

# Reduced Fear Memory and Anxiety-like Behavior in Mice Lacking Formylpeptide Receptor 1

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**Abstract** *N*-formylpeptide receptor 1 (FPR1) is a G protein-coupled receptor that mediates pro-inflammatory chemotactic responses by phagocytic leukocytes to *N*-formylpeptides produced by bacteria or mitochondria. Mice lacking *Fpr1* (*Fpr1*<sup>-/-</sup> mice) have increased susceptibility to challenge with certain bacteria. FPR1 is also a receptor for annexin-1, which mediates the anti-inflammatory effects of glucocorticoids as well as negative feedback by glucocorticoids of the hypothalamic-pituitary-adrenocortical axis. However, homeostatic functions of FPR1 in the neuroendocrine system have not previously been defined. Here we show that in systematic behavioral testing *Fpr1*<sup>-/-</sup> mice exhibited increased exploratory activity, reduced anxiety-like behavior, and impaired fear memory, but normal spatial memory and learning capacity. Consistent with this, the homeostatic serum level of corticosterone in *Fpr1*<sup>-/-</sup> mice was significantly lower compared with wild-type mice.

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The data implicate *Fpr1* in modulation of anxiety-like behavior and fear memory by regulating glucocorticoid production.

**Keywords** Chemoattractant receptor · *Fpr1* · Behavior · Anxiety · Knockout mice · Corticosterone

## Introduction

*N*-formylpeptide receptor 1 (FPR1) is the founding member of a small family of G protein-coupled receptors with seven-transmembrane domains, designated the FPR family. In human, the FPR family consists of three genes encoding three functional receptors (FPR1, FPR2 and FPR3), whereas in mouse the family consists of three genes encoding functional receptors (including *Fpr1*, the homologue of FPR1) and six genes encoding orphan receptors and possible pseudogenes (Ye et al. 2009; Gao et al. 1998). *N*-formylpeptide receptor family members are mainly expressed in mammalian phagocytic leukocytes and bind structurally diverse ligands, including pro-inflammatory bacterial and mitochondrial *N*-formylpeptides, and the anti-inflammatory agonists annexin-1 and lipoxin A4. Treatment of phagocytes with the prototypical FPR ligand *N*-formylmethionyl-leucyl-phenylalanine (fMLF) may stimulate multiple pro-inflammatory and antimicrobial responses, including chemotactic migration, phagocytosis, degranulation, and superoxide anion production (Ye et al. 2009). Mice lacking *Fpr1* (*Fpr1*<sup>-/-</sup> mice) have increased susceptibility to challenge with certain bacteria (Gao et al. 1999). Expression of human *FPR1* has also been reported on endocrine cells, epithelial cells, and neurons in the central nervous system (Becker et al. 1998). However, mouse *Fpr1* was not detected by in situ hybridization of

whole brain (Lein et al. 2007). Expression of several Fpr family members, but not *Fpr1*, has been detected by in situ hybridization in neurons of the mouse vomeronasal organ (Liberles et al. 2009; Riviere et al. 2009). Despite expression beyond the immune system, evidence for non-immunologic functions in vivo has been lacking for FPR family members in both human and mouse. In this regard, while working with *Fpr1*<sup>-/-</sup> mice, multiple independent observers noticed that they appeared to be more active than wild-type littermates. Therefore, we conducted systematic behavioral testing.

## Materials and methods

### Mice

Construction of the *Fpr1*<sup>-/-</sup> mouse has been previously described (Gao et al. 1999). *Fpr1*<sup>-/-</sup> mice were backcrossed to Taconic C57Bl/6 mice for 12 generations. Male wild-type and N12 *Fpr1*<sup>-/-</sup> mice were produced at Taconic Farms and shipped to the NIH or the Karolinska Institutet Animal Facility at 2–3 months of age. Animals were housed separately by genotype, with five mice per cage. The housing room was maintained at 22°C on a 12-h light/dark cycle (lights off at 6 pm). Behavioral testing was carried out from 9 am to 2 pm, during the light phase of the mouse light/dark cycle. In the first experiment, five behavior tests (open-field, elevated plus-maze, hole-board, Y-maze, and inhibitory avoidance) were performed sequentially with 20 male mice in each group. In the second experiment, four behavior tests (open-field, elevated plus-maze, Y-maze, and water-maze) were performed with 20 additional male mice in each group. In each individual test, *Fpr1*<sup>-/-</sup> and wild-type mice were age-matched. All procedures were conducted in strict compliance with the policies on animal ethics and welfare of the Southern Stockholm Animals' Ethics Committee and the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases.

### Open-field test

The open-field test was performed in two 30-min sessions in a photocell-equipped automated open field arena (35 × 35 × 20 cm). The sessions were conducted 24 h apart. Locomotion (ambulation in the horizontal dimension) and rearing (movement in the vertical dimension) were measured. Four mice were tested simultaneously in four different activity cages, with equal numbers of animals from all groups tested in each of the four arenas. Loco-

motion and rearing activities were recorded in 5 min bins. At the end of the test session the arenas were cleaned with 70% alcohol followed by water to remove odor cues between each subject.

### Elevated plus-maze (EPM) test

The plus-maze was constructed with two closed arms, with grey tinted transparent Plexiglas walls 20 cm in height, and two open arms. The maze was elevated 60 cm from the floor, and the arms were 30 cm long and 5 cm wide. Animals were placed on the center section (5 × 5 cm), and allowed to freely explore the maze for 5 min. The EPM was set up under a camera to permit automated tracking measurements, using the Ethovision video-tracking system (Noldus, Wageningen, the Netherlands). The following measurements were taken: (1) number of entries into the open and closed arms; (2) time spent in open and closed arms; (3) distance covered in open and closed arms; and (4) number of head dips (scanning the floor area by protrusion of the head over the edge of an open arm and from the center area).

### Hole-board exploration test

The hole-board test apparatus consisted of a Perspex board (Ugo Basle 6652, Comerio VA, Italy), 40 × 40 × 27 cm, with 16 holes, each 3 cm in diameter, equally spaced on the board. The mouse was placed in the center of the board and its activity was assessed for 9 min. Head dips into the holes were automatically registered by photocell beam breaks in each hole. The number of head dips and peering around the periphery were recorded (edge peering was defined as when the mouse extends its head and neck beyond the edge of the board).

### Step-through inhibitory avoidance test

The passive avoidance apparatus consists of two compartments separated by a guillotine door (6 × 10 cm). The lighted, white square compartment (45 × 45 × 18 cm) is made of white solid Plexiglas and illuminated from directly above with an 80 W light bulb (~1 m above the floor of the compartment). The dark square compartment (26 × 15 × 18 cm) consists of black Plexiglas walls and lid, and a metal grid floor connected to a shock generator. On day 1 each mouse was given a habituation trial of 100 s, during which it was placed in the center of the white chamber, with the guillotine door removed to allow free passage into and out of the dark chamber. On day 2 each mouse was given three trials, each with a maximum duration of 60 s and with a 5 min inter-trial interval. For

the first two trials, the mouse was placed in the white chamber with its head facing away from the door, and the latency to enter the dark chamber was recorded. Once it entered the dark chamber, the mouse was allowed to remain there with the door closed for 10 s. Any mouse failing to enter the dark chamber within 60 s was eliminated from the third trial. On the third trial, once the mouse entered the dark chamber and the door closed, an electrical (0.5 mA for 2 s) scramble current was delivered through the grid floor, and the mouse remained in the dark chamber for 10 s before it was returned to its home cage. A retention test was conducted 24 h later, and again after 7 days. On this retention test the mouse was placed in the light compartment, and latency to enter the dark chamber with all four paws was recorded. A maximum retention latency of 60 s was assigned if the mouse did not enter the dark chamber.

#### Hot-plate test

The hot-plate test was performed as described before with some modifications (Dimitrov et al. 2010). The hot-plate (Hot-Plate Analgesia Meter, Columbus Instruments, OH) was set at 50°C with a cut-off time of 50 s. Mice were placed on the hot surface under a transparent bottomless box that allowed unrestrained movement of the animal. The latency to the first bite, vigorous lift or shake of the hindpaw or jump before the cut-off time was recorded.

#### Spontaneous Y-maze test

Mice were tested in random order in a symmetrical Y-maze apparatus made of gray PVC material. Each arm measured 15 × 36 cm with 27 cm-high side walls. The maze arms were positioned 120° from each other, and were designated as A, B or C. The maze was set up under a camera connected to a computer and video recorder to permit automated measurements (Ethovision, Noldus, Wageningen, the Netherlands). Mice were allowed to roam freely through the maze during a 5-min trial. The number of arm entries and sequence of entries were recorded. An arm entry required that all four of the animal's feet cross into the arm. Alternation behavior was defined as consecutive entries into each arm in order (i.e., triad entries ABC, ACB, CAB, etc.). The percentage of alternations was used as a memory index ( $\% \text{ alternation} = [(\text{number of alternations}) / (\text{total arm entries} - 2)] \times 100$ ). For example if a mouse entered the arms in the sequence ABCBACABACBCBAC, the number of alternation triads would be 8, while the total number of arm entries would be 15. Percent alternation would therefore be 61.5. Mice that exhibited fewer than five arm entries during the test were not included in data analysis.

#### Water-maze test

The water-maze is a circular pool constructed of grey PVC, 100 cm in diameter and 45 cm in height. The pool is filled with water mixed with white powdered milk to make the water opaque. The water is kept at a temperature of  $27 \pm 2^\circ\text{C}$ . A plastic transparent platform (9 × 9 cm) is placed approximately 0.7 cm below the water surface and 10 cm from the edge of the pool. Distal visual cues consist of several wall posters approximately 0.50 × 0.75 m in size that surround the pool. The whole experiment consists of four phases. (1) Water adaptation trial: the day before the first day of the acquisition phase of this test, the mice are habituated to the apparatus by being given three swim trials, each 60 s long, with 10 min inter-trial intervals. (2) Acquisition learning test: the entire acquisition training procedure occurs over the next 7 days. On each of these days, mice are transferred from their home cage to a non-transparent transfer cage to be taken to the testing room, to avoid visual orientation prior to release into the pool. Release points are balanced across four symmetrical positions on the pool perimeter. For the acquisition training days (four trials per day), the maximum time allowed to locate the hidden platform was 60 s/trial with inter-trial intervals of 10–15 min. Between trials the mice were kept in a holding cage under a heat lamp. The position of the hidden platform remained fixed for this acquisition phase. During the acquisition phase, mice that did not find the platform during the 60 s trial period were placed on the platform for 10 s at the end of the trial, to assist their learning. On the day following the 7-day acquisition phase, a probe trial occurred. On this trial, the platform was removed from the pool and each mouse was placed in the pool for 60 s. (3) Reversal learning test: the day after the acquisition test, the platform is relocated to a second position and the reversal phase of the test followed over 4 days. Then, the platform was removed again and a second probe trial conducted. (4) Visual cue tests: immediately after the reversal test, the hidden platform is raised above the water surface and becomes visible. Two visual cue tests were performed to let the mice find the visible platform. The swim path of the mice is recorded by means of a computer-based video-tracking system (Ethovision, Noldus, Wageningen, Netherlands). For the acquisition and reversal phases the variables recorded were latency to reach the platform, mean swimming speed, and swim path distance. For the probe trials, the variables recorded were time to swim to the prior location of the hidden platform (latency), number of crossings (frequency) over the prior location of the hidden platform, time spent in the vicinity (quadrant of the circular pool) where the platform had been located, number of entries into the quadrant where the platform had been located, and mean swim speed.

## Serum corticosterone level measurement

To measure serum corticosterone levels in mice, mouse blood was collected at 5 AM, just before the beginning of the light cycle, and at ~11 AM from different animals. The concentration of serum corticosterone was measured by ELISA (Ani Lytics, Gaithersburg, MD).

## Statistical analysis

Open-field and water-maze tests were analyzed with repeated measurements analysis of variance (ANOVA). Bonferroni post-tests were performed when ANOVA yielded significant interactions. Two-tailed unpaired t-test was used to analyze results from the elevated plus-maze, hole-board, hot-plate, and Y-maze tests. Two-tailed non-parametric Mann–Whitney test was used to analyze results from the inhibitory avoidance test. The level of statistical significance was set at  $p < 0.05$  for all parameters. All data are presented as group mean values  $\pm$  SEM.

## Results

### Increased exploratory activity and reduced anxiety-like behavior in *Fpr1*<sup>-/-</sup> mice

In the open-field test, which measures locomotor activity, hyperactivity, and exploratory behaviors (Crawley 1999), *Fpr1*<sup>-/-</sup> mice were significantly more active than wild-type mice on both days of testing with respect to both locomotion ( $p < 0.002$  on both days; Fig. 1a, b) and rearing ( $p < 0.01$  on day 2; Fig. 1c, d). Greater exploratory activity in an open arena, especially rearing, may also reflect reduced anxiety.

To quantitate anxiety-like behavior, mice were subjected to the elevated plus-maze test (EPM), which is based on the conflict between the natural tendency of mice to actively explore a new environment versus the aversive properties of an elevated open runway. The number of open arm entries and time spent on the open arms are considered as measures of anxiety-like behavior (File 2001). *Fpr1*<sup>-/-</sup> mice entered the open arms more frequently ( $p < 0.02$ ; Fig. 2a), and spent more time ( $p < 0.005$ ; Fig. 2b) and covered more distance ( $p < 0.0007$ ; Fig. 2c) on the open arms than wild-type mice. The increased activity of *Fpr1*<sup>-/-</sup> mice on the open arms is not due to a general increase in motor activity because both strains made a similar number of total arm entries, an independent measure of spontaneous motor activity (File 1995). Furthermore, *Fpr1*<sup>-/-</sup> mice made significantly more head dips

from the open arms than wild-type mice ( $p < 0.004$ ; Fig. 2d), an indication of risk-taking behavior (File 1995).

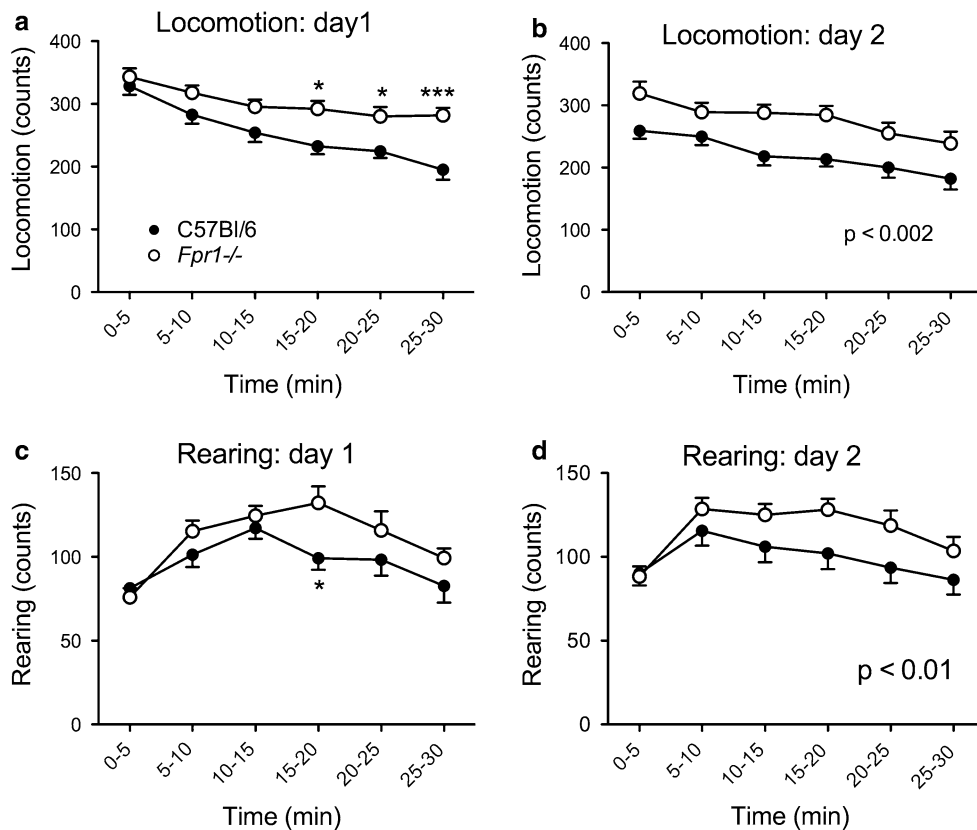
The hole-board is an independent test to assess exploratory activity and anxiety-like behavior (File 2001). In this test, the mouse explores its environment by plunging its head in and out of one of multiple holes a few times, peering, and then moving on to the next hole. This is a natural exploratory behavior that may be suppressed by anxiety. In addition, going to the edge of the hole-board to peer over (head peering) is classically interpreted as risk-taking behavior (File 2001). The *Fpr1*<sup>-/-</sup> mice displayed more head peering behavior (Fig. 3b,  $p < 0.006$ ) than wild-type mice.

Defecation during behavioral testing has been used as a physiological measure of anxiety. The number of fecal boli produced during EPM, the open-field test and the Y-maze test (see below) was significantly lower for *Fpr1*<sup>-/-</sup> mice than wild-type mice ( $8.40 \pm 0.67$  vs.  $11.85 \pm 1.20$  boli,  $p < 0.02$ ), consistent with a lower state of anxiety. There was no weight or food consumption difference observed between *Fpr1*<sup>-/-</sup> mice and wild-type mice at any age.

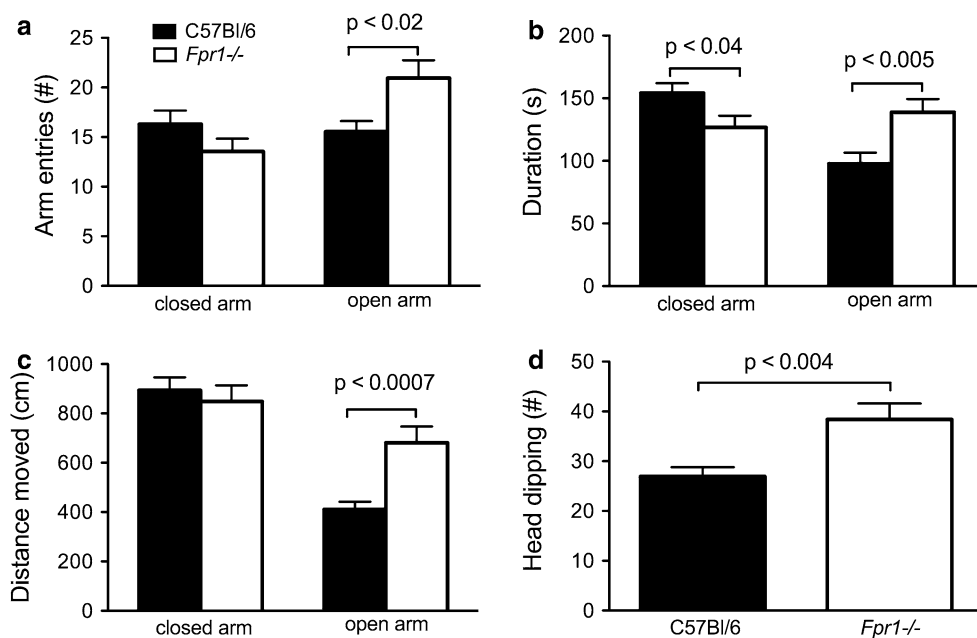
### Reduced fear memory in *Fpr1*<sup>-/-</sup> mice

To address whether the observed increased exploratory activity and decreased anxiety-like behavior in *Fpr1*<sup>-/-</sup> mice is related to altered memory and learning ability, we first performed the step-through inhibitory avoidance test (Izquierdo and McGaugh 1987). This test uses an apparatus consisting of a light box connected to a dark box. Because mice prefer a dark place, they move into the dark box when placed in the light box. However, when a mouse is given an electric shock in the dark box, the mouse hesitates to move into the dark box. The ability of mice to hesitate moving into the dark box is attributed to memory of the fearful experience. Hence, this test evaluates the ability of a mouse to learn and remember fear, which can be measured as the time to enter the dark box (latency). In the habituation trials (day 1 and day 2), there were no significant differences between *Fpr1*<sup>-/-</sup> mice and wild-type mice in latency to enter the dark chamber. However, after the electric shock in the dark box, *Fpr1*<sup>-/-</sup> mice showed a far greater tendency to enter the dark, aversive chamber than wild-type mice (Fig. 4a, b). Twenty-four hours after the training, none of the wild-type mice entered the dark chamber when tested for 60 s, whereas 42% of *Fpr1*<sup>-/-</sup> mice entered the dark compartment in this time frame, with an average retention time of 51 s ( $p < 0.04$ ). Seven days later, 5% of wild-type mice and 68% of *Fpr1*<sup>-/-</sup> mice entered the dark compartment with average retention time of 59 and 39 s respectively ( $p < 0.0004$ ). To address whether the observed difference between *Fpr1*<sup>-/-</sup> mice and wild-type

**Fig. 1** Increased exploratory activity and reduced anxiety-like behavior in *Fpr1*<sup>-/-</sup> mice in the open-field test. The open-field test was performed in two 30-min sessions (day 1 and day 2) with 20 male mice per group. Locomotion (ambulation in the horizontal dimension, **a, b**) and rearing (movement in the vertical dimension, **c, d**) activities were recorded in 5 min bins. Data were analyzed with repeated measurements analysis of variance (ANOVA). Bonferroni post-tests were performed when ANOVA yielded significant interactions ( $*p < 0.05$ ,  $***p < 0.001$ ). Data are presented as mean  $\pm$  SEM. The data shown are representative of two independent experiments with different animals but with the same age, sex and number per group



**Fig. 2** Increased exploratory activity and reduced anxiety-like behavior in *Fpr1*<sup>-/-</sup> mice in the elevated plus-maze test. Male mice ( $n = 20$  per group) were placed on the centre section of an elevated plus-maze and allowed to freely explore the maze for 5 min. The number of arm entries (**a**), time duration (**b**) and distance moved (**c**) in each arm, and head dips (**d**) over the open arms were recorded. Data were analyzed with the two-tailed unpaired  $t$  test, and are presented as mean  $\pm$  SEM. The data shown are representative of two independent experiments with different animals but with the same age, sex and number per group

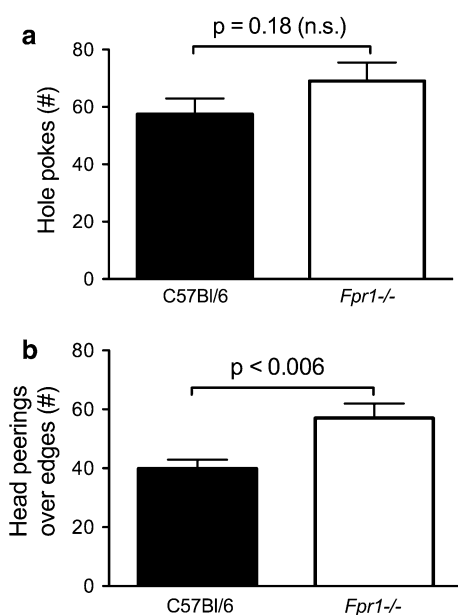


mice is due to decreased pain sensitivity of the *Fpr1*<sup>-/-</sup> mice to the electric shock, we performed a hot-plate test. The result (Fig. 4c) showed no difference between the two groups. Together, the results are consistent with the interpretation that *Fpr1*<sup>-/-</sup> mice have persistently impaired fear memory.

Normal spatial memory and learning capacity in *Fpr1*<sup>-/-</sup> mice

To test whether spatial memory and learning ability in *Fpr1*<sup>-/-</sup> mice were also altered, we performed the Y-maze and Water-maze tests. The Y-maze test is performed in a





**Fig. 3** Increased exploratory activity and reduced anxiety-like behavior in  $Fpr1^{-/-}$  mice in the hole-board test. Male mice ( $n = 20$  per group) were placed in the centre of the hole-board and activity was assessed for 9 min. The number of head dips into the holes (a) and the number of times the mouse peered around the periphery (b) was recorded. Data were analyzed with a two-tailed unpaired  $t$  test, and are presented as mean  $\pm$  SEM

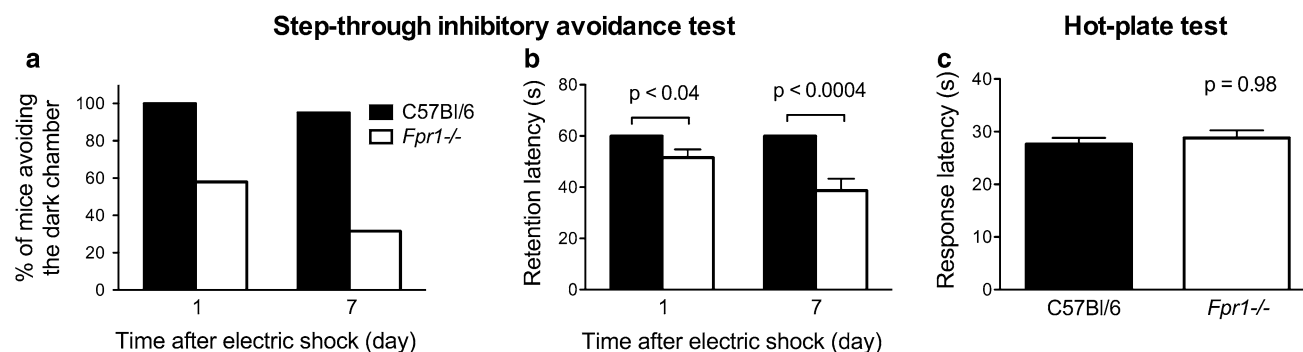
symmetrical Y-shaped maze with three arms. Normally, mice tend to enter the maze arm that was explored least-most recently, implying they remember the order of the arm entry. This test is therefore considered as a measure of spatial memory and general activity (Sarter et al. 1988).  $Fpr1^{-/-}$  mice showed the same percentage of alternation as wild-type mice (Fig. 5a), suggesting that they have normal spatial memory. However, they did have more arm

entries ( $p < 0.002$ ; Fig. 5b) and more distance moved ( $p < 0.002$ ; Fig. 5c), demonstrating increased overall exploratory activity, which is consistent with the findings in the open-field, EPM, and hole-board tests.

Next, we performed the water-maze test, a frequently used test of spatial learning, memory capability and cognitive flexibility in rodents (Morris 1984).  $Fpr1^{-/-}$  and wild-type mice improved their ability to find the hidden platform at the same rate in both acquisition and reversal learning phases of the test. This was true when the parameter measured was either latency (Fig. 6a, b) or distance (Fig. 6c, d), indicating that  $Fpr1$  deficiency did not impair learning capability and cognitive flexibility. Memory for the learned spatial locations was assessed by two probe trials at the end of the acquisition and reversal learning protocols with the platform removed. There were no significant differences between the two groups for the numbers of platform site crossings, the frequency of entry to the area around platform site, and duration in the area around the platform site (data not shown). Visual learning ability was assessed using the visible platform test, in which the platform was placed above the water level. In two consecutive trials, no significant group differences were observed (data not shown). It is notable that  $Fpr1^{-/-}$  mice were better able to find the hidden platform, especially in the acquisition learning phase (Fig. 6a, e).

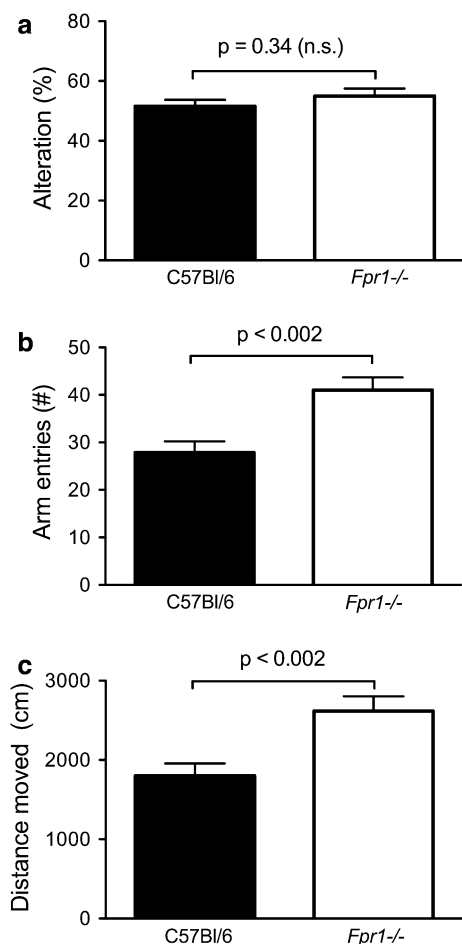
#### Lower level of serum corticosterone in $Fpr1^{-/-}$ mice

Since  $Fpr1$  mRNA has not been detected in mouse brain (Lein et al. 2007), we considered whether  $Fpr1$  deficiency may modulate mouse behavior by affecting homeostatic serum corticosterone levels, which have been reported to be regulated by annexin-1 (Buckingham et al. 2003; John



**Fig. 4** Impaired fear memory in  $Fpr1^{-/-}$  mice in the step-through inhibitory avoidance test but normal pain sensitivity in the hot-plate test. **a, b** Step-through inhibitory avoidance test. The inhibitory avoidance apparatus consists of a lighted and a dark chamber separated by a guillotine door. After a mild electric shock in the dark chamber, retention tests were conducted 24 h later, and again after 7 days. **a** Percentage of mice avoiding the dark aversive chamber; **b** the retention latency to enter the dark aversive chamber.  $Fpr1^{-/-}$

mice,  $n = 19$ ; wild-type C57Bl/6 mice,  $n = 20$ . Data were analyzed with a two-tailed nonparametric Mann–Whitney test, and are presented as mean  $\pm$  SEM. **c** Hot-plate test. Mice ( $n = 10$  per group) were placed on a hot-plate ( $50^{\circ}\text{C}$ ) with a transparent bottomless box and allowed to move without restraining. The latency to the first hindpaw bite, vigorous lift and shake of a hindpaw, or jump was recorded. Data were analyzed with a two-tailed unpaired  $t$  test, and are presented as mean  $\pm$  SEM



**Fig. 5** Normal spatial memory in *Fpr1*<sup>-/-</sup> mice in the Y-maze test. Male mice ( $n = 20$  per group) were allowed to roam freely through a symmetrical Y-shaped maze during a 5-min trial. The alterations of arm entries (a), the number of arm entries (b) and distance moved (c) were recorded. Alterations are defined as consecutive entries into each arm in order (i.e., triad entries ABC, ACB, CAB, etc.). The data were expressed as % alternation ( $[(\text{number of alternations})/(\text{total arm entries}-2)] \times 100$ ). Data were analyzed with a two-tailed unpaired t-test, and are presented as mean  $\pm$  SEM. The data shown are representative of two independent experiments with different animals but with the same age, sex and number per group

et al. 2007), a ligand for *Fpr1* and *Fpr2*, and which are known to affect behavior (Tronche et al. 1999; Roozendaal 2000). Figure 7 shows that *Fpr1*<sup>-/-</sup> mice had significantly lower homeostatic serum corticosterone levels compared with wild-type mice (80 ng/ml vs. 120 ng/ml,  $p < 0.008$ ) in the light phase, the time when behavior tests were performed, but not in the dark phase.

## Discussion

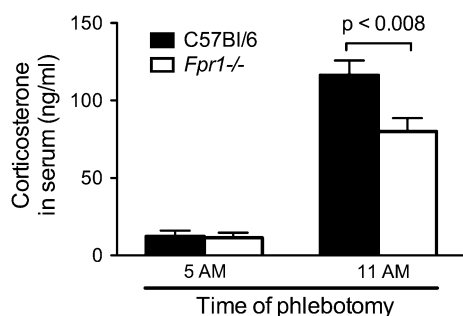
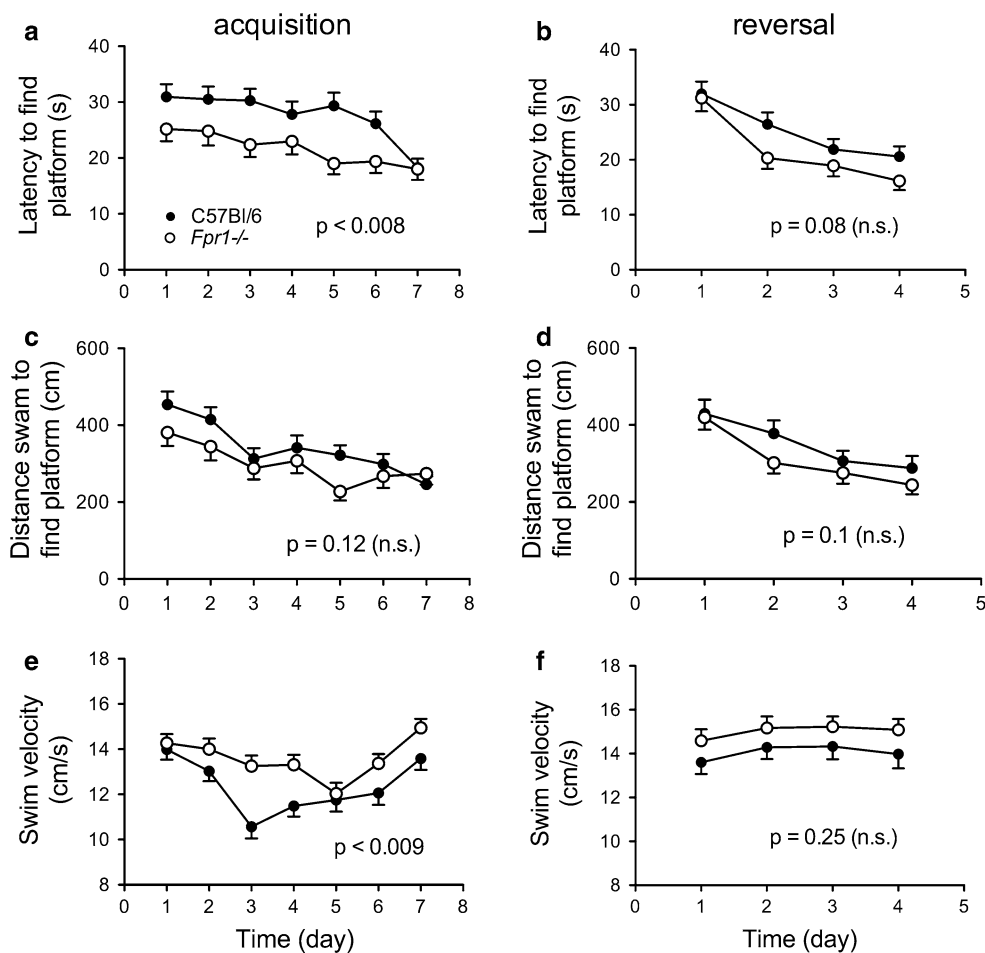
In the present study we have demonstrated that *Fpr1*<sup>-/-</sup> mice have overall increased exploratory activity and reduced anxiety-like behavior in the open-field, elevated

plus-maze, and hole-board tests. In the Y-maze test, *Fpr1*<sup>-/-</sup> mice showed normal spatial memory but increased overall activity. In the water-maze test, *Fpr1*<sup>-/-</sup> mice showed normal memory and learning capacity but were better able to find the hidden platform, especially in the acquisition learning phase (Fig. 6a, e). A possible explanation is that *Fpr1*<sup>-/-</sup> mice, having less anxiety, may be more focused on learning the task. This interpretation would be consistent with their greater exploratory activity in the open field, EPM, hole-board, and Y-maze tests. The inhibitory avoidance test is typically regarded as a test of fear memory. However, differences in pain sensitivity thresholds between the groups can confound the interpretation. To address whether the observed difference between *Fpr1*<sup>-/-</sup> mice and wild-type mice (Fig. 4a, b) is due to decreased pain sensitivity in *Fpr1*<sup>-/-</sup> mice, we performed a hot-plate test. The similar thermosensitivity of the two groups of mice (Fig. 4c) supports the conclusion that *Fpr1*<sup>-/-</sup> mice have impaired fear memory. Thus, with systematic behavioral testing, we have defined novel phenotypes for *Fpr1*<sup>-/-</sup> mice: increased exploratory activity, reduced anxiety-like behavior, and impaired fear memory.

Emotional behavior is regulated by steroid hormones, such as glucocorticoids. Normally, glucocorticoid levels fluctuate in the blood according to the circadian rhythm, and further changes may occur in response to many factors including stress (Ader et al. 1967). In healthy rodents, behavior is influenced by the circadian rhythm in the light/dark cycle. Rodents are most active and least anxious in their dark phase, when the lowest levels of corticosterone are found in the blood (Oishi et al. 2006). High levels of cortisol have been linked to exaggerated fear and anxiety responses in monkeys (Kalin et al. 1998), and raising corticosterone levels by exogenous administration can potentiate fear-induced freezing in rats (Corodimas et al. 1994), and induce anxiogenic effects in mice (Vafaei et al. 2008). In contrast, adrenalectomy decreases contextual fear conditioning in rats (Fleshner et al. 1997), and disruption of the glucocorticoid receptor gene in the nervous system in mice results in reduced anxiety (Tronche et al. 1999). In humans, disturbances in glucocorticoid secretion have been associated with mental health disorders (Holsboer and Barden 1996). Thus, hypocorticosteronemia in *Fpr1*<sup>-/-</sup> mice is a plausible mechanism to explain at least in part the reduced anxiety-like behavior and impaired fear memory that further cause increased exploratory activity.

How *Fpr1* deficiency causes hypocorticosteronemia may relate to its ability to compete with *Fpr2* to bind annexin-1, which is known to mediate negative feedback of the HPA axis by glucocorticoids (Buckingham et al. 2003; Buckingham et al. 2006; Taylor et al. 1993; Morris et al. 2006). Annexin-1 is produced by both leukocytes and the neuroendocrine system, particularly in the anterior pituitary

**Fig. 6** Normal spatial memory and learning capacity in  $Fpr1^{-/-}$  mice in the water-maze test. In the acquisition learning phase (a, c, e), male mice ( $n = 20$  per group) were allowed to locate the hidden platform for 60 s. The mice were tested four times per day for 7 days. The reversal learning phase (b, d, f), in which the platform was relocated to a new position, immediately followed the acquisition learning test. The latency (a, b) and distance swam (c, d) to find platform, and swim velocity (e, f) were recorded. Data were analyzed with repeated measurements analysis of variance (ANOVA), and are presented as mean  $\pm$  SEM



**Fig. 7** Lower level of serum corticosterone in  $Fpr1^{-/-}$  mice. Blood from male mice with the indicated genotypes was collected at 5 AM, just before the beginning of the light cycle, and at  $\sim$ 11 AM (different animals were analyzed at each time point;  $n = 17$  for each genotype at each time point). The concentration of serum corticosterone was measured with ELISA. Data were analyzed with a two-tailed unpaired  $t$  test, and are presented as mean  $\pm$  SEM

gland and in specific loci in the hypothalamus (John et al. 2007; Gerke and Moss 2002). Glucocorticoids stimulate expression and release of annexin-1, which can be found at high concentrations in serum and other body fluids (Walther et al. 2000). Annexin-1 binds to both Fpr1 and Fpr2, and is able to inhibit neutrophil extravasation (Walther et al. 2000;

Dufton et al. 2010; Perretti et al. 2002). Annexin-1 can activate Fpr2 expressed on corticotrophs to prevent release of adrenocorticotrophic hormone (ACTH) thereby reducing levels of glucocorticoid (John et al. 2007; Morris et al. 2006). *N*-formylpeptide receptor 1 could potentially buffer this effect of Fpr2 by competing for annexin-1. There is published evidence that in addition to expression on phagocytic leukocytes, Fpr1 is expressed on non-phagocytic cells in multiple organs (Becker et al. 1998). Whether collectively this represents enough binding sites to protect pituitary corticotrophs from annexin 1 action at Fpr2 is unknown. Further systematic studies are needed to delineate the role of Fpr1 in HPA axis, and to explore possible direct role of Fpr1 in regulating corticosterone level.

Abnormal behavior is the first non-immunologic phenotype discovered for  $Fpr1^{-/-}$  mice. It may also be considered the first spontaneous phenotype for this knockout, since the test results were consistent with casual observations, made by multiple independent observers, of hyperactivity in these mice unstressed in their cages. A limitation of the study, however, is that it requires placing the animal in a novel and stressful environment. Future studies will be needed to address this by analyzing behavior in the home



cage in the absence of stress and anxiety induced by the test environment. This could also address whether *Fpr1*<sup>-/-</sup> mice exhibit increased locomotor activity in the dark phase. We predict that they would not since they have similar corticosterone levels to wild-type mice in the dark phase. Restraint stress is another relevant test to consider for future study, as it is possible that *Fpr1*<sup>-/-</sup> mice are less susceptible than control mice to stress-induced corticosterone level fluctuations across various time points. This may provide valuable information regarding HPA axis activation. Our data also provide preclinical evidence in support of FPR1 as a potential target for development of novel therapeutic agents for the treatment of anxiety-related diseases. Currently, all known agonists and antagonists for Fpr1 also bind to Fpr2 (Ye et al. 2009), and none has been entered into clinical trials.

Our results were unexpected and are unprecedented for the large family of leukocyte chemoattractants and chemoattractant receptors. However, there is precedent for behavioral phenotypes among other types of immunoregulatory factors. In particular, both *IL6*<sup>-/-</sup> mice (Armario et al. 1998) and *IFN $\gamma$* <sup>-/-</sup> mice (Kustova et al. 1998) have been reported to have increased emotionality. *TNFA*<sup>-/-</sup> mice have increased anxiety-like behavior, possibly through alterations of serotonin metabolism (Yamada et al. 2000). Nautiyal et al. (2008) have reported that mast cell-deficient mice have an increased anxiety-like phenotype and have suggested a role for brain mast cells in modulation of anxiety-like behavior.

In conclusion, we have identified the first evidence that a leukocyte chemoattractant receptor may also regulate behavior. Our data support the hypothesis that Fpr1 may be involved in modulation of anxiety-like behavior and fear memory by regulating glucocorticoid levels.

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