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Neurobehavioral abnormalities in a brain-specific NADPH-cytochrome P450 reductase knockout mouse model

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Abstract

The aim of the present study was to test a new hypothesis that brain cytochrome P450 reductase (CPR) and CPR-dependent enzymes play important roles in behavioral performance. A mouse model with brain neuron-specific deletion of the *Cpr* gene (brain-*Cpr*-null) was recently generated. Brain-*Cpr*-null mice and wild-type (WT) littermates were compared in a variety of behavioral assays. Notable differences were found in the exploratory behavior assay: for both males and females, activity in the center of the chamber was significantly higher for brain-*Cpr*-null than for WT mice on days 2 and 3 of the assay, although no significant difference was found between the two groups in anxiety-like behavior in the elevated zero maze. Furthermore, in the fear-conditioning assay, brain-*Cpr*-null mice exhibited significantly less activity suppression than did WT controls. This deficit in activity suppression was not accompanied by any difference between WT and brain-*Cpr*-null mice in nociceptive responses to foot shocks. Abnormal activity suppression was also observed in both male and female brain-*Cpr*-null mice during the contextual memory test. However, in the Morris water maze assay, the brain-*Cpr*-null and WT mice were indistinguishable, indicating normal spatial memory in the mutant mice. These data collectively indicate a novel role of the *Cpr* gene in fear conditioning and memory.

Keywords

Cytochrome P450; NADPH-cytochrome P450 reductase; fear conditioning; memory; brain; mice

1. Introduction

Cytochrome P450 monooxygenases (P450s) metabolize drugs and other xenobiotic compounds, as well as endogenous compounds such as steroids and eicosanoids (Porter and Coon, 1991). P450 enzymes are expressed both in portal-of-entry organs, such as the lung

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and small intestine, and in internal organs, including the brain (Miksys and Tyndale, 2002; Strobel et al., 2001). The amount of P450s expressed in brain as a whole is relatively low, reportedly ~3–10% of that in liver, but higher levels may be found in select regions and cells (Miksys and Tyndale, 2002; Strobel et al., 2001). The biological functions of most P450 enzymes in brain remain largely unknown. It has been suggested that brain P450s are involved in the local metabolism of psychoactive drugs, synthesis and metabolism of neurosteroids, metabolism of neurotransmitters, and etiology of neurodegenerative diseases (Hedlund et al., 2001; Meyer et al., 2007; Miksys and Tyndale, 2002; Rose et al., 2001; Yau et al., 2006). For example, hippocampal neuronal cells not only express CYP17A1, CYP19A1, and P450_{scc}, enzymes indispensable for steroid synthesis, but also functionally synthesize steroids in situ (Hojo et al., 2004; Kretz et al., 2004; Zwain and Yen, 1999). Interestingly, the levels of testosterone, 17 β -estradiol, and dihydrotestosterone measured in rat hippocampus were higher than those in systemic circulation, and hippocampal estradiol was detected at relatively high levels even after castration (Hojo et al., 2009). Additionally, behavioral deficits in learning and memory were found in *Cyp46a1*-null mice (Kotti et al., 2006), while human *CYP46* genetic polymorphisms have been linked to increased risk for Alzheimer disease (Borroni et al., 2004).

To further explore the biological and pharmacological functions of microsomal P450 enzymes in the brain, we recently generated a brain neuron-specific cytochrome P450 reductase (CPR or POR) knockout (brain-*Cpr*-null) mouse (Conroy et al., 2010). CPR, the required electron provider to all microsomal P450 enzymes and heme oxygenases (HOs) (Black and Coon, 1987; Gu et al., 2003; Schacter et al., 1972; Strobel et al., 1995), is indispensable for fetal development (Otto et al., 2003; Shen et al., 2002). However, tissue-specific *Cpr*-null mouse models, such as brain-*Cpr*-null, liver-*Cpr*-null, lung-*Cpr*-null, cardiomyocyte-*Cpr*-null, and intestinal epithelium-*Cpr*-null (Conroy et al., 2010; Fang et al., 2008; Gu et al., 2003; Henderson et al., 2003; Weng et al., 2007; Zhang et al., 2009) are generally viable and thus suitable for in vivo studies on the combined functions of all microsomal P450 enzymes in specific cell types, regions, or organs.

The brain-*Cpr*-null mouse was generated by cross-breeding between the *Cpr*^{lox/lox} mouse (Wu et al., 2003) and a Cre recombinase (Cre) transgenic mouse in which Cre expression was driven by the calcium/calmodulin-dependent protein kinase IIa (CaMKIIa) gene promoter (Dragatsis and Zeitlin, 2000), for brain neuron-specific *Cpr* gene deletion (Conroy et al., 2010). In adult brain-*Cpr*-null mouse, neuronal CPR loss was detected in multiple brain regions, including hippocampus, cortex, and ventrolateral periaqueductal gray. The loss of CPR expression resulted in large decreases in P450 and HO enzymatic activities in brain microsomes. Furthermore, brain-*Cpr*-null mice exhibited a blunted response to morphine-induced analgesic effect, a finding that led to the conclusion that brain P450 enzymes mediate pain-relieving properties of morphine, possibly through the production of epoxyeicosanoids (Conroy et al., 2010).

In the current study, brain-*Cpr*-null mice and wild-type (WT) littermates were compared in a battery of behavioral assays in order to systemically explore the role of CPR/P450 in advanced brain functioning, including learning and memory. The Morris water maze assay was used to test learning and spatial memory; the open field assay and the elevated zero maze assay were used to test for anxiety-like behavior, and the fear conditioning assay was used to test for associative memory. Our results indicate a novel role of the *Cpr* gene in fear conditioning and memory.

2. Experimental Procedures

2.1. Animals

The generation of the brain-*Cpr*-null mice has been reported recently (Conroy et al., 2010). Briefly, CaMKII α -Cre (also known as L7ag13-Cre) mice (Dragatsis and Zeitlin, 2000) (at least 6 generations on the C57BL/6 background) were mated with *Cpr*^{lox/lox} mice (Wu et al., 2003) (originally produced on a mixed C57BL/6 and 129/Sv genetic background, then backcrossed to C57BL/6 for over 10 generations) to generate a conditional knockout mouse line with brain-specific *Cpr* deletion. The brain-*Cpr*-null mice (CaMKII α -Cre^{+/+}/*Cpr*^{lox/lox}) and WT littermates (CaMKII α -Cre^{-/-}/*Cpr*^{lox/lox}) were generated from CaMKII α -Cre^{+/+}/*Cpr*^{lox/lox} and *Cpr*^{lox/lox} breeding pairs.

Adult male and female mice were used for all neurobehavioral assays. Mice were housed in same-sex, mixed-genotype groups in clear Plexiglas cages (29 cm L, 18 cm W, 12.5 cm H) with stainless steel wire lids and filter tops, in a temperature-controlled (68–72°F) room with food (LabDiet, Prolab RMH 3500, Purina Mills, St. Louis, MO) and water available *ad libitum* under standard 12-hr light, 12-hr dark cycle (lights on at 7:00 am). Mice were evaluated for exploratory behavior in the open field and performance in elevated zero maze, fear conditioning, and Morris water maze assays. All animal studies were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

2.2. Exploratory behavior in the open field

This protocol has been described in detail previously and will only be briefly reviewed here (Bolivar, 2009). Mice were tested over three consecutive days, and behavior over a 15-min testing session each day was measured with Digiscan 16-beam automated activity monitors (AccuScan Instruments, Columbus, OH) contained within sound-attenuating chambers (Med Associates, St. Albans, VT). After a 1-h period of acclimation, each mouse was weighed, placed in a clear Plexiglas holding cage for 5 min, and then placed in the center of the dark activity monitor for testing. The total distance traveled was recorded. Center time and the percentage of center relative to total distance traveled were also calculated as measures of anxiety-like and/or fear behaviors.

2.3. Elevated zero maze assay

Anxiety-like behavior was evaluated in a darkened room (<1.0 lux) using the elevated Zero Maze Digital Monitoring system (AccuScan Instruments). The elevated zero maze assay has been described elsewhere (Eisener-Dorman et al., 2010) and will only be briefly outlined here. The maze consists of an elevated circular platform with two “open” and two “closed” quadrants and, at the beginning of the 5-min test session, each mouse was placed on the threshold of one of the closed quadrants. The total amount of time each mouse spent in the open and closed quadrants was measured.

2.4. Morris water maze assay

The Morris water maze assay consisted of training and probe sessions. In the first 7 days (training session), mice were trained to search and find a hidden platform in a water pool by environmental cues. The pool (140 cm in diameter and 40 cm high) was filled to a depth of 21 cm with water and arbitrarily divided into four equal quadrants (or zones). A circular platform (10 cm in diameter) was placed in the center of a quadrant, and was kept 0.5 cm under the surface of the water. The water, maintained at room temperature (~22°C), was made opaque by nontoxic white paint (Living Colors, J.L Hammett Co., Braintree, MA), which kept the target platform invisible to the mice. There were 4 training trials per day, which were divided into 2 blocks of 2 trials, with a 3-h rest period between blocks. The inter-trial time was 1 min. For each trial, mice were given 60 s to locate the target platform.

If a mouse failed to find the platform in 60 s, it was placed on the platform by the experimenter, and allowed to rest there for 30 s before being returned to the cage. During training trials, the platform was placed in zone 1 (Southwest), and mice were introduced into the water in zones 4, 3, 2, and 1, sequentially, in a clockwise fashion, in the four trials of each day. During trials, the movements of the mice in the pool were captured by an overhead video camera linked to a video tracking system (Videocan model 2000, AccuScan Instruments). The latency to find the platform during training and time spent in each quadrant during probe trials were recorded.

On the eighth day, a probe trial was conducted, during which the target platform was removed from the pool. All mice were placed in the water at zone 3, which is opposite to zone 1, where the target platform was originally located. Mice were allowed to swim for 60 s, and the fraction of the total time spent in each zone was determined. Starting on the 9th day, the mice were retrained for 3 days. One week after the final training, memory was tested again in a second probe trial. Beginning on the day following the second probe trial, mice were retrained again to find the platform, for 3 additional days. The third probe trial was conducted one month after the final training.

2.5. Fear conditioning test

To evaluate fear-related learning and memory, mice were tested in a two-day protocol (Bolivar et al., 2003; Bolivar et al., 2001), using fear conditioning monitors (San Diego, CA). Each mouse was placed in a test chamber with inside dimensions of 25 cm (L) X 21 cm (W) X 18 cm (H). Mouse activities inside the chamber were monitored by an array of infrared photo beams (12 X 10, spaced 2 cm apart). Electronic shocks were conducted by a stainless steel grid floor, consisting of rods (0.25 cm in diameter) spaced 1.0 cm apart. On day one, baseline activity (as indicated by beam-breaks) and activity after each of three 2-s 0.5 mA foot shocks (2.5 min apart), which co-terminated (i.e., was paired) with a 5-s tone, were recorded in a conditioning chamber. On day two, contextual memory was assessed by measuring activity during a five-minute period in the same environment without shock or tone. At the end of testing the mice were returned to the home cage for 4 hours before completing the cued memory test. The cued memory test was conducted in the same test chamber, but with altered environmental features, e.g., the metal grid floor of the test chamber was covered by a rubber plate to provide a smooth surface, the wallpapers were changed, and orange scent was added. Those changes were designed to create an altered environment for evaluating cued memory. The activity level during the first three minutes in the altered environment was recorded and used as the baseline for measuring cued memory. The activity in the following 3 minutes, during which three 5-s tones were delivered to the test chamber (one minute apart), was used as a measure of cued memory. For the intense training experiment, 5 paired, 2-s shocks over 15 minutes, instead of three paired shocks, were delivered to mice on day 1. Contextual and cued memory were measured as previously described.

2.6. Pain threshold test

The pain threshold test was conducted according to previously published methods (Bourtchuladze et al., 1994). Mice were given a series of 1-s foot shocks, starting at 0.075 mA, and increasing in intensity every 30 s (0.1, 0.25, 0.35, 0.45, 0.55, 0.65, and 0.75 mA) in succeeding shocks. During the test, three typical responses (flinching, jumping, and vocalizing) were observed, and the lowest shock intensity that elicited any of these responses was recorded. The experiment was stopped prior to reaching a shock current of 0.75 mA if the animal vocalized.

2.7. Statistical analysis

Behavioral data were analyzed by either Student's *t* test or two-way ANOVA or three-way ANOVA with repeated measures. *P* value less than 0.05 was set as the level of statistical significance.

3. Results

3.1. Activity in the open field

Mice were evaluated in the open field to measure exploratory activity and anxiety-like behavior. Both males and females were studied, in order to detect potential sex differences. Indices measured included total distance traveled, the percentage of center versus total distance traveled, and time spent in the center of the chamber. Significant differences between WT and brain-*Cpr*-null mice were found in the percentage of center versus total distance traveled ($F_{(1, 37)} = 9.988$, $P < 0.01$) and time spent in the center of the chamber ($F_{(1, 37)} = 4.169$, $P < 0.05$), but not in total distance traveled ($F_{(1, 37)} = 3.585$, $P = 0.066$); differences between sexes for those indices were not statistically significant (three-way ANOVA with repeated measures using sex and genotype as the main between-subjects factors; $F_{(1, 37)} = 0.240$, 0.123, and 0.062 for total distance traveled, the percentage of center versus total distance traveled, and time spent in the center of the chamber, respectively, $P > 0.05$). There was no interaction between sex and genotype for any of the measures (three-way ANOVA with repeated measures; $F_{(1, 37)} = 1.932$, 0.001, and 1.637, respectively, $P > 0.05$). Further statistical analyses stratifying the genotype groups by sex revealed subtle sex-specific changes in the aforementioned indices. There was no difference in total distance traveled or in time spent in the center area, between WT and brain-*Cpr*-null male mice (3- to 5-month old; Fig. 1A, 1B). However, on days 2 and 3, male brain-*Cpr*-null mice exhibited a significantly higher percentage of center to total distance, compared with WT mice ($P < 0.05$, two-tailed *t*-test; between groups, two-way ANOVA with repeated measures $F_{(1, 15)} = 6.417$, $P < 0.05$), indicating that more of the total distance they traveled in the chamber was in the center (Fig. 1C). In female mice (3- to 4-month old), starting from day 1, brain-*Cpr*-null mice showed a trend of increased total distance traveled relative to WT littermates, although this did not reach statistical significance ($P = 0.091$; two-tailed *t* test) until days 2 and 3 ($P < 0.05$, two-tailed *t*-test; between groups, two-way ANOVA with repeated measures, $F_{(1, 22)} = 5.467$, $P < 0.05$) (Fig. 1D). The time spent in the center of the chamber showed the same trend. Female brain-*Cpr*-null mice spent significantly more time in the center area during the test on days 2 and 3 than did WT mice ($P < 0.05$, two-tailed *t*-test; between groups, two-way ANOVA with repeated measures, $F_{(1, 22)} = 6.443$, $P < 0.05$) (Fig. 1E). These data were further supported by the measurement of the percentage of center distance versus total distance traveled (Fig. 1F): on days 2 and 3, female brain-*Cpr*-null mice had significantly higher percentages than did WT mice ($P < 0.05$, two-tailed *t*-test; between groups, two-way ANOVA with repeated measures, $F_{(1, 22)} = 4.971$, $P < 0.05$), which suggested that the activity of brain-*Cpr*-null mice was more focused in the center area of the test chamber. Taken together, these results seem to indicate that brain-*Cpr*-null mice, especially females, are less anxious than WT controls.

3.2. Elevated zero maze

The elevated zero maze assay was used to further evaluate the anxiety-like behaviors observed in the open field. Females are used, given their more robust response in the open field assay, than that of males, to the loss of CPR. We found that female (3- to 4-month old) brain-*Cpr*-null mice ($n = 14$) and WT littermates ($n = 10$) spent equal amounts of time (35.8 ± 4.9 s and 34.4 ± 5.8 s, respectively; means $\pm$ S.E.) in the open quadrants during testing. This finding indicated that there was no difference in the level of anxiety, at least as measured by this assay, between the two groups.

3.3. The fear-conditioning assay

The fear-conditioning assay was used to evaluate associative memory. In this task, mice were exposed to three foot shocks (0.5 mA, 2 s each) paired with tone, 2.5 min apart, over 10 min. As a natural response to the aversive stimulus, mice would reduce their level of activity (freezing response). After training, mice learned to associate the aversive stimulus with the specific environment (contextual memory). Unexpectedly, baseline activity levels, measured by the number of beam-breaks made during the first 2.5 minute prior to foot shocks, were significantly different between WT and brain-*Cpr*-null mice (147.84 ± 4.11 and 134.56 ± 4.41 beam breaks for males ($n = 20\text{--}21$, pooled from both three-shock and five-shock experiments, means $\pm$ S.E.; $P < 0.05$, two-tailed *t*-test), and 170.44 ± 6.21 and 137.06 ± 6.63 beam breaks for females ($n = 20\text{--}21$, pooled from both three-shock and five-shock experiments, means $\pm$ S.E.; $P < 0.05$, two-tailed *t*-test), respectively). Brain-*Cpr*-null mice exhibited lower baseline activity levels than did WT mice. Therefore, the level of activity immediately after shocks and, on the second day, during the contextual memory test, was presented as the percentage relative to pre-shock baseline. Activity level during cued memory testing was presented as the percentage relative to altered activity baseline on day 2. Measuring percentage of activity against baseline, rather than the actual number of beam breaks, essentially eliminated the possible interference from unequal baseline activity levels among individual animals (Huynh et al., 2009). Fear conditioning, e.g. activity suppression relative to baseline after foot shocks, was readily observed in WT mice. Usually, the second shock brought the activity suppression response to a plateau. Both male and female brain-*Cpr*-null groups were significantly more active immediately after the 2nd and 3rd foot shocks, compared with corresponding WT groups ($P < 0.05$, two-tailed *t*-test) (Fig. 2A, 2C). There were also significant differences in fear conditioning between WT and brain-*Cpr*-null mice, as well as between male and female mice (three-way ANOVA with repeated measures using genotype and sex as the main between-subjects factors; for genotype, $F_{(1, 36)} = 17.514$, $P < 0.01$; for sex, $F_{(1, 36)} = 10.021$, $P < 0.01$). There was no interaction between genotype and sex (three-way ANOVA with repeated measures; $F_{(1, 36)} = 0.661$, $P > 0.05$). Further statistical analyses were conducted by subdividing the mice into male and female groups. Significant differences between WT and brain-*Cpr*-null mice were found in both groups (two-way ANOVA with repeated measures; for the male, $F_{(1, 19)} = 8.079$, $P < 0.05$; for the female, $F_{(1, 17)} = 11.888$, $P < 0.01$). In the contextual memory test, significant differences were found between WT and brain-*Cpr*-null mice, and between male and female mice (two-way ANOVA; for genotype, $F_{(1, 36)} = 18.498$, $P < 0.01$; for sex, $F_{(1, 36)} = 17.055$, $P < 0.01$). There was no interaction between genotype and sex (two-way ANOVA; $F_{(1, 36)} = 0.661$, $P > 0.05$). Both male and female brain-*Cpr*-null mice were more active than were WT controls ($P < 0.01$, two-tailed *t*-test), while no significant differences were observed in the cued memory test between those two groups (two-way ANOVA; for genotype, $F_{(1, 36)} = 1.002$, $P > 0.05$; for sex, $F_{(1, 36)} = 0.032$, $P > 0.05$; for interaction between genotype and sex, $F_{(1, 36)} = 0.087$, $P > 0.05$) (Fig. 2B, 2D). These data suggest that a deficit exists in brain-*Cpr*-null mice for fear conditioning and contextual memory.

3.4. Nociception

A potential confounding factor in the fear conditioning performance differences between brain-*Cpr*-null and WT mice is a difference in nociceptive responses to foot shock between the two groups of mice. To rule out that possibility, a pain threshold test was conducted. During the experiment, mice were exposed to a series of foot shocks of increasing intensity. Several different responses to foot shocks, including flinching, jumping, and vocalizing, were measured. In both male and female mice (15–16-month-old and 4–7-month-old, respectively), the brain-*Cpr*-null group did not differ from WT in pain threshold responses (Fig. 3A). In fact, female brain-*Cpr*-null mice showed a trend toward a higher level of sensitivity (Fig. 3B).

3.5. Fear conditioning with an intensified training paradigm

An intensified training paradigm, as previously used to evaluate an Alzheimer's disease mouse model (Dineley et al., 2002), was adopted in an effort to distinguish contextual memory from conditioning deficit in the fear-conditioning assay. Naive, WT and brain-*Cpr*-null mice (3–6-month-old, both males and females, $n = 9$ –11) were given 5 foot shocks over 15 min, instead of the standard paradigm of 3 shocks over 10 min. Consistent with our previous experiment, immediately after the 3rd shock, brain-*Cpr*-null mice were still more active (relative to baseline) than were WT mice ($P < 0.01$, two-tailed t -test in both males and females) (Fig. 4A, 4C). However, more shocks only slightly decreased post-shock activity levels, and significant differences were still observed between WT and brain-*Cpr*-null mice after the fifth shock (Fig. 4A, 4C) ($P < 0.05$ for the male and $P < 0.01$ for the female, two-tailed t -test). Again, there were significant differences in fear conditioning between WT and brain-*Cpr*-null mice as well as between male and female mice (three-way ANOVA with repeated measures; for genotype, $F_{(1, 36)} = 34.340$, $P < 0.01$; for sex, $F_{(1, 36)} = 4.705$, $P < 0.05$). There was no interaction between genotype and sex (three-way ANOVA with repeated measures; $F_{(1, 36)} = 0.078$, $P > 0.05$). During the contextual memory test on the next day, brain-*Cpr*-null mice were again more active than were WT mice ($P < 0.05$ for the male and $P < 0.01$ for the female, two-tailed t -test; two-way ANOVA; for genotype, $F_{(1, 36)} = 13.992$, $P < 0.01$; for sex, $F_{(1, 36)} = 12.242$, $P < 0.01$), but with no difference found in cued memory (two-way ANOVA; for genotype, $F_{(1, 35)} = 0.066$, $P > 0.05$; for sex, $F_{(1, 35)} = 0.242$, $P > 0.05$; one WT male was excluded due to abnormal behavior.) (Fig. 4B, 4D). There was no interaction between genotype and sex in either contextual or cue memory test (two-way ANOVA; $F_{(1, 36)} < 0.0001$ and $F_{(1, 36)} = 0.528$, respectively, $P > 0.05$).

3.6. Morris water maze

Spatial learning and memory were evaluated in the Morris water maze assay, in which a number of parameters, including latency to find the hidden platform during training and time spent in each zone during the probe trials, were measured (see Fig. 5A for a summary of the experimental design). Only male mice were studied. Both brain-*Cpr*-null and WT mice (8- to 12-month old) learned the assay, gradually decreasing the latency to find the hidden platform. There was no difference between WT and brain-*Cpr*-null mice in terms of escape latency (two-way ANOVA with repeated measures; $F_{(1, 98)} = 0.031$, $P = 0.86$) (Fig. 5B). Probe trials were conducted at several time points to evaluate long-term spatial memory. In the probe trial given on the 8th day, WT mice spent significantly more time in the target zone than in other zones (37%, one sample t -test against 25%, $P = 0.012$) (Fig. 5C). The brain-*Cpr*-null mice also spent more time in the target zone; although the zonal time difference was not statistically significant (Fig. 5C) (32%, one sample t -test against 25%, $P = 0.09$), and there was no significant difference when the percentages of time spent in target zone were compared between WT and brain-*Cpr*-null mice (t -test, $P = 0.49$). In the one-week probe trial, mice were first retrained for 3 days to find the hidden platform (Fig. 5A). At the end of the third day, it took both groups ~20 s to find the platform, an indication of good spatial learning (Fig. 5B). A probe trial was conducted one week later to evaluate long-term memory. WT mice again spent significantly more time in the target zone than in the other zones (Fig. 5D) (35%, one sample t -test against 25%, $P = 0.007$). The brain-*Cpr*-null mice did not spend more time (31%) in the target zone than in the other zones (Fig. 5D) (one sample t -test against 25%, $P = 0.15$); however, there was no significant difference when the percentages of time spent in target zone were compared between WT and brain-*Cpr*-null mice (35% vs. 31%) (t -test, $P = 0.49$). Mice were trained again for another three days. Both groups quickly reestablished spatial learning, taking only ~18 s to find the platform by the end of training session (Fig. 5B). One month later, another probe test was conducted. Both groups spent significantly more time in the target zone (Fig. 5E) (36%, t -test against 25%, $P < 0.05$). There was no significant difference in the percentages of time spent in the target

zone between WT and brain-*Cpr*-null mice (*t*-test, $P = 0.98$). Notably, although there appeared to be a significant zonal time difference in zones 1, 2, and 3 in Probe3 (Fig. 5E), the time spent in these zones was not relevant to assessment of spatial memory, given that the animals were trained to locate the hidden platform in zone 4.

4. Discussion

In the current study, we evaluated behavioral performance in male and female brain neuron-specific *Cpr* knockout mice using a battery of assays. We confirmed postnatal deletion of *Cpr* in neurons located in both the hippocampus and basolateral amygdala, which could result in learning and memory deficits. However, loss of CPR in neurons did not result in gross changes in brain morphology (Conroy et al., 2010). Brain-*Cpr*-null mice displayed significantly less fear-related responses both immediately after foot shocks and when returned to the chamber 24 h later (contextual memory). However, the nociceptive responses to foot shocks were similar between brain-*Cpr*-null and WT mice. The deficit in fear conditioning and contextual memory seen in brain-*Cpr*-null mice cannot be rescued by intense training.

The exploratory behavior in the open field assay has been widely used to measure animal locomotor activity, as well as anxiety. Rodents naturally prefer to move along the peripheral area of a novel, anxiety-inducing environment (Pruet and Belzung, 2003; Ragnauth et al., 2001). Increased thigmotaxis (wall-hugging) activity is deemed as an indication of anxiety in animals (Pruet and Belzung, 2003). In our study, both male and female brain-*Cpr*-null mice traveled proportionally more than did age- and gender-matched WT mice in the center area of the chamber on days 2 and 3, an observation suggesting that brain-*Cpr*-null mice are less anxious and fearful, compared with WT littermates. The mechanism underlying the observed sex differences in total distance traveled and time spent in the center area is currently unknown; but the behavioral differences may be somehow related to the known sex differences in the levels of steroid hormones in the brain (e.g., levels of both testosterone and estradiol in the hippocampus are higher in males than in females; Hojo et al., 2009).

In contrast to the results of the open field assays, there were no differences between the two mouse strains in the elevated zero maze assay. This is not surprising in that the open field assay requires daily exposure of mice to the test chamber in a 15-min session for three consecutive days; in contrast, the elevated zero maze assay involves only a single 5-min session. Therefore, the findings are consistent that there was no difference in time spent in elevated zero maze between brain-*Cpr*-null and WT mice and that the apparent decrease in anxiety/fear in brain-*Cpr*-null mice in the open field assay was observed only on days 2 and 3, but not day 1. The low anxiety-like behavior seen in the mutant mice only becomes evident upon repeated exposures to the apparatus.

Notably, in the current study, we only assessed the classical “spatial” parameters, such as duration and number of open/closed arm entries, in the elevated zero maze assay. Discordant results from the open field assay and the elevated zero maze assay have been observed in previous studies (Carola et al., 2002, Carola et al., 2004); these earlier studies also demonstrated that the additional inclusion of ethological parameters (e.g., head-dipping, rearing, and sniffing) and multifactorial statistical analysis might be useful for resolving differences between results from the two assays. Thus, further behavioral testing of the brain-*Cpr*-null mouse, using both spatial parameters and ethological parameters, may be worthwhile in future studies.

The idea that brain-*Cpr*-null mice have impaired fear conditioning and contextual memory is supported by the striking difference between brain-*Cpr*-null and WT mice in the fear-conditioning assay. In the current study, using the percentage of post- versus pre-shock

activity nullified the difference observed in baseline activity among individual mice. Furthermore, while brain-*Cpr*-null mice displayed lower baseline activity compared with WT mice, in the following conditioning test, the opposite trend became evident. The occurrence of an apparent fear conditioning deficit in the brain-*Cpr*-null mice made it complicated to determine the potential impact of the *Cpr* deletion on memory. It is noteworthy that both WT and brain-*Cpr*-null demonstrated the similar level of activity suppression during the cued memory test. If the difference in post-shock activity between WT and brain-*Cpr*-null mice resulted in the higher levels of activity observed during contextual memory test in brain-*Cpr*-null mice, it would have generated similar effects on cued memory, since both memory tests measure associative memory to the same aversive stimulus. In other words, it implies that the deficit of contextual memory in brain-*Cpr*-null mice could be independent of that observed during fear conditioning on day 1. Importantly, the apparent fear conditioning deficit in the null mice was not because of a change in threshold of the shock response; there was no difference between WT and brain-*Cpr*-null mice in nociceptive responses to the shocks, which was in agreement with the previous finding of normal nociceptive responses of the brain-*Cpr*-null mice in hot-plate and tail-flick tests (Conroy et al., 2010). Finally, further studies are needed to assess potential contributions of anxiety to the observed deficit in fear conditioning and memory. As demonstrated in the open field test, there might be an inherent difference in innate anxiety between WT and brain-*Cpr*-null mice, which could lead to the differing levels of learned fear responses observed in those two groups of mice.

In contrast to the deficits in fear conditioning and memory, we found no difference in spatial learning and memory in the water maze between brain-*Cpr*-null and WT mice. Previous research suggests that the process of fear conditioning and memory might involve different pathways than those used to establish spatial learning and memory, with fear-related cognitive function relying more on synaptic activities in the basolateral amygdala (Huynh et al., 2009; LeDoux, 2000). In that connection, neuronal CPR deletion in the amygdala of the brain-*Cpr*-null mouse was confirmed (data not shown). The expression of CPR protein in mouse amygdala had not been described previously. We found strong neuronal expression of CPR protein in both basolateral and central nuclei of amygdala in WT mice. The loss of CPR expression in the basolateral nucleus of the amygdala, as well as hippocampus (Conroy et al., 2010), of the brain-*Cpr*-null mice was consistent with reported Cre expression pattern in the CaMKII α -Cre transgenic mouse brain (Oliveira et al., 2011). However, the mechanisms underlying the differential effects of *Cpr* deletion on fear conditioning and contextual memory, and spatial learning and memory, remain to be determined.

The behavioral phenotype in the brain-*Cpr*-null mouse differs from that of the *Cyp46a1*-null mouse. CYP46A1 is a microsomal P450 enzyme (thus dependent on CPR for activity), and it is critical for cholesterol degradation in the brain (Russell et al., 2009). In *Cyp46a1*-null mice, spatial learning and memory were severely impaired; fear memory was also impaired, although it was not indicated whether fear conditioning was affected (Kotti et al., 2006). CPR and CYP46A1 are both important for maintaining levels of cholesterol in the brain, but the loss of these two enzymes would have differing impacts on the homeostasis of bioactive intermediates in cholesterol biosynthesis pathways. In the *Cyp46a1*-null mice, the loss of CYP46A1-mediated cholesterol degradation was compensated by active suppression of cholesterol synthesis, which led to altered homeostasis of metabolic intermediates that are critical for hippocampal long-term potentiation, and learning and memory (Russell et al., 2009). In the brain-*Cpr*-null mice, *Cpr* deletion would lead to suppression of the functions of two CPR-dependent enzymes in the cholesterol biosynthesis pathway, lanosterol 14 α -demethylase (CYP51) and squalene monooxygenase (Li and Porter, 2007; Weng et al., 2005); the resultant changes in intermediate metabolites, such as 24,25-dihydrolanosterol, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are expected to differ, at least to

some extent, from those occurring in the *Cyp46a1*-null mice. Further studies to clarify the impact of CPR loss on the cholesterol synthesis pathway in the brain are needed in order to understand the behavioral consequences.

The loss of HO activity might also have contributed to the observed fear conditioning/memory deficits in the brain-*Cpr*-null model. It had long been proposed that gaseous neurotransmitters such as NO and CO serve specific functions in the brain (Verma et al., 1993). CO can bind to heme domain of soluble guanylyl cyclase and regulate the production of cGMP in neurons, which further regulates formation of long-term potentiation (LTP) (Zhuo et al., 1999). CO in brain is generated mainly through the enzymatic action of heme oxygenase 2 (HO2), an enzyme requiring CPR for its normal function. Previous characterization of brain-*Cpr*-null mice confirmed the critical role of CPR to heme oxygenases, since HO enzymatic activities were greatly reduced in hippocampus and cortex of brain-*Cpr*-null mice (Conroy et al., 2010). Characterization of HO2-knockout mice did not reveal any major deficit in behavioral performance, while loss of HO2 did cause the impairment in the peripheral nervous system (Burnett et al., 1998; Poss et al., 1995). It is intriguing that HO2 knockout mice did show a tendency of less fear of falling in a test for forelimb strength, and increased activity in the open field assay (Burnett et al., 1998).

Finally, apart from CYP46, CYP51, and SQLE, other microsomal P450 enzymes in brain might also play a role in fear conditioning and memory. The loss of CPR will affect key enzymes in androgen and estrogen synthesis and metabolism (such as CYP17A1 and CYP19A1); thus, further studies are warranted to determine whether brain levels of androgens and estrogens, which may contribute directly to sex-differences in anxiety or fear conditioning (Pinna et al., 2008), are altered in the brain-*Cpr*-null mice. A recent study, using a microarray methodology to explore gene changes in the hippocampus during fear conditioning, revealed that *Cyp3a11* was one of 1539 “*learning-regulated genes*” (Peleg et al., 2010). CYP3A11 expression in mouse hippocampus was reported previously (Hagemeyer et al., 2003). Our preliminary study confirmed the expression of CYP3As in mouse cortex and hippocampus. CYP3A enzymes can metabolize cholesterol as well as sex steroids (Li et al., 1995; Pikuleva, 2006); the loss of activity of this and other microsomal P450 enzymes (such as CYP2B and CYP2C) capable of metabolizing neurosteroids or eicosanoids in the *Cpr*-null brain might have contributed to the observed fear conditioning and memory phenotype.

In summary, through behavioral studies on a mouse model with brain neuron-specific deletion of the *Cpr* gene, we discovered a novel function of CPR in fear conditioning and memory. The behavioral deficit occurred in the absence of any notable changes in brain morphology (Conroy et al., 2010, and data not shown), and was not accompanied by changes in threshold of nociceptive responses or deficits in spatial learning or memory. Further studies are warranted to elucidate the details of the underlying mechanisms.

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Abbreviations

P450	cytochrome P450
CPR	NADPH-cytochrome P450 reductase
PBS	phosphate-buffered saline
WT	wild-type
brain-<i>Cpr</i>-null	brain specific <i>Cpr</i> -knockout

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- Brain P450 reductase (CPR) is important in fear conditioning and memory
- Brain-*Cpr*-null mice are more active than are wild-type in exploratory behavior assay
- Brain-*Cpr*-null mice show deficit in activity suppression in fear-conditioning assay
- Brain-*Cpr*-null mice have no change in pain threshold or spatial learning or memory

Fig. 1. Exploratory behavior in brain-*Cpr*-null mice

Both male (A–C; 3- to 5-month old) and female (D–F; 3- to 4-month old) mice were examined. A. Male WT ($n = 6$) and brain-*Cpr*-null ($n = 11$) mice traveled similar distances in the open field across the three days of testing; B. Male WT and brain-*Cpr*-null mice spent similar lengths of time in the center of the chamber; C. The percentages of center to total distance were significantly higher in male brain-*Cpr*-null mice, compared with WT littermates, on days 2 and 3; D. Female brain-*Cpr*-null mice ($n = 14$) traveled significant longer distances on days 2 and 3 than did WT littermates ($n = 10$); E. Female brain-*Cpr*-null mice also spent more time in center of the chamber on days 2 and 3; F. The percentages of center to total distance were significantly higher in female brain-*Cpr*-null mice, compared with WT littermates on days 2 and 3. Values represent means $\pm$ S.E. *, $P < 0.05$; **, $P < 0.01$.

Fig. 2. Impaired fear conditioning and memory in brain-*Cpr*-null mice

Male WT ($n = 12$, 3–6 months) and brain-*Cpr*-null ($n = 9$, 3–6 months) mice (A and B) and female WT ($n = 9$, 3–6 months) and brain-*Cpr*-null ($n = 10$, 3–6 month) mice (C and D) were subjected to three tone-foot shock pairings over a 10-min session. Activity levels immediately after shocks were recorded and expressed as percentage of baseline activities for WT and brain-*Cpr*-null mice, respectively. The male (C) and female (D) mice were returned to the test chamber 24 h later to measure activity levels without foot shock for contextual memory and without foot shock but with three presentations of tone for cued memory, as described in the Methods. Values represent means $\pm$ S.E. *, $P < 0.05$; **, $P < 0.01$.

Fig. 3. Normal nociceptive responses in brain-*Cpr*-null mice

WT and brain-*Cpr*-null mice were subjected to a series of foot shocks of increasing intensities. The various responses, including flinching, jumping, and vocalizing, were measured. A. Male (15–16-month-old) WT ($n = 6$) and brain-*Cpr*-null mice ($n = 5$) did not show a significant difference in the threshold of nociceptive response (the intensity of foot shocks to which they first responded to); B. Female (4–7-month-old) WT ($n = 5$) and brain-*Cpr*-null mice ($n = 6$) did not show a significant difference in the threshold of nociceptive response to foot shocks. Values represent means $\pm$ S.D.

Fig. 4. Intense training failed to rescue deficits of fear conditioning and contextual memory in brain-*Cpr*-null mice

Male WT ($n = 9$, 3–6 months) and brain-*Cpr*-null ($n = 10$, 3–6 months) mice (A and B) and female WT ($n = 10$, 3–6 months) and brain-*Cpr*-null ($n = 11$, 3–6 month) mice (C and D) were subjected to five tone-paired, 2-second, foot shocks (0.5 mA) over a 15-min session. Activity immediately after the shocks was recorded and expressed as percentage of baseline activities. Brain-*Cpr*-null mice persistently displayed significantly more after-shock activity compared with WT mice, starting from the second shock. Intensive training did not effectively mitigate the deficit of fear conditioning observed in brain-*Cpr*-null mice. Brain-*Cpr*-null mice were also significantly more active than WT during the contextual memory test, while no significant difference in cued memory was found between those two groups, in both the males (C) and females (D). Values represent means $\pm$ S.E. *, $P < 0.05$; **, $P < 0.01$.

Fig. 5. Normal spatial learning and spatial memory in brain-*Cpr*-null mice

A. Diagram of experimental design. B. Escape latency of WT and brain-*Cpr*-null mice during training. Adult (8–12-month-old; age-matched) male WT ($n = 13$) and brain-*Cpr*-null ($n = 12$) mice were trained to navigate to a hidden platform submerged below the water surface. There was no difference in escape latency between brain-*Cpr*-null and WT mice during any of the training sessions. Spatial memory was measured in three probe tests. Brain-*Cpr*-null and WT mice spent equal amount of time in the target zone during one-day (C), one-week (D), and one-month (E) probe tests. Values represent means $\pm$ S.E.