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GPR26-DEFICIENT MICE DISPLAY INCREASED ANXIETY- AND DEPRESSION-LIKE BEHAVIORS ACCOMPANIED BY REDUCED PHOSPHORYLATED CYCLIC AMP RESPONSIVE ELEMENT-BINDING PROTEIN LEVEL IN CENTRAL AMYGDALA

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Abstract—Anxiety disorders are among the most common and well studied psychiatric disorders in humans. A number of animal models have been established to study the mechanisms of anxiety and to test putative anxiolytic drugs. *Gpr26* belongs to the G-protein-coupled receptor family and is exclusively expressed in brain tissue. To investigate the biological function of *Gpr26 in vivo*, we have generated *Gpr26* knockout mice. The mutant mice grew and developed normally but displayed increased levels of anxiety-like behaviors in the open field and elevated plus maze tests, as well as a higher level of depression-like behaviors in the forced-swim and tail-suspension tests. Meanwhile, no significant alteration in spatial learning and memory abilities were found for *Gpr26*-deficient mice in the Morris water maze test. Previous studies demonstrated that lower protein kinase A (PKA)–cAMP responsive element-binding protein (CREB)–neuropeptide Y (NPY) signaling in the amygdala is linked to higher anxiety and excessive alcohol-drinking behaviors in rats. Therefore, we further examined the phosphorylated CREB (pCREB) and CREB levels in the brains of *Gpr26*-deficient mice. Reduced pCREB levels were observed in the central amygdala but not in the other regions, while total CREB levels remained comparable between wild-type and mutant mice. Combined, our data indicate that Gpr26 is important for emotion regulation in mice, a function probably mediated by the phosphorylation of CREB in the central amygdala.

Key words: *Gpr26* knockout mice, anxiety, depression, CREB, pCREB.

Anxiety disorders are the most common of all mental health problems that affect human beings. Recent surveys have found that as many as 18% of Americans may be affected by a variety of anxiety disorders (Kessler et al., 2005). Genetic factors are important in the predisposition to anxiety behaviors. Animal models with certain gene deficiencies have been developed to facilitate the discovery of genetic and neurobiological substrates of anxiety and to test putative anxiolytic drugs (Ramboz et al., 1998; Marsch et al., 2007; van den Buuse et al., 2007; George et al., 2008; Zanettini et al., 2010).

The transcription factor cAMP responsive element-binding protein (CREB) is one of the most studied genes relevant to anxiety and represents an important linker between a number of neurotransmitters and downstream gene expression (Silva et al., 1998; Nestler, 2001; Lonze and Ginty, 2002). CREB is regulated via phosphorylation at serine 133 by cAMP-dependent protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinases, and mitogen-activated protein kinases (Okuno and Fujisawa, 1996; Silva et al., 1998; Impey et al., 1999). Phosphorylated CREB (pCREB) regulates downstream cAMP-inducible gene expression of such molecules as neuropeptide Y (NPY) and brain-derived neurotrophic factor (BDNF) (Shieh et al., 1998; Mayr and Montminy, 2001; Lonze and Ginty, 2002; McClung and Nestler, 2003). It was found that CREB-haplodeficient mice, which have significantly decreased total CREB/pCREB levels and also downstream NPY and BDNF levels, displayed more anxiety-like behaviors and a higher preference for ethanol than did wild-type littermates (Pandey et al., 2004). It was also found that the levels of PKA, total CREB, pCREB, and NPY were innately lower in the central amygdala (CeA) and medial amygdala (MeA) of alcohol-preferring rats (Pandey et al., 2005b). Therefore, lower PKA-CREB-NPY signaling in the amygdala may be linked to higher anxiety and excessive alcohol-drinking behaviors.

G-protein-coupled receptors (GPCRs) comprise the largest family of membrane proteins, and they mediate most of our physiological responses to hormones, neurotransmitters, and environmental stimulants. Thus, they have great potential as therapeutic targets for a broad

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Abbreviations: ANCOVA, analysis of covariance; ANOVA, analysis of variance; BLA, basolateral amygdala; cAMP, cyclic AMP; CeA, central amygdala; CREB, cAMP responsive element-binding protein; EPM, elevated plus maze; ES cell, embryonic stem cell; FST, forced-swim test; GPCR, G-protein-coupled receptor; HSV, herpes simplex virus-1; KO, knockout; MeA, medial amygdala; MWM, Morris water maze; Neo, neomycin resistance cassette; NPY, neuropeptide Y; OPT, open field test; PCR, polymerase chain reaction; pCREB, phosphorylated CREB; PGK, phosphoglycerate kinase; SD, standard deviation; SEM, standard error of the mean; TK, thymidine kinase cassette; TST, tail-suspension test; WT, wild type.

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spectrum of diseases (Milligan, 1998; Fredriksson et al., 2003; Rosenbaum et al., 2009). GPCRs that are extensively expressed in human brain play an important role in the pathophysiology and pharmacotherapy of neuropsychiatric disorders (including schizophrenia, anxiety, depression, addiction, and eating disorders) and neurodegenerative disorders (Alzheimer's and Parkinson's disease) (Bockaert et al., 2010). The classical role of GPCRs is to couple the binding of agonists to the activation of specific heterotrimeric G proteins, leading to the modulation of downstream effecter proteins (Milligan, 1998; Rosenbaum et al., 2009). G proteins are classified as Gs, Gi, or Gp, each with distinct functions. Activation of Gs-coupled receptor would increase the cellular cAMP level, and an important consequence of this process is the activation and translocation of PKA. Activated PKA in turn regulates many cellular functions including the phosphorylation of CREB, and increases downstream cAMP-inducible gene expression (Asher et al., 2002; Yao et al., 2002).

Gpr26, first described in 2000 (Lee et al., 2000), belongs to the GPCR family. *Gpr26* is an orphan GPCR without known endogenous ligands (Chung et al., 2008). The amino acid sequence of Gpr26 is highly conserved among species. For example, there is 95% sequence identity between human and mouse orthologs, indicating strong phylogenetic conservation of the protein structure and associated functional properties. Previous work revealed that *Gpr26* mRNA is highly expressed in the mouse central nervous system. *In situ* hybridization shows that *Gpr26* is expressed in many regions of the mouse brain, with high levels in olfactory area, hippocampus, amygdala, and cortex (Jones et al., 2007). Our data also indicate that *Gpr26* is exclusively expressed in adult mouse brain tissue. Other studies have shown that *Gpr26* is coupled to Gs, which means that the activation of *Gpr26* would lead to an increase in cAMP levels in target cells (Lee et al., 2000; Jones et al., 2007).

In the present study, we explore the role of Gpr26 in emotion regulation by generating a *Gpr26* knockout (KO) mouse model. We found that Gpr26 plays an important role in regulating anxiety- and depression-like behaviors, and that these effects may be mediated through the phosphorylation of CREB, indicating that Gpr26 may be a potential target for anxiolytic or antidepressant drugs.

EXPERIMENTAL PROCEDURES

Gpr26 KO mouse generation and maintenance

A targeting vector was constructed by replacing the mouse *Gpr26* genomic 3250 bp fragment covering exon 1, with the 1904 bp phosphoglycerate kinase–neomycin resistance cassette (PGK–Neo cassette) for positive selection, and was laid with an external herpes simplex virus-1–thymidine kinase cassette (HSV–TK cassette) for negative selection (Fig. 2A). The targeting vector contained 3.8 kb homologous DNA upstream to the PGK–Neo cassette and 5.0 kb downstream as homologous recombination arms. The embryonic stem (ES) cells harboring the homologous recombination were determined by polymerase chain reaction (PCR) (Fig. 2B) using two pairs of primers with direction and position depicted in Fig. 2A with triangles. The primers for 5′ arm recombination are (a), 5′-GGCCTCTTCAGTCTGGTGTTC-3′ and (b), 5′-GGCCTACCCGCTTCCATTGCTC-3′. 3′ arm primers are (c), 5′-CCGTGCCTTCCTTGACCCTGG-3′ and (d), 5′-CAGGAATGGACCGTAAGAGCAC-3′. The correctly recombined ES cells (clone 7) were subsequently microinjected into blastocysts and implanted into pseudopregnant female recipients to generate chimeric mice. The F1 mice with germ line transmission of the *Gpr26* KO allele were heterozygotes. These heterozygotes were then intercrossed to generate homozygous, heterozygotes, and wild-type mice for further experimentation. Genotypes were determined with PCR using mouse tail DNA (Fig. 2C).

The mutant mice were maintained on a mixed 129Sv/C57BL/6 background. Mice were generated and group housed (three to five per cage) under specific pathogen-free (SPF) conditions at a constant room temperature of 22–24 °C with a 12-h light/dark cycle (lights on: 8 AM, off: 8 PM) and provided *ad libitum* access to food and water. Mice used in this study were housed in a facility with animal care and use programs accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and animal protocols and experiments were approved by the Animal Use and Care Committee of Shanghai Jiao Tong University School of Medicine.

RT- and real-time PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's instructions. The first-strand cDNAs were synthesized from 400 ng of total RNA with oligo (dT) primer and random 6 mer primer using PrimerScript™ RTase (Takara, Dalian, China) at 37 °C for 15 min. One microliter of the reverse transcription reaction products was used as PCR template. *Gpr26* expression was detected with semi-quantitative RT-PCR using primers 5′-TCTCCTTCATCGTGCTCTG-3′ and 5′-CTCCTGCGGTATTGGTGT-3′. Real-time PCR was carried out using SYBR® Premix Ex Taq™ (Takara) in 96-well optical reaction plates on an Eppendorf Mastercycler® system according to the manufacturer's protocol. The cycle threshold (C_T) values of *Gpr26* primers were compared with those of *β-actin*-specific primers using the comparative C_T method. The primers used in real-time PCR are listed as follows: *Gpr26* sense, 5′-CTTCCTGCTCTCCTTCATCG-3′, anti-sense, 5′-CAGACACCGTTCCCTCAC-3′.

Histology and immunohistochemistry

Mice were anesthetized with ketamine/xylazine and perfused with ice cold saline followed by 4% ice cold paraformaldehyde (PFA) in PBS. Brains were postfixed overnight in 4% PFA at 4 °C, then cryoprotected in 30% sucrose, and stored at 4 °C. For Nissl-staining, sections were stained with 0.1% Cresyl Violet/0.5% acetic acid. For immunohistochemistry, the sections were blocked for 1 h in PBS containing 5% normal goat serum (v/v) at room temperature, and then incubated with antibodies against GPR26 (ab12558, Abcam Biotechnology, Cambridge, UK, 1: 200 dilution), pCREB (#9198, Cell Signaling Technology, Beverly, MA, 1:200 dilution), or CREB (#9197, Cell Signaling, 1:500 dilution) in blocking buffer overnight at 4 °C. After washing, the sections were incubated with biotin-labeled goat-anti-rabbit antibody, and signal was developed using an ABC kit (VECTOR Laboratories, Burlingame, CA). For quantitative analysis, fields measuring 200× magnification within brain sections were digitally captured, and the mean optical density of certain amygdala regions was automatically counted using Image Pro Plus software.

Behavioral testing

Age- (10- to 12-week-old) and body weight-matched (body weight range: 28–33 g) male littermates with different genotypes were used for phenotypic analyses, and the same numbers of KO, heterozygous and wild-type (WT) mice were randomly chosen for

subsequent behavioral tests. The behavioral tests were conducted in an isolated behavioral testing room in the animal facility to avoid external distractions. Investigators observed animal behaviors through a video monitor in another room. The apparatus and analysis software used in behavior tests were supplied by Shanghai Mobile Datum Information Technology Co., Ltd. In order to facilitate adaptation to the experimental environment, mice were housed in the testing room for at least 1 h before the experiments. The animals were naive to the test situation and were used only once. All behavioral tests were conducted during the light phase of their activity cycle (between 1 and 5 PM) except for the Morris water maze, which was conducted between 10 AM and 5 PM, and all behavioral tests were carried out under bright environmental light. Between experiments, the apparatus was cleaned with 75% ethanol and dried using clean paper.

Open field test (OFT)

Mice were tested for 10 min in an open field ($24\times24\times38$ cm^3) equipped with a video camera. A square region of 12×12 cm^2 in the center of the chamber was defined as the "central zone." Horizontal locomotion (total distance moved), average movement speed, and time spent in the central zone were recorded by a video/computer system and analyzed by custom-written computer software.

Elevated plus maze (EPM)

The elevated plus maze is constructed with white chipboards. The arms are 6-cm wide and 36-cm long, and two opposing arms have 30-cm-high walls (closed arm), while the other arms are left open (open arm). The maze was elevated 50 cm off the floor. The animals were placed onto the central compartment facing the open arm. During a 5-min exposure, the animal behavior was recorded by a video/computer system, and custom-written computer software was used to calculate the amount of time that the mice spent on each arm.

Forced-swim test (FST)

The mice were put into a container of 45 cm in height and 19 cm in diameter, filled with water (23 ± 1 °C) to a depth of 22 cm. The animals were left to swim in the water for 6 min and then dried and put back in their cages. The experiment was recorded on a videotape for later manual analysis of immobility time. A mouse was judged to be immobile when it floated in the water and made only small movements to keep its head above water. Since little immobility was observed during the first 2 min, the duration of immobility was recorded during the last 4 min of the 6-min test.

Tail-suspension test (TST)

Each mouse was suspended individually by its tail on a metal rod. The rod was fixed 50 cm above the surface of a table covered with soft cloth in a sound-proof room. The tip of the mouse tail was fixed on the rod using adhesive scotch tape. The duration of the test was 5 min. The immobility time of the tail-suspended mice was measured as previously described (Bilkei-Gorzo et al., 2002).

Morris water maze (MWM)

The Morris water maze consists of a circular black swim tank (120 cm in diameter and 45 cm in depth) filled with water (23 ± 1 °C) to a depth of 27 cm, and the water was made opaque by the addition of non-toxic white powder paint. A circular escape platform (8 cm in diameter) was placed 1 cm below the water surface. A full experiment consisted of a learning period (platform in place) with four trials per day for 7 consecutive days and a test period (platform moved) on the 8th day. In the learning period, the platform was always located in the center of the same quadrant (target quadrant) for all animals. Each trial consisted of a maximum of 90 s starting from one of the four quadrants with the animal facing the wall. If an animal did not reach the platform after 60 s, it was guided to the platform. After reaching the platform, animals were allowed to remain there for 30 s, and then mice were quickly dried with a towel and put under a heating lamp set at exactly 37 °C between each trial to avoid hypothermia. In the learning period, the latencies, path lengths, and swims speeds for a single day were averaged to produce a daily mean. At day 8, the platform was removed, and mice were allowed to swim for 90 s. The swimming trials for each mouse were recorded with a video camera, and data were analyzed using custom-written software. The time spent in the target quadrant and the number of times crossing the original platform position were compared between mice of different genotypes.

Ethanol preference procedure

Ethanol preference was measured by the two-bottle free-choice paradigm used by previous investigators (Thiele et al., 2000; Misra and Pandey, 2003; Pandey et al., 2004, 2005a). Mice had *ad libitum* access to food and to water from two bottles and were habituated to drink water from either bottle. Bottle positions were changed daily so that the mice would not develop a position habit. Once they started drinking water equally from either bottle, mice were provided with a 7% ethanol solution in one bottle and water in the other bottle for 3 days. The concentration of ethanol was then increased to 9% for 3 days and to 12% for another 3 days. Consumption of ethanol and water was measured daily at 6 PM, and fresh water and ethanol (7, 9, 12%) solutions were provided every day. The mean percentage of ethanol intake was calculated from the total fluid intake for 3 days for 7, 9, and 12% ethanol solutions.

Western blot analysis

Nuclear protein extracts from different brain regions were separately prepared according to the handbook of NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL). The protein content of the nuclear extracts was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Extracts were subjected to electrophoresis on a 12% SDS polyacrylamide gel. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes at 23 V for 1 h. The membranes were blocked and then incubated overnight with the primary antibody [anti-pCREB (#9198, Cell Signaling), anti-CREB (#9197, Cell Signaling), or anti-Lamin B (3807-100, BioVision, Mountain View, CA)]. Alexa Fluor 800 goat-anti-rabbit IgG (LI-COR) was used as the secondary antibody. Infrared fluorescence on membranes was detected using the Odyssey infrared imaging system (LI-COR Biotechnology, Nebraska, USA). For quantitative determinations, a densitometric analysis of signal bands was performed using Gel-Pro Analyzer software.

Statistical analysis

To determine deviations from the expected genotype frequencies and sex ratio in the offspring, goodness-of-fit χ^2 tests were performed. Quantitative results are represented graphically as mean$\pm$standard error (SEM). Outlier values that fell above or below the mean$\pm3\times$standard deviation (SD) were excluded. For OFT, EPM, FST, and the test period of MWM, the statistical differences in the observed data were compared by one-way ANOVA (analysis of variance) followed by Tukey's test for post hoc comparisons among groups. For TST, the data were analyzed using one-way ANCOVA (analysis of covariance) with body weight as a covariant, followed by Tukey's test. For the alcohol intake test, the data were analyzed by fitting a linear mixed model

as follows. For the *j*-th observation of alcohol intake percentage of the *i*-th mouse:

$$intake_{ij} = \beta_0 + \beta_1 c1_{ij} + \beta_2 c2_{ij} + \beta_3 g_{ij} + \beta_4 c1_{ij} g_{ij} + \beta_5 c2_{ij} g_{ij} + \mu_i + \varepsilon_{ij},$$

where β_0 is the intercept; β_1, β_2, β_3, β_4, and β_5 are the fixed effects of alcohol concentration *c*1 and *c*2 (two dummy variables for the concentration of 9% and 12% relative to 7%), genotype *g*, and the interactions between *c*1/*c*2 and *g*, respectively; μ_i denotes the random effect of the *i*-th mouse; ε_{ij} is the error term. For the training period of MWM, the data were analyzed using the same method as above, except that two dummy variables were used for the genotype factor with three levels (WT, KO heterozygotes, and KO), and the training day was considered as one ordinal variable. For all tests above, $P<0.05$ was considered statistically significant. All statistical analyses were performed with the R statistical software (http://www.r-project.org/).

RESULTS

Gpr26 is specifically expressed in mouse brain

Semi-quantitative RT-PCR and quantitative real-time PCR were used to determine the tissue distribution of *Gpr26* mRNA. *Gpr26* mRNA was highly and exclusively detected in brain (Fig. 1). The distribution of Gpr26 protein in the mouse brain was examined using immunohistochemistry (Fig. 2E). The Gpr26 protein was detectable in most of the regions of adult mouse brain, including the amygdala regions (BLA, CeA, and MeA), and the highest expression was seen in cortex and hippocampus (both CA2 and CA3 regions). In the cerebellum, *Gpr26* expression was noted in the Purkinje cells (as indicated in Fig. 2E), which are the only known neurons that send output from the cerebellar cortex and have been implicated in motor coordination, learning, and cognitive functions (Ito, 2002).

Gpr26 KO mice are born alive and appear grossly normal

Gpr26 KO mice were generated using a standard homologous recombination strategy as described in "*Gpr26* KO mouse generation and maintenance." PCR was used to genotype mouse tail DNA samples (Fig. 2C). The genotype of the *Gpr26* KO mice was confirmed by absence of detectable *Gpr26* transcripts in brain by RT-PCR (Fig. 2D), and there was also no detectable expression of Gpr26 protein in the mutant mouse brain sections as determined by immunohistochemistry using anti-GPR26 antibody (Fig. 2E). However, the *Gpr26* mRNA levels of heterozygous mice fluctuated between those of wild-type and KO mice (data not shown). After the ablation of *Gpr26*, we examined the basic phenotypes of *Gpr26* KO mice, and found no abnormalities in genotype distribution, sex ratio, and body weights as compared to their wild-type littermates. Further, no significant impairment in reproduction capability was observed (data not shown). Histologically, Nissl-stained brain sections of mutant mice showed no evidence of gross changes in brain organization or connectivity when compared to those of wild-type mice (Fig. 3).

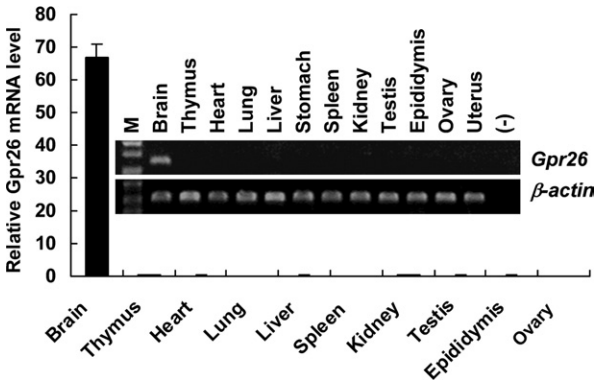

Fig. 1. *Gpr26* was exclusively expressed in mouse brain. Expression levels of *Gpr26* mRNA were examined in major tissues of normal mice using semi-quantitative RT-PCR (upper) and quantitative real-time PCR (lower), the expression level of *β-actin* was used as endogenous control. The results are from two independent experiments while each group was analyzed in triplicate. M, marker lane; (−), negative control without template.

Gpr26 KO mice show anxiety-like behaviors

To identify the potential function of *Gpr26 in vivo*, we carried out a series of behavioral tests. We first examined the differences in behaviors in the open field test, which is widely used in laboratories to quantify anxiety-like and locomotion behaviors in mice. Mice prefer to move around the periphery of an apparatus when they are placed in an open field of a novel environment. The time spent in the central area of the open field is considered to be inversely correlated to their level of anxiety-related proneness. We found that all three genotypes of mice displayed similar locomotion activity when placed in the novel environment of the open field, as all mice showed similar total horizontal movement distance and average movement speed ($P>0.05$) during the test (Fig. 4A, B). However, we found a significant genotypic effect on the time spent in the central zone of the novel open field ($F(2, 35)=6.84$, $P=0.003$, Fig. 4C). Post hoc comparisons showed that both KO mice and heterozygotes spent less time in the central zone of the open field as compared to WT controls ($P=0.040$ and $P=0.003$, respectively), suggesting an enhanced level of anxiety due to Gpr26 deficiency. The difference between KO mice and heterozygotes was not statistically significant. To further confirm this observation, we performed an EPM test, a rodent model for anxiety evaluation which is widely used as a screening test for putative anxiolytic compounds, as well as a general research tool in neurobiological anxiety research. Reduced activities of mice in the open arms in the EPM test are thought to be associated with high levels of anxiety. The results revealed a significant genotypic effect on open-arm distance ($F(2, 27)=4.79$, $P=0.017$, Fig. 5B), and a marginally significant genotypic effect on open-arm time ($F(2, 29)=3.16$, $P=0.057$, Fig. 5A). Post hoc comparisons showed that KO mice exhibited reduced open-arm exploration in terms of both open-arm distance and open-arm time as compared to their WT controls ($P=0.015$ and $P=0.046$, respectively). Compared with the results of the open field test, the heterozygous mice showed an

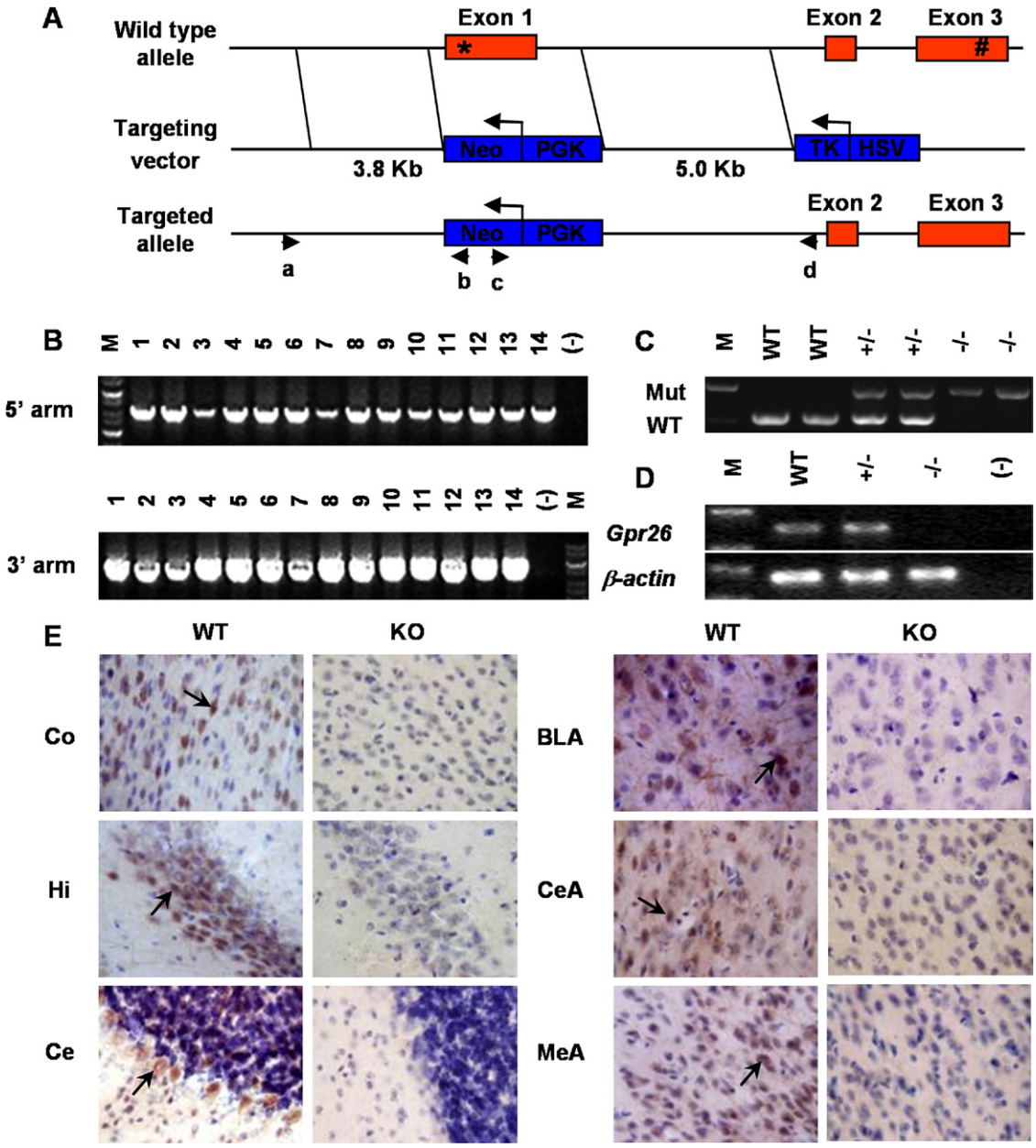

Fig. 2. Generation of *Gpr26* KO mice. (A) Graphic representation of the *Gpr26* targeting strategy. Exons 1–3 are shown in boxes. Positions of the start codon and the stop codon are indicated by star and pound sign, respectively. The targeting vector was designed to delete exon 1 encoding the ATG site. The targeting vector contained a 3.8-kb 5′ arm and a 5.0-kb 3′ arm. PGK–Neo and HSV-TK cassettes were used for positive and negative selections, respectively. The genomic positions of the PCR primers for genotyping are indicated by triangles (5′ arm: a, b; 3′ arm: c, d). (B) Genomic DNA from ES cell clones was isolated and analyzed by PCR with primers shown in (A). The successful targeted ES cell DNA can be amplified into 6.5-kb and 4.6-kb products for the 5′ arm and 3′ arm, respectively. (C) The PCR analysis for the genotyping of $Gpr26^{+/+}$, $Gpr26^{+/-}$, and $Gpr26^{-/-}$ mice. The sizes of amplification products of WT and KO alleles are 526 bp and 738 bp, respectively. (D) RT-PCR was performed using total RNA isolated from $Gpr26^{+/+}$, $Gpr26^{+/-}$, and $Gpr26^{-/-}$ mouse brain with primers specific for *Gpr26*. Transcript for *β-actin* was examined as a control for RNA loading and integrity. (E) Expression pattern of Gpr26 protein in WT and *Gpr26* KO adult mouse brain revealed by immunohistochemistry (original magnification ×1000). Gpr26 positive signals are indicated by black arrows. Co, cortex; Hi, hippocampus; Ce, cerebellum; CeA, central amygdale; BLA, basolateral amygdale; MeA, medial amygdale; M, marker lane; (−), negative control without template; Mut, mutant allele.

intermediate anxiety level between the other two genotypes, although the differences were not statistically significant (all $P>0.05$). In addition, consistent with the results from the open field test, all mice exhibited similar total travel distance ($P>0.05$, Fig. 5C).

Gpr26 KO mice display a higher preference for ethanol

We used male *Gpr26* KO mice and their matched WT littermates to measure alcohol-drinking behaviors in the

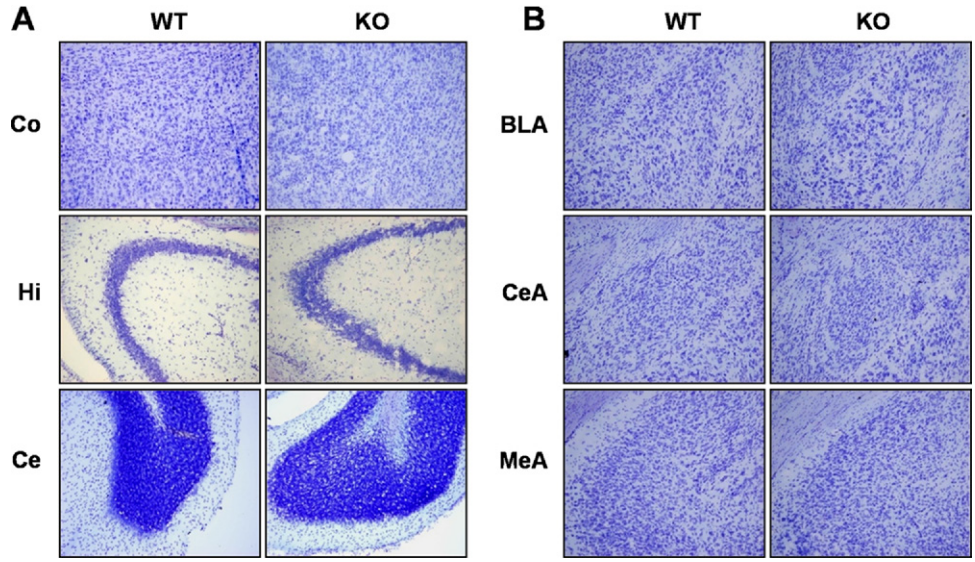

Fig. 3. (A, B) Nissl-stained sections of different portions of mouse brain revealed no significant morphologic differences between WT and *Gpr26* KO mice (original magnification ×200). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

two-bottle free-choice paradigm. According to the linear mixed model described in "Statistical analysis," it was found that KO mice consumed significantly higher overall amounts of alcohol solution as compared to their WT littermates (genotypic main effect, $t(13)=9.054$, $P=0.010$, Fig. 6). In addition, we also observed a reduced genotypic effect for an alcohol concentration of 9% as compared to 7% (interaction effect between genotype KO/WT and concentration 7%/9%, $t(26)=-2.060$, $P=0.049$), as well as 12% compared to 7% ($t(26)=-2.061$, $P=0.049$). Other terms in the model were not significant. Stratified by different alcohol concentrations, we found that KO mice consumed a higher amount of 7% alcohol solution than did WT mice (*t*-test $P=0.024$), but this was not true for the alcohol solutions of 9% or 12% ($P>0.05$). These results indicate that *Gpr26* KO mice have a higher preference for ethanol than their WT littermates, particularly with regard to a low ethanol concentration.

Gpr26 KO mice display higher depression-like behaviors

To evaluate the tendency toward depression-like behaviors in mice of different genotypes, the forced-swim and tail-suspension tests were employed in this study. Both experimental schemes are based on the observation that rodents, when forced into an aversive situation from which they cannot escape, will cease attempts to escape and become immobile. In the forced-swim test, there was a significant genotypic effect on the immobility time ($F(2, 35)=3.85$, $P=0.031$, Fig. 7A). However, post hoc comparisons did not reveal significant differences among the genotypes (all $P>0.05$), although we observed a marginally significant difference between WT and KO mice ($P=0.051$). In the tail-suspension test, we found a highly significant genotypic effect on the immobility time ($F(2, 32)=10.17$, $P=3.8\times10^{-4}$, Fig. 7B). Post hoc comparisons

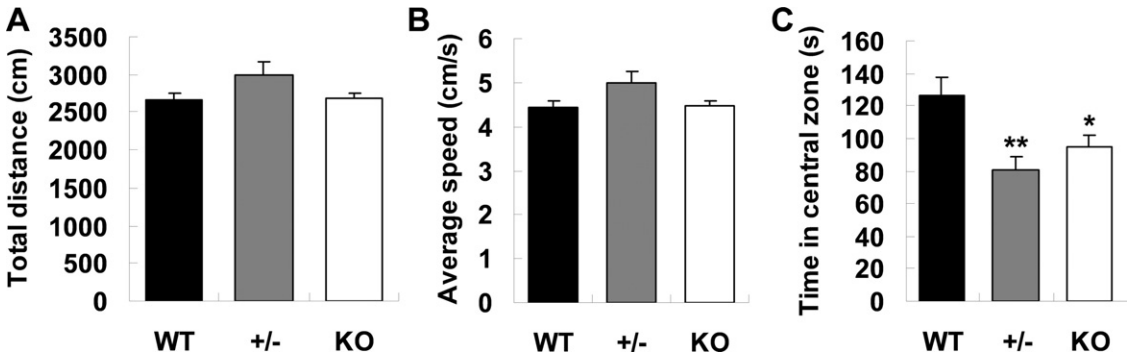

Fig. 4. *Gpr26* KO mice displayed an increased level of anxiety behaviors in the open field test. Sample sizes: WT, $n=13$; $Gpr26^{+/-}$, $n=12$; $Gpr26^{-/-}$, $n=13$. (A) Mice of all three genotypes showed a similar total travel distance in the open field test ($F(2, 35)=2.63$, $P=0.086$). (B) No difference in average speed was observed between genotypes ($F(2, 35)=2.63$, $P=0.086$). (C) $Gpr26^{+/-}$ and $Gpr26^{-/-}$ mice spent less time in the center zone as compared to WT controls (post hoc test, $P=0.040$ and $P=0.003$, respectively), which suggests an enhanced level of anxiety. * $P<0.05$, ** $P<0.01$ vs. wild-type group.

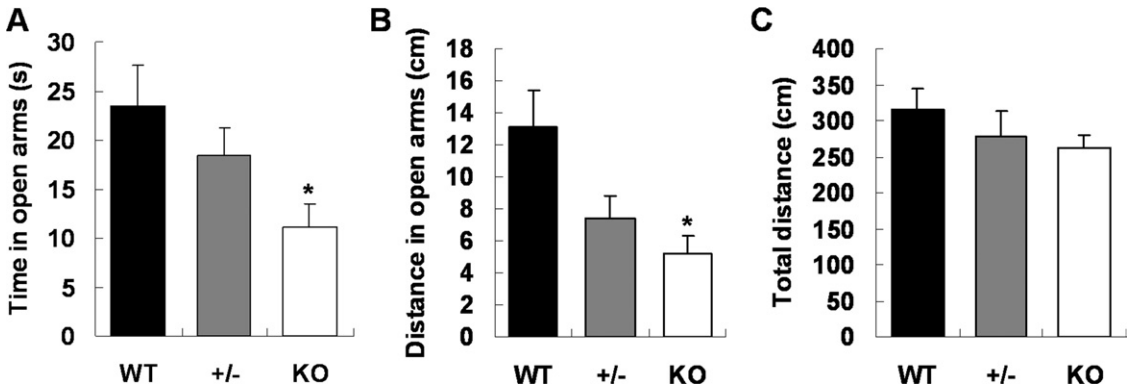

Fig. 5. *Gpr26* KO mice displayed an increased level of anxiety behaviors in the elevated plus maze. Sample sizes: WT, $n=12$; $Gpr26^{+/-}$, $n=12$; $Gpr26^{-/-}$, $n=12$. *Gpr26* KO mice exhibited reduced open-arm exploration in terms of both open-arm time (A) and open-arm distance (B) as compared to WT controls (post hoc test, $P=0.046$ and $P=0.015$, respectively). (C) Mice of all three genotypes exhibited similar total travel distance ($F(2, 33)=0.86$, $P=0.431$). * $P<0.05$ vs. wild-type group.

showed that immobility time of KO mice was significantly higher than that of WT littermates and heterozygotes ($P=8.2\times10^{-4}$ and $P=2.2\times10^{-3}$, respectively, Fig. 7B). The difference between the heterozygotes and WT mice was not significant ($P>0.05$). In addition, a positive correlation between body weight and immobility time was observed ($F(1, 32)=6.35$, $P=0.017$). Combining the results of FST and TST, we conclude that the *Gpr26* KO mice displayed higher depression-like behaviors than did WT controls.

Gpr26 KO mice do not exhibit significant impairment in learning and memory

As *Gpr26* exhibits a relatively high expression level in the hippocampus, we investigated whether *Gpr26* KO mice exhibited altered hippocampal-dependent learning and memory in the MWM test. During the training period, all mice exhibited progressive improvement in finding the hidden platform, as indicated by the significant decrease in escape latency time during the training (main effect of training day, $t(231)=-6.884$, $P=5.4\times10^{-11}$, Fig. 8A).

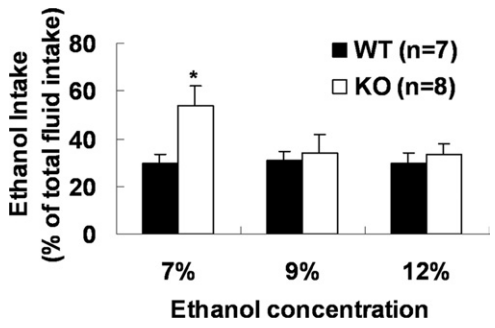

Fig. 6. *Gpr26* KO mice have a higher preference for ethanol than WT controls. Sample sizes: WT, $n=7$; $Gpr26^{-/-}$, $n=8$. Ethanol intake (7% for 3 days, 10% for 3 days, and 12% for 3 days) was measured using the two-bottle free-choice paradigm. Alcohol drinking percentages of total fluid intake (v/v) were recorded and represented. KO mice consumed significantly higher overall amounts of alcohol solution as compared with their wild-type littermates (β_3, $t(13)=9.05$, $P=0.010$). KO mice consumed a higher amount of 7% alcohol solution than WT mice (*t*-test $P=0.024$). * $P<0.05$ vs. wild-type group.

However, the genotypic effects and the interaction effects between genotypes and training day were not significant (all $P>0.05$), and similar results were found for swimming distance and swimming velocity (data not shown). In the test period when the platform was removed, all mice spent significantly more time in the target quadrant (data not shown); however, for mice of different genotypes, the time spent in the target quadrant and the number of times crossing over the original platform position did not differ significantly (both $P>0.05$, Fig. 8B, C, respectively). Therefore, we considered that *Gpr26* KO mice were not significantly impaired in learning and/or memory behaviors.

Decreased pCREB level in the central amygdala of Gpr26 KO mice

The transcription factor CREB is one of the components of many signaling cascades activated by neurotransmitter receptor engagement, and it is regulated by the GPCR-cAMP-PKA pathway. It has been reported that rodents with decreased phosphorylation of CREB in the amygdala display higher baseline anxiety-like behaviors and consume greater amounts of alcohol (Pandey et al., 2004, 2005b). To determine whether the increased anxiety-like behaviors observed are relative to a reduced pCREB level in amygdalas of *Gpr26* KO mice, we examined the pCREB level in *Gpr26* KO mouse brain. Quantitative analysis was performed on the whole brain, cortex, hippocampus, cerebellum (Fig. 9A), and amygdala (Fig. 9D), all selected on the basis of their relatively high expression of *Gpr26* or their possible involvement in emotion regulation. As shown in Fig. 9B, C, the levels of nucleic pCREB and CREB in the whole brain, cortex, hippocampus, and cerebellum showed no significant difference between WT and KO mice as measured by Western blot analysis. We next examined the levels of pCREB and CREB in the amygdalas of KO mice using immunohistochemistry. As a result, the mean optical density for pCREB immunostaining in the CeA of KO mice was found to be significantly lower (42%) than that of WT mice ($P=0.027$ by *t*-test), indicating a decreased pCREB level, while the total CREB level was not affected (Fig. 9E,

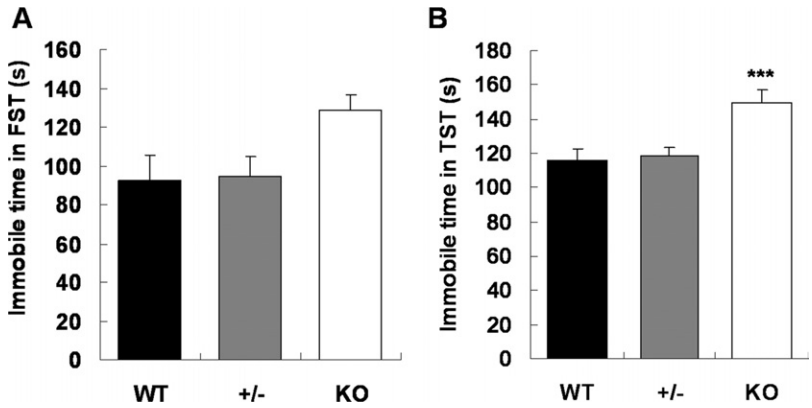

Fig. 7. *Gpr26* KO mice displayed increased depression-like behaviors in the forced-swim test and tail-suspension test. (A) Forced-swim test. Sample sizes: WT, $n=13$; $Gpr26^{+/-}$, $n=12$; $Gpr26^{-/-}$, $n=13$. There was a significant genotypic effect on the immobility time ($F(2, 35)=3.85$, $P=0.031$). The *Gpr26* KO mice displayed marginally significantly more immobility time than WT mice (post hoc test, $P=0.051$). (B) Tail-suspension test. Sample sizes: WT, $n=12$; $Gpr26^{+/-}$, $n=12$; $Gpr26^{-/-}$, $n=12$. There was a significant genotypic effect on the immobility time ($F(2, 32)=10.17$, $P=3.8\times10^{-4}$). The *Gpr26* KO mice displayed significantly more immobility time than WT mice (post hoc test, $P=8.2\times10^{-4}$). *** $P<0.001$ vs. wild-type group.

F). Therefore, we speculate that ablation of *Gpr26* inhibited the phosphorylation process of CREB in central amygdala. On the other hand, neither the total CREB nor the pCREB level of the KO mice in the other portions of the amygdala (BLA, MeA) was significantly altered (Fig. 9E, F).

DISCUSSION

Anxiety and depression disorders are major public health concerns. These disorders are often resistant to current therapeutic approaches such as anxiolytic or anti-depression drug treatment and cognitive behavior therapy. Accordingly, novel treatment strategies are required. In the present study, we explored the function of *Gpr26* in emotion regulation and showed that *Gpr26* ablation leads to higher anxiety- and depression-like behaviors. We further examined the pCREB and CREB levels in the brains of *Gpr26*-deficient mice. A reduced pCREB level was observed in the central amygdala but not in the other regions, while the total CREB level remains comparable between wild-type and mutant mice. Our findings are of particular importance in review of the neurobiological mechanisms underlying the conceptually diverse phenomena of anxiety and depression, and their possible implications for the psychopharmacological and behavioral treatment of anxiety and depression disorders.

Previous work and the present study found that *Gpr26* is exclusively expressed in mouse brain. After the ablation of *Gpr26*, we examined the gross phenotypes of *Gpr26* KO mice and found no abnormalities in growth, development, body weight, or reproduction capability (data not shown).

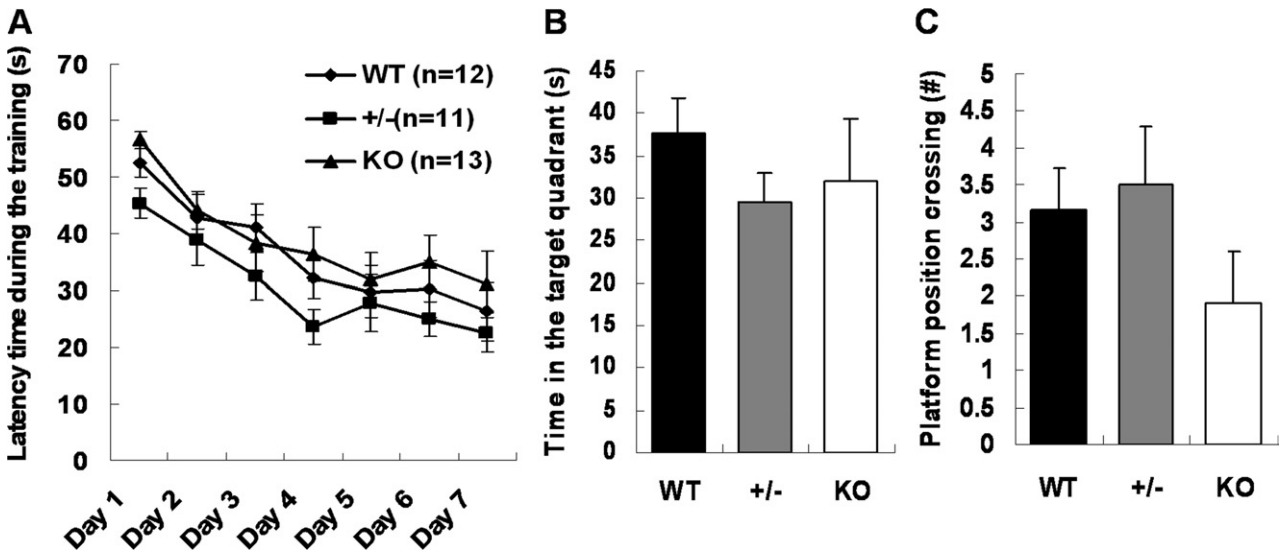

Fig. 8. No significant alteration in spatial learning and memory abilities in the Morris water maze was found in *Gpr26* KO mice. Sample sizes: WT, $n=12$; $Gpr26^{+/-}$, $n=14$; $Gpr26^{-/-}$, $n=13$. (A) Learning curves during the training period. There was no significant genotypic effect on the latency time ($F(1, 36)=1.50$, $P=0.236$). Swimming profiles in the test period were measured by the time spent in the target quadrant (B) and the number of times crossing over the original platform position (C).

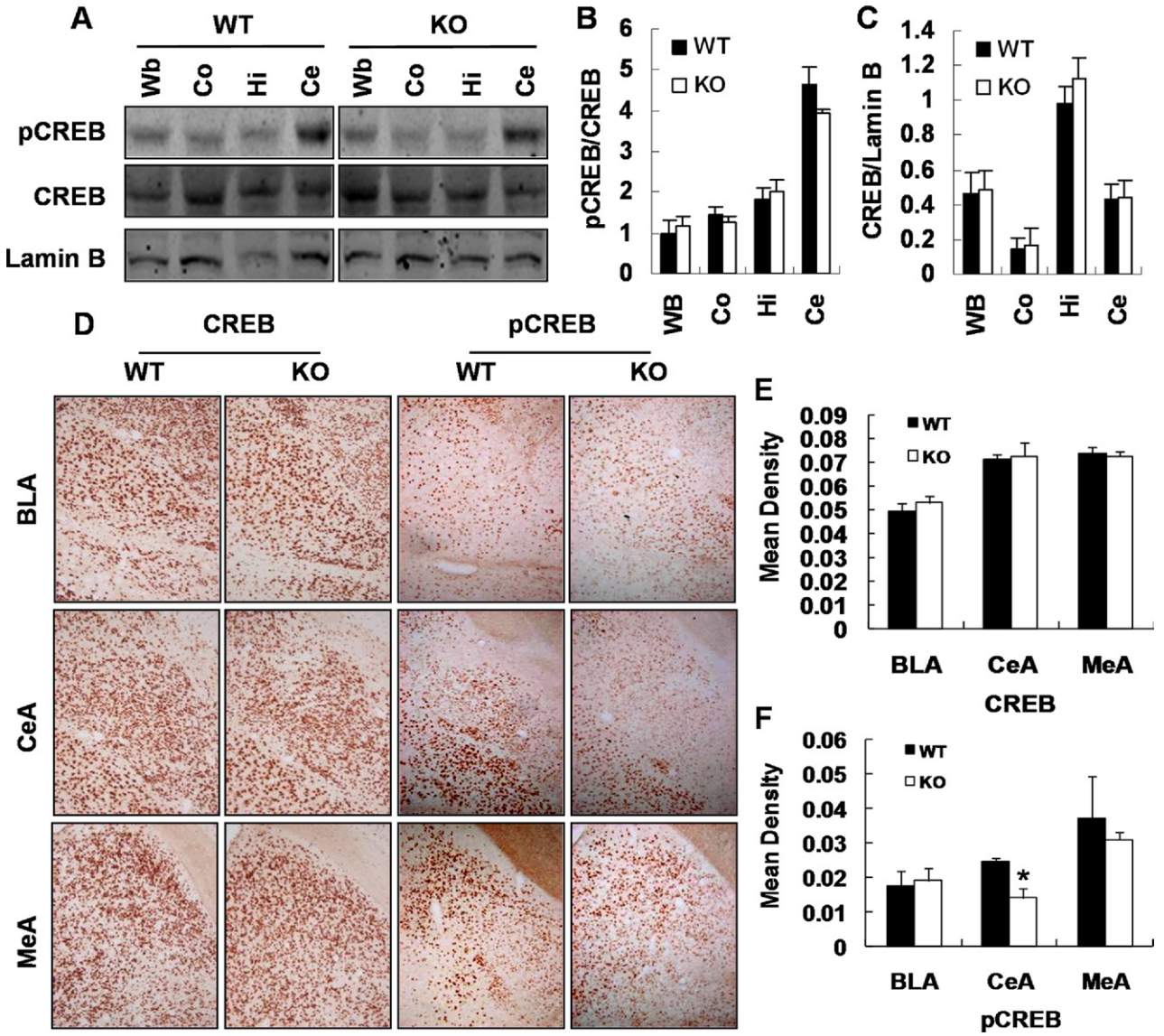

Fig. 9. pCREB level in the central amygdala was affected in *Gpr26* KO mice. (A) Western blot images representing the expression of pCREB and total CREB in WT and KO mouse brain. Nuclear protein isolated from the whole brain (Wb), cortex (Co), hippocampus (Hi), and cerebellum (Ce) was examined, with the mouse nuclear housekeeping protein Lamin B as equal protein loading control. Quantitative analysis results for relative expression levels of pCREB/CREB and CREB/Lamin B are shown in (B) and (C), respectively (WT: $n=4$; KO: $n=4$). (D) Photomicrographs of total CREB- and pCREB-immunoreactivity cells in WT and KO mouse amygdala regions (BLA, CeA, and MeA; original magnification ×200). Quantitative immunohistochemistry analysis results for total CREB and pCREB are shown in (E) and (F), respectively (Sample sizes: WT: $n=4$; KO: $n=5$). The pCREB level of *Gpr26* KO mice in CeA was down-regulated as compared to WT mice ($P=0.027$). * $P<0.05$ vs. wild-type group. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Moreover, we found no evidence of gross changes in brain organization or connectivity in *Gpr26* KO mice. In addition, no significant impairment of learning or memory was observed. These features make *Gpr26* a potential ideal target for the treatment of anxiety and depression, since the ablation of *Gpr26* specifically affects the regulation of anxiety and depression.

In a comprehensive behavioral test battery, we found that *Gpr26* KO mice displayed higher levels of anxiety and depression. In the normal brain, both *Gpr26* mRNA and protein are present in the emotion regulation regions, including hippocampus, amygdala, and cortex (Jones et al., 2007). The amygdala is an important structure for regulating anxiety with the central amygdala that facilitates the activation of the hypothalamic–pituitary–adrenal axis and the autonomic nervous system in response to stress (Myers and Greenwood-Van Meerveld, 2009). A large body of human imaging studies and animal experiments indicate that depression-like behaviors are associated with a neuronal circuit composed of the anterior cingulate cortex and its connections with the limbic system that includes the amygdala and the hippocampus among other brain regions (Fonberg, 1989; Whalen et al., 2002; Walf and Frye, 2006). Our findings are consistent with the prominent pres-

ence of *Gpr26* expression in regions of the brain that regulate emotion, and the well-recognized fact that alteration in the signal transduction through GPCR pathways (Pierce et al., 2002) have been reported in the etiopathology of mood disorders (Manji and Lenox, 2000; Manji et al., 2001; Stockmeier, 2003; González-Maeso and Meana, 2006).

In the first set of experiments, we assessed anxiety-related behavior in the open field and elevated plus maze tests. The open field test is a widely used test of anxiety as well as of exploration and locomotor activity (Archer, 1973; Walsh and Cummins, 1976; Lipkind et al., 2004) and is one of the most commonly used behavioral tests in genetically engineered mouse research (Bolivar et al., 2000). The elevated plus maze is a generally used behavioral assay for rodents, and it has been validated to assess the anti-anxiety effects of pharmacological agents and steroid hormones, and to define brain regions and mechanisms underlying anxiety-related behavior (Lister, 1987; Dawson and Tricklebank, 1995; Rodgers and Dalvi, 1997; Belzung and Griebel, 2001; Walf and Frye, 2007). Both paradigms are used for measuring states of anxiety based on the conflict between exploration of novel environments and the avoidance of potentially dangerous situations (Hogg, 1996; Rodgers and Dalvi, 1997; Prut and Belzung, 2003; Sousa et al., 2006). The results of both tests indicate that *Gpr26* KO mice were more anxious. It has been suggested that some alcoholics may be predisposed to alcohol-drinking behaviors because of innately high anxiety levels (Schuckit and Hesselbrock, 1994; Pandey, 2003). Many studies have shown that a relationship exists between the G-protein-mediated signaling cascade and responses to alcohol (Misra and Pandey, 2003; Pandey, 2003; Pandey et al., 2005a; Krahe et al., 2009). To examine the effect of *Gpr26* on alcohol abuse, we further assessed the alcohol-drinking behaviors in the two-bottle free-choice paradigm, which has been a commonly used test for alcohol-preferring behaviors (Thiele et al., 2000; Misra and Pandey, 2003; Pandey et al., 2004, 2005a). It is important to note that *Gpr26* KO mice drank significantly more alcohol than wild-type mice (53% vs. 29%) when the concentration of ethanol was low (7%), but when the concentration was elevated (9% and 12%), the higher alcohol-drinking behaviors could no longer be observed. The discordant results observed with different ethanol concentrations might be explained by taste aversion, which occurs at high ethanol concentrations (Spanagel et al., 1995). An earlier study also found that a positive correlation between anxiety levels and ethanol intake was observed only at low ethanol concentrations ($<$6%), and in a follow-up study, consumption of higher ethanol concentrations (8% and 10%) did not correlate with anxiety-related behavior (Spanagel and Hölter, 1999). It is also known that rats usually prefer alcohol solutions of lower concentrations ($<$6%) over those of higher concentrations (Henniger et al., 2002).

In a second set of experiments, we assessed the consequences of *Gpr26* ablation on depression-like behaviors in forced-swim and tail-suspension tests, which are widely used paradigms to assess depression and anti-depression phenotypes in genetically altered mice (Porsolt et al., 1977; Cryan and Mombereau, 2004). In both tests, the immobility time, a posture thought to reflect a state of "behavioral despair" in which animals have given up the hope of escape, is thought to be related to depression, and drugs with anti-depressant activity reduce the time that the animals remain immobile (Porsolt et al., 1977; Borsini et al., 1988; Bilkei-Gorzo et al., 2002). In our present study, we found evidence of an increased depression level due to ablation of *Gpr26*. These behavioral effects are similar to those that other investigators have observed in genetically altered mice (Zeng et al., 2007; Sun et al., 2009). Our data are also coincident with the previous observation that depression is associated with either enhanced (Singewald et al., 2004; Zeng et al., 2007; Bahi et al., 2009) or reduced anxiety-like behaviors (Sun et al., 2009).

According to the behavioral tests described above, our results indicate that inactivating *Gpr26* induces anxiety- and depression-like phenotypes. Thus, we conclude that *Gpr26* plays an important role in modulation of mood regulation. We next examined the CREB/pCREB expression level in different brain components. We chose CREB based upon the GPCRs–cAMP–PKA–pCREB signaling pathway. In the nervous system, GPCR functions as a receptor for functionally important ligands including many neurotransmitters, and CREB is one of the downstream genes of GPCR. CREB is activated by a phosphorylation at Ser-133 (Gonzalez et al., 1989). The kinase responsible for this activating phosphorylation was identified as the cAMP-dependent kinase, PKA (Regier et al., 1990). PKA activity is regulated by molecules that can alter cAMP levels, and hence by GPCRs, which regulate adenylate cyclase activity. Nevertheless, the ability to activate CREB is not restricted to a GPCR signaling pathway (Impey et al., 1999; Lonze and Ginty, 2002). Recent evidence indicated a strong association between anxiety and alcohol abuse (Regier et al., 1990; Schuckit and Hesselbrock, 1994; Spanagel et al., 1995; Pandey et al., 2003), and the link between anxiety and alcohol abuse might involve abnormalities in CREB signaling cascades. It was found that reduced phosphorylation of CREB was associated with decreased expression of NPY, and it is possible that CREB regulates anxiety and alcohol abuse behaviors via NPY (Pandey, 2003; Pandey et al., 2003, 2005b; Johnson et al., 2010). Based on the above reasoning, we examined the phosphorylation level of CREB in *Gpr26* KO mice and found a reduced phosphorylation of CREB in the central amygdala, while the pCREB levels were not significantly affected in other brain components. These observations are in accordance with the previous findings that the amygdaloid structures are the primary ones involved in mood control (Johnson et al., 2010), affecting fear, anxiety, and depression. Furthermore, the central amygdala appears to be an important regulator of anxiety and a mediator of the motivational aspects of alcohol drinking behaviors (Pandey, 2003; Pandey et al., 2003, 2005b).

In sum, we have generated a *Gpr26* KO mouse line and found that ablation of *Gpr26* leads to anxiety- and depression-like behaviors. Furthermore, we found that

the phosphorylation level of CREB is down-regulated in the CeA region of *Gpr26* KO mice. Therefore, we reach a conclusion that *Gpr26* plays an important role in emotion regulation and may function through the CREB signaling pathway. Our findings will amplify our knowledge in understanding the mechanism of anxiety and provide a potential pharmacological target for anxiety and depression treatment.

AUTHOR CONTRIBUTIONS

This study was conceived and designed by Z.-G. WANG, L.-L. ZHANG and J. FEI. The experiments were performed by L.-L. ZHANG, J.-J. WANG, Y. LIU, X.-B. LU, Y. KUANG, Y. CHEN, Y.-H. WAN, H.-M. YAN. The data were analyzed by L.-L. ZHANG. The paper was written by L.-L. ZHANG and Z.-G. WANG.

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