Supporting Information

Joseph et al. 10.1073/pnas.1219601110

SI Materials and Methods

Experimental Animals. The procedures for the generation of cholecystokinin receptor-2 transgenic (IF-*CCKR-2* tg; or simply dtg) mice were described in our previous publication (1), Briefly, a tetracycline transactivator (tTA)/ tet operator (tetO) system (pTet-off; Clontech) was used, in which two independent single transgenic mouse strains, ∂-Ca^{2+}-calmodulin kinase II (CaMK-II)-tTA and tetO-*CCKR-2*, were required. The tTA was flanked by an upstream 0.6-kb splicing signal (p265) and a downstream 0.5-kb SV-40 poly-A signal (p265). *CCKR-2* cDNA (1.3 kb), which was amplified by RT-PCR with total RNA extracted from the brain of a male B6/CBA F_1 mouse (Jackson Laboratory) with the primers of 5′-CGGGATCCATGGATCTGCTCAAGCTG-3′ and 5′-GCTCTAGATCAGCCAGGTCCCAGCGT-3′, was flanked by the same upstream 0.6-kb splicing signal (p265) but a downstream 1.1-kb β-globin poly-A signal and was subcloned into a pTRE2 vector (Clontech). These expression cassettes were separately injected into the pronucleoli of B6/CBA F_1 zygotes. Founders and their transgene copy numbers were determined by Southern blot. Founders with a suitable transgene copy number were crossed into B6/CBA F_1 mice (Jackson Laboratory) to produce single transgenic mice and then to produce hemizygous double transgenic (IF-*CCKR-2* tg mice; simply called dtg hereafter) mice by breeding these two single transgenic mice together. The genotypes were determined by PCR amplification of the tTA (5′-AGGCTTGAGATCTGGCCATAC-3′ and 5′-AGGAAAAG-TGAGTATGGTG-3′) and *CCKR-2* transgene (5′-ACGGTGG-GAGGCCTATATAA-3′ and 5′-GAGTGTGAAGGGCATGCA-A-3′) with genomic DNA from tails. The dtg mice used here were at 12–16 generations since they were generated. However, these mice were outbred into the B6/CBAF1 mice in every 5–6 generations, in order to avoid an inbreed effect. Single transgenic (tTA or tetO-*CCKR-2* only) and WT littermates of dtg mice were used as controls and are collectively and simply called WT mice hereafter. Mice were kept in standard laboratory mouse cages under standard conditions (12-h light/dark cycle, temperature at 22 ± 1 °C, humility at 75%) with food and water ad libitum.

Real-Time RT-PCR and In Situ Hybridization. Real-time RT-PCR and in situ hybridization were used to detect the expression level and pattern of the *CCKR-2* transgene in the brain, respectively. For real-time RT-PCR, doxycycline (doxy) treatment indicated that mice were treated with doxy (2 mg/100 mL in drinking water; Sigma) for 5 d. Doxy withdrawal indicated that doxy was withdrawn for 7 d after this 5-d treatment. The whole forebrains were dissected for the total RNA extraction with TRIzol (Invitrogen), and then the total RNA was purified by using RNEasy columns (Qiagen). RT was performed with a SuperScript III First-Strand synthesis system (Invitrogen). A fluoresce probe recognizing the total (endogenous and transgenic) *CCKR-2* mRNAs was used to amplify the mRNAs with a 40-cycle PCR amplification (Applied Biosystems). The expression level of the total *CCKR-2* mRNAs was normalized by the 18S rRNA expression, and the experiments were repeated three times in three mice. Mice were killed at the age of P25 or P60 to determine whether there was any difference in the expression of the *CCKR-2* transgene between these ages. For in situ hybridization, brains from both WT and dtg mice were collected by decapitation and were frozen with powered dry ice immediately. Sagittal sections (20 μm) were made with a Cryostat (CM 1900; Leica). An oligo probe for tTA and a cRNA probe for the total *CCKR-2* mRNAs were labeled with ^{35}S UTP (>1,000 Ci/mmol; NEN) by a random labeling kit and in vitro transcription kit (Invitrogen), respectively. The hybridization was performed overnight at 55 °C, and after washing, slides were exposed to Kodak BioMax film (NEN) for the same time of the same experiments from 3 to 30 d, depending on the probe used.

CCKR Binding Assay. CCKR binding assays were conducted by using ^{3}H-CCK-8 (propiony-^{3}H-sulfated and propionylated CCK-8, 93.0 Ci/mmol; NEN), as described in our previous study (1). Because we already confirmed the saturated binding curve in the forebrain of dtg mice (1), we did not repeat these experiments again here. Instead, only two concentrations of ^{3}H-CCK-8, 2 and 20 nM, were examined. Mice were examined at two ages: P25 and P60. Each assay was conducted in triple samples from each brain, and three assays in total were repeated in three mice. Nonspecific binding was determined by using 1 μM cold CCK-8 under the same incubating conditions as used in experiments with hot CCK-8. The specific binding was calculated as follows: total binding (cpm) − nonspecific binding (cpm).

ELISA. Commercially available kits for both the adrenocorticotropic hormone (ACTH; MD Bioproducts, St. Paul, MN) and corticosteroid hormone (CORT; R&D Systems) were used to determine the serum level of these hormones. Experimental procedures followed the recommended steps. To have enough samples for triplicate measurements, blood was collected with a retroorbital eye bleeding method. To minimize nonspecific effects, blood collection was conducted at 9:00 AM, and the procedure was completed within 30 s, by which time any possible change that might be produced by the sampling procedure was not yet measurable.

Adolescent Trauma. Both WT and dtg mice at the age of P25 were individually put into a small shock-box (4 × 4 × 10 in. in height) that was modified from the shock box from a fear-conditioning system (Coulbourn Instruments), to ensure that the mice did not have much space for escaping during shocking. The current of the footshock was higher (1.0 mA) than commonly used in the fear-conditioning test (0.6–0.8 mA). The footshock was conducted five times (trials) in total, during a period of 1 min, and each trial lasted for 2 s, with an interval of 10 s between trials. "Naïve trauma" indicates that mice underwent the same procedures but did not receive the footshock.

Adult Acute Stressor. After adolescent trauma (AT), mice were returned to their homecages until P60 (2 mo in age), and then these mice were subjected to additional stress (0.8 mA for 2 s for one trial), namely acute stressor (AS) in this study, with a standard fear-conditioning paradigm as described below. The AS used here was to trigger posttraumatic stress disorder (PTSD)-like behavior as described elsewhere (2).

PTSD-Like Behavior. PTSD-like behavior was examined using the following five behavioral tests.

Fear-conditioning test. A standard fear-conditioning paradigm from the TruScan and Coulbourn Instruments was used. The experimental procedures were described in our previous study (3). Briefly, mice were individually put into a shock chamber (10 × 10 × 15 in. in height) and were allowed to freely explore the chamber for 2.5 min. A tone at 90 dB and 2,800 Hz [conditioned stimulus (CS)] was then delivered for 30 s, and at the last 2 s, an inescapable footshock at 0.8 mA [unconditioned stimulus (US)] was delivered for 2 s. The US was considered as the AS here. After the CS/US(AS) pairing,

the mouse was allowed to stay in the chamber for another 30 s. Contextual conditioning was examined 24 h after the pairing, during which mice were individually put back to the same chamber where they received the AS, and freezing was recorded for 3 min by using a 5-s sampling method. After the completion of the contextual conditioning, cued conditioning was examined, during which mice were individually placed into a novel chamber and were allowed to freely explore the chamber for 3 min (pretone stage). Immediately after this, the same tone used in the CS/US(AS) pairing was delivered for 3 min (during-tone stage). Freezing was recorded in both stages by using the same 5-s sampling method. The freezing rate was calculated as freezing sampling number/total sampling number × 100%.

Fear-extinction test. The mice used in fear-conditioning test above were further examined for their fear extinction by using a protocol of five trials of extinction as described in our previous publication (4). Briefly, mice were individually and repeatedly reexposed to the same shock chamber, but did not receive any footshock, five times (trials), with an interval of 2 h between trials. Each reexposure lasted for 3 min, and freezing was recorded in all these trials by using the same 5-s sampling method. The freezing rate was calculated as described above. In addition, a freezing extinction rate was calculated: 1 − (fear response/next fear response × 100%). This rate indicates how fast the freezing response could be extinguished following extinction trials.

Open-field test. Open-field behaviors were examined by using an automatic-recording open-field station (MED Associates). The bottom of the open-field box (40 × 40 × 30 cm in height) was divided into 16 identical squares by lines that were eye-invisible but computer-detectable. The box was illuminated by a dim light (20 lx). Two sets of 16 pulse-modulated infrared photobeams were placed on opposite walls 2.5 cm apart from the wall to record *X-Y* ambulatory movements. Exploratory behavior in the box was computer-interfaced at a sampling rate of 100-ms resolution. Mice were transported to the behavioral room to adapt to the environment for at least 1 h before the experiment. Behavioral indices including total distance traveled, ambulation counts, and number of rearing were automatically recorded by the photobeam scanning system for 60 min.

Elevated-plus maze test. The apparatus (MED Associates) consisted of a platform (7 × 7 cm) and four dark gray Plexiglas arms, of which two were opened (67 × 7 cm) and the other two were closed (67 × 7 × 17 cm). Open arms and closed arms formed a cross shape with two open arms opposite each other and two closed arms opposite each other also. The maze was set at 55 cm above the floor and was dimly illuminated (20 lx). Photobeam cells, connected to a computer, were placed at two different directions along the length of each arm and were able to detect the passage of the animal from the central platform to the end of any arm. A video tracking system (EthoVision; Noldus) was placed above the apparatus to record behavioral responses, and data were automatically analyzed by the tracking system. During testing, mice were individually placed in the center of the platform by facing to a closed arm and were allowed to freely explore the maze for 5 min. Numbers of open-arm and closed-arm visits and time spent in open arms and closed arms were separately recorded and analyzed statistically.

Modified tone-conditioning test. In this study, a unique behavioral paradigm was developed, based on the principles of the cued conditioning test. Briefly, mice were first individually trained to associate an US (inescapable footshock at 0.8 mA for 2 s, one trial) with a CS (tone at 95 dB and 2,800 Hz for 30 s). Twenty-four hours later, mice were individually put into a novel context (open box, same as described above), and then a changed tone (80 dB and 1,800 Hz) was delivered for 6 min, during which period the behavioral responses including total distance traveled, total nonmovement time, and number of groomings were automatically recorded by a photobeam scanning system. The changed tone was used as a related clue to trigger fear response, and thus a higher fearing rate in this test is considered as a hyperarousal status to a stress-related cue.

Cognitive Function. Cognitive function was examined in terms of mnemonic function. Two behavioral tests were used.

Fear-conditioning test. The details are described in above.

Morris water-maze test. A Morris water maze was used to evaluate spatial learning and memory as described previously (4). Briefly, a circular water tank (diameter, 100 cm; 75 cm in height) was filled with water that was made opaque with nontoxic white paint (Reeves & Poole group) by three-quarters of the tank in depth. The water tank was surrounded by a black curtain 1 m away, with three visible signs on the curtain. A round platform (diameter 15 cm), which was located in the center of a given quadrant of the pool, was hidden 1 cm beneath the surface of water. Training was conducted for 6 d (six sessions), and each session consisted of four trials. In every trial, the mouse was released from the wall of the tank by facing against the wall into water and then was allowed to freely swim (search/find) in the pool and to stand on the platform for 10 s within the 90-s testing period. An interval of 2 h was set between the two trials. In each training session, a starting quadrant and the order of quadrants from where the mouse was put into the water was randomly chosen so that both the starting quadrant and the quadrant order were different in different sessions in each animal and were different between animals. Navigation was recorded by a video camera, and the task performances including swimming paths, swimming speed, and time spent in each quadrant were recorded and analyzed by the same EthoVision video tracking system. A probe test was conducted 24 h after the completion of the training. In this test, the platform was removed from the pool, and the task performances were recorded for 1 min. The time spent in each quadrant was considered as the index for their memory retention.

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2. Golub Y, Mauch CP, Dahlhoff M, Wotjak CT (2009) Consequences of extinction training on associative and non-associative fear in a mouse model of posttraumatic stress disorder (PTSD). *Behav Brain Res* 205(2):544–549.
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4. Tang YP, et al. (1999) Genetic enhancement of learning and memory in mice. *Nature* 401(6748):63–69.

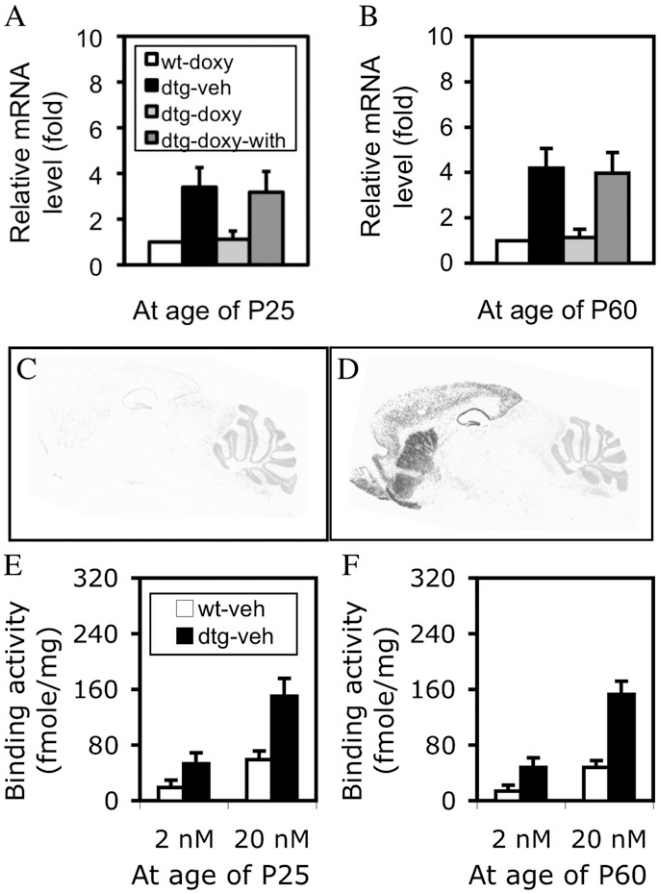

Fig. S1. *CCKR-2* transgene expression and its function. (*A* and *B*) The expression level of the total *CCKR-2* (endogenous and transgenic) mRNAs in the forebrain of mice at age of P25 (*A*) and P60 (*B*) detected by using real-time RT-PCR. veh, vehicle; with, withdrawal. Data are expressed as mean ± SD. (*C* and *D*) Expression pattern of the tTA mRNA detected by using in situ hybridization with mouse saggital brain sections. No tTA mRNA expression is detected in WT mice (*C*). In dtg mice (*D*), a forebrain-specific expression of tTA is observed, and the highest level is observed in the hippocampus, striatum, and amygdala and a moderate level in the other forebrain regions. The expression is not found in the thalamus/hypothalamus, brainstem, or cerebellum. (*E* and *F*) CCKR binding activity in the forebrain of mice at age of P25 (*E*) and P60 (*F*) in the presence of a low (2 nM) or a high (20 nM) dose of the ligand. Data are expressed as mean ± SD.

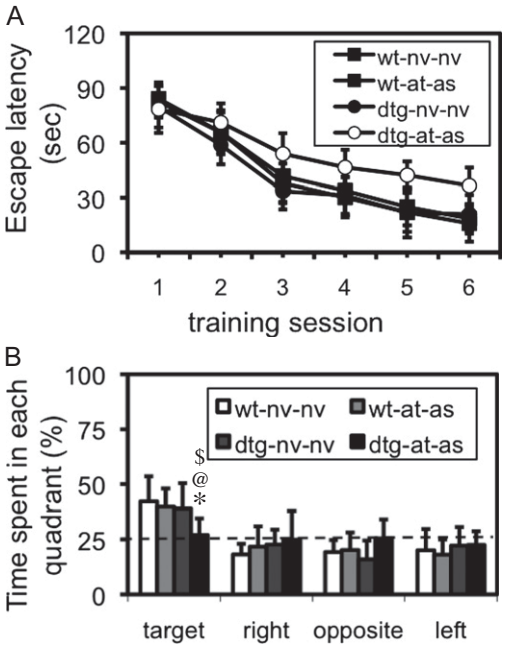

Fig. S2. Spatial leaning and memory deficit in dtg mice with AT/AS in a Morris water-maze test. Our previous study has shown that naïve dtg mice have normal spatial learning and memory (data not shown). As the aim here was to determine whether the AT, together with the transgene, impaired learning and memory, we only examined three groups of mice: WT-naïve-naive ($n = 12$), WT-AT-AS ($n = 10$), and dtg-AT-AS ($n = 12$). Training was conducted 24 h after the AS for six sessions. As shown in *A*, the escape latency was decreased dramatically following training trails, and a within-group one-way ANOVA revealed a highly significant difference in WT-naïve-naïve mice [$F(5,66) = 29.44$, $P < 0.001$], WT-AT-AS mice [$F(5,54) = 21.37$, $P < 0.001$], and dtg-AT-AS mice [$F(5,66) = 9.12$, $P < 0.001$], indicating that all these mice were able to learn the task. However, a repeated ANOVA revealed a significant effect of the AT and the *CCKR-2* transgene on escape latency [$F(2,31) = 8.97$, $P < 0.01$], and post hoc tests revealed a significant difference ($P < 0.05$–0.01) between dtg-AT-AS mice and either WT-naïve-naïve mice or WT-AT-AS mice from the fourth trial to the last trial of training but not between WT-naïve-naïve mice and WT-AT-AS mice, indicating an interaction between the AT and CCKR-2 transgene in impairing spatial learning and memory. As shown in *B*, a probe test revealed a significant difference in time spent in the targeting quadrant. Dotted line represents the performance at the chance level (25%). *, $P < 0.05$, compared to WT-naïve-naïve mice; @, $P < 0.05$, compared to WT-AT-AS mice, and $, $P < 0.05$, compared to dtg naïve-naïve mice, of which all were Student's *t* test. Data are expressed as mean ± SD.

Table S1. ACTH level (pg/mL) in a time course study following AS

Group	Before AS (basal level)	After AS			
		1 h	2 h	4 h	8 h
WT-naïve	110.7	638.1	95.3	70.3	63.9
dtg-naïve	84.8	745.1	81.2	56.8	58.1
WT-AT	91.4	759.6	101.7	51.1	69.2
dtg-AT	80.1	872.6	266.7	178.9	163.3

Table S2. CORT level (ng/mL) in a time course study following AS

Group	Before AS (basal level)	After AS			
		1 h	2 h	4 h	8 h
WT-naïve	64.6	417.5	106.6	69	54.7
dtg-naïve	49.5	539.1	136.3	55.8	71.7
WT-AT	47.8	454.5	119.7	59.9	67.1
dtg-AT	44.5	586.8	233.9	166.1	110.8