicator of anxiety-like behavior.

2.2.3 Morris Water Maze—Previously untested male p50−/− and p50+/+ littermates were trained to locate a submerged escape platform using salient extramaze cues found in the testing room. Testing was conducted in a circular pool (120-cm diameter) filled with white-opaque tap water (25–27°C) to a level that covered a stationary transparent escape platform by 1 cm. Hidden platform training consisted of four trials per session, with the mouse starting facing the pool edge, in a new quadrant on each trial. Trials lasted for 60 sec. If a mouse did not successfully locate the platform by the completion of the trial, it was guided to the platform by the experimenter. Mice remained on the platform for 15 sec before being placed under a warming light for the 30–45 sec intertrial interval. Mice were given a block of four trials per day for up to 5 days to learn the location of the submerged platform. The criterion for learning was an average latency of 15 sec or less to locate the platform across a block of four consecutive trials per day. A 1-min probe trial was conducted 3 h after completing hidden platform testing either on day 5 or on the day the 15-sec criterion for learning was met. Trials were videotaped and scored with Actimetrics video tracking software (Actimetrics, Inc. Wilmette, IL). For analysis of spatial learning in the MWM, escape latencies for each animal were averaged per block of trials. The effectiveness of the probe trial search was evaluated by time spent in each quadrant (dwell time) and the number of crossings over the trained quadrant platform location

compared to the analogous locations in the non-trained quadrants. Spatial learning was demonstrated by greater dwell times in the quadrant where the platform had been previously located, in comparison to other areas of the pool. To assess memory performance in the 3-h probe trial, dwell times for each quadrant were averaged by group.

A second experiment measured serum corticosterone and biochemical changes in hippocampus of p50−/− and p50+/+ mice after spatial learning in MWM. Using parameters similar to the first MWM experiment, a naïve cohort of mice was exposed to a maximum of five days of hidden platform trials. Mice were decapitated 30 or 60 min after testing on day 1, 3, or 5. Trunk blood was collected for investigation of HPA response to MWM.

A third experiment measured hippocampal NF-κB signaling by electrophoretic mobility shift assay in the hippocampus of p50−/− and p50+/+ mice after spatial learning in MWM. NF-κB p50−/− mice and their WT counterparts were exposed to four 1 min trials of MWM training for up to 5 days. On MWM training day 1, 3, or 5 mice were sacked 15 min after the first trial and hippocampus removed. A 15 min time point was based on work performed by Boccia et al (Boccia et al., 2007) that showed an increase of hippocampal NF-κB activity 15 min after memory reactivation. A swim only (yoked control) group was used as an internal control. The hidden platform was removed in the yoked control group and mice were placed in the MWM and allowed to swim for the same amount of time as their spatially trained counterparts.

2.2.4 Barnes Maze—The Barnes maze is similar to the MWM in that it tests spatial learning ability but without the inherent high-stress swim component. For this test, a 100-cm diameter circular maze with 21 possible exit holes along the periphery was set up in a low-light arena, with minimum noise and no air current. One of the holes opens into a burrow, made of opaque Plexiglas. A naive set of previously untested animals were placed under an opaque dome in the middle of the maze and allowed to acclimate to the surface for 1 min. Once the dome is lifted, the animal is able to orient to distant spatial cues and find the hole with a hidden burrow. On the first day of testing, a training session was performed. The mouse was placed in the middle of the maze in a start chamber. After 10 sec had elapsed, the chamber was lifted, and the mouse was set free to explore the maze. The session ended when the mouse entered the escape burrow or after 5 min elapsed. If the mouse did not enter the burrow by itself, it was guided there and allowed to remain there for 2 min. Following the pre-training trial, the first trial started. Acquisition phase consisted of five consecutive training days with four trials per day. Each trial lasted 5 min. The maze was rotated between trials and the surface cleaned with 70% EtOH between trials to remove any odor trails, although the burrow was kept in the same spatial position relative to the room and external objects within it. The following parameters were recorded: number of primary errors, total errors, primary latency to find exit hole, and total latency to enter exit hole. Errors were defined as nose pokes and head deflections over any hole that did not have the burrow. Because the Barnes maze lacks strong reinforcement stimuli, mice may lack motivation and occasionally explore the maze after finding the target hole without entering it. To rectify this confounding factor, we measured both total and primary errors to better understand and interpret data during the acquisition phase. The number of errors and latency to the first encounter of the escape burrow were called primary errors and primary latency, respectively. Three h after the acquisition phase, subjects received a probe trial for 90 sec to check the short-retention memory. During probe trials, the tunnel leading to the target box was closed. Mice were allowed to explore the maze, and visits in to the target hole, adjacent holes, and primary latency to reach the target hole were recorded.

2.2.5 Plasma corticosterone determination—Collected trunk blood was centrifuged at 800 g for 30 min, and plasma was frozen at −70°C until analysis. Plasma corticosterone was quantified by radioimmunoassay using ICN kits (ICN Radiochemicals, Irvine, CA).

2.2.6 Hippocampal mRNA expression—The expression of immediate early genes in the hippocampus was analyzed after MWM exposure on Days 1, 3, and 5. A control group was added to separate IEG responses induced by non-learning stimuli (ie, sensorimotor stimulation, stress, swimming behavior) and IEG responses caused by spatial learning. In the control group, free swimming mice were placed in the MWM and allowed to swim for the same amount of time as their spatially trained counterparts. Thirty or sixty min after the first MWM swim trial, mice were rapidly decapitated, and the brain was removed from the skull. The brain was rapidly sectioned into 0.2 mm thick slices and placed into RNAlater (Qiagen, Valencia, CA) for 24 h at 4°C. Hippocampus (without choroid plexus) was micro-dissected from the sections and total RNA was extracted using a commercial kit (Qiagen, Valencia, CA). Total RNA was quantified on a Nanodrop spectrophotometer (Thermo Scientific, USA) and reverse transcribed using a Superscript III First Strand cDNA Synthesis Kit (Invitrogen, USA). Two-step real-time RT-PCR with 2× SYBR Green Master Mix (Bio-Rad)was performed using the Bio-Rad iCycler (Hercules, CA). The endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used to normalize quantification of the mRNA target. RT-PCR was performed using the following primers: GAPDH; Forward 5′ CAAAATGGTGAAGGTCGGTGTG Reverse 5′ TGATGTTAGTGGGGTCTCGCTC, FOS; Forward GGGGCAAAGTAGAGCAGCTA Reverse TGCAACGCAGACTTCTCATC, ARC; Forward GGTACACTCTCCCGTGAAGC Reverse CTCCTCAGCGTCCACATACA, EGR1; Forward GAGCGAACAACCCTATGAGC Reverse GAGTCGTTTGGCTGGGATAA, JunB; Forward GCAGCTACTTTTCGGGTCAG Reverse TTCATCTTGTGCAGGTCGTC. We also chose to examine the expression of several chemokines known to be regulated by NF-κB, including: CCL2; Forward 5′ ATCCCAATGAGTAGGCTGGAGAGC Reverse 5′ GGTGGTTGTGGAAAAGGTAGTGG, CXCL1; Forward GCTGGGATTCACCTCAAGAA, Reverse TGGGGACACCTTTTAGCATC, CX3CL1; Forward AAGGTCTTCCAATGTGGCGG Reverse ATCCCAGTGGCTTTGCTCATC. All primers were validated prior to this experiment. Absolute quantitative measurement of immediate early gene (IEG) mRNA was as follows: following RT-PCR, products were run on an agarose gel, product bands visualized with UV light, excised and then extracted using a QIAquick Gel extraction kit (Qiagen, USA). Isolated cDNA was quantified on a Nanodrop, serially diluted, and used as a template for RT-PCR. Dilution curves confirmed the linear dependence of the threshold cycle number on the concentration of template RNA. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their Ct values (Heid et al., 1996).

2.2.7 Electrohoretic mobility shift assay (EMSA)—Hippocampal tissue samples were homogenized on ice in the cytosolic extraction buffer provided in a cytosolic-nuclear extraction kit (Pierce). Cytosolic and nuclear extracts were then prepared using this kit according to the manufacturor's instructions. All buffers used were supplemented with both protease (Pierce) and phosphatase (Sigma) inhibitors. Twenty (for hippocampal samples) micrograms of nuclear extracts were incubated in EMSA binding buffer (10 nM Tris,-PH 7.4, 20 mM KCL, 1 mM MgCL2, 0.5 mM EDTA, 2.5% glycerol, 20 ug BSA, 0.5% NP40, 1 ug Poly dI:dC) for 30 min at room temperature in the presence of 32P-labeled double-stranded NF-κB oligonucleotide (AGTTGAGGGGACTTTCCCAGGC, Promega Corp Madison, Wis.). DNA-protein complexes were resolved on a 6% non-denaturing polyacrylamide gel in Tris-borate buffer. The gel was then frozen and the DNA-protein complexes visualized by autoradiography. Pre-incubation of the tissue extracts for 20 minutes at room temperature prior to the addition of the 32P-labeled double-stranded NF-κB oligonucleotide with either unlabeled double-stranded NF-κB oligonucleotide, or NCI 1263 (anti-p50) and NIH #7057 (anti-p65) antisera was done to either determine the specificity of the binding reaction or the components of the DNA-protein complex. Gel band density was determined using ImageJ Gel Analysis Macro and analyzed

with Two-way ANOVA (genotype × time). Optical density values were relativized to the mean optical density values of the WT yoked control group to estimate the NF-κB activity in hippocampal nuclear extracts.

2.3 Statistics

All the models of spatial learning tasks and ambulatory counts in the open field were analyzed using two-way ANOVA (Genotype × Trial Time) with repeated measures (trial days) and Bonferonni post-hoc tests when significant main effects or interactions were detected. Data collected from plasma corticosterone and hippocampal mRNA expression assays were assessed by a two-way ANOVA and Bonferonni post-test comparisons, where applicable. Data from mRNA analysis were further examined by one-way ANOVA and where applicable Student-Newman-Keuls *post-hoc* comparisons. A t-test was used to analyze basal anxiety between genotypes in Light/Dark test. Statistics were analyzed using software packages from SPSS 16.0 for Macintosh, and significance was determined at $p < 0.05$. Data were expressed as mean ± S.E.M.

3. Results

3.1. Basal anxiety measurements

The characterization of anxiety-like behavior was based on performance in the open-field test and light/dark test. Both tests use conflicting innate tendencies—avoidance of bright light and open spaces (that ethologically mimic a situation of predator risk) measured against the motivational drive to explore a novel environment—to determine adaptive emotional responses to mild stressors. Reduced exploratory behaviors characterized by thigmotactic (wall-hugging) behavior in the open field test and avoidance of the brightly lit area in the light/dark test are classically interpreted as anxiety-like behavior (Crawley, 1985; Crawley et al., 1997).

3.1.1. Open field test—NF-κB1 p50−/− and WT p50+/+ littermates were assessed for exploratory activity and anxiety-like behavior in the open field test. Two-way repeated measures ANOVA (genotype × time) revealed a significant genotype x time interaction effect on horizontal locomotor activity [$F_{(2,56)} = 4.478$, $p < 0.02$] and center time exploration [$F_{(2,56)} = 7.843$, $p < 0.001$]. There was also a significant time effect on horizontal locomotor activity [$F_{(2,56)} = 16.74$, $p < 0.0001$, Fig 1a] and center time exploration [$F_{(2,56)} = 18.88$, $p < 0.0001$, Fig 1b]. There was also a significant effect of genotype on horizontal locomotor activity [$F_{(1,28)} = 39.28$, $p < 0.0001$] and center time exploration [$F_{(1,28)} = 26.06$, $p < 0.0001$]. Bonferroni posttest analysis showed that p50−/− animals traveled significantly less than p50 +/+ littermates across all measured time periods (0–5 min: $t = 4.77$ $p < 0.001$; 5–10 min: $t = 3.27$ $p < 0.01$; 10–15 min: $t = 2.82$ $p < 0.05$) and they spent significantly less time in the center arena between 0–5 min ($t = 2.73$, $p < 0.05$) and 10–15 min ($t = 5.38$, $p < 0.001$), indicating an anxiogenic-like phenotype. A significant interaction effect [$F_{(2,56)} = 11.78$, $p < 0.0001$, Fig 1c] and a significant genotype effect [$F_{(1,28)} = 19.2$, $p < 0.001$, Fig 1c] on vertical activity (rearing behavior) was also detected. Bonferroni post-hoc analysis showed that p50−/− mice exhibited significantly less rearing behavior during the first 10 min of open field exposure (0–5 min: $t = 4.14$ $p < 0.001$; 5–10 min: $t = 2.90$ $p < 0.05$).

3.1.2. Light/dark exploration test—As shown in Fig. 1d, p50−/− mice spent significantly less time in the light chamber compared to WT littermates ($t = 1.90$, $df = 18$, $p < 0.05$), indicating increased anxiety in the p50−/− mice. No significant difference between genotypes on the number of transitions between light and dark chambers was detected (Fig 1e).

3.2. Spatial learning tasks

3.2.1 5 day Morris water maze trial—The MWM is a test of spatial learning for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform. The MWM has proven to be a robust and reliable test that is strongly correlated with hippocampal synaptic plasticity and NMDA receptor function. As shown in Fig. 2a, a two-way ANOVA with repeated measures revealed significant variability in regards to latencies to find the hidden platform [genotype × day interaction effect [$F_{(4,88)} = 4.389$, $p < 0.005$]; genotype effect [$F_{(1,22)} = 31.33$, $P = < 0.0001$]; and a day effect [$F_{(4,88)} = 82.4$, $P < 0.0001$]. Bonferroni posthoc analysis showed that on days 2, 3, and 4, p50−/− mice had significantly shorter escape latencies than did their p50+/+ littermates (day 2: $t = 4.62$ $p < 0.05$; day3: $t = 5.75$ $p < 0.001$; day 4: $t = 5.36$ $p < 0.001$). Swim velocities were also analyzed to remove the possibility that enhanced performance observed in p50−/− mice was due to swim speeds differences between genotypes (Fig. 2b). Although both groups showed increased swim velocity as the trial progressed [$F_{(4,88)} = 4.439$, $p < 0.005$], no significant differences between genotypes were detected.

3.2.1.1. Probe trial: During probe trial testing, all genotypes spent significantly more time (Fig. 2c) [$F_{(3,47)} = 5.914$, $p < 0.001$], [$F_{(3,47)} = 7.147$, $p < 0.0005$] (p50−/−, p50+/+ respectively) (Newman-Keuls Post hoc, $p < 0.001$) and made significantly more platform crosses (Fig. 2d) [$F_{(3,47)} = 21.89$, $p < 0.0001$], [$F_{(3,47)} = 26.61$, $p < 0.0001$] (p50−/−, p50+/+ respectively) (Newman-Keuls Post hoc, $p < 0.001$) in the trained quadrant than all other quadrants. No significant difference between genotypes was observed.

3.2.2. Three-day Morris Water Maze Trial—The findings presented above suggest that p50−/− mice show enhanced learning in MWM, p50−/− mice reach criterion on Day 3 compared to Day 5 for WT mice. However, no significant differences in probe test performance were detected between genotypes, most likely because the probe test was performed after five days of training, when no noticeable difference in latencies was observed. To confirm that differences in latency indicate an enhancement in spatial learning a second cohort of mice was exposed to MWM training for three days (Fig. 3a) and probe tested on the third trial day (Fig 3c and 3d). A two-way ANOVA with repeated measures revealed a significant effect of genotype [$F_{(1,18)} = 9.93$, $p < 0.005$]; and trial day [$F_{(2,36)} = 53.73$, $p < 0.0001$], but no interaction effects. For swim velocity, no significant effects of time or genotype were detected (Fig. 3b)

3.2.2.1. Probe trial: A one-way ANOVA of p50−/− performance in the probe trial revealed a significant effect of time [$F_{(3,39)} = 50.29$, $p < 0.005$]; (Fig. 3d) and platform crosses [$F_{(3,39)} = 12.43$, $p < 0.005$]; (Fig. 3c). After three days of acquisition training, p50−/− mice exhibited a strong bias for the training quadrant during the probe trial as determined by a significant increase in time spent and number of platform crosses made in the training quadrant compared to all other quadrants (Newman-Keuls Post hoc, $p<0.001$). In contrast, WT mice showed no significant preference for the training quadrant.

3.2.3. Hippocampal mRNA expression after MWM—Based on the observed differences in spatial learning acquisition between genotypes, we examined the expression of several IEGs within the hippocampus 30 min and 60 min after MWM exposure (Table 1). An additional control group was added to separate mRNA expression caused by swim stress and expression due to spatial learning. In this group, the hidden platform was removed from the MWM and mice were exposed to conditions similar to the experimental groups. Two-way ANOVA showed no significant variances at 30 min or 60 min for *fos* or *arc* expression. However, 30 min after MWM, significant variability of trial day was found for *junb* [$F_{(3,40)} = 30.45$, $p < 0.0005$] and *egr1* [$F_{(3,40)} = 47.05$, $p < 0.001$]. In Hippocampal samples collected 60 min after MWM, two-way ANOVA of *junb* expression detected significant variability of trial day

[$F_{(3,40)}$ = 23.86, $p < 0.001$], genotype [$F_{(1,40)}$ = 7.00, $p < 0.02$], and a significant interaction (day x genotype) [$F_{(3,40)}$ = 24.89, $p < 0.0005$]. Hippocampal expression of *junb* mRNA was significantly higher in p50−/− mice compared to wt control mice 60 min after MWM. For *egr1*, significant variability of genotype [$F_{(1,40)}$ = 5.34, $p < 0.05$] and trial day [$F_{(3,40)}$ = 43.03, $p < 0.0001$] was detected 60 min after MWM; however no interaction effects were observed. Because trial day as a factor was statistically significant for *egr1* and *junb* expression, the respective significances for single trial days are shown in Table 1. Although *fos* and *arc* show no statistical change, *egr1* and *junb* show significant increases during acquisition compared to swim-only control group, suggesting that *egr1* and *junb* may be involved in acquisition of spatial memory.

We also examined the mRNA expression of several NF-κB responsive genes within hippocampus after MWM, including *cxcl1*, *ccl2*, and *cx3cl1*. No significant changes were found in *ccl2* or *cx3cl1* expression, data not shown. However, a two-way ANOVA of *cxcl1* revealed significant variability of genotype [$F_{(1,40)}$ = 77.82, $p < 0.0001$] and trial day [$F_{(3,40)}$ = 4.537, $p < 0.01$]. For *cxcl1*, respective differences for single trial days are shown in Table 1. Interestingly, *cxcl1* mRNA was significantly increased 30 min after the first day of MWM training compared to a similar time point in the swim only group, suggesting that spatial learning alters *cxcl1* expression in p50−/− mice.

3.2.4. Plasma Corticosterone after MWM—We measured plasma corticosterone secretion 30 min (Fig. 4a) and 60 min (Fig. 4b) after MWM exposure. A two-way ANOVA (day x genotype) revealed a significant main effect of trial day on plasma corticosterone levels at both 30 min [$F_{(2,24)}$ = 30.47, $p < 0.0001$] and 60 min [$F_{(2,24)}$ = 7.456, $p < 0.005$] after completion of MWM. Two-way ANOVA also detected a significant main effect of genotype on plasma corticosterone [$F_{(1,24)}$ = 55.57, $p < 0.0001$] 30 min after completion of MWM. Interestingly, Bonferonni posthoc analysis showed that p50−/− mice had significantly higher plasma corticosterone levels 30 min after MWM testing compared to p50+/+ mice on all examined days (Day 1: $t = 2.231$, $p<0.05$; Day 3: $t = 3.831$ $p < 0.005$; Day 5: $t = 15.52$, $p<0.0001$) (Fig. 4A). However, no significant differences in plasma corticosterone levels were detected between genotypes 60 min after MWM (Fig. 4B).

3.2.5. Characterization of NF-κB activity in hippocampus after MWM—We next evaluated the presence of activated NF-κB in the hippocampus of WT and p50−/− mice 15 min after 1, 3, or 5 days of MWM training and in a yoked control group. The gel shift of the different samples and the densitometric analyses of the gel shift are shown in Fig. 5A and 5B, respectively. Two-way ANOVA found a strong effect of genotype [$F_{(3,15)}$ = 17.64, $p < 0.001$], NF-κB mediated gel shifts were detected in WT control mice after water maze training and after free swimming (yoked control). In contrast, hippocampal nuclear extracts from trained and yoked control p50−/− mice did not contain activated NF-κB complexes. In either genotype no significant difference in band intensity was detected between yoked control groups and mice exposed to spatial learning. The gel supershift shown in Fig. 5C demonstrates that the ability to detect DNA binding of p50 to κB sites was intact and provides specificity of the binding reaction and the components of the DNA-protein complex. The lack of difference between yoked-control and spatially trained groups suggests that hippocampal NF-κB DNA binding may be caused by a non-specific (non-learning) stress response in WT mice.

3.2.6. Barnes maze—The Barnes maze is a learning task requiring use of spatial cues similar to the MWM, but the task itself is much less aversive. A two-way ANOVA with repeated measures was used to analyze primary latency to find and total latency to enter the target burrow during the acquisition phase. Spatial learning is reflected by significant reduction in the number of primary and total errors. Two-way repeated measures ANOVA revealed significant variability in the number of primary (Fig 6a); [$F_{(4,88)}$ = 110.7, $p < 0.0001$] and total errors (Fig

6b); [$F_{(4,88)} = 86.71$, $p < 0.0001$] across trial days. Furthermore, a two-way ANOVA with repeated measures showed significant variability in the latency to find (Fig 6c); [$F_{(4,88)} = 36.16$, $p < 0.0001$] and latency to enter (Fig 6d); [$F_{(4,88)} = 98.21$, $p < 0.0001$] the target burrow across the five trial days. No significant differences between genotypes or interaction effects between genotype x trial day were observed.

3.2.6.1 Barnes maze probe trial: During the probe trial on day five, the number of visits to each hole was recorded (Fig 7a). Repeated-measurements ANOVA found no significant difference between strains. Paired Students' t-test used to examine the difference between average visits to the target hole (Hole 0) and adjacent holes revealed significant differences in each genotype, indicating that both groups learned the task. Unpaired Student's t-test showed no significant difference between strains for visits to the target hole. The primary latency to find the target hole was also analyzed (Fig 7b); no significant difference between genotypes was detected.

3.2.7. Adrenalectomy (ADX) and Morris water maze—The p50−/− mice relative to WT mice show enhanced spatial learning ability and higher corticosterone secretion after MWM exposure. We tested whether the elevated corticosterone secretion is responsible for enhanced spatial learning ability displayed in p50−/− mice. Adrenalectomized p50+/+ and p50 −/− mice receiving corticosterone-treated water ad libitum and sham surgery control mice were exposed to hidden platform MWM testing over 5 days. As shown in Fig. 8a, ADX had no effect on acquisition performance in WT mice. In comparing acquisition performance between WT Sham and WT ADX groups, a two-way ANOVA with repeated measures revealed significant variability in regards to latencies to find the hidden platform only across trial days [$F_{(4,48)} = 22.12$, $p < 0.0001$], suggesting that both groups acquired the training task at a similar rate. Interestingly, adrenalectomy in p50−/− mice had a significant effect on acquisition performance across trial days, shown in Fig. 8b. a two-way repeated measures ANOVA for acquisition performance of Sham and p50−/− ADX groups revealed significant variability in regards to latencies to find the hidden platform [surgery effect: [$F_{(1,14)} = 12.55$, $p < 0.05$]; trial day effect: [$F_{(4,56)} = 66.39$, $p < 0.0001$]; a trial day x surgery interaction: [$F_{(4,56)} = 4.291$, $p < 0.05$]. Bonferroni post-hoc analysis showed that on days 3 and 5, Sham p50−/− mice had significantly shorter escape latencies compared to ADX p50−/− mice (day3: $t = 4.59$ $p < 0.001$; day 5: $t = 3.43$ $p < 0.01$). For swim velocity, two-way repeated measures ANOVA revealed no significant effect of surgery in either genotype group, and a significant effect of trial day in both groups; p50−/− [$F_{(4,56)} = 4.915$, $p < 0.05$], WT [$F_{(4,48)} = 5.954$, $p < 0.01$]. Recorded values for swim velocity (cm/s ± S.E.M.) are as follows: for p50−/− SHAM: 14.34 ± 0.682, 15.62 ± 0.51, 16.06 ± 0.44, 16.49 ± 0.66, 17.06 ± 0.84, Days 1,2,3,4, and 5 respectively. For p50−/− ADX mice swim velocities (cm/s ± S.E.M.) are as follows: 14.06 ± 0.32, 15.33 ± 0.92, 16.36 ± 0.61, 16.41 ± 0.28, 16.58 ± 0.86 for days 1,2,3,4, and 5 respectively. For WT SHAM mice swim velocities (cm/s ± S.E.M.) are as follows: 14.09 ± 0.25, 14.79 ± 0.41, 15.67 ± 0.26, 16.14 ± 0.74, 16.87 ± 0.47, Days 1,2,3,4, and 5 respectively. For WT ADX mice swim velocities (cm/s ± S.E.M.) are as follows: 14.34 ± 0.97, 14.38 ± 0.84, 15.09 ± 0.90, 15.59 ± 0.54, 16.02 ± 0.77 for days 1,2,3,4, and 5 respectively.

3.2.7.1. Probe trial: During probe trial on day 5, time spent in target quadrants was measured and analyzed (each strain separately). As shown in figures 8c and 8d, all groups showed significant preference for training quadrant (all comparison *p* values < 0.05) and made significantly more platform crosses (Fig. 8d) in the trained quadrant vs. all other quadrants (all comparison *p* values < 0.05). No significant difference between groups was observed.

4. Discussion

In the present study, we characterized the behavioral phenotype and evaluated spatial learning and memory ability of Nfkb1-null (p50−/−) mutants. Principal findings emerging from this work were that p50−/− relative to WT p50+/+ mice show 1) a situation-specific anxiogenic phenotype, 2) enhanced spatial learning ability in cognitive tasks with inherent high stress (Morris water maze) but no significant enhanced learning ability in tasks with low stress (Barnes maze) components, and 3) significantly elevated serum corticosterone levels 30 min after MWM exposure. Lastly, the enhanced learning ability displayed by p50−/− mice in the MWM task is abolished following adrenalectomy and corticosterone replacement. Altogether, these findings suggest that p50−/− mice are resistant to the adverse effects of anxiety on spatial learning and that elevated corticosterone release resulting from MWM exposure is, in part at least, responsible for the enhanced spatial learning displayed in p50−/− mice.

Previous work from our lab (Kassed and Herkenham, 2004) demonstrated an involvement of the p50 subunit in the manifestation of affective behavior using a p50−/− mouse bred on a mixed B6;129P2 genetic background, purchased from The Jackson Laboratory. Mice from that study displayed a strong anxiolytic phenotype, and we predicted a similar phenotype would be observed in p50−/− mice, with an identical deletion of the Nfkb1 gene, that had been backcrossed onto a pure C57BL/6 background. The striking phenotypic differences observed between these groups of mice, even though the studies were performed in the same laboratory, are most likely due to the different background strains that were utilized. A wealth of information has demonstrated that genotypic differences in the inbred strains used to generate targeted mutant mouse lines can affect the behavioral phenotype of the mutant line, including anxiety profiles (Crawley and Davis, 1982; Voikar et al., 2001; Bouwknecht and Paylor, 2002) and learning and memory (Crawley et al., 1997; Nguyen et al., 2000; Patil et al., 2009). Indeed, strain differences in murine anxiety paradigms affect not only the outcome of experiments with mouse mutants but also pharmacological and lesion studies in all animals (Wahlsten et al., 2006) for review).

The present results of our experiment indicate that p50−/− and p50+/+ mice perform similarly during the acquisition and retention phase of the Barnes maze but that p50−/− showed superior performance during the acquisition phase of the MWM, demonstrating that performance is task-dependent. The Barnes maze and MWM both assess spatial learning and memory in a similar manner; fixed extra-maze visible cues located around the testing arena permit animals to learn the location of the target zone (Morris, 1984; Pompl et al., 1999). Recent reports have shown that water maze training induces greater increases in plasma corticosterone compared to Barnes maze (Harrison et al., 2009). In comparison to the MWM, the Barnes maze is considered less aversive and less anxiogenic; it allows testing of spatial learning under less stressful conditions, without physical exertion (Barnes, 1979; Harrison et al., 2009; Patil et al., 2009).

The response to stress involves increased production of adrenal glucocorticoids that bind to mineralcorticoid and glucocorticoid receptors in hippocampus and elsewhere to influence the biochemical cascades triggered by associative learning. It is known that cognitive function, neuronal plasticity, and long-term potentiation are influenced by stress and glucocorticoids. The relationship between stress-induced arousal and performance in a learning task follows an inverted U-shaped function (Yerkes-Dodson law). Thus, low and high levels of arousal may impair learning, whereas moderate levels of stress-induced arousal are optimal for learning. The heightened anxiety measures observed in p50−/− mice during the open field and light/dark tests suggest higher baseline levels of arousal, a proposition further corroborated by the exaggerated corticosterone response to MWM in p50−/− relative to WT mice. Moreover, it is likely that the higher level of arousal exhibited by p50−/− mice is correlated with the superior

performance observed in MWM, because adrenalectomized p50−/− mice receiving corticosterone supplements, which clamped the stress-induced corticosterone spike, showed impaired acquisition relative to sham-operated p50−/− mice. In contrast, adrenalectomized, corticosterone-replaced WT mice performed only slightly more poorly than their unoperated WT counterparts, consistent with the fact that the corticosterone response had not been so greatly elevated in the normal WT mice in the MWM.

There is an important body of literature showing that NF-κB is involved in learning and memory. NF-κB activation in the brain has been shown to be required for consolidation of fear memories (Yeh et al., 2002; Yeh et al., 2004; Freudenthal et al., 2005; Lubin and Sweatt, 2007). Nuclear translocation of NF-κB subunits and DNA binding activity at κB sites were temporally linked to associative learning (Yeh et al., 2002) (Freudenthal et al., 2004). Knockdown of NF-κB activity with κB decoy DNA (Yeh et al., 2002; Yeh et al., 2004; Freudenthal et al., 2005; Boccia et al., 2007) blocked fear memory consolidation in a time- and context-specific manner. Furthermore, the disruption of the NF-κB p50 subunit has resulted in a number of interesting, albeit conflicting, findings. For instance, some groups have found p50-deficient mice to experience deleterious effects including; increased oxidative stress and striatal neuron damage following treatment with mitochondrial toxin (Yu et al., 1999), impaired learning ability (Kassed et al., 2002; Vernon et al., 2006), increased hippocampal neuronal degeneration following toxicant (Kassed et al., 2002) and excitotoxic injury (Yu et al., 1999), and age-related neuronal degeneration (Schneider et al., 1999; Lu et al., 2006). To the contrary, other studies have found p50-null mice to show increased hippocampal neuronal survival and reduced ischemic damage following mid-cerebral artery occlusion (Schneider et al., 1999), reduced oxidative stress biomarkers in brain (Owen et al., 2008), and in the current study, enhanced spatial learning ability. The lack of difference between yoked-control and spatially trained groups suggests that hippocampal NF-κB DNA binding may be caused by a non-specific (non-learning) stress response in WT mice. Interestingly this stress-induced activation was not detected in p50KO mice and may underlie the differential stress responses observed between the two strains. The difficult task of elucidating the exact mechanisms of p50 may be due to this key transcription factor's ability to act as both a transcriptional repressor when homodimerized and a transcriptional activator when heterodimerized with p65. In addition, nuclear p50 interacts with separate inhibitory proteins that recruit corepressor complexes containing histone deacetylases to various NF-κB target genes, and it functions as a general repressor of NF-κB-dependent transcription in resting cells (Zhong et al., 2002; Oakley et al., 2005). Indeed, the loss of this repressive mechanism may explain the augmented immune response to inflammatory responses (Sha et al., 1995; Udalova et al., 2000; Wessells et al., 2004; Campbell et al., 2008) in p50-null mice and may also contribute to alterations in anxiety and emotional learning and memory. In the current study, the loss of this epigenetic repressor could also explain elevated hippocampal *cxcl1* mRNA expression observed in p50−/− mice.

The hippocampal expression of immediate early genes is integral to spatial learning and make IEGs likely candidates for examining whether alterations in hippocampal activity correlate with enhanced spatial acquisition displayed by p50−/− mice. Compared to swim-yoked controls, both WT and p50−/− mice that acquired the spatial task displayed an increase in *zif268* and *junb* mRNA levels. The augmented expression of *junb* detected in p50−/− mice after the first day of MWM training may be due to the increased sensitivity to glutamate receptor activation (Yu et al., 1999) displayed in p50−/− mice since glutamate and NMDA receptor activation are known to induce the rapid expression of IEGs (Sharp et al., 1995). However the lack of differences in other IEGs and at other time points suggests that other unknown factors are at play.

The present study accentuates the complexities surrounding the mechanisms and involvement of NF-κB in emotional behavior and memory. Although the likely major consequence of loss

of the NF-κB p50 subunit in mice is the absence of its repressor function, our interpretations of the results are limited because the ultimate biological effect of NF-κB is highly dependent on the stimulus used for its activation, the subunit composition of its gene targets, and other unknown compensatory factors often present in unconditional knockout animal models. As far as we know, this is the first study to analyze spatial learning ability in p50-null mice using tasks with varying aversive components. Compared to wildtype littermates, p50−/− mice performed significantly better in the MWM, a task with high-stress component, but showed no significant differences of spatial learning in the Barnes maze, a less aversive task. Because of the strong correlation between enhanced corticosterone secretion and elevated acquisition displayed by p50−/− mice we hypothesized that corticosterone might facilitate spatial learning in p50−/− mice. We found that adrenalectomy significantly decreased spatial learning ability in p50−/− mice but had little effect of WT mice. Thus p50 is involved in modulating HPA responsiveness to stressful stimuli, and it influences learning under stressful situations.

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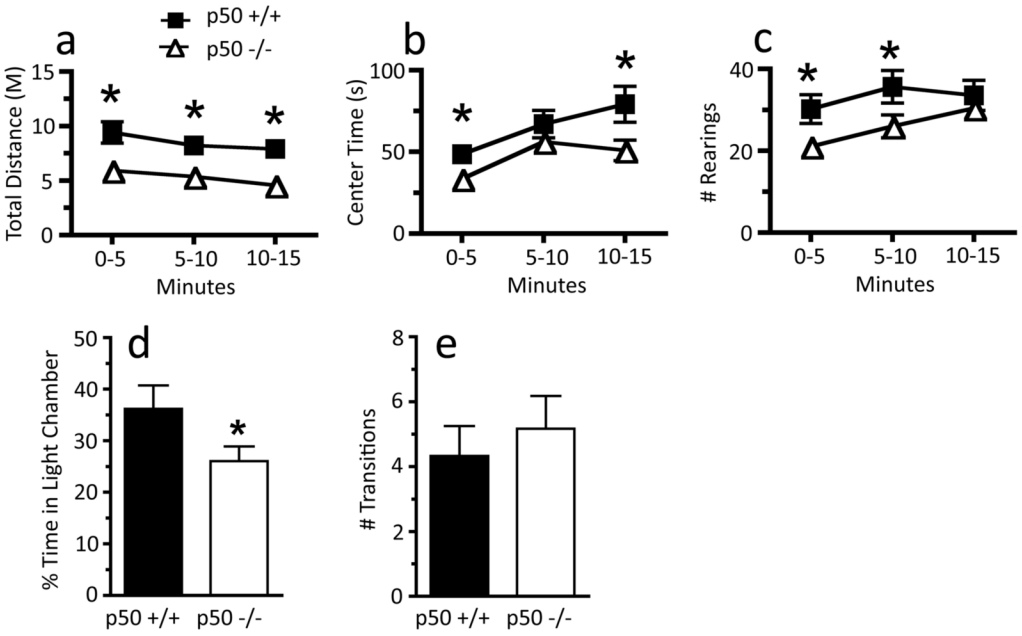

Figure 1.

Anxiogenic-like phenotype of NFkB1 p50−/− mice was observed in open field exploration and light/dark exploration tests. In the 15-min open field exploration test (a–c), p50−/− mice showed a significant decrease in distance traveled (a), spent significantly less time in the center quadrant (b), and showed a significant decrease in rearing behavior (c) than WT p50+/+ mice (n = 15 for each group). In the light/dark exploration test, p50−/− mice spent a higher percentage of time in the dark compartment compared to WT mice (d). The genotypes did not differ significantly on the number of transitions between the light and dark compartments (e) (n = 10 for each group). (Mean ± S.E.M.) (*$p < 0.05$)

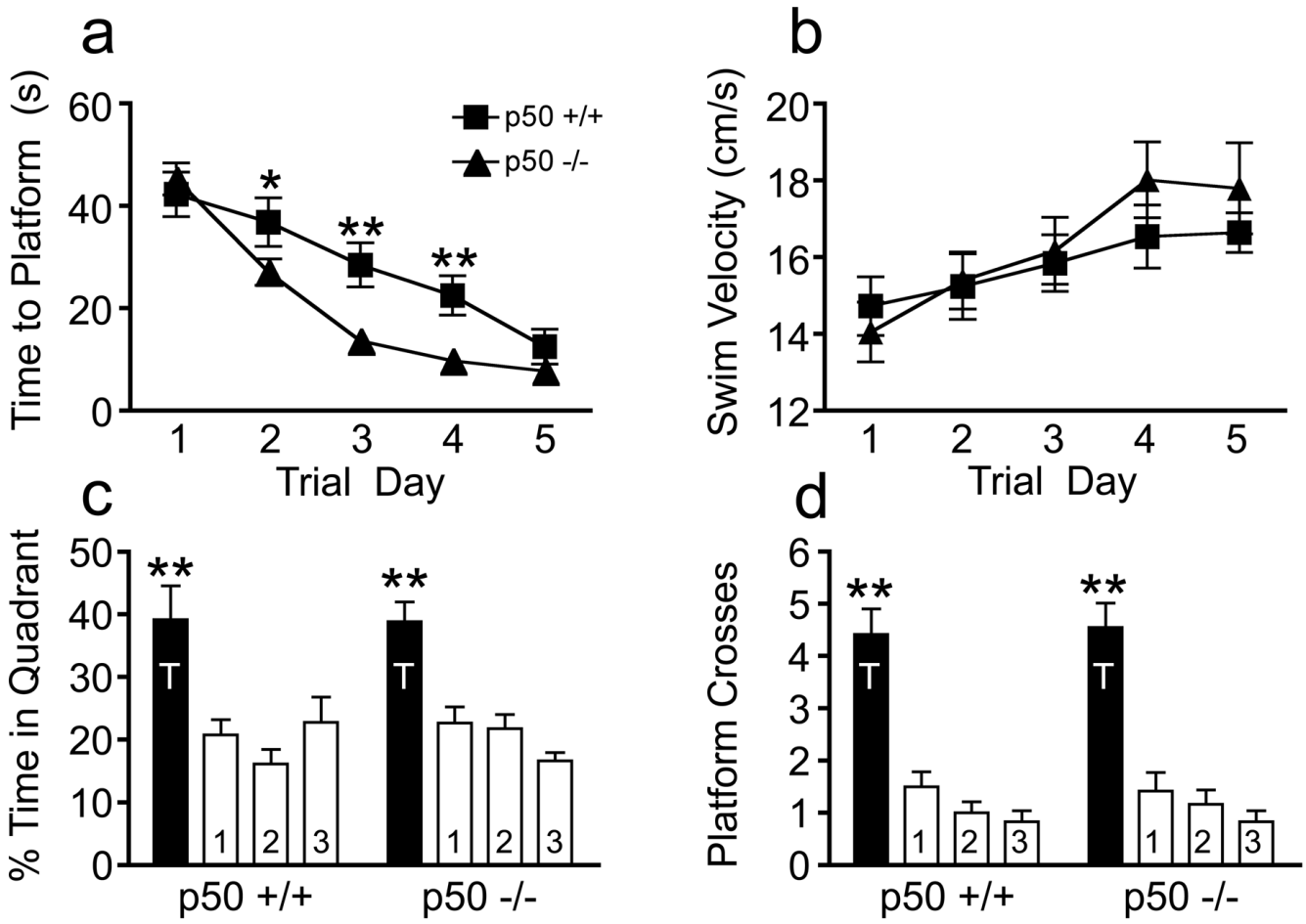

Figure 2.

In the Morris water maze, p50−/− mice performed significantly better than their WT p50+/+ littermates during acquisition, showing reduced total latency (sec) to reach the hidden platform. Latency to platform (a), and average swim speed (b) during 5 days of acquisition trials in MWM. Both genotypes demonstrated spatial learning during the probe trial. Significantly more time was spent in the trained quadrant (c) and significantly more platform crossings were made in the trained quadrant (d) than in the other three quadrants (n = 12 for each group). (Mean ± S.E.M.) (*$p < 0.05$)(**$p<0.01$)

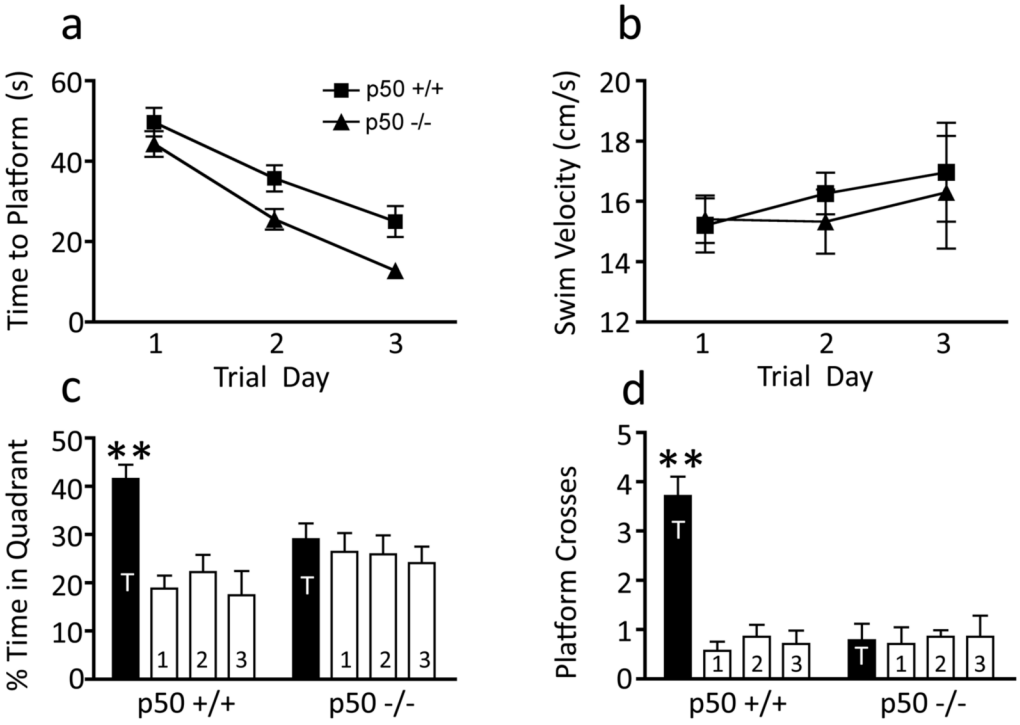

Figure 3.

In the Morris water maze, p50−/− mice performed significantly better than their WT p50+/+ littermates during acquisition, showing reduced total latency (sec) to reach the hidden platform. Latency to platform (a), and average swim speed (b) during 3 days of acquisition trials in MWM. During the probe trial, p50−/− mice spent significantly more time (c) and made significantly more platform crossings (d) in the trained quadrant than in the other three quadrants (n = 10 for each group). (Mean ± S.E.M.) (*$p < 0.05$)

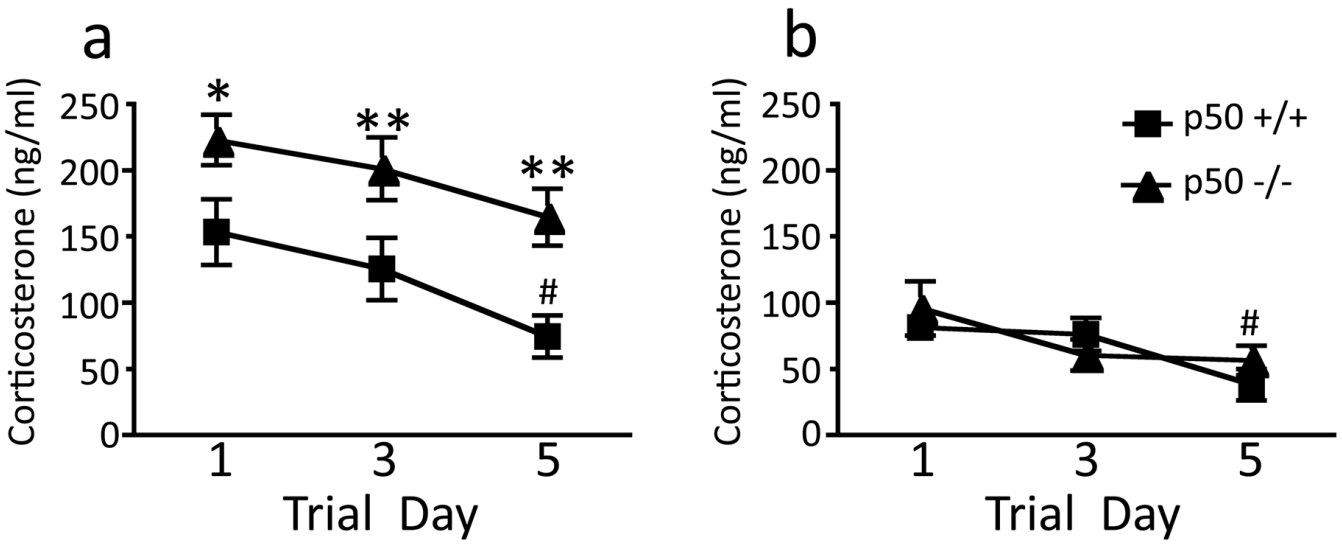

Figure 4.

Examination of plasma corticosterone revealed differential stress reactions between p50−/− and WT p50+/+ mice after Morris water maze exposure. p50−/− mice showed significantly higher plasma corticosterone levels 30 min after MWM testing compared to WT mice on all examined days (a). By 60 min, no significant difference between genotypes was detected (b). Furthermore, WT but not p50−/− mice showed a significant decrease in plasma corticosterone across trials at both 30 and 60 min after MWM. (n = 6 per measurement point) (Mean ± S.E.M.) Within day comparisons; *p<0.05, **p<0.01. Within genotype comparison; #p < 0.05 vs Day 1.

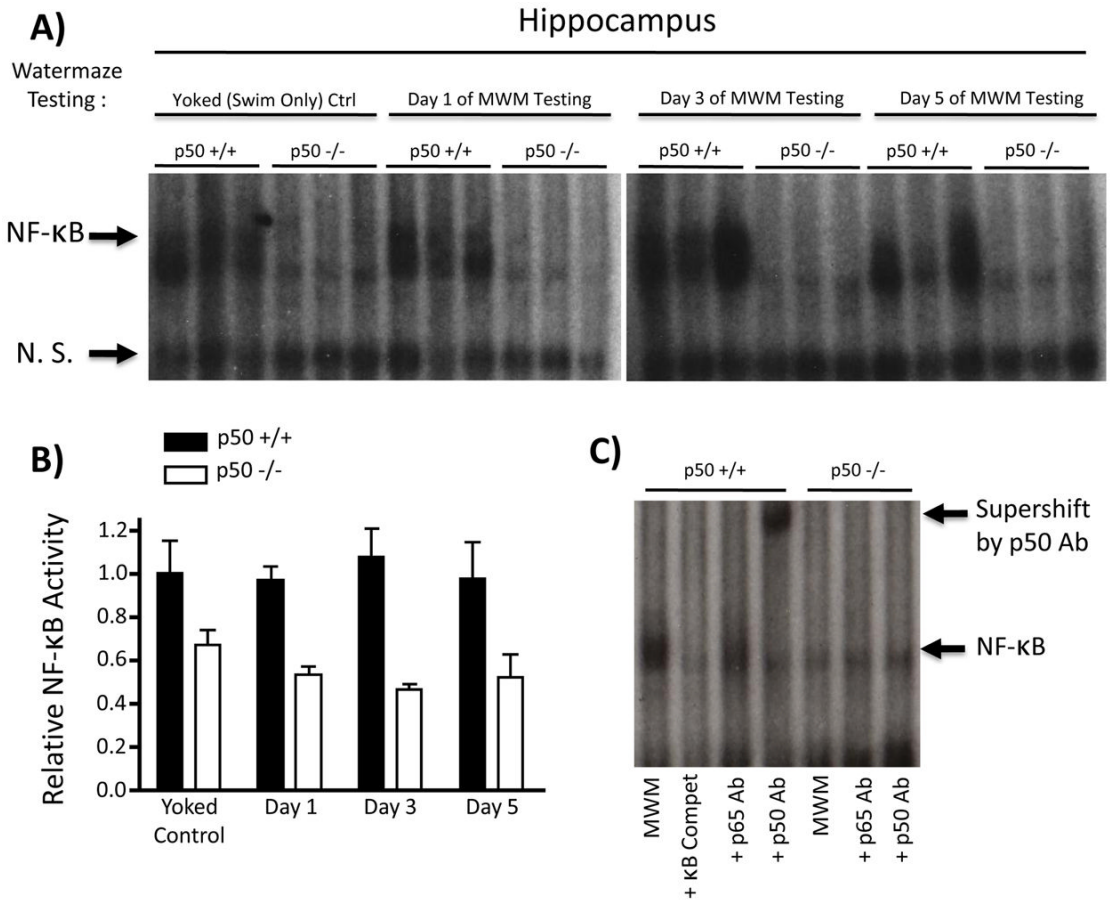

Figure 5.

Detection of activated NF-κB induced by spatial training was evaluated by EMSA using NF-κB specific radiolabeled probes. (A) NF-κB mediated gel shifts were detected in WT control mice after water maze training on Days 1, 3, and 5, and after free swimming (yoked control). In contrast, hippocampal nuclear extracts from trained and yoked control p50−/− mice did not contain activated NF-κB complexes. (B) NF-κB activity observed in swim-only and spatially trained WT and p50−/− mice estimated by densitometric analysis of the indicated band. Values for each group were relativized to the mean optical density of the WT yoked control group. (C) Gel supershifts illustrate that the ability to detect DNA binding of p50 to κB sites was intact. The specificity of the binding reaction and the components of the DNA-protein complex was determined by pre-incubation of the hippocampal nuclear extracts from MWM exposed p50−/− and WT p50+/+ mice with the following treatments: Control MWM exposure/no treatments (MWM), unlabeled double-stranded NF-κB oligonucleotide (+κB compet), NCI 1263 (+ anti-p50 antisera), or NIH #7057 (+ anti-p65 antisera).

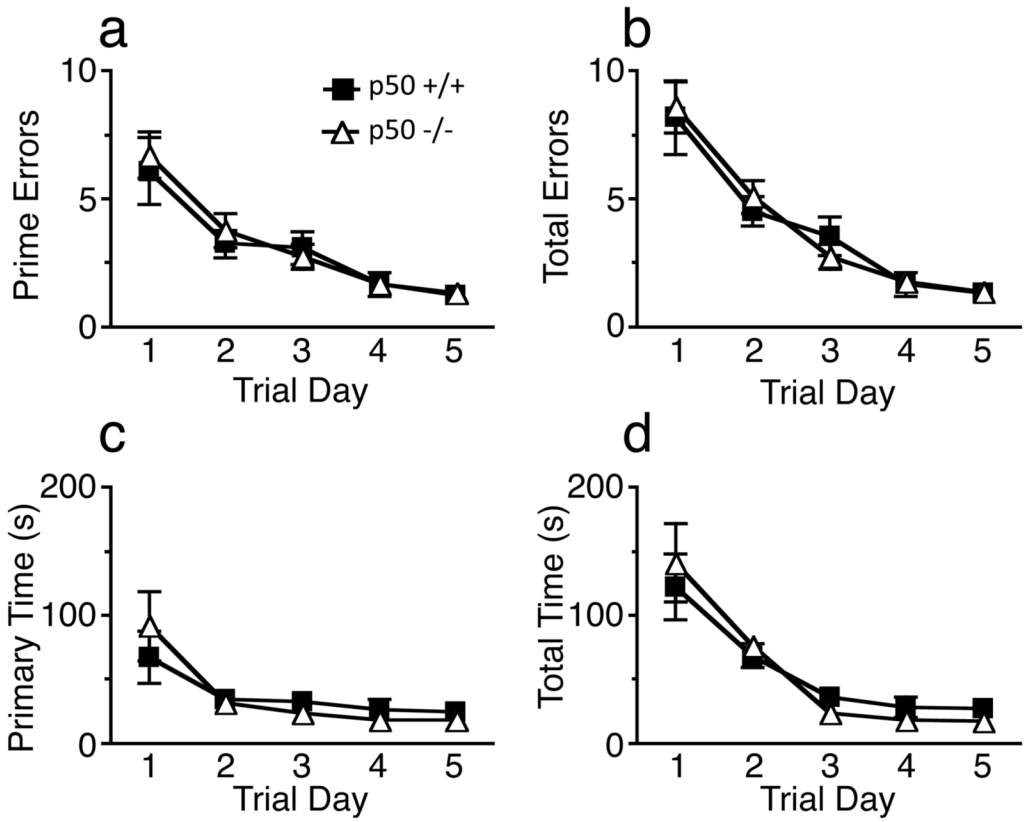

Figure 6.

In the Barnes maze, a minimally aversive spatial learning task, acquisition rates between genotypes were not different. In the acquisition phase, p50−/− and WT p50+/+ mice showed similar number of primary errors to reach the target burrow (a), total errors before entering the target burrow (b), and total latency to reach (c) and enter target burrow (d) recorded as prime time and total time (sec), respectively (n = 12 per genotype). (Mean ± S.E.M.)

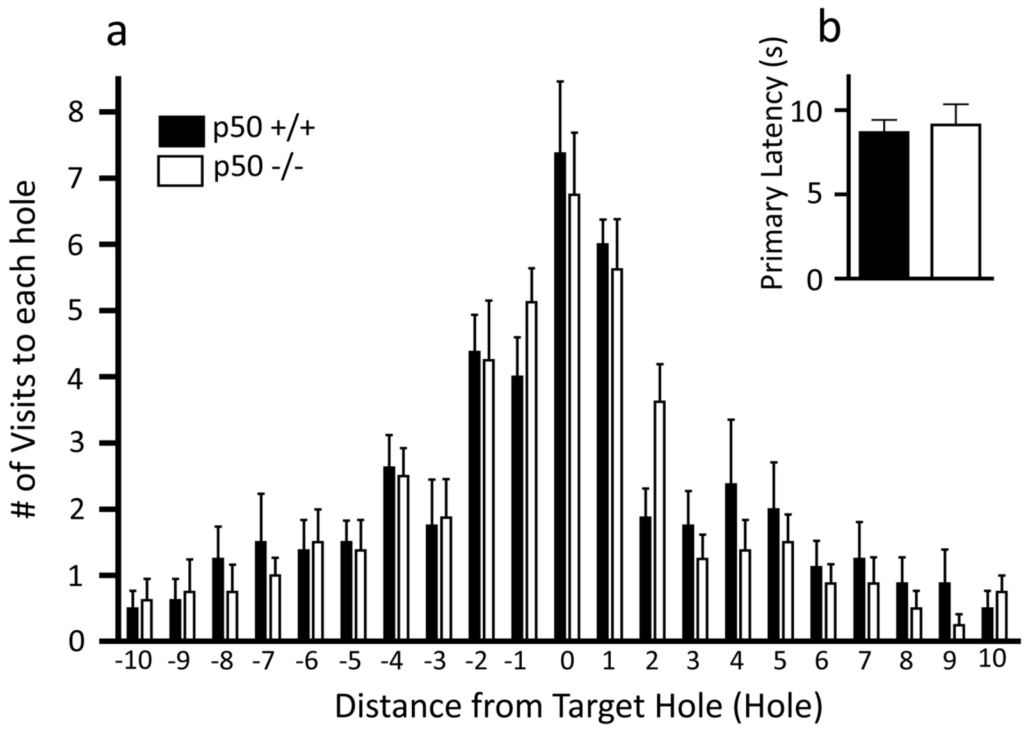

Figure 7.
Retention phase in Barnes maze on Day 5. Number of visits to each hole (a) and primary latency (sec) to reach target burrow were similar for both genotypes (b) (n = 12 per genotype). (Mean ± S.E.M.)

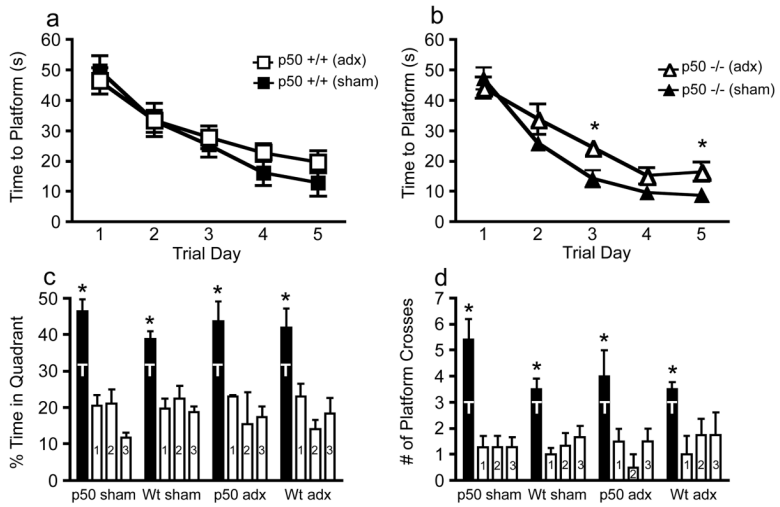

Figure 8.

Adrenalectomy (ADX) altered the enhanced acquisition performance of p50−/− mice but had no effect on WT p50+/+ mice during 5d acquisition trials in the water maze. The effects of ADX on latency to platform in WT p50+/+ mice (a) and p50−/− mice (b) during 5 days of acquisition trials in the water maze. During the probe trial, all groups spent significantly more time (c) and made significantly more platform crossings (d) in the trained quadrant than in the other three quadrants (*$p < 0.05$). (N=; WT sham= 6, WT ADX = 8, p50−/− sham = 7, p50−/− ADX = 9) (Mean ± S.E.M.)

Table 1

The expression of immediate early genes (FOS, ARC, JUNb, and EGR1) and the chemokine CXCL1 in hippocampi of WT p50+/+ mice and p50−/− mice 30 or 60 min after exposure to 1, 3, or 5 days of acquisition trials in the water maze. Values are expressed as attograms of gene of interest/picograms of GAPDH. *F* values for one-way ANOVA (trial day) for each gene of interest, 30 or 60 min after water maze exposure of days 1, 3, and 5.

		Free Swimming		Day 1		Day 3		Day 5		ANOVA *F* (3,23) *p*	
		30 min	**60 min**	**30 min**	**60 min**	**30 min**	**60 min**	**30 min**	**60 min**	**30 min**	**60 min**
Arc	p50+/+	154.43 ± 33.54	131.11 ± 16.19	114.13 ± 23.77	101.47 ± 14.43	140.47 ± 29.98	121.92 ± 36.67	114.132 ± 59.95	120.7 ± 22.79	0.260, p>0.8	0.266, p>0.8
	p50−/−	205.72 ± 29.95	169.04 ± 47.31	142.85 ± 28.27	125.33 ± 39.11	148.71 ± 30.23	125.01 ± 26.23	181.4 ± 41.19	152.24 ± 25.69	0.864, p>0.5	0.365, p>0.7
Egr1	p50+/+	260.41 ± 30.21	199.89 ± 46.58	348.71 ± 57.90	392.39 ± 42.47[a]	423.21 ± 37.10[a]	344.23 ± 54.15	467.40 ± 45.88[a]	396.32 ± 45.76[a]	4.249, p<0.001	4.546, p<0.02
	p50−/−	288.33 ± 20.67	245.26 ± 37.70	368.02 ± 36.49	417.6 ± 41.47[a]	466.09 ± 31.83[a]	446.93 ± 24.54[a]	490.59 ± 22.54[a,b]	451.35 ± 32.85[a]	10.58, p<0.001	7.940, p<0.001
Fos	p50+/+	253.04 ± 36.23	266.62 ± 55.83	205.41 ± 52.38	237.11 ± 30.5	289.37 ± 65.3	197.31 ± 12.75	263.44 ± 38.4	174.57 ± 45.12	0.504, p>0.6	1.025, p>0.4
	p50−/−	304.17 ± 24.65	272.35 ± 47.26	320.56 ± 49.02	323.1 ± 52.96	295.58 ± 20.63	255.71 ± 20.37	266.1 ± 37.84	216.63 ± 31.59	0.428, p>0.7	1.242, p>0.3
JunB	p50+/+	1.69 ± 0.31	1.49 ± 0.40	1.83 ± 0.41	2.69 ± 27	5.05 ± 0.85[a,b]	3.53 ± 0.75	2.72 ± 0.25	7.12 ± 1.28[a,b,c]	4.492, p<0.02	9.658, p<0.001
	p50−/−	1.85 ± 0.12	2.73 ± 0.49	2.69 ± 0.39	7.61 ± 1.61[a]	3.1 ± 0.42[a,c]	6.23 ± 0.57	1.73 ± 0.03	4.27 ± 0.35	5.089, p<0.01	5.624, p<0.005
Cxcl1	p50+/+	5.64 ± 1.25	2.361 + 0.72	6.37 ± 2.54	3.07 ± 81	3.45 ± 0.48	1.96 ± 1.16	3.44 ± 1.01	2.86 ± 1.43	1.608, p>0.2	1.314, p>0.2
	p50−/−	10.74 ± 1.74	9.92 ± 1.41	15.47 ± 0.84[a,d]	10.44 ± 2.23	12.95 ± 0.97	11.15 ± 2.02	10.15 ± 1.67	12.41 ± 0.89	4.264, p<0.02	2.359, p>0.1

Values are expressed as attograms of gene of interest/picograms of GAPDH; mean ± S.E.M.

[a] p <0.05 compared to swim only;

[b] p <0.05 compared to Day 1;

[c] p <0.05 compared to Day 3

[d] p<0.05 compared to Day 5

(n=6 per sample)