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Anxiogenic-Like Behavioral Phenotype of Mice Deficient in Phosphodiesterase 4B (PDE4B)

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Abstract

Phosphodiesterase-4 (PDE4), an enzyme that catalyzes the hydrolysis of cyclic AMP and plays a critical role in controlling its intracellular concentration, has been implicated in depression- and anxiety-like behaviors. However, the functions of the four PDE4 subfamilies (PDE4A, PDE4B, PDE4C, and PDE4D) remain largely unknown. In animal tests sensitive to anxiolytics, antidepressants, memory enhancers, or analgesics, we examined the behavioral phenotype of mice deficient in PDE4B (PDE4B−/−). Immunoblot analysis revealed loss of PDE4B expression in the cerebral cortex and amygdala of PDE4B−/− mice. The reduction of PDE4B expression was accompanied by decreases in PDE4 activity in the brain regions of PDE4B−/− mice. Compared to PDE4B + / + littermates, PDE4B−/− mice displayed anxiogenic-like behavior, as evidenced by decreased head-dips and time spent in head-dipping in the holeboard test, reduced transitions and time on the light side in the light–dark transition test, and decreased initial exploration and rears in the open-field test. Consistent with anxiogenic-like behavior, PDE4B−/− mice displayed increased levels of plasma corticosterone. In addition, these mice also showed a modest increase in the proliferation of neuronal cells in the hippocampal dentate gyrus. In the forced-swim test, PDE4B −/− mice exhibited decreased immobility; however, this was not supported by the results from the tail-suspension test. PDE4B−/− mice did not display changes in memory, locomotor activity, or nociceptive responses. Taken together, these results suggest that the PDE4B subfamily is involved in signaling pathways that contribute to anxiogenic-like effects on behavior

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DISCLOSURE/CONFLICT OF INTEREST

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Keywords

phosphodiesterase 4B(PDE4B); gene knockout; anxiogenic; antidepressant; neurogenesis; rolipram

INTRODUCTION

Phosphodiesterase-4 (PDE4), an enzyme that specifically catalyzes the hydrolysis of cyclic AMP (cAMP) and is critical in controlling its intracellular concentration, is involved in various central nervous system (CNS) processes, including depression (O'Donnell and Zhang, 2004), learning and memory (Barad *et al*, 1998), anxiety (Beer *et al*, 1972), and analgesia (Bradaia *et al*, 2005). Administration of PDE4 inhibitors such as rolipram produces antidepressant-like effects (Zhang *et al*, 2002, 2006), reverses memory deficits induced pharmacologically, physically, or genetically (Bourtchouladze *et al*, 2003; Imanishi *et al*, 1997; Zhang *et al*, 2000, 2004), alters anxiogenic-like behavior (Imaizumi *et al*, 1994; Silvestre *et al*, 1999a), and induces analgesia in rodents (Kumar *et al*, 2000). Inhibition of PDE4 also increases adult neurogenesis (Nakagawa *et al*, 2002), which is believed to be involved in antidepressant activity (Dranovsky and Hen, 2006; Malberg and Duman, 2003; Santarelli *et al*, 2003). However, the complexity of the PDE4 family, which includes four subfamilies (PDE4A, PDE4B, PDE4C, and PDE4D) and 21 splice variants (O'Donnell and Zhang, 2004), makes it unclear which subfamilies are involved in these actions.

PDE4 subfamilies may exert different functions based on their differential distributions in the brain (Cherry and Davis, 1999; Perez-Torres *et al*, 2000) and the **compartmentation of cAMP signaling, which is controlled by different PDE4s (Baillie and Houslay, 2005; Fischmeister, 2006; Rich *et al*, 2007; Terrin *et al*, 2006). While the lack of selective inhibitors of PDE4 isoforms has made it particularly difficult in understanding the roles of PDE4 subfamilies, gene mutation and RNA interference (RNAi) have been shown to be feasible approaches to identifying functions of PDE4 subfamilies and even their variants (Lehnart *et al*, 2005; Lynch *et al*, 2005; Terrin *et al*, 2006; Zhang *et al*, 2002). Using genetic deletion mutants of specific PDE4 subtypes (Hansen *et al*, 2000; Jin *et al*, 2005b), it has been shown that PDE4D is important for antidepressant activity (Zhang *et al*, 2002). Mice deficient in PDE4D display antidepressant-like behavior in tests sensitive to antidepressants; the antidepressant-like effect of rolipram is abolished in these mice, which do not show any changes in a test sensitive to anxiolytic treatment. PDE4D may be involved in the mediation of memory, as indicated by memory-enhancing effects of PDE4 inhibitors exhibiting modest PDE4D selectivity (Zhang *et al*, 2005). More recently, PDE4D has been shown to be involved in the pathogenesis of stroke (Gretarsdottir *et al*, 2003; Zee *et al*, 2006). By contrast, very little is known regarding the role of PDE4B in CNS functions.

PDE4B has five splice variants (PDE4B1, 4B2, 4B3, 4B4, and 4B5; Bolger *et al*, 1993, 1994; Cheung *et al*, 2007; Huston *et al*, 1997; Shepherd *et al*, 2003). While the roles of these variants are still not known, recent studies using mice deficient in PDE4B have shown that this subfamily plays a complementary role in the control of neutrophil function (Ariga *et al*, 2004) and is required for antipsychotic effects of rolipram (Siuciak *et al*, 2007). In addition, PDE4B also is involved in schizophrenia by interacting with the DISC1 gene, a candidate susceptibility factor for schizophrenia (Clapcote *et al*, 2007; Millar *et al*, 2005). These results indicate that PDE4B plays a significant role in CNS functions.

Studies to date indicate that PDE4B may be involved in the regulation of anxiety and depression. First, PDE4B is the predominant PDE4 subtype in the amygdala, hypothalamus, and striatum; it also is expressed at relatively high levels in the frontal cortex and olfactory

bulb (Cherry and Davis, 1999; Perez-Torres *et al*, 2000). The amygdala and hypothalamus are key regions in the mediation of anxiety and stress responses (Charney and Deutch, 1996), while the frontal cortex and olfactory bulb contribute in the mediation of antidepressant effects on behavior (Soares and Mann, 1997; Webster *et al*, 2000). Second, the benzodiazepine anxiolytic diazepam inhibits PDE4 expressed in HEK293 cells *in vitro*; PDE4B is more sensitive to inhibition by diazepam than PDE4A (Cherry *et al*, 2001). Third, chronic nicotine treatment, which appears to induce anxiolytic- and antidepressant-like effects (Biala and Budzynska, 2006; Semba *et al*, 1998), produces down-regulation of PDE4B in the brain (Polesskaya *et al*, 2007). Finally, chronic treatment with antidepressants decreases the expression of PDE4B in the mouse hippocampus (Dlaboga *et al*, 2006) and increases adult neurogenesis (Malberg *et al*, 2000). However, since there are no highly selective inhibitors of individual PDE4 subtypes, direct evidence for the potential involvement of PDE4B in anxiety-and depression-like behaviors is lacking. Hence, we examined behaviors of mice deficient in PDE4B using a battery of animal tests sensitive to anxiolytics, antidepressants, memory enhancers, and analgesics. In addition, we compared the expression of PDE4 subtypes and PDE activity in the brain, neuronal cell proliferation in the hippocampus, and plasma corticosterone levels between PDE4B−/− mice and their wild-type controls.

MATERIALS AND METHODS

Animals

Adult male mice with the same mixed genetic background (C57BL/6 × 129/Ola) were used in experiments involving PDE4B−/− mice, which were generated by homologous recombination as described previously (Jin *et al*, 1999).Wild-type (PDE4B+ /+) and PDE4B −/− offspring used in the experiments were derived from mating of heterozygous (PDE4B+/ −) mice. Genotype was determined by Southern blot analysis of genomic DNA. All mice were housed in groups of 1–4 per cage in a temperature-controlled room (22–23°C) with a 12-h on/12-h off light cycle (lights on at 0600 hours). Water and food were freely available in their home cages. Blind observations were used throughout all the experiments, which were performed from 0900 to 1300 hours in a quiet room. All procedures were approved by the Animal Care and Use Committee of West Virginia University Health Sciences Center, and were performed according to the ‘NIH Guide for the Care and Use of Laboratory Animals’ (NIH Publications No. 80–23, revised 1996).

Immunoblotting of PDE4 Subtypes in the Mouse Brain

The experiment was performed as described previously (Dlaboga *et al*, 2006; Richter *et al*, 2005). In brief, PDE4B+/+ and PDE4B−/− mice were decapitated and brain regions (cerebral cortex, amygdala, and hippocampus) were dissected and kept frozen (−80°C) until testing. Brain tissues were homogenized in RIPA buffer (50mM Tris, pH 8.0, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and centrifuged at 16 000 g for 20 min. One half of the supernatant was immunoprecipitated with specific polyclonal PDE4A, PDE4B, or PDE4D antibodies (FabGennix Inc., Frisco, TX), which were generated against the unique C-terminal of each isoform and detected all known variants within any particular PDE4 subfamily without crossreaction (Dlaboga *et al*, 2006). The immunoprecipitated PDE4A, PDE4B, and PDE4D proteins were separated by SDS–PAGE, transferred to nitrocellulose, and detected by immunoblotting using the respective PDE4 subtype-specific antibodies.

PDE Assay

The other half of the above supernatants from the cerebral cortex, amygdala, and hippocampus were used for PDE assay, as described previously (Zhang *et al*, 2002). PDE

activity was determined by the detection of the [^{3}H]cAMP converting rate, using unlabeled cAMP (1 μM) as the substrate, in the absence (total PDE activity) and presence (non-PDE4 activity) of 10 μM rolipram; PDE4 activity was determined by subtracting non-PDE4 activity from the total PDE activity.

Cell Proliferation by Immunohistochemistry

This was performed as described previously (Nakagawa *et al*, 2002). In brief, groups of PDE4B + / + and PDE4B−/− mice were injected (i.p.) with 50 mg/kg BrdU (Sigma, St Louis, MO) three times at 1 h intervals. Thirty minutes after the last injection, mice were killed via intracardial perfusion, the brains were post-fixed, and serial coronal sections (30 μm) through the entire hippocampus were collected using a freezing microtome. Every 9th section was slide-mounted for peroxidase BrdU/Tuj1 (neuronal-specific protein for immature neurons) immunolabeling. The sections were incubated with mouse monoclonal antibody against BrdU (1 : 1000; Sigma) and murine anti-Tuj1 antibody (1 : 500; Babco, Richmond, CA) followed by goat anti-mouse secondary antibody labeled with Alexa 488 (green fluorescence for BrdU) or Alexa 568 (red fluorescence for Tuj1). The sections were analyzed by confocal laser microscopy.

For immunofluorescence staining, free-floating sections were incubated with the primary antibody (mouse anti-BrdU monoclonal IgG; 1 : 1000) and then FITC-conjugated goat anti-mouse IgG (1 : 200, Jackson ImmunoResearch, West Grove, PA) following the procedures reported previously (Nakagawa *et al*, 2002). The number of BrdU-positive cells in bilateral, entire hippocampi was counted through a × 40 objective (Zeiss, Oberkochen, Germany). Ten sections were analyzed to cover the entire hippocampus. The total number of BrdU-positive cells in both sides of the granule cell layer was multiplied by 9 and reported as the total number of cells.

Determination of Plasma Corticosterone Concentrations

One week after the holeboard test (Figure 1c), mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) before blood collection. Blood was harvested by exsanguinations using heparinized syringes and collected in Eppendorf tubes. Plasma corticosterone concentrations were quantified by ELISA following the manufacturer's instructions (OCTEIA Corticosterone EIA, IDS, Boldon, UK).

Behavioral Test Procedures

Three batches of PDE4B + / + and PDE4B−/− mice, 15- to 18-week-olds at the beginning of tests and 25- to 28-week- (Figure 1a), 24- to 27-week- (Figure 1b), or 18- to 21-week-olds (Figure 1c) by the end of tests, were used for batteries of behavioral tests. More than one batch of mice was used to limit the repetition of tests using the same animals. In addition, to reduce potential influence of stress and/or the previous test, all mice were handled for 1 week before the beginning of tests and given 1–2weeks break between the tests; tests involving less stress (eg open-field and water-maze tests) were performed before those that were more stressful (eg hot-plate and writhing tests). In the drug treatment group, mice were tested with saline 1 week before drug tests to monitor the baseline of immobility in the FST (Figure 1b); the lower dose of desipramine and acute rolipram were tested 1 week before the higher dose of desipramine and chronic rolipram, respectively.

Anxiogenic-Like Effects on Behavior

The open-field exploration test—This test utilizes behavioral changes in rodents exposed to a novel environment and has been used to evaluate anxiogenic and anxiolytic activity under identical situations (Pellow, 1986). The test used was modified slightly from

the previous procedure (Moreira *et al*, 2000). The floor of the open-field chamber (50 × 50 × 25 cm), which was made of white Plexiglas, was divided into 16 identical squares. The naive mouse was placed at one corner of the chamber and allowed to explore for 5 min. The latency (ie the time taken to move from the corner square to the next adjacent square), exploration (ie the number of squares crossed), and rears (ie the number of times the animals stood on its rear paws) were recorded.

The holeboard test—This was performed as described previously (Hilakivi and Lister, 1990). The holeboard apparatus consisted of an open Plexiglas box (40 × 40 × 30 cm) with four holes (3 cm in diameter, 2 cm in depth) in the black floor. The test was performed in a dimly lit room. Mice were placed individually in the center of the floor and allowed to explore for 5 min. The number of head-dips and the time spent in head-dipping were recorded.

The light–dark transition test—The test was performed as described previously (MacNeil *et al*, 1997). Mice were individually placed in the dark compartment (15 × 23 cm) of the light–dark chamber. The latency to cross through the hole (8 × 6 cm) into the light compartment (30 × 23 cm; illuminated with a 60-W bulb positioned 50 cm above), the time spent in the light compartment, and transitions, defined as the number of crossings from the dark compartment to the light side, were recorded for 5 min.

Antidepressant-Like Effects on Behavior

Tail-suspension test (TST)—The TST was performed as described previously (Zhang *et al*, 2002). Each mouse was suspended using adhesive tape placed approximately 1 cm from the tip of its tail. The duration of immobility (ie passive hanging and complete motionlessness) was recorded during the 6 min test period.

The forced-swim test (FST)—This was performed as described previously (Zhang *et al*, 2002). Mice were individually placed in a plastic cylinder (45 cm high × 20 cm diameter) filled with water (22–23°C; 28 cm in depth), allowing for free swimming. The duration of immobility, which was defined as floating in an upright position without additional moving other than that necessary for the animal to keep its head above water, was recorded for 6 min. The test was repeated 7 weeks later to confirm the behavioral observations.

Measurement of Memory Performance

Step-down passive avoidance task—The experiment was performed as described previously (Maurice and Privat, 1997), with minor modifications. During the training, each mouse was placed on a wooden platform (3.5 × 3.5 × 2.5 cm) that was fixed at the center of the grid floor (connected to an isolated pulse stimulator; Model E13-08, Coulbourn, Allentown, PA) of a Plexiglas chamber (25 × 15 × 10 cm). The mouse was subjected to a footshock (0.4 mA, 5 s) as soon as it completely descended to the grid floor. This procedure was repeated immediately and again 1 h after the initial training. Retention tests (no shocks) were performed for 3 and 24 h and then, for assessing the extinction of memory, 8 days after the last training, with an upper cut-off time of 300 s.

Morris water-maze task—Spatial learning and memory were assessed using the Morris water-maze task as described previously (El-Ghundi *et al*, 1999), with minor modifications. In brief, during the acquisition training, mice were trained (6 trials × 2 days plus 4 trials × 1 day) in a pool (65 cm high × 59 cm diameter) filled with water (22 ± 1°C, 15 cm in depth) to escape by swimming to the platform (12 cm high × 7.5 cm diameter) hidden 1 cm under the water in one of four quadrants. The escape latency (ie the time required to locate and climb onto the platform) for each mouse was recorded. Twenty-four hours after the last trial, the

probe trial was started. The platform was removed, and the number of entries into and time spent in the target quadrant (the previous location of the platform) were recorded during the 60 s test.

Measurement of Nociceptive Responses

The hot-plate test—This was performed as described previously (Ballou *et al*, 2000). Mice were individually placed at the center of the heated flat surface (25 × 25 cm; 55°C) of an Analgesia Meter (Columbus Instrument, Columbus, OH) and removed immediately after they licked the footpad of the hind paw. The latency (ie the time spent before paw licking) was recorded with a cut-off time of 30 s.

The tail-flick test—This was performed as described previously (Bohn *et al*, 2000). Latency to respond to warm water (50°C) was measured to assess the nociceptive response; this was defined as the removal of the tail from the warm water.

The writhing test—Mice were injected (i.p.) with 1% acetic acid (10 ml/kg) and placed separately in transparent, plastic cages (25 × 50 × 25 cm). Five minutes later, a 5-min observation was initiated, during which the number of writhes (defined as constriction of the abdomen with stretching of the hind legs) was recorded.

Measurement of Locomotor Activity

This was performed using the open-field chamber. The floor was divided into four identical squares. Naive mice were individually placed in the center of the chamber and allowed to explore for 30 min, during which the number of line crossings (with all four paws crossed the line) and rears (ie the number of times the animals stood on its rear paws) were recorded every 5 min.

Statistical Analyses

All data were analyzed using Student's *t*-tests except for those in the forced-swim, step-down, and water-maze tests, in which two-way ANOVA was used followed by Newman–Keuls comparison tests.

RESULTS

Loss of PDE4B Expression and Reduction of PDE4 Activity in PDE4B−/− Mice

To confirm the deficiency of PDE4B in the brains of PDE4B−/− mice, western blotting was used to analyze the expression of the PDE4 subtypes in the cerebral cortex, amygdala, neostriatum, hippocampus, and cerebellum from PDE4B + / +, PDE4B +/− (heterozygous knockout), and PDE4B−/− mice. The results revealed that the PDE4B1/3 isoforms were not expressed in the cerebral cortex of PDE4B−/− mice; by contrast, the expression of PDE4A (4A5) and PDE4D (4D3) was not changed (Figure 2a). PDE4B +/− mice exhibited intermediate expression of PDE4B relative to the PDE4B + / + and PDE4B−/− mice. Similar patterns of expression also were observed in the other regions of the brain (data not shown). Thus, PDE4B was selectively knocked out in PDE4B−/− mice. Activities of PDE4, non-PDE4, and total PDEs were determined in the brain regions. Consistent with the reduced PDE4B expression, PDE4 activity (ie rolipram-sensitive PDE activity) was decreased in the brains of PDE4B−/− mice; it was reduced by 33% in the cerebral cortex ($P < 0.001$; Figure 2b) and 68% in the amygdala ($P = 0.002$; Figure 2c). In contrast, non-PDE4 activity was not significantly different from that in the PDE4B + / + mice. Total PDE activity was decreased in the amygdala ($P < 0.01$) and tended to be decreased in the cerebral cortex ($P = 0.06$) in PDE4B−/− mice. Similar decreases in PDE4 activity also were

observed in the other regions tested except for the hippocampus, in which PDE4 activity tended toward a decrease (ie by 22%) but this was not significant (data not shown).

PDE4B−/− Mice Displayed Increases in Hippocampal Neurogenesis

To determine changes in neurogenesis, the number of newborn cells, an index of proliferation (Duman *et al*, 2001), in the dentate gyrus of the hippocampus was examined by immunohistochemical detection of bromodeoxyuridine (BrdU) within nuclei of dividing cells. The immature neuron marker Tuj1 was used to label newly born cells. BrdU-positive nuclei of proliferating cells were localized predominantly in the subgranular layer, ie the border between granule cell layer (GCL) and hilus, and within GCL in both PDE4B + / + and PDE4B−/− mice (Figure 2d and e). Cells double-labeled for Tuj1 and BrdU in the GCL appeared in clusters of two or more (Figure 2f–h), indicating incorporation of BrdU occurs during proliferation of granule cells. BrdU-positive cells were slightly, but significantly increased in the GCL of hippocampal dentate gyrus from PDE4B−/− mice relative to those from PDE4B + / + controls ($P < 0.05$; Figure 2i).

Anxiogenic-Like Behavior of PDE4B−/− Mice

To determine the role of PDE4B in the regulation of anxiety, we examined behaviors of PDE4B−/− mice and their wild-type controls in a battery of anxiety tests, including the holeboard, light–dark transition, and open-field exploration tests. In the holeboard test, PDE4B−/− mice displayed significant decreases in the number of head-dips ($P < 0.001$) and the time spent in head-dipping relative to PDE4B + / + controls ($P < 0.01$; Figure 3a), indicating an anxiogenic-like effect. Consistent with this, in the light–dark transition test, PDE4B−/− mice exhibited an increase in the latency to cross from the dark to the light compartment ($P < 0.05$; Figure 3b) and decreases in the number of transitions ($P < 0.05$; Figure 3c) and duration, ie the time spent in the light compartment, compared to the PDE4B + / + mice ($P < 0.05$; Figure 3d). Similarly, in the open-field test, PDE4B−/− mice showed decreases in exploration, ie an increase in the latency to exit the initial quadrant ($P < 0.05$; Figure 4a) and decreases in initial exploration ($P < 0.01$; Figure 4b) and rears ($P < 0.001$; Figure 4c). These data suggest that mice deficient in PDE4B display anxiogenic-like behavior. These effects appeared not to be associated with changes in general activity since PDE4B−/− mice did not show altered locomotor activity (see below).

Consistent with the behavioral observations, PDE4B−/− mice displayed a significant increase in plasma corticoster-one levels relative to PDE4B + / + controls ($P < 0.001$; Figure 4d), suggesting a greater susceptibility of mice deficient in PDE4B to stress.

PDE4B−/− Mice Displayed Antidepressant-Like Behavior

To determine the potential role of PDE4B in antidepressant-like activity, PDE4B−/− mice and their wild-type controls were first tested in the TST followed by the FST with or without treatment with rolipram or desipramine. In the TST, PDE4B−/− mice tended to show an increase in immobility compared to the wild-type control ($P = 0.11$; Figure 5a). By contrast, in the FST, PDE4B−/− mice displayed decreases in the duration of immobility relative to PDE4B + / + littermates ($P < 0.05$; Figure 5b, Session 1); this was verified in a second test performed 7 weeks later using the same mice ($P < 0.01$; Figure 5b, Session 2). While acute treatment with rolipram did not alter forced-swim behavior in either genotype, repeated treatment with rolipram (0.5 mg/kg, i.p. for 8 days) decreased immobility in the PDE4B + / +, but not PDE4B−/− mice ($P < 0.05$; Figure 5c). Similarly, acute administration of desipramine at doses of 20 and 40 mg/kg reduced immobility only in PDE4B + / + mice ($P < 0.01$ and $P < 0.001$, respectively; Figure 5d).

Memory Performance of PDE4B−/− Mice

To determine whether PDE4B deficiency altered memory, we examined the performance of PDE4B−/− mice and their wild-type controls in step-down passive avoidance and Morris water-maze tasks. In the step-down test, compared to PDE4B + / + controls, PDE4B−/− mice did not display any changes in either short-term (3 h) or long-term (1 and 8 days) retention (Table 1). Similarly, in the water-maze test, while both PDE4B + / + and PDE4B−/− mice displayed progressive decreases in escape latency during the acquisition training, there was no difference between the genotypes (Figure 6a). In addition, PDE4B−/− mice did not show any different changes in entries into and time spent in the target quadrant in the probe trial (Figure 6b). Negative results also were obtained in the radial-arm maze test (data not shown).

Nociceptive Responses of PDE4B−/− Mice

To determine the potential role of PDE4B in nociception, we examined the responses of PDE4B−/− mice and their wild-type littermates in three nociceptive tests: the hot-plate, tail-flick, and acetic acid-induced writhing tests. Compared to PDE4B + / + controls, PDE4B−/− mice did not exhibit altered nociceptive responses, as evidenced by unchanged latencies of paw licking and tail-flick and number of writhes in the three tests, respectively (Table 2).

Locomotor Activity in PDE4B−/− Mice

In the open-field test, PDE4B−/− mice displayed a decrease in line crossings in the first 5 min relative to the PDE4B +/ + controls ($P < 0.05$). However, this was not repeated at the other time points (Figure 7a); the total line crossings in the 30 min test period were not altered either (Figure 7c). PDE4B−/− mice tended to show decreases in rears over the test times (Figure 7b) and displayed a significant decrease in the total rears compared to the wild types ($P < 0.05$; Figure 7d). These results were consistent with the decreased open-field exploration and anxiogenic behavior described above. In addition, the overall unaltered line crossings suggest unchanged locomotor activity in PDE4B−/− mice.

DISCUSSION

It is well established that PDE4 inhibitors such as rolipram produce antidepressant-like and memory-enhancing effects (Barad *et al*, 1998; O'Donnell and Zhang, 2004; Zhang *et al*, 2004, 2006; Zhang and O'Donnell, 2007). They also may induce anxiogenic-like effects and sedation (Heaslip and Evans, 1995; Imaizumi *et al*, 1994; Silvestre *et al*, 1999b), although an anxiolytic-like effect also has been reported (Silvestre *et al*, 1999a). While studies have shown that PDE4D plays an important role in the mediation of antidepressant-like effect (Zhang *et al*, 2002) and may be involved in memory (Zhang *et al*, 2004, 2005), the role of PDE4B has not been investigated due to the lack of highly selective inhibitors of individual PDE4 subtypes and the complexity of the PDE4 family in terms of its numerous variants and compartmentation (Conti *et al*, 2003; Houslay *et al*, 2005; Terrin *et al*, 2006). Using mice deficient in PDE4B, it has been demonstrated that this subtype plays a critical role in lipopolysaccharide-induced signaling and inflammatory responses (Ariga *et al*, 2004; Jin *et al*, 2005a). Most recently, it has been shown that PDE4B is required for the antipsychotic effect of rolipram (Siuciak *et al*, 2007), which is consistent with the association of this subtype with schizophrenia (Millar *et al*, 2005). Nevertheless, little is known about the CNS function of PDE4B. In the present study, it was found that PDE4B was involved in behaviors associated with anxiety, but not memory and nociception.

While it is relatively well established that PDE4 is involved in antidepressant-like and memory-enhancing effects, the regulation of anxiety by PDE4 remains largely unknown. The reports to date are inconsistent. One study showed that the PDE4 inhibitor rolipram

produced an anxiolytic-like effect in rats (Silvestre *et al*, 1999a), whereas other studies have shown that PDE4 inhibitors, including rolipram, result in anxiogenic-like behavior in dogs and mice (Heaslip and Evans, 1995; Imaizumi *et al*, 1994). The reasons for the inconsistent results may be due, at least partially, to the sedative effect of PDE4 inhibitors (Griebel *et al*, 1991; Silvestre *et al*, 1999b) and/or the different contributions of PDE4 subtypes to anxiety regulation. Knockout of individual PDE4 subtypes helps to address these issues.

Mice deficient in PDE4B exhibited behavioral changes in tests sensitive to anxiolytic drugs. PDE4B−/− mice displayed decreases in head-dips and time spent in head-dipping in the holeboard test, an increase in latency before crossing to the light side and decreases in transitions and time spent in the light compartment in the light–dark transition test, and an increase in latency to explore and decreases in exploration and rears in a novel and lighted open-field chamber. All these changes represent typical anxiogenic-like behavior (MacNeil *et al*, 1997; Pellow, 1986; Suaudeau *et al*, 2000). Given that PDE4B−/− mice did not display significant changes in locomotor activity, as evidenced by unaltered total line crossings in the open-field test, the behavioral effects of PDE4B deficiency on anxiety tests appeared not to be attributed to sedation. Consistent with the behavioral data, the plasma corticosterone concentrations were increased, but the size of adrenal glands was not changed (data not shown) in PDE4B−/− mice. These are supported by rolipram-induced activation of the hypothalamo–pituitary–adrenal (HPA) axis and increases in plasma corticosterone (Kumari *et al*, 1997). This pattern of behavior and biochemistry is indicative of an anxiogenic-like effect (Hascoet and Bourin, 1998; Liebsch *et al*, 1998; Suaudeau *et al*, 2000).

Anxiogenic-like behavior of PDE4B−/− mice appears to be in agreement with the unique distribution of PDE4B in the mouse brain; this subtype is highly expressed in the hypothalamus and locus coeruleus, relative to the other PDE4 subtypes (Cherry and Davis, 1999). Noradrenergic systems in the hypothalamus, amygdala, and locus coeruleus are involved in the regulation of anxiety. Increases in the release of norepinephrine, which stimulates cAMP formation in the brain (Bettahi *et al*, 1998; Quesada and Etgen, 2000), produce anxiogenic-like behavior in animals (Tanaka *et al*, 2000). Although there is no evidence showing a high expression of PDE4B in the mouse amygdala, significantly decreased activity of PDE4 in PDE4B−/− mice indicates that PDE4B most likely is the predominant PDE4 subtype in this region, which is important in anxiety (Wand, 2005). Loss of PDE4B leads to increases in cAMP in these brain regions and results in anxiogenic-like behavior.

PDE4B−/− mice also displayed ‘antidepressant-like’ behavior, ie reduced immobility in the initial exposure to forced-swim, a behavioral test that is widely used for evaluating antidepressants. Antidepressant treatment typically decreases the immobility duration of animals that are repeatedly exposed to the forced-swim test, even though the baseline of the immobility is increased (Raghavendra *et al*, 2000; Zhang *et al*, 2002). This was verified using the same mice 7 weeks later, showing that this was a persistent behavioral change. Interestingly, while acute desipramine or repeated rolipram produced antidepressant-like effects on forced-swim behavior in the wild-type animals, neither treatment significantly altered the immobility duration in PDE4B−/− mice, although both tended to produce decreases in immobility. However, ‘antidepressant-like’ behavior of PDE4B−/− mice was not supported by the TST, which also is a test sensitive to antidepressant drugs and commonly used for assessing antidepressant activity (Cryan *et al*, 2005). Since PDE4B−/− mice displayed anxiety-like behavior, it is possible that the ‘antidepressant-like’ effect in the FST resulted from anxiety-related struggling, leading to decreased immobility. This is supported by studies showing that treatment causing anxiogenic-like effects (eg acute pentylenetetrazol or the CRF1 agonist cortagine) results in antidepressant-like behavior in

the FST (Cannizzaro *et al*, 1993; Tezval *et al*, 2004). Therefore, the 'antidepressant-like' effect of PDE4B−/− mice observed in the FST may be a 'false positive' result. Given that the effects of rolipram and desipramine were attenuated in PDE4B−/− mice, further studies are needed to clarify the effect of PDE4B deficiency on FST behavior and depression-related behaviors in general.

PDE4B−/− mice also displayed a slight, but significant, increase in adult neurogenesis relative to the wild-type controls, as evidenced by increased proliferation of neuronal cells in the subgranular zone of the hippocampal dentate gyrus. Since stress decreases hippocampal neurogenesis, in particular cell proliferation (Warner-Schmidt and Duman, 2006), PDE4B−/− mice might be expected to display a decrease, rather than an increase, in cell proliferation. The inconsistent results may be attributed to the persistent stimulation of cAMP signaling in PDE4B−/− mice. It was noted that PDE4 activity in the hippocampus was decreased by 22%, although it was not significantly changed relative to the wild-type controls. While this effect was subtle, probably due to the relatively low levels of PDE4B in the hippocampus (Perez-Torres *et al*, 2000), long-term decreases in PDE4 activity in PDE4B−/− mice may mimic the effect of PDE4 inhibition by chronic treatment with rolipram, which increases adult hippocampal neurogenesis (Nakagawa *et al*, 2002). Since rolipram plays its role in neurogenesis via activation of the cAMP/CREB cascade, neurogenesis induced by PDE4B deficiency likely is also mediated by cAMP signaling. Interestingly, chronic administration of the antidepressant fluoxetine, a selective serotonin reuptake inhibitor, not only increases hippocampal neurogenesis (Encinas *et al*, 2006; Malberg *et al*, 2000), but also decreases the expression of PDE4B in the brains of mice and rats (Dlaboga *et al*, 2006; Miro *et al*, 2002). However, while these findings appear to be consistent with FST behavior and hippocampal neurogenesis observed in PDE4B−/− mice, further studies are needed to determine their relationship to antidepressant activity. In addition, these results do not rule out the contributions of other PDE4 subtypes, such as PDE4A and PDE4D, given the consideration of their roles in or association with antidepressant-like behavior (Takahashi *et al*, 1999; Ye *et al*, 2000; Zhang *et al*, 2002).

PDE4B appears not critical for memory processes. While overall inhibition of PDE4 enhances memory performance in animals (Barad *et al*, 1998; Zhang *et al*, 2004, 2005), PDE4B−/− mice did not show significant changes in either short- or long-term memory in step-down passive avoidance and Morris water-maze tasks (Table 1 and Figure 5), two tests measuring hippocampus-based memory (Bevilaqua *et al*, 1997). This is consistent with the limited expression of PDE4B in the hippocampus (Cherry and Davis, 1999). Further studies need to be performed in amygdala- and striatum-based memory models, given that PDE4B is highly expressed in these two regions (Perez-Torres *et al*, 2000). Similar to the results observed in the memory tests, PDE4B−/− mice did not display any nociceptive responses in the tests sensitive to analgesics (Table 2), although overall inhibition of PDE4 has been reported to produce an analgesic effect (Levy and Goldstein, 1981).

In conclusion, our results indicate that PDE4B is particularly involved in the mediation of behaviors associated with anxiety. Deficiency of PDE4B produces anxiogenic-like behavior in the tests used in the present study. They also showed a slight but significant increase in hippocampal neurogenesis. The behavioral pattern of the anxiogenic-like effect and reduced immobility in the FST observed in PDE4B−/− mice appears not uncommon; it also occurs in BDNF transgenic mice (Govindarajan *et al*, 2006) and in rats treated acutely with pentylenetetrazol (Cannizzaro *et al*, 1993) or fluoxetine (Zienowicz *et al*, 2006). Thus, the present data suggest that PDE4B plays a role in the anxiogenic-like effects of acute rolipram (Heaslip and Evans, 1995; Imaizumi *et al*, 1994), which also exerts antidepressant activity.

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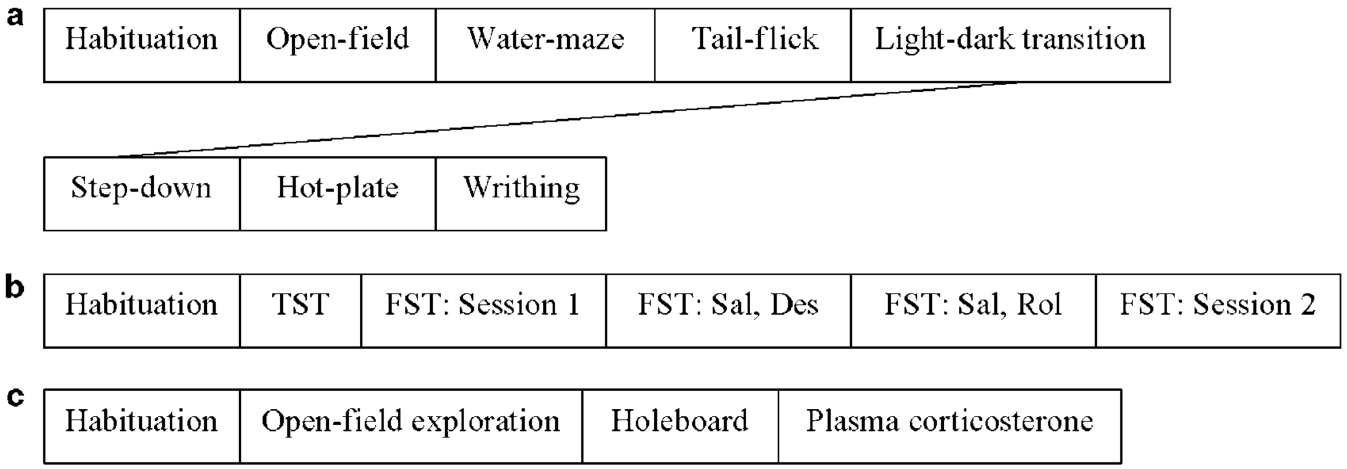

Figure 1.

The schedule of behavioral tests for three groups (a, b, and c) of PDE4B+ / + and PDE4B−/ − mice. Six to eight mice per genotype in each group were used in the batteries of behavioral tests and in the examination of plasma corticosterone (c). All animals were habituated for 1 week before tests. Intervals between tests were 1–2 weeks. TST, the tail-suspension test; FST, the forced-swim test; Des, desipramine; Sal, saline; Rol, rolipram (acute and repeated administration).

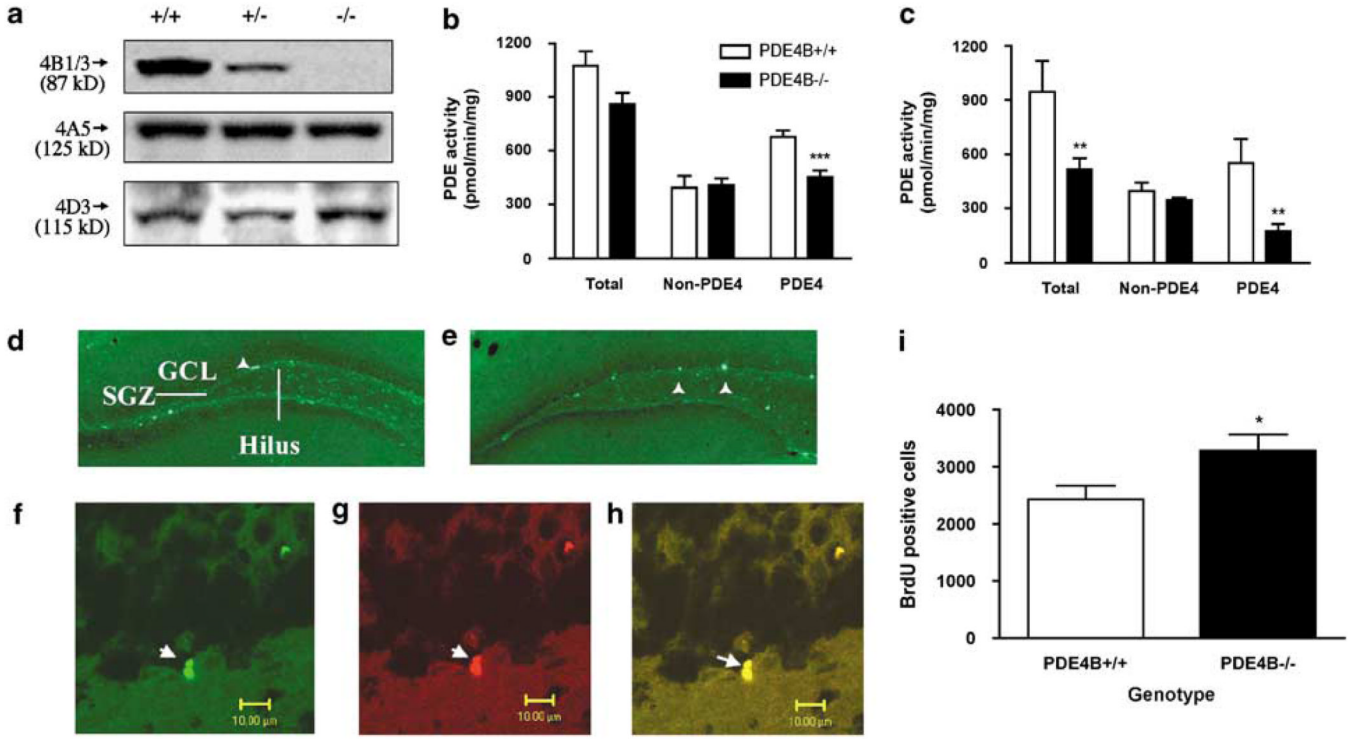

Figure 2.

PDE4B−/− mice displayed decreases in PDE4B expression and PDE4 activity and an increase in neurogenesis. (a) Mice deficient in PDE4B (−/−) displayed a decrease in the expression of PDE4B1/3, but did not show any changes in that of PDE4A (A5) and PDE4D (D3) in cerebral cortices, relative to PDE4B wild-type (+ / +) controls. PDE4B heterozygous (+/ −) mice displayed intermediate expression in PDE4B1/3 relative to PDE4B + / + and PDE4B −/− mice. The tissues were homogenized and immunoprecipitated as described in the Methods section. The proteins of PDE4B1/3, PDE4A5, and PDE4D3 were detected by western blot analysis. Molecular weights of the PDE4 variants are shown in parentheses (Dlaboga *et al*, 2006). (b and c) PDE4B−/− mice displayed decreased PDE4 activity, but no change in non-PDE4 activity in both the cerebral cortex (b) and the amygdala (c). In contrast, these mice exhibited decreases in total PDE activity in the amygdala and a tendency toward decreased total PDE activity in the cerebral cortex. PDE4 activity was obtained by subtracting non-PDE4 (ie rolipram-sensitive PDE) activity from total PDE activity. (d and e) Representative photomicrographs of the hippocampal dentate gyrus from PDE4B+ / + (d) and PDE4B−/− (e) immunofluorescent staining for BrdU-positive cells (arrowhead), which were predominantly localized in the subgranular zone (SGZ) and within the granule cell layer (GCL). (f–h) Representative photomicrographs of cells double immunostaining (h; yellow) for Tuj1 (f; green) and BrdU (g; red) in the GCL of the dentate gyrus of the hippocampus from PDE4B−/− mice. (i) Quantification of BrdU and Tuj1 double-positive cells. PDE4B−/− mice displayed an increase in cell proliferation. Data shown represent the mean±SEM of 7–8 mice per group. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 vs PDE4B + / + (two-tailed Student's *t*-test).

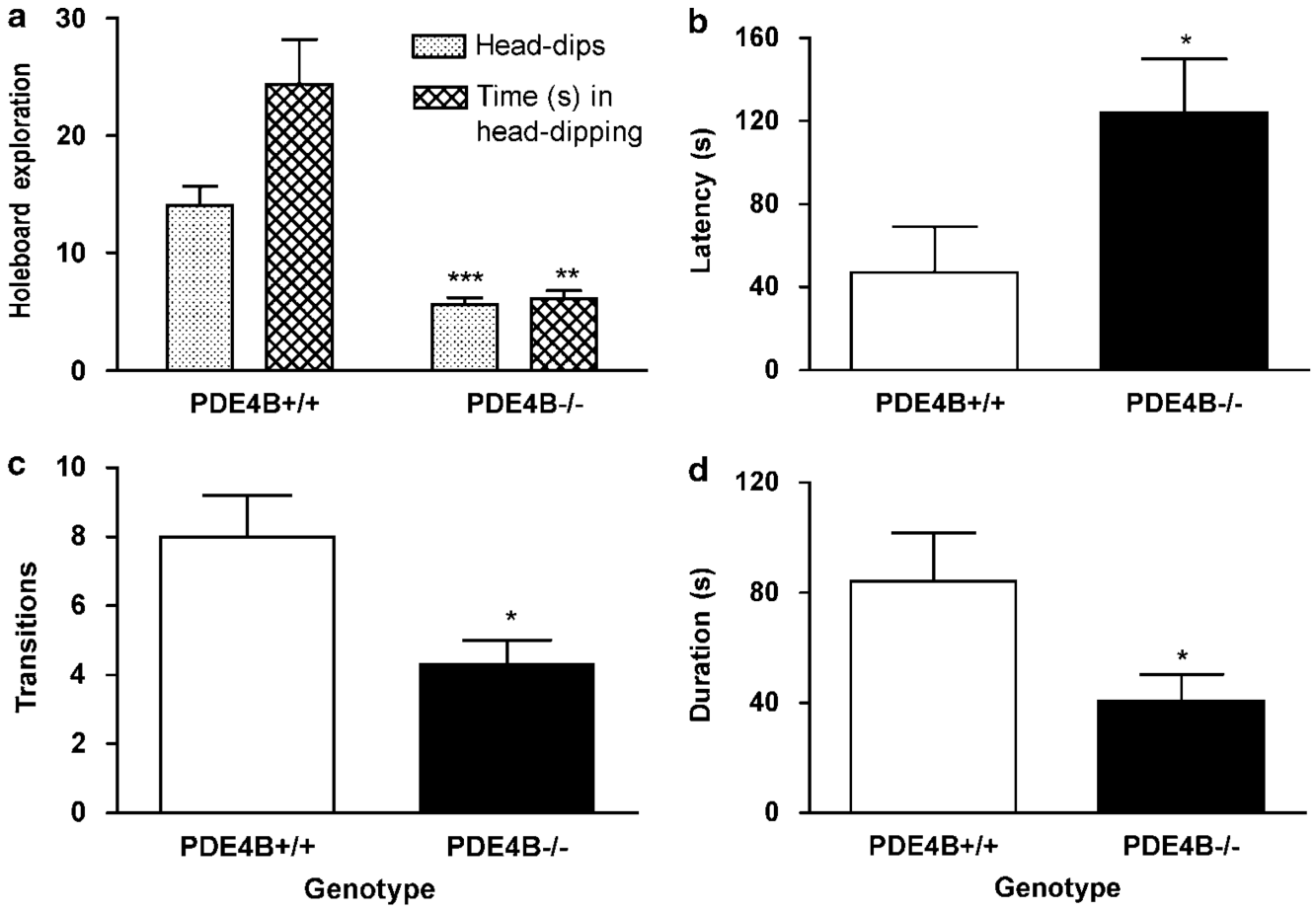

Figure 3.

PDE4B−/− mice displayed anxiogenic-like effects on behavior in the holeboard and light–dark transition tests. (a) PDE4B−/− mice displayed decreases in head-dips and time spent in head-dipping in the holeboard test. (b–d) PDE4B−/− mice displayed decreases in latency (b) before crossing over to the light compartment, transitions (c) from the dark compartment to the light side, and duration (d), ie the time spent in the light compartment. Data shown represent the mean±SEM of 7–10 mice per group; **P* < 0.05; ***P* < 0.01; ****P* < 0.001 vs PDE4B + / + (two-tailed Student's *t*-test).

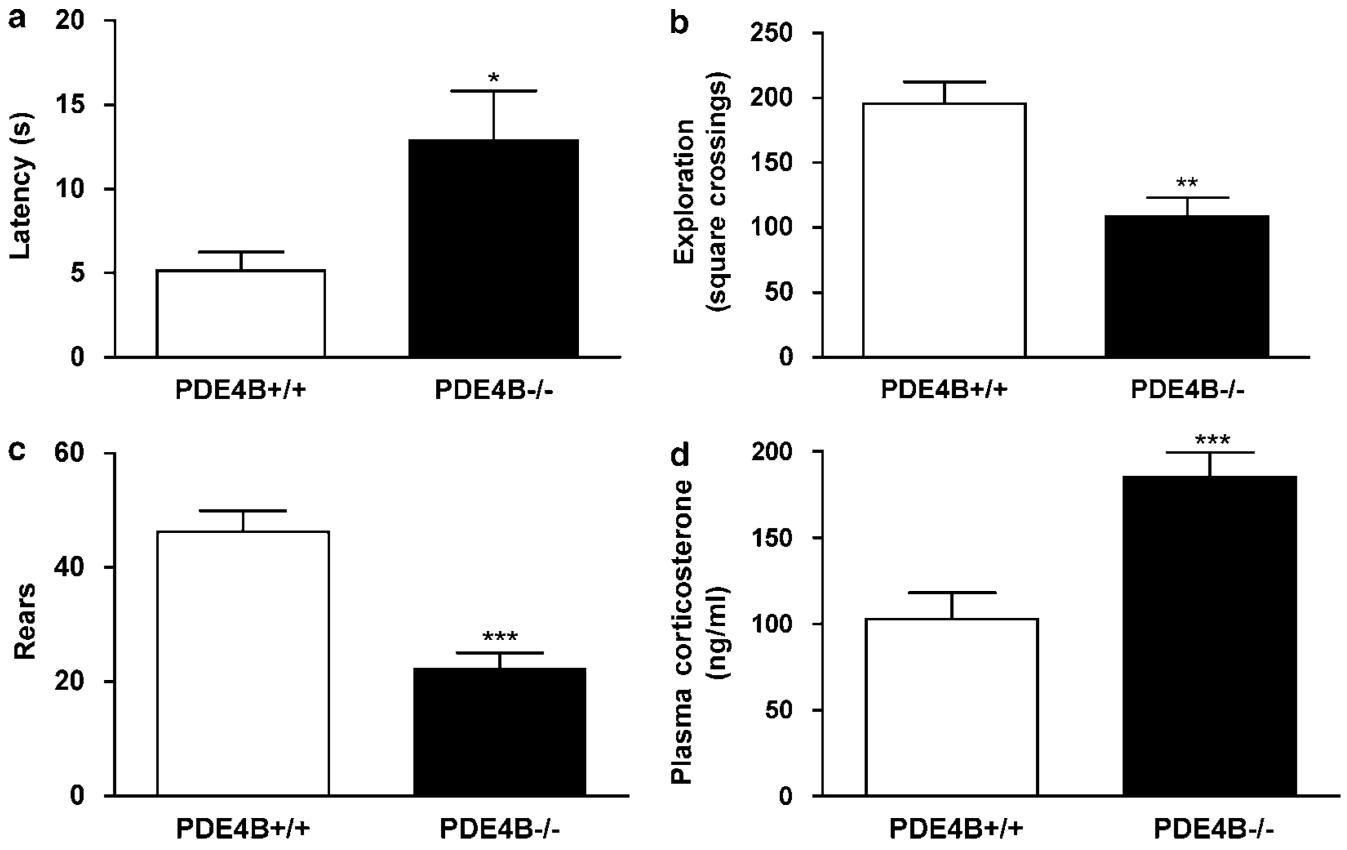

Figure 4.

PDE4B−/− mice displayed anxiogenic-like effects on open-field behavior and increases in plasma corticosterone levels. (a–c) PDE4B−/− mice showed increased latency to explore, decreases in initial exploration (b), ie the number of squares crossed, and rears (c) in the novel open-field test. (d) PDE4B−/− mice displayed increases in plasma corticosterone levels. Data shown represent the mean±SEM of 7–10 mice per group. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 vs PDE4B + / + (two-tailed Student's *t*-test).

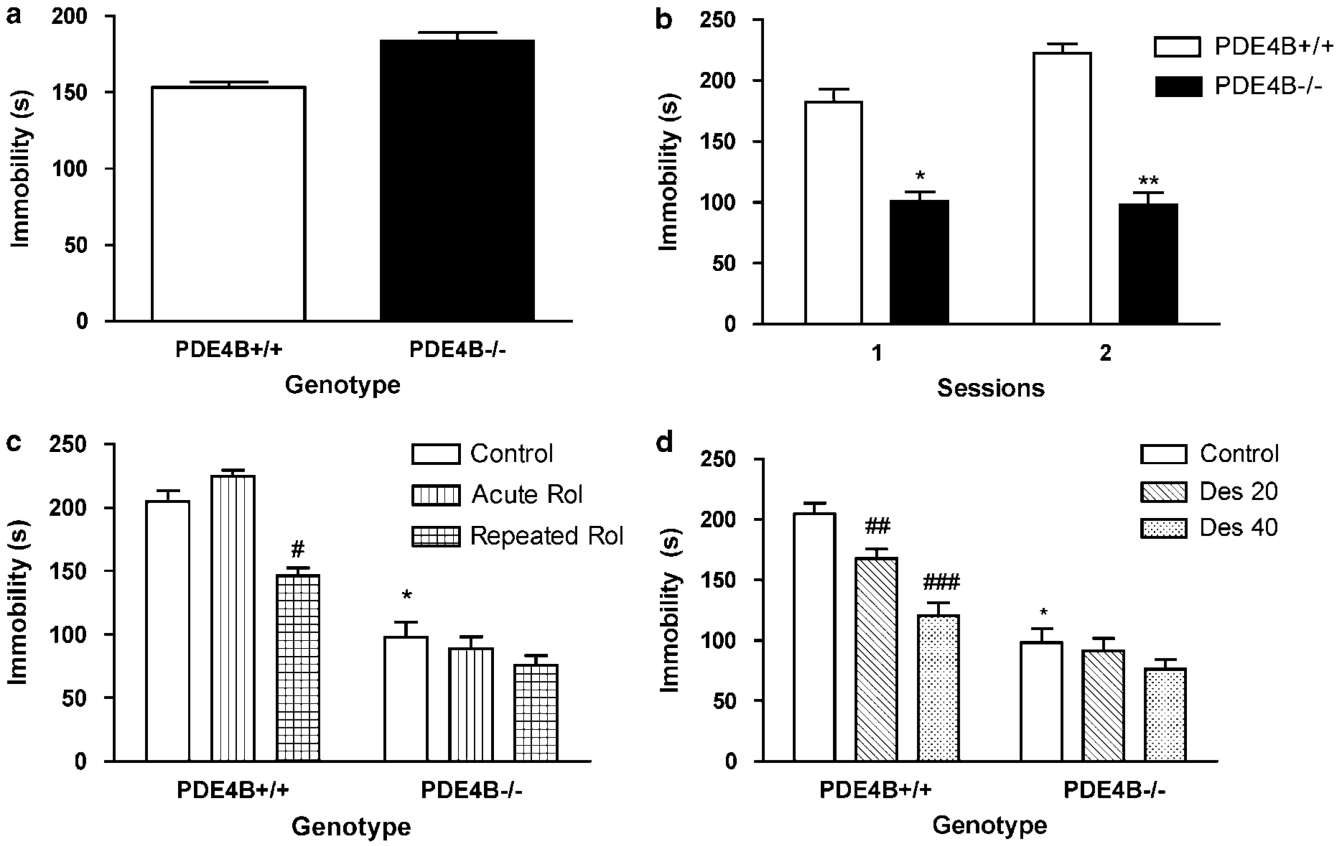

Figure 5.

Behaviors of PDE4B−/− mice in the TST (a) and FST (b–c). (a) PDE4B−/− mice did not show significant changes in immobility in the TST; (b) PDE4B−/− mice displayed decreases in immobility duration in Session 1 and 2 of FST (there was a 7 weeks interval between the sessions). (c) Repeated treatment with rolipram (Rol) decreased immobility in PDE4B + / +, but not PDE4B−/− mice. Rol (0.5 mg/kg, i.p.) was given singly (Acute Rol) or once a day for 8 days (Repeated Rol). (d) Desipramine (Des) decreased immobility in PDE4B + / +, but not PDE4B−/− mice. Mice were tested 30 min after the single Rol or Des or the last Rol injection. Data shown represent the mean±SEM of 7–8 mice per group. $^{*}P < 0.05$; $^{**}P < 0.01$ vs corresponding PDE4B+/+ mice and $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs control in the same genotype (two-tailed Student's *t*-test).

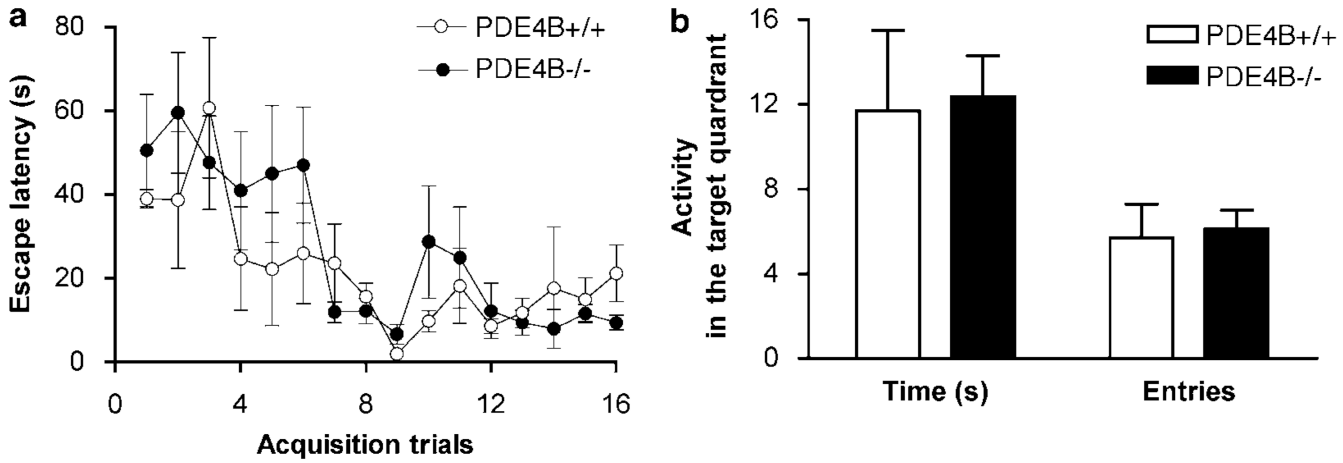

Figure 6.

PDE4B−/− mice did not exhibit altered performance in the Morris water-maze task. (a) PDE4B−/− mice did not show significant changes in escape latencies in the acquisition training trials relative to PDE4B+/+ controls. (b) PDE4B−/− mice did not display any changes in either entries into or time spent in the target quadrant in Probe Trial 1 given 24 h after the last acquisition trial. All the observations were performed for 60 s. Data shown represent mean ± SEM of 6–7 mice per group. There was no difference between the genotypes as revealed by two-way ANOVA (for a) or unpaired *t*-tests (b).

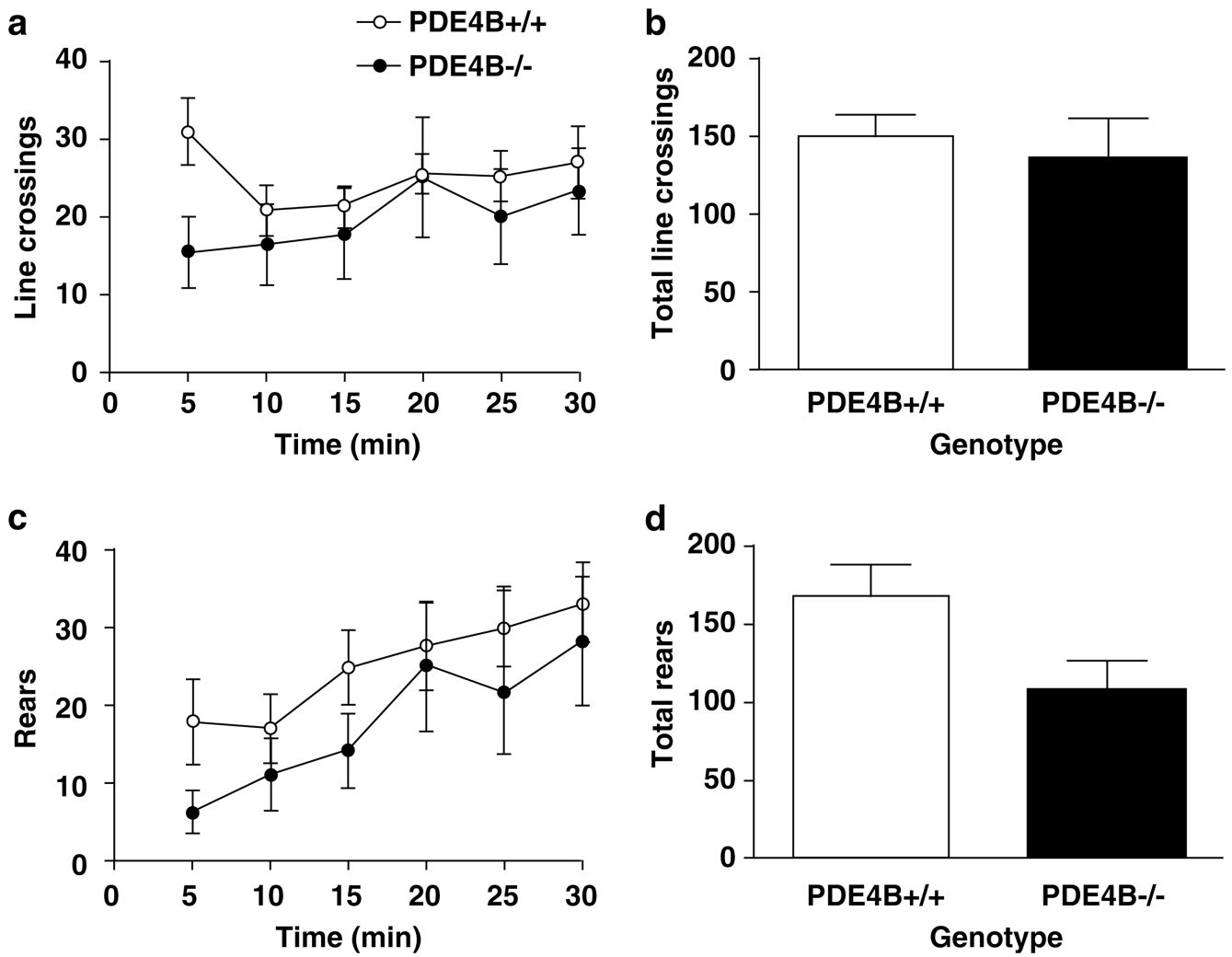

Figure 7.

Locomotor activity in the open-field test in PDE4B + /+ and PDE4B−/− mice. PDE4B−/− mice displayed decreases in line crossings during the first 5 min (a) but no change for the 30-min test (b), while rears were not reduced significantly for any 5-min time period (c), they were decreased in the 30-min test (d). Line crossings and rears were recorded for every 5-min period for 30 min. Data shown represent mean ± SEM of 7–8 mice per group. **P* < 0.05 vs PDE4B + / + mice at the corresponding time point (two-tailed Student's *t*-test).

Table 1

Retention Latency of PDE4B+/+ and PDE4B−/− at the Time Points of 3 h, 1 day, and 8 days after Initial Training in the Step-Down Passive Avoidance Test

	Latency (s)		
	3 h	**1 day**	**8 day**
PDE4B+/+	163.7 ± 46.9	218.2 ± 44.3	192.8 ± 41.4
PDE4B−/−	189.1 ± 40.5	223.0 ± 38.9	203.1 ± 47.3

Data shown represent the mean ± SEM of 6–7 mice per group. The latency was the time before the animal descended to the grid floor where they had previously received a mild, inescapable footshock. Compared to PDE4B+/+ controls, PDE4B−/− mice did not display any changes in either short-term (3 h) or long-term (1 and 8 days) retention, as revealed by two-way ANOVA and unpaired *t*-tests.

Table 2

Comparison of Nociceptive Responses Between PDE4B+/+ and PDE4B−/− Mice in the Hot-Plate, Tail-Flick, and Acetic Acid–Induced Writhing Tests

	Hindpaw licking latency (s)	Tail-flick latency (s)	Number of writhes
PDE4B+/+	19.3 ± 1.3	3.0 ± 0.1	6.5 ± 2.0
PDE4B−/−	20.6 ± 3.2	3.4 ± 0.4	8.6 ± 1.4

Data shown represent the mean ± SEM of 7–8 mice per group. The latency was the time before the animal licked its hind paw in the hot-plate test or removed its tail from the water in the tail-flick test with a cut-off time of 30s. The number of writhes, which was defined as constriction of the abdomen with stretching of the hind legs, was recorded for 5min starting from 5 min after the injection of 1% acetic acid (10ml/kg, i.p.). Compared to PDE4B+/+ controls, PDE4B−/− mice did not exhibit altered nociceptive responses, as evidenced by unchanged latencies of hindpaw licking and tail-flick and number of writhes in the three tests, as revealed by unpaired *t*-tests.