Anxiety in liver X receptor β knockout female mice with loss of glutamic acid decarboxylase in ventromedial prefrontal cortex

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Anxiety disorders are the most prevalent mental disorders in adolescents in the United States. Female adolescents are more likely than males to be affected with anxiety disorders, but less likely to have behavioral and substance abuse disorders. The prefrontal cortex (PFC), amygdala, and dorsal raphe are known to be involved in anxiety disorders. Inhibitory input from the PFC to the amygdala controls fear and anxiety typically originating in the amygdala, and disruption of the inhibitory input from the PFC leads to anxiety, fear, and personality changes. Recent studies have implicated liver X receptor β (LXRβ) in key neurodevelopmental processes and neurodegenerative diseases. In the present study, we used elevated plus-maze, startle and prepulse inhibition, open field, and novel object recognition tests to evaluate behavior in female LXRβ KO (LXRβ$^{-/-}$) mice. We found that the female LXRβ$^{-/-}$ mice were anxious with impaired behavioral responses but normal locomotion and memory. Immunohistochemistry analysis revealed decreased expression of the enzyme responsible for GABA synthesis, glutamic acid decarboxylase (65+67), in the ventromedial PFC. Expression of tryptophan hydroxylase 2 in the dorsal raphe was normal. We conclude that the anxiogenic phenotype in female LXRβ$^{-/-}$ mice is caused by reduced GABAergic input from the ventromedial PFC to the amygdala.

psychiatric diseases | inhibitory interneuron | neurotransmitters

Anxiety disorders are the most common psychiatric disorders (28% lifetime prevalence) (1) and contribute to the etiology of major depression and substance abuse (2, 3). Anxiety disorders share common features involving, for example, feelings of fear and worry that can lead to avoidant or compulsive behaviors (4). Although the ventromedial prefrontal cortex (vmPFC) (5–8), amygdala (4), and dorsal raphe (9), important for emotional processing, have been proposed to have roles in anxiety, the neural mechanisms that control anxiety remain unclear.

Abnormal activity in the vmPFC is probably the most widely reported anomaly in the involved brain regions (5–8). The PFC is responsible for executive functions, such as planning, decision making, predicting consequences for potential behaviors, and understanding and moderating social behavior. In the healthy brain, the frontal cortex regulates impulses, emotions, and behavior via inhibitory top-down control of emotion-processing structures (10, 11). Quidé et al. (4) hypothesized that reduced inhibitory function in the PFC results in loss of inhibition of the amygdala, leading to generally exaggerated amygdala activity and anxiety output. The PFC has an extensive interrelationship with the serotonergic system (12–14), and the serotonergic system can exert control over PFC excitability (15–19).

Liver X receptor (LXR) is a subfamily of the nuclear receptor family of transcription factors. LXRβ is one of the two members of this subfamily, which has irreplaceable functions in the central nervous system (20–24). We previously showed that LXRβ expression is essential for the maintenance of motor neurons in the spinal cord and dopaminergic neurons in the substantia nigra (25, 26). More recently, we found that LXRβ plays a specific role in cortical lamination and is essential for radial migration of later-generated neocortical neurons in embryonic mice (27, 28).

In the present study, we compared the behavior of LXRβ KO (LXRβ$^{-/-}$) female mice and WT littermates using a comprehensive approach to evaluate anxiety, motor function, cognition, and memory incorporating the elevated plus maze (EPM), startle and prepulse inhibition, open field, and novel object recognition tests. We found that female LXRβ$^{-/-}$ mice display anxious behavior and impaired behavioral response with no abnormalities in locomotion and memory.

Results

Increased Anxiety-Related Behaviors. In the EPM and the startle and prepulse inhibition tests, the LXRβ$^{-/-}$ mice exhibited more anxious behavior compared with the WT control littermates. The LXRβ$^{-/-}$ mice avoided the open arm and spent less time and less percent time in this anxiety-inducing exposed area ($P = 0.0048$), but did not differ in the total number of entries and percent entries visiting the open arm compared with the WT mice (Fig. 1*A*). On repeat testing performed 4 wk later, the same WT mice exhibited a significantly reduced percent number of entries ($P = 0.0011$), total time ($P = 0.0486$), and percent time ($P = 0.0486$) in the open arms of the maze, with no difference in the total number of entries (Fig. 1*B*). However, the LXRβ$^{-/-}$ mice displayed almost the same response as in the first test, with no evident adjustment from the previous experience (Fig. 1). In the startle and prepulse inhibition test, the LXRβ$^{-/-}$ mice demonstrated no abnormalities in either the initial test or the repeat test (Fig. 2 *A* and *B*).

No Abnormal Locomotion in LXRβ$^{-/-}$ Mice. Although the LXRβ$^{-/-}$ mice clearly exhibited anxiety behavior on the EPM test, they demonstrated no motor dysfunction. No differences in path length in 5 min, total path length, and percent path in center between WT and LXRβ$^{-/-}$ littermates were observed in either the first test or the second test (Fig. 3 *A* and *B*).

Normal Learning and Memory in Mice with Loss of LXRβ. As noted above, in the repeat EPM test, the LXRβ$^{-/-}$ mice, unlike the WT mice, did not adjust their behavior, indicating abnormal memory function or impaired behavioral response. The novel object recognition test is used to evaluate cognition and particularly learning and recognition memory. The test revealed no differences in exploration time, discrimination index, or preference between WT and LXRβ$^{-/-}$ littermates (Fig. 4*B*). A repeat novel object recognition test of the same mice 4 wk later showed no abnormal learning or memory in the LXRβ$^{-/-}$ mice (Fig. 4*C*).

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The authors declare no conflict of interest.

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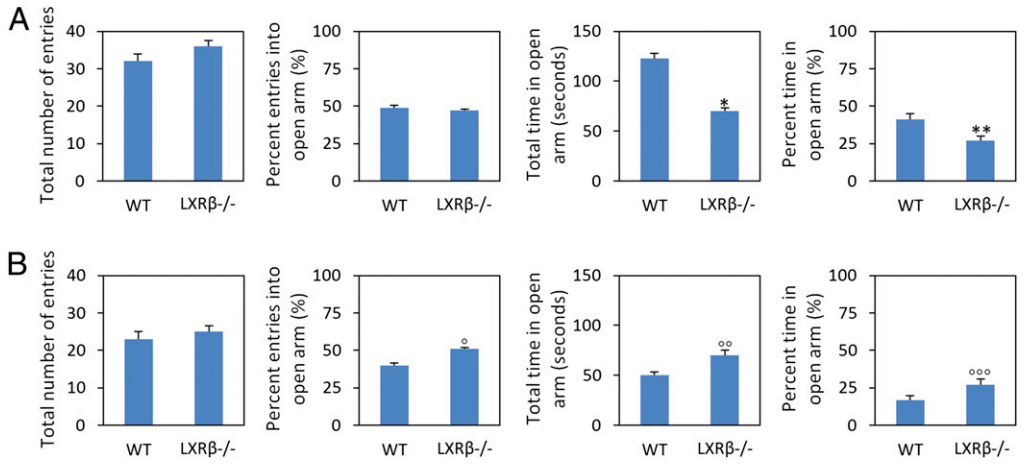

Fig. 1. Anxious behavior of LXRβ$^{-/-}$ female mice. (*A* and *B*) Behavior of 10 groups of female WT and LXRβ$^{-/-}$ littermates in the EPM test. (*A*) Compared with WT littermates, the LXRβ$^{-/-}$ mice had significantly lower total time (*P = 0.0048) and percent time (**P = 0.0048) in the open arms of the maze, with no differences in the total number of entries or percent entries into open arms. (*B*) In a repeat EPM test performed 4 wk after the first test, LXRβ$^{-/-}$ mice had a similar response as in the first test; however, the WT mice exhibited significantly reduced percent entries ($^{\circ}P$ = 0.0011), total time ($^{\circ\circ}P$ = 0.0486), and percent time ($^{\circ\circ\circ}P$ = 0.0486) in the open arms of the maze, with no difference in the total number of entries.

Decreased Glutamic Acid Decarboxylase Expression in the vmPFC of LXRβ$^{-/-}$ Mice. The PFC's inhibitory function is involved in the cross-talk between the PFC and the amygdala, an important mechanism in the pathophysiology of anxiety and depression disorders (4). Glutamic acid decarboxylase (GAD) is the enzyme that catalyzes the synthesis of GABA from glutamate (29). GAD (65+67) is expressed in the cytoplasm and the projections of inhibitory interneurons (Fig. 5). The LXRβ$^{-/-}$ mice had a significantly reduced number of GAD(65+67)-positive interneurons in the vmPFC, as shown in both sagittal (Fig. 5 *A–F*) and coronal (Fig. 5 *G–L*) sections. This decrease was confined to the PFC; abundant GAD expression remained in other areas of the cortex, the hippocampus, and Purkinje cells in the cerebellum.

Tryptophan Hydroxylase Expression in the Dorsal Raphe. Tryptophan hydroxylase (TPH) is the enzyme responsible for the synthesis of serotonin. TPH1 is expressed mainly in peripheral tissues, whereas TPH2 is preferentially expressed in the brain serotonergic neurons from the raphe nuclei (9). The dorsal raphe was cut completely from its rostral margin to its caudal margin, and comparable sections were selected according to the position and size of blood vessels and the morphology of the cerebral aqueduct (Fig. 6*A*). TPH2-positive serotonergic neurons in the dorsal raphe can be divided into two gross types, those in the dorsal part and those in the ventral part. No significant difference in either type was seen between the WT and LXRβ$^{-/-}$ littermates (Fig. 6 *A* and *B*).

Discussion

Anxiety disorders are the most common mental disorders affecting US adolescents at 31.9% (30). Types of anxiety disorder include panic disorder, agoraphobia, social phobia, specific phobia, generalized anxiety disorder, posttraumatic stress disorder,

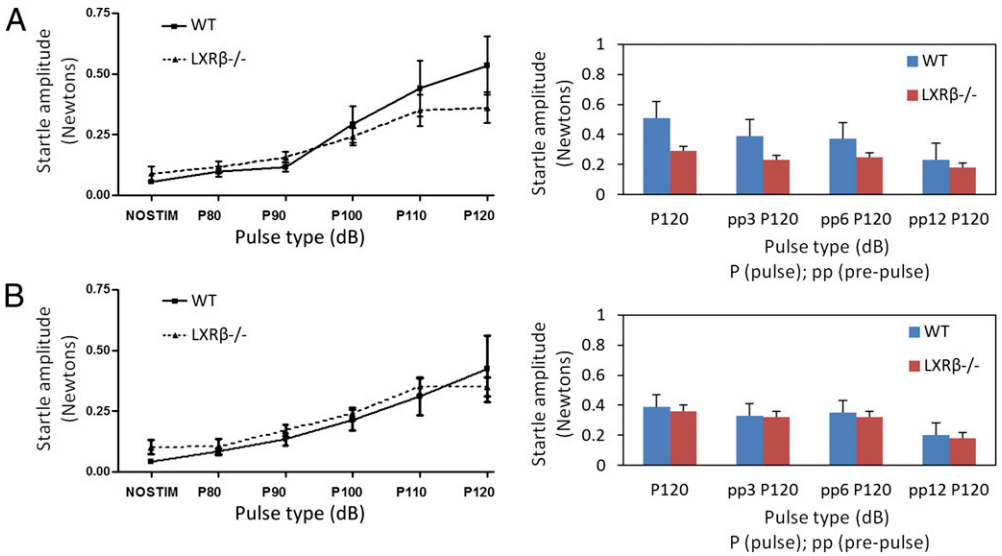

Fig. 2. Startle and prepulse inhibition test in LXRβ$^{-/-}$ female mice. (*A* and *B*) Behavior of 10 groups of female WT and LXRβ$^{-/-}$ littermates in the startle and prepulse inhibition test. (*A*) No differences in startle amplitude (P = 0.187) or prepulse inhibition (P = 0.092) were observed between WT and LXRβ$^{-/-}$ littermates. (*B*) A repeat of the startle and prepulse inhibition test in the same mice 4 wk later revealed no significant differences in startle amplitude (P = 0.381) or prepulse inhibition (P = 0.25) between littermates.

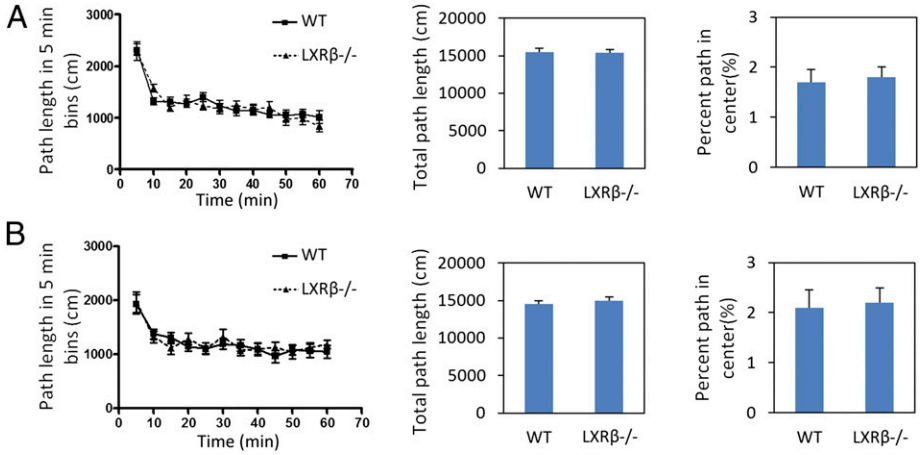

Fig. 3. Open field test of locomotion in LXRβ$^{-/-}$ female mice. (*A* and *B*) Behavior of 10 groups of female WT and LXRβ$^{-/-}$ littermates in the open field test. (*A*) No differences in path length in 5 min, total path length, or percent path in center were observed between WT and LXRβ$^{-/-}$ littermates. (*B*) Repeated open field test of the same mice 4 wk later revealed no significant difference in locomotor activity between the littermates.

and separation anxiety disorder. The EPM test was validated as a test of anxiety-related behavior initially in rats by Pellow and File (31) and later in mice by Lister (32). The test is sensitive to the actions of both anxiolytic and anxiogenic agents and is currently considered the gold standard test for anxiety-related behaviors (33). The LXRβ$^{-/-}$ mice demonstrated greater anxiety behavior in the EPM test compared with their WT littermates. In a published study, mice presented with previous test experience in the EPM exhibited open arm avoidance (34). Interestingly, the LXRβ$^{-/-}$ mice showed no such adjustment to the second round of testing. This difference between the LXRβ$^{-/-}$ and WT mice might be related to the impaired memory or altered behavioral responses of the LXRβ$^{-/-}$ mice; however, the normal response of the LXRβ$^{-/-}$ mice to the novel object recognition test argues against impaired cognition and memory.

The startle and prepulse inhibition test provides another way to measure anxiety behavior. The acoustic startle response is characterized by a startle reflex to an unexpected auditory stimulus. In a prepulse inhibition protocol, the startle response can be attenuated by a weaker prestimulus. Whereas the startle response is a parameter of anxiety and fear, the prepulse inhibition paradigm provides an operational measure of sensorimotor gating, which reflects an animal's ability to integrate sensory information (35). No differences between the LXRβ$^{-/-}$ mice and WT mice were demonstrated in either the first or second round of this test.

The open field test is widely used for evaluation of motor function (36, 37). The LXRβ$^{-/-}$ mice exhibited no motor dysfunction on the open field test, although they clearly showed anxiety behavior on the EPM test. The novel object recognition test is a hippocampal-dependent task designed to test the integrity of recognition memory (38, 39). Both the first and second object recognition tests showed no difference in discrimination index or preference between the WT and LXRβ$^{-/-}$ mice. Taken

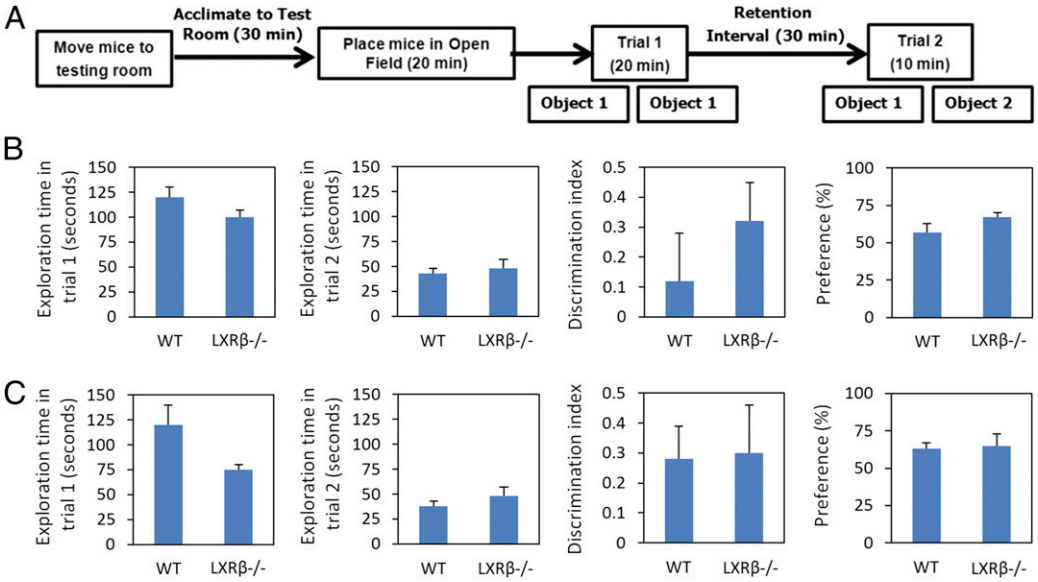

Fig. 4. Novel object recognition test in LXRβ$^{-/-}$ female mice. (*A*) Experiment time line and procedure. (*B*) Behavior of 10 groups of female WT and LXRβ$^{-/-}$ littermates in the test. No difference was observed between WT and LXRβ$^{-/-}$ littermates in exploration time, discrimination index, or preference. (*C*) Repeated novel object recognition test of the same mice 4 wk later revealed no significant differences in exploration time, discrimination index, or preference between the littermates.

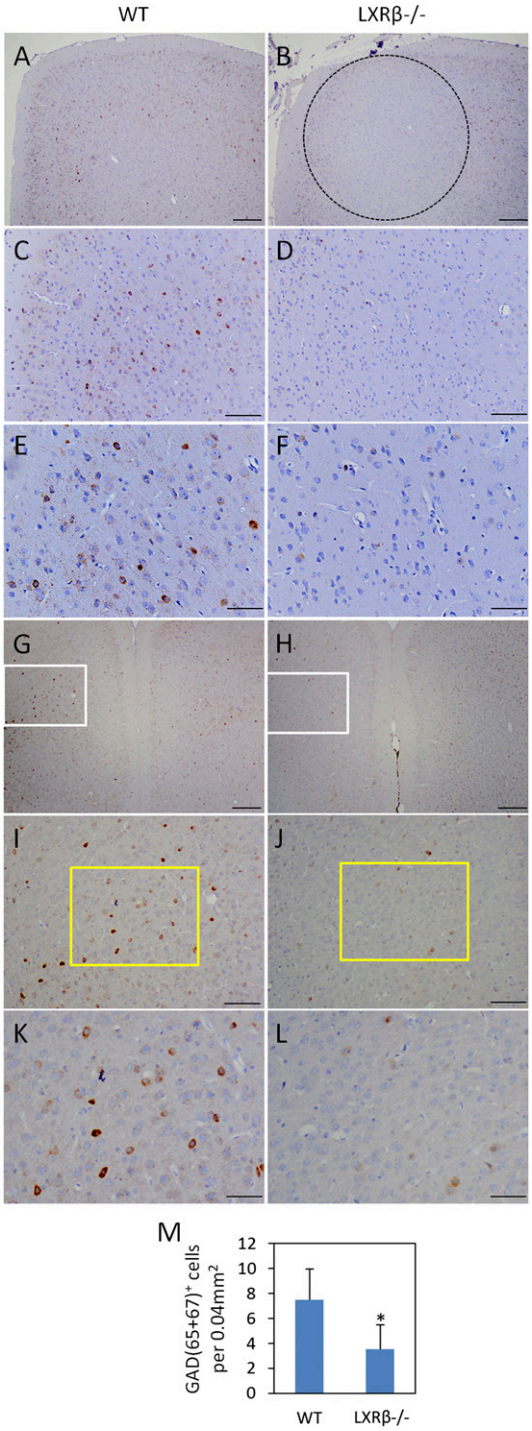

Fig. 5. Expression of GAD in the sagittal and coronal vmPFC of LXRβ$^{-/-}$ mice. (*A–F*) Fewer GAD(65+67)-positive interneurons (brown color) were detected in the sagittal vmPFC in LXRβ$^{-/-}$ mice compared with WT littermates. The black dotted circle indicates the outline of the vm PFC. (*G–L*) In coronal sections of the vmPFC, the number of GAD(65+67)-positive interneurons was significantly lower in LXRβ$^{-/-}$ mice compared with WT littermates. (*I* and *J*) Amplified views of the boxed areas in *G* and *H*. (*K* and *L*) Amplified views of the boxed areas in *I* and *J*. (*B* and *H*) GAD-positive cells were abundant in other areas of the cortex in the LXRβ$^{-/-}$ mouse brain. (*M*) The number of GAD(65+67)-positive cells per 0.04 mm^2 was significantly lower (*$P < 0.001$; $n = 5$) in the vmPFC of LXRβ$^{-/-}$ mice (3.53 ± 1.96) compared with WT littermates (7.47 ± 2.47). (Scale bars: *A*, *B*, *G*, and *H*: 200 μm; *C*, *D*, *I*, and *J*: 100 μm; *E*, *F*, *K*, and *L*: 50 μm.)

together, the test findings indicate that the female LXRβ$^{-/-}$ mice displayed anxiety-like behavior and impaired behavior response with no abnormalities in locomotion or memory.

Inhibitory output from the PFC is involved in the repression of fear and anxiety signals originating in the amygdala (4). Functional neuroimaging studies have identified disruption of PFC-amygdala circuitry as a common lesion underlying anxiety-related behaviors (40). Exaggerated amygdala activation is demonstrated in trait anxiety (41), posttraumatic stress disorder (42, 43), social anxiety (44, 45), and generalized anxiety disorder (46). Thus, inhibitory top-down control of the emotion-processing functions of the amygdala via GABAergic input from the frontal cortex regulates impulsive behavior and emotions (10, 11). In the LXRβ$^{-/-}$ mice, the number of GAD(65+67)-positive interneurons was significantly reduced in the vmPFC, whereas abundant GAD(65+67) expression was seen in other areas of the cortex, the hippocampus, and Purkinje cells in the cerebellum. This reduced GABAergic output together with the behavioral test results support the idea that decreased inhibitory function of the vmPFC led to exaggerated output from the amygdala and produced the resulting anxiety phenotype in the LXRβ$^{-/-}$ mice (40, 47, 48).

TPH is an enzyme involved in the synthesis of serotonin in serotonergic neurons located in the dorsal raphe. Serotonin and its receptors play critical roles in controlling PFC excitability (49). No significant differences in the dorsal or ventral parts of the dorsal raphe, or in the density or morphology of the TPH2-positive serotonergic neurons, were seen between the WT and LXRβ$^{-/-}$ littermates. Given the crosstalk between the vmPFC and the amygdala and between the vmPFC and the dorsal raphe, it is possible that in LXRβ$^{-/-}$ mice, activation of the vmPFC inhibits the hyperactivity of the amygdala without interrupting the function of serotonergic neurons (49–51).

Because all anxiety disorder subtypes are more frequent in females (30) and the anxiolytic effect of fluoxetine is often ineffective in postmenopausal females (52), there is a critical need for a mouse model for use in the study of anxiety in female humans. In light of the altered GABAergic system in the vmPFC and the resulting anxiety phenotype, our findings indicate that LXRβ$^{-/-}$ female mice may provide such a model.

Materials and Methods

Animals and Housing. Ten groups of female LXRβ$^{-/-}$ and WT littermates, aged 3–4 mo, were used for the experiments. Each group comprised one WT mouse and one LXRβ$^{-/-}$ mouse. The mice were maintained on a 12-h light/12-h dark cycle (6:00 AM to 6:00 PM) in a barrier facility (Taconic). Food and water were available ad libitum. Testing was performed during the middle of the light cycle after the mice had been acclimated to the testing room for at least 30 min. After behavioral testing, all of the mice were anesthetized with Avertin (tribromoethanol) 0.06 mL/g i.p. and then perfused with PBS followed by 4% (wt/vol) paraformaldehyde in 0.1 M PBS (pH 7.4). All brains were collected and processed for paraffin sections (5 μm). All of the behavioral testing was done at Taconic's laboratories. All animal protocols were approved by the Taconic NY-NJ Institutional Animal Care and Use Committee. The animal care and use program complied with the standards and recommendations set forth in the National Research Council's 1996 Guide for the Care and Use of Laboratory Animals.

Behavioral Testing Equipment and Procedures. ***EPM test.*** The EPM test setup involves black Plexiglass in a cross/plus sign shape with a pair of 30-cm-long open arms surrounded by a short protective barrier of adjustable height (0.5 cm) and a pair of closed arms surrounded by 30-cm-high walls. The four arms meet at a 6 × 6 cm central platform. Four metal legs elevate the maze 50 cm above the floor. The maze is illuminated from the bottom by infrared light. The movements of the experimental animal are monitored by an infrared-sensitive video camera with filters to block visible light. The camera is fixed on the ceiling of the experimental room. Each camera is connected to a computer equipped with Videotrack software (Viewpoint Life Sciences) for data analysis. The time spent in the open arms and closed arms and the number of entries into the arms were determined using the Videotrack software.

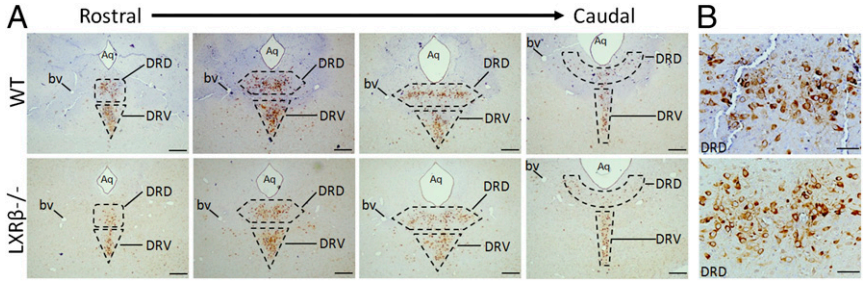

Fig. 6. Expression of TPH2 in the dorsal raphe in female WT and LXR$\beta^{-/-}$ littermates. (*A*) The dorsal raphe was completely sectioned from its rostral margin to its caudal margin. Sections were selected according to the position and size of blood vessels and morphology of the cerebral aqueduct. No significant differences in the dorsal or ventral parts of the dorsal raphe were seen between WT and LXR$\beta^{-/-}$ littermates. (*B*) There was no significant difference in density or cell morphology in the caudal part of the dorsal raphe nucleus between WT and LXR$\beta^{-/-}$ littermates. Aq, cerebral aqueduct; bv, blood vessel; DRD, dorsal raphe nucleus, caudal part; DRV, dorsal raphe nucleus, ventral part. (Scale bars: *A*, 200 μm; *B*, 50 μm.)

Startle and prepulse Inhibition test. In this test, the mice were weighed and placed in the animal enclosures of sound-attenuating chambers (StartleMonitor; Kinder Scientific). The effects on baseline startle response were tested on the first day. The mice were allowed to acclimate for another 5 min inside the chambers and were then presented with six 40-msec acoustic stimuli of varying intensity (0, 80, 90, 100, 110, and 120 dB). A total of 30 trials were presented in a pseudorandomized manner with an average intertribal interval (ITI) of 15 s (range, 10–20 s). Background noise of 70 dB was presented during the ITI. The StartleMonitor system and computer recorded the average amplitude of the startle of mice within a 150-msec window after each of the acoustic stimuli. The two groups of mice were compared using ANOVA with Bonferroni post hoc analysis. The mice were returned to their cages after the first day.

The effects on prepulse inhibition were examined the next day. The mice were acclimated to the chamber for 5 min and then presented with five different trials: no stimulus, with only background (70 dB) noise; a 120-dB pulse alone; and prepulses of 3, 6, or 12 dB above background followed by a 120-dB pulse. Each prepulse lasted 20 msec and preceded the 40-msec 120-dB pulse by 100 msec. The test session was presented in 12 blocks of 10 trials each. The first and last blocks consisted of 10 presentations of 120-dB pulses at an average ITI of 15 s (range, 10–20 s). Each of the remaining blocks (2–11) consisted of two presentations of each pulse type (no stimulation, a 120-dB pulse alone, or prepulses of 3, 6, or 12 dB above background followed by a 120-dB pulse) presented in a pseudorandom manner at an average ITI of 15 s (range, 10–20 s).

Open field test. The open field test was performed in a custom-made testing apparatus comprising 50 × 50 cm chambers. The experiment was recorded and tracked using a Viewpoint tracking system. The time and path length in the center of the open field were determined. The center of the open field was defined as a 13.5 × 13.5 cm square in the geometric center of the arena. The percentage of path in the center was calculated using the following formula: path length in the center/total path length × 100%. For each mouse, the total path length and path length for 60 min at 5-min intervals were determined as measures of locomotor activity. Each chamber was cleaned between individual mouse testing.

Novel object recognition test. The novel object recognition test was performed in a 50 cm × 50 cm open field arena made of white Plexiglas. The mouse's nose was tracked using a Viewpoint tracking system. Exploration time was recorded for an object if the mouse's nose touched or was within 2 cm of the object. All pairs of objects were placed in the opposite quadrants of the open field 5 cm from each wall. To evaluate nonspatial memory, the following parameters were recorded: e1, total time spent exploring objects in trial 1; e2, total time spent exploring objects in trial 2; discrimination index (D2 index), calculated as time spent exploring a novel object − time spent exploring a familiar object)/e2; and percent preference, calculated as time spent exploring a novel object/time spent exploring a familiar object) × 100. Superior performance on the test was demonstrated by a higher D2 index and percent preference values. The objects used in the study were plastic culture flasks, toothbrush holders, and salt and pepper shakers. Each mouse was handled for 2 min on the day before the test. On the day of the test, the mouse was acclimated to the testing room for at least 30 min before testing. For this acclimation, the mouse was placed in the open field with two objects placed in the opposite quadrants. The objects used for acclimation were not used in trial 1 or 2 of the test. After 20 min of acclimation, the mouse was removed from the open field and placed in its home cage.

For trial 1, the mouse was once again placed in the open field with two similar objects (familiar objects) in the opposite quadrants of the open field for 20 min, and the time spent exploring each object was recorded (e1). The mouse was then removed from the open field arena and returned to its home cage. For trial 2, after a retention interval of 30 min, the mouse was placed back in the open field with one copy of the object presented in trial 1 and one novel object that the mouse had not been exposed to previously. To avoid olfactory cues, a third copy of the familiar object was used in trial 2. The time spent exploring the familiar and novel object, along with the total time spent exploring the objects (e2), were recorded.

Immunohistochemistry. Paraffin sections were deparaffinized in xylene, rehydrated through graded alcohol, and processed for antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 2–3 min. The sections were incubated in 1% H_2O_2 in PBS for 15 min at room temperature to quench endogenous peroxidase. To block nonspecific binding, sections were incubated in 3% BSA for 10 min, after which a biotin blocking system (Dako) was used to block endogenous biotin. Sections were then incubated with rabbit anti-TPH (1:1,000 dilution; Millipore) and rabbit anti-GAD(65+67) (1:500 dilution; Abcam) in 1% BSA overnight at room temperature. BSA replaced the primary antibodies in the negative controls. After washing, sections were incubated with the corresponding secondary antibodies using a rabbit-on-rodent HRP-polymer kit (RMR622; Biocare Medical) for 15–20 min at room temperature, followed by 3,3′-diaminobenzidine tetrahydrochloride as a chromogen.

Data analysis. The percent of entries into the open arms in the EPM test was calculated using the following formula: number of entries in the open arm/total number of entries × 100%. Data were compared between groups using the unpaired *t* test. ANOVA with Bonferroni post hoc analysis was used to compare the effect of three prepulses on the startle amplitude and the extent of the prepulse inhibition. The path length for 60 min at 5-min intervals was analyzed by repeated-measures ANOVA using SPSS software, and all other parameters were compared using one-way ANOVA in the open field test. The discrimination index and percent preference were compared between the groups using the unpaired *t* test. An outlier was defined as a value 2 SDs less than or greater than the mean of the group in the novel object recognition test. MicroSuite Basic Edition (Olympus) and Image-Pro Plus 6.0 (Media Cybernetics) were used for the analysis of GAD(65+67). Data are presented as mean ± SD. The statistical significance of differences between the WT and LXR$\beta^{-/-}$ mice was assessed using the unpaired *t* test.

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