ORIGINAL ARTICLE

Dentate nNOS accounts for stress-induced 5-HT$_{1A}$ receptor deficiency: Implication in anxiety behaviors

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Abstract

Background: Anxiety is a common disorder with high social burden worldwide. Dysfunction of serotonin-$_{1A}$ receptor (5-HT$_{1A}$ receptor) in the dentate gyrus (DG) of the hippocampus has been predominantly implicated in the anxiety behavior. However, the molecular mechanism underlying the deficiency of postsynaptic 5-HT$_{1A}$ receptor in regulating anxiety behavior remains unclear.

Methods: Using pharmacological and genetic methods, we investigated the role of detate nNOS in 5-HT$_{1A}$ receptor decline and anxiety behavior induced by chronic mild stress (CMS) in mice.

Results: Here we showed that local elevation of glucocorticoids in the DG accounted for chronic stress-induced anxiety behavior. Neuronal nitric oxide synthase (nNOS) mediated chronic stress-induced downregulation of 5-HT$_{1A}$ receptor in the DG through peroxynitrite anion (ONOO•) pathway but not cyclic guanosine monophosphate (cGMP) pathway. By using pharmacological tool drugs and nNOS knockout mice, we found that nNOS in the DG played a key role in chronic stress-induced anxiety behavior.

Conclusions: These findings uncovered an important role of nNOS-5-HT$_{1A}$ receptor pathway in the DG of the hippocampus in chronic stress-induced anxiety. Accordingly, we developed a "dentate nNOS-5-HT$_{1A}$ receptor closed-loop" theory (stress-glucocorticoids-nNOS-Nitric oxide-ONOO•-5-HT$_{1A}$ receptor -nNOS) of stress-related anxiety.

KEYWORDS
5-HT$_{1A}$ receptor, anxiety, chronic stress, glucocorticoids, neuronal nitric oxide synthase

1 | INTRODUCTION

Anxiety disorders are among the most disabling of all medical disorders.[1] It has been well demonstrated that serotonin (5-HT) neurotransmission plays important roles in modulation of numerous diseases.[2,3] These functions are mediated by at least 15 well identified different 5-HT receptors.[4-6] Among these receptors, 5-HT$_{1A}$ receptor, in particular, is proved to be implicated in the etiology of anxiety disorders.[7] Several lines of evidence supported that 5-HT$_{1A}$ receptor level in the hippocampus of patients with anxiety disorders is in deficit and that partial 5-HT$_{1A}$ receptor agonists are anxiolytics.[8-11] Moreover, 5-HT$_{1A}$ receptor knockout mice represent a genetic animal model of anxiety.[7,12]

Reportedly, stress is closely correlated with depression and anxiety.[13] Chronic stress increases corticosterone (CORT, glucocorticoids in rodents), decreases 5-HT$_{1A}$ receptor in the dentate gyrus (DG), and gives rise to anxiety-related behaviors.[14,15] Hence, glucocorticoids may be the predominant substance that mediates the impact of stress on the hippocampus in the development of anxiety. However, the molecular mechanisms underlying stress and glucocorticoids-induced decrease in expression of hippocampal 5-HT$_{1A}$ receptor are still unclear.

Neuronal nitric oxide synthase (nNOS) is enriched in the hippocampus.[16] Our previous study revealed that chronic stress upregulates the level and activity of hippocampal nNOS and thus contributes to stress-induced depressive behaviors.[17] There are some reports that nNOS inhibitors produce anxiolytic-like effects, and 5-HT system is dysfunctional in nNOS knockout mice.[18-20] However, it remains unknown whether nNOS-NO pathway contributes to 5-HT$_{1A}$ receptor deficiency in the development of anxiety. In physiological condition, NO functions through the soluble guanylate cyclase/cyclic guanosine monophosphate/protein kinase G (sGC/cGMP/PKG) pathway.[17,21] Besides, excessive NO, synthesized by increased nNOS, may react with O2· and thereby form peroxynitrite anion (ONOO•). Both NO itself and ONOO• can regulate a variety of molecular expression and function.[17,21-23] Importantly, it was found that sodium nitroprusside (SNP), a type of NO donors, causes anxiogenic effect at high doses but exerts anxiolytic property at low doses, implicating ONOO• in the induction of anxiety behaviors.[24-26] Here, using genetic and pharmacological methods, we investigated the link among glucocorticoids, nNOS, and 5-HT$_{1A}$ receptor. We report that nNOS-NO-ONOO• pathway accounts for the high level of glucocorticoids-induced decline in 5-HT$_{1A}$ receptor in the DG of the hippocampus and probably contributes to anxiety behavior, uncovering a novel molecular mechanism for anxiety pathogenesis.

2 | MATERIALS AND METHODS

2.1 | Animals

Young adult (6- to 7-week-old) male homozygous nNOS-deficient mice (B6.129S4-Nos1^{tm1Plh}/J, KO, stock number: 002986) and their wild-type controls (C57BL/6J, WT, stock number: 000664) (both from The Jackson Laboratory; maintained at Model Animal Research Center of Nanjing University, Nanjing, China), young adult (6- to 8-week-old) ICR mice were used for behavioral analysis. Littermates were used in a same individual experiment in this study. Animals were housed in an air-conditioned room (20 ± 2°C), with food and water ad libitum, except when specified otherwise. Five mice were housed in each cage with 12 hours light/dark cycle. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (protocol number: IACUC-1704010). Every effort was made to minimize the number of animals used and their suffering.

2.2 | Drugs

For generating consistent elevated plasma level of CORT in mice, CORT (20 mg/kg/d) were injected subcutaneously. We selected the dosage due to that a low dose of CORT treatment induced anxiety-like phenotype in mice.[27] To block the synthesis of CORT continuously, we treated mice subcutaneously with metyrapone (100 mg/kg/d), the dosage of which was proved blocking the synthesis of CORT.[17] To know the systemic effect of 7-nitroindazole (7-NI), 7-NI was intraperitoneally injected at a dosage of 30 mg/kg/d which produced antidepressant-like effect in mice.[28] To study the effect of drugs in local sites, stereotaxic injection was used to deliver 10 μM corticosterone (CORT; 2 μL; Sigma-Aldrich), 1 mM 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN-1; 2μl; Sigma-Aldrich), 10 μM 7-NI (2 μl; Sigma-Aldrich), or 20 μM water-soluble carboxy-2-phenyl-4,4,5,5-tetramethylimidazolineoxyl-1-oxyl-3-oxide (cPTIO; 2 μL; Sigma-Aldrich) into the bilateral DGs of the hippocampi. The designated drug solution (vehicle) in 2 μL of volume was microinjected (0.2 μL/min) into control mice. To avoid backflow of injected chemicals, the needle was kept in the injection site for 5 minutes before withdrawing of the needle. Diethylenetriamine NONOate (DETA/NONOate) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and spironolactone were purchased from Tocris Bioscience. For stereotaxic surgery, adult mice were anesthetized with 0.07 mL of a mixture of ketamine (90.9 mg/mL) and xylazine (9.1 mg/mL).

2.3 | Chronic unpredicted mild stress

The procedure of chronic unpredictable mild stress (CUMS) was designed as described previously.[28] Briefly, the CUMS protocol consists of the sequential application of a variety of mild stressors, including restraint in tubes, forced swimming in cold water, water and/or food deprivation, and pairing with another stressed animal in wet sawdust, reversal of the light/dark cycle, housing in wet sawdust, housing in constant illumination or darkness each for a period ranging from

10 minutes to 24 hours, in a schedule that lasts for 3 weeks. The schedule of stressors in the protocol is unpredicted to mice.

2.4 | Behavioral measurements

For the open-field test (OFT), the test apparatus was constructed of a plastic plate (72 × 72 cm). The under plate was into 256 squares by lines drawn on the plate. The plate was surrounded by a 35.18-cm-high plastic wall. The 64 squares in the center of the test arena were referred to as the central squares (36 × 36 cm). Each mouse was placed on a corner square of the arena, facing the corner, and allowed to freely explore the open field for 5 minutes per trial, recorded by Motor-Monitor System SF16R. In the system, the squares are recognized and analyzed automatically. No stressor was applied to the animals for at least 12 hours before the test. After each trial, the plate was cleaned with 70% ethyl alcohol (EtOH). Mobility was scored when an animal crossed a sector border with both its hindlimbs. Parameters assessed were the number of square crossings (horizontal) and the times of standing (vertical) during the 5 minutes test. The value of parameters of each tested mouse was calculated by the Motor-Monitor System SF16R automatically after tests. All the mice were measured about 24 hours after last injection of drugs. The experimental environment was evenly illuminated with white light (25 lux). Animals were group housed in a 12-hour dark, 12-hour light cycle and tests were performed during the light phase. Experimenter was blind as to the genotype of the mice in the behavior experiments.

2.5 | Corticosterone level measurement

For measurement of CORT level, all mice were decapitated between 9:00 and 11:00 AM. Blood from angulus oculi vessels was collected in heparinized tubes, and CORT in plasma was measured with a CORT ELISA Kit according to the instructions of the manufacturer (Cayman Chemical Company). The detection limit of this kit is approximately 40 pg/mL. Mice were euthanized for plasma collection at least 15 hours after the end of behavior test.[29]

2.6 | Cell culture

Primary hippocampal neurons were cultured as reported previously with minor modifications. In brief, the hippocampi of embryonic day 18 mice were freshly isolated and enzymatically dissociated in calcium- and magnesium-free HBSS containing 0.125% trypsin at 37°C for 10 min. Then, the digestion was terminated with DMEM/F12 (1:1) containing 10% FBS. Dissociated cells were centrifuged, resuspended in Neurobasal medium (Invitrogen) containing 2% B27 supplement, 0.5 mM L-glutamine, 5 IU penicillin, and 5 μg/mL streptomycin, and plated on 10 μg/mL polyornithine-coated dishes (diameter, 3.5 cm) at 5×10^4 cells/cm^2. Half

of the medium was replaced with fresh Neurobasal/B27 medium every 2-3 days.

2.7 | Western blot analysis

Western bolt analysis of samples from cultured hippocampal neurons and hippocampal tissues of animals was performed as described previously.[17] In this study, we dissected the DG from the hippocampus for Western blot. The primary antibodies were as follows: rabbit anti-nNOS (1:1000; Millipore Bioscience Research Reagents), mouse anti-nitrotyrosine (1:3000; Millipore Bioscience Research Reagents), and rabbit anti-5-HT$_{1A}$ receptor (1:500; Santa Cruz Biotechnology). Appropriate horseradish peroxidase-linked secondary antibodies were used for detection by enhanced chemilumi-nescence (Pierce; Thermo Fisher Scientific). When we prepared the samples, all the hippocampus was checked to find the needle track of infusion. Only the samples with obvious needle track of Alzet osmotic minipumps or infusion needles were remained for further measurement.

2.8 | RNA extraction and reverse transcription-PCR

Total RNA was extracted from the DG of the hippocampus using TRIzol reagent according to the manufacture's instructions (Sigma-Aldrich). The primers for 5-HT$_{1A}$ receptor and GAPDH were as follows: for 5-HT$_{1A}$ receptor: Forward 5′-GACTGCCACCCTCTGCCCTATATC-3′ and Reverse 5′-TCAGCAAGGCAAACAATTCCAG-3′; For GAPDH: Forward 5′-CAAGGTCATCCATGACAACTTTG-3′ and Reverse 5′-GTCCACCACCCTGTTGCTGTAG-3′. PCR conditions were 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 45 seconds. PCR products were separated by electrophoresis through 1.5% agarose gel containing 0.5% μg/mL ethidium bromide and imaged using a BioDoc-IT imaging system (Bio-Rad); band intensities were determined using GS-710 calibrated imaging Densitometer (Bio-Rad). The mRNA for GAPDH was included in the PCR mixture as a standard.

2.9 | Microinjection

Adult mice were anesthetized with 0.07 mL of a mixture of ketamine (90.9 mg/mL) and xylazine (9.1 mg/mL) and placed in a stereotaxic apparatus (Stoelting). The drug solutions in 2 μL volume were microinjected into the DGs (0.2 μL/min) at coordinates 2.3 mm posterior to bregma, 1.3 mm lateral to the midline, and 2.0 mm below dura. Osmotic pump was used for continuous delivery of drug. For the osmotic pump implantation, two 7d or 28d Alzet osmotic minipumps (DURECT Corporation) containing cPTIO solution were placed subcutaneously in the back of mice, and two brain infusion cannulas connected to the pump were positioned at the following coordinates: 2.3 mm posterior to bregma, 1.3 mm lateral to the midline, and 2.0 mm below dura. The infusion rate of the osmotic pump was 0.25 μL/h. We anesthetized
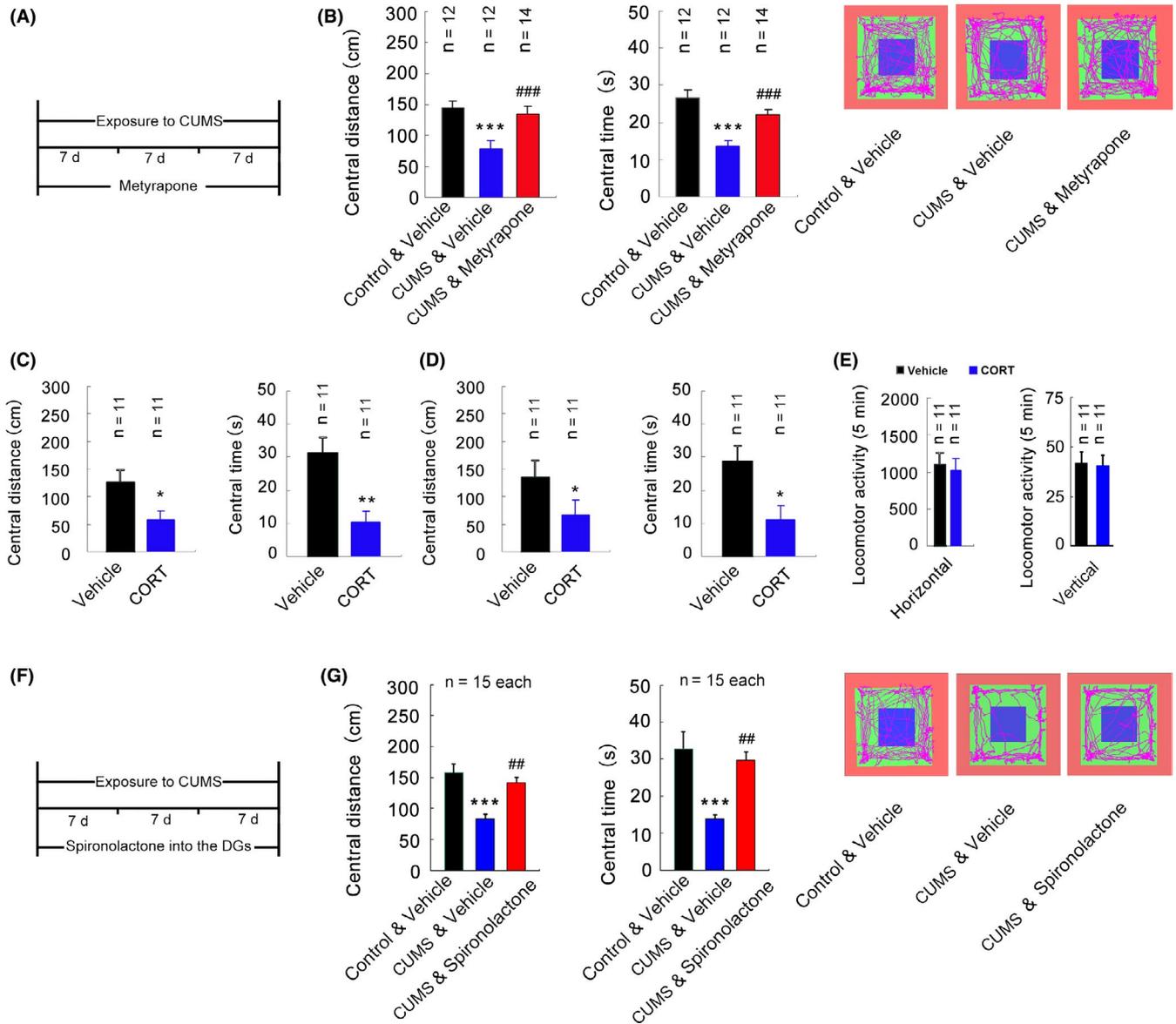
FIGURE 1 Elevated level of glucocorticoids in the DG causes anxiety behavior. (A) Schedule designed. Mice were administrated with or without metyrapone (100 mg/kg/d, s.c., 1 time/d, 21 d) during 21 d of exposure to CUMS. (B) Central distance (left) and central time (middle) spent by mice in the OFT after 21-d CUMS with or without metyrapone administration (100 mg/kg, s.c., 1 time/d, 21 d). Representative tracks of mice in each group in the OFT are shown on the right. (C) Central distance (left) and central time (right) spent by mice in the OFT after 21 d CORT treatment (20 mg/kg, s.c. 1 time/d). (D) Central distance (left) and central time (right) spent by mice in the OFT 21 days after infusion of CORT (10 μM, 2 μL) into the DG. (E) The locomotor activity of mice in the OFT 21 days after infusion of CORT into the DG. Parameters assessed were the number of square crossings (horizontal) and the times of standing (vertical). (F) Schedule designed. Chronic continuouse delivery of spironolactone (50 μM, 0.25 μL/h) into the DGs using osmotic minipumps during 21 d of exposure to CUMS. (G) Central distance (left) and central time (middle) spent by mice in the OFT. Representative tracks of mice in each group in the OFT are shown on the right. Mean ± SEM, *$P < .05$, **$P < .01$, ***$P < .001$, compared with Vehicle, #$P < .05$, ##$P < .01$, ###$P < .001$, compared with CUMS + Vehicle in B and G, one-way ANOVA for B and G. Student's t test for C, D, and E

mice with a mixture of ketamine (90.9 mg/mL) and xylazine (9.1 mg/mL) and removed the osmotic pumps 1 day before behavioral tests.

2.10 | Statistical analysis

Statistical analyses were performed using commercial software (GraphPad Prism; GraphPad Software Inc) Data are presented as Means ± SEM. To compare 3 or more independent groups passed normality test, evaluated by Shapiro-Wilk normality test, were analyzed by one-way ANOVA or two-way ANOVA analysis. Comparisons among multiple groups were made by Bonferroni's post hoc test. To compare 3 or more independent groups which did not pass normality test, Kruskal-Wallis tests with Dunn's multiple comparison corrections were used. To compare the effect of two factors with respect to abnormal distribution, we used two-way ANOVA with Bonferroni's

multiple comparison corrections. Comparisons between two groups were made with two-tail Student's t test. To compare two independent groups with respect to abnormal distribution, Mann-Whitney U tests were used. For all results, differences were considered significant when $P < .05$.

3 | RESULTS

3.1 | Elevated glucocorticoids in the hippocampus induce anxiety behaviors

CUMS paradigms, modeling the stressful life events, are applied as a typical model of depression and also induce anxiety behavior changes in rodents such as reduced time spent in open arm in elevated plus-maze test, decreased time entering in central place in the OFT[30-32]. To know the role of glucocorticoids in stress-induced anxiety-like behaviors, we used metyrapone (at daily dose of 100 mg/kg, s.c. during 21-day CUMS exposure, Figure 1A), a corticosteroids synthesis inhibitor,[17] to block CORT synthesis and to test behavioral changes in the OFT. In consistent with our previous study, the mice, which underwent 21-day CUMS, possessed a markedly a higher concentration of CORT in the plasma ($F_{2,14}$ = 28.98, $P < .001$; Vehicle: 73.59 ± 21.90 ng/mL; CUMS + Vehicle: 236.40 ± 55.80 ng/mL; Control + Vehicle vs. CUMS + Vehicle: $P < .001$, n = 5-6, one-way ANOVA) and exhibited reduced central distance and central time in the OFT (Figure 1B, central distance: $F_{2,35}$ = 36.90, $P < .001$, Control + Vehicle vs. CUMS + Vehicle: $P < .05$; central time: $F_{2,35}$ = 35.24, $P < .001$, CUMS + Vehicle vs. Control + Vehicle vs. CUMS + Vehicle: $P < .05$), reflecting anxiety phenotype in mice. Indeed, metyrapone treatment blocked the increase in CUMS-induced CORT synthesis ($F_{2,14}$ = 28.98, $P < .001$; CUMS + Vehicle: 236.40 ± 55.80 ng/mL; CUMS + Metyrapone: 77.64 ± 30.09 ng/mL; CUMS + Vehicle vs. CUMS + Metyrapone: $P < .001$. n = 5-6, one-way ANOVA). Importantly, metyrapone normalized the central distance and central time spent by the mice exposed to CUMS in the OFT (Figure 1B, central distance: $F_{2,35}$ = 36.90, $P < .001$, CUMS + Vehicle vs. CUMS + Metyrapone: $P < .05$; central time: $F_{2,35}$ = 35.24, $P < .001$, CUMS + Vehicle vs. CUMS + Metyrapone: $P < .05$). Additionally, all treatments did not change the locomotor activity of mice (data not shown). Together, these results indicate that the elevated level of CORT in the body accounts for chronic stress exposure-induced anxiety behavior.

To further investigate whether high level of glucocorticoids per se can directly induce anxiety-like behavior, we treated mice with CORT (20 mg/kg, s.c., once per day) for 21 days and found that chronic administration with CORT caused a significant decrease in central distance and central time spent by the mice in the OFT (Figure 1C, Student's t test, central distance: $P < .05$; central time: $P < .01$). However, treatments did not change the locomotor activity of mice (data not shown). Next, to study whether local elevation of CORT in the DG induce anxiety-like behavior, we microinjected CORT (10 μM, 2 μL) into the bilateral DGs of the hippocampi and measured the anxiety-related behaviors 21 days later. As expected,

CORT microinjection resulted in a significant decrease in central distance and central time spent by the mice in open-field test (Figure 1D, Student's t test, central distance: $P < .05$; central time: $P < .05$) but did not change the locomotor activity (Figure 1E, Student's t test, Horizontal: $P > .05$; Vertical: $P > .05$). To know whether blocking the function of corticosteroids locally in bilateral DGs can affect the effect of stress on anxiety behavior, we infused the glucocorticoids receptor antagonist, spironolactone (50 μM, 0.25 μL/h), into the DGs using osmotic minipumps during CUMS. Local administration of spironolactone into the DGs reversed CUMS-induced decreased central distance and central time spent by the mice exposed to CUMS in the OFT (Figure 1B, central distance: $F_{2,42}$ = 12.73, $P < .001$, CUMS + Vehicle vs. CUMS + Spironolactone: $P < .01$; central time: $F_{2,42}$ = 2.407, $P < .001$, CUMS + Vehicle vs. CUMS + Spironolactone: $P < .01$). Collectively, these data demonstrate that elevated glucocorticoids in the DG of the hippocampus play a critical role in the induction of stress-related anxiety behavior.

3.2 | Hippocampal nNOS mediates the glucocorticoids-induced 5-HT$_{1A}$ receptor expression decline

Downregulation of 5-HT$_{1A}$ receptor in DG granule cells of the hippocampus is essential for induction of anxiety.[33] By Western blot and RT-PCR methods, we found that a high dose of CORT (20 mg/kg, s.c., 5 days) but not lower doses (10 and 5 mg/kg, s.c., 5 days) of CORT significantly reduced the protein and mRNA level of 5-HT$_{1A}$ receptor in the DG of the hippocampus compared with vehicle-treatment group (Figure 2A, one-way ANOVA, Western blot: $F_{3,8}$ = 10.36, $P < .01$, n = 3; RT-PCR: $F_{3,8}$ = 11.55, $P < .01$, n = 3). Notably, persistent inhibition of 5-HT$_{1A}$ receptor protein expression in the DG was also observed after long-term administration of CORT (20 mg/kg, s.c., 21 days, Figure 2B, Western blot: Student's t test, $P < .05$, n = 4).

To know whether nNOS is involved in the regulation of 5-HT$_{1A}$ receptor by stress and glucocorticoids, first, we treated mice with 7-NI (30 mg/kg, i.p., 5 days), a selective nNOS activity inhibitor,[28] and found that hippocampal nNOS negatively regulated both the protein and mRNA level of 5-HT$_{1A}$ receptor in the DG (Figure 2C, Student's t test, Western blot: $P < .05$, n = 3, RT-PCR: $P < .05$, n = 3). Second, we selectively infused 7-NI (10 μM, 2 μL) into the bilateral DGs of the hippocampi followed by high concentration of CORT administration (20 mg/kg, s.c., Figure 2D) or CUMS exposure for 5 days (Figure 2E). The results indicated that inhibition of nNOS catalytic function in the DG blocked the negative regulation of 5-HT$_{1A}$ receptor expression in the hippocampus by CORT (Figure 2D, one-way ANOVA, $F_{2,6}$ = 70.44, $P < .001$, n = 3) and CUMS (Figure 2E, one-way ANOVA, $F_{2,6}$ = 10.89, $P < .05$, n = 3). The precision of the coordinates used for local infusion into the DG was validated by microinjection of DiIC18(3) (DiI), a type of red color dye, into the DG (data not shown). Moreover, we found that CORT administration (20 mg/kg, s.c., 21 days) significantly reduced the hippocampal 5-HT$_{1A}$ receptor expression in wild-type
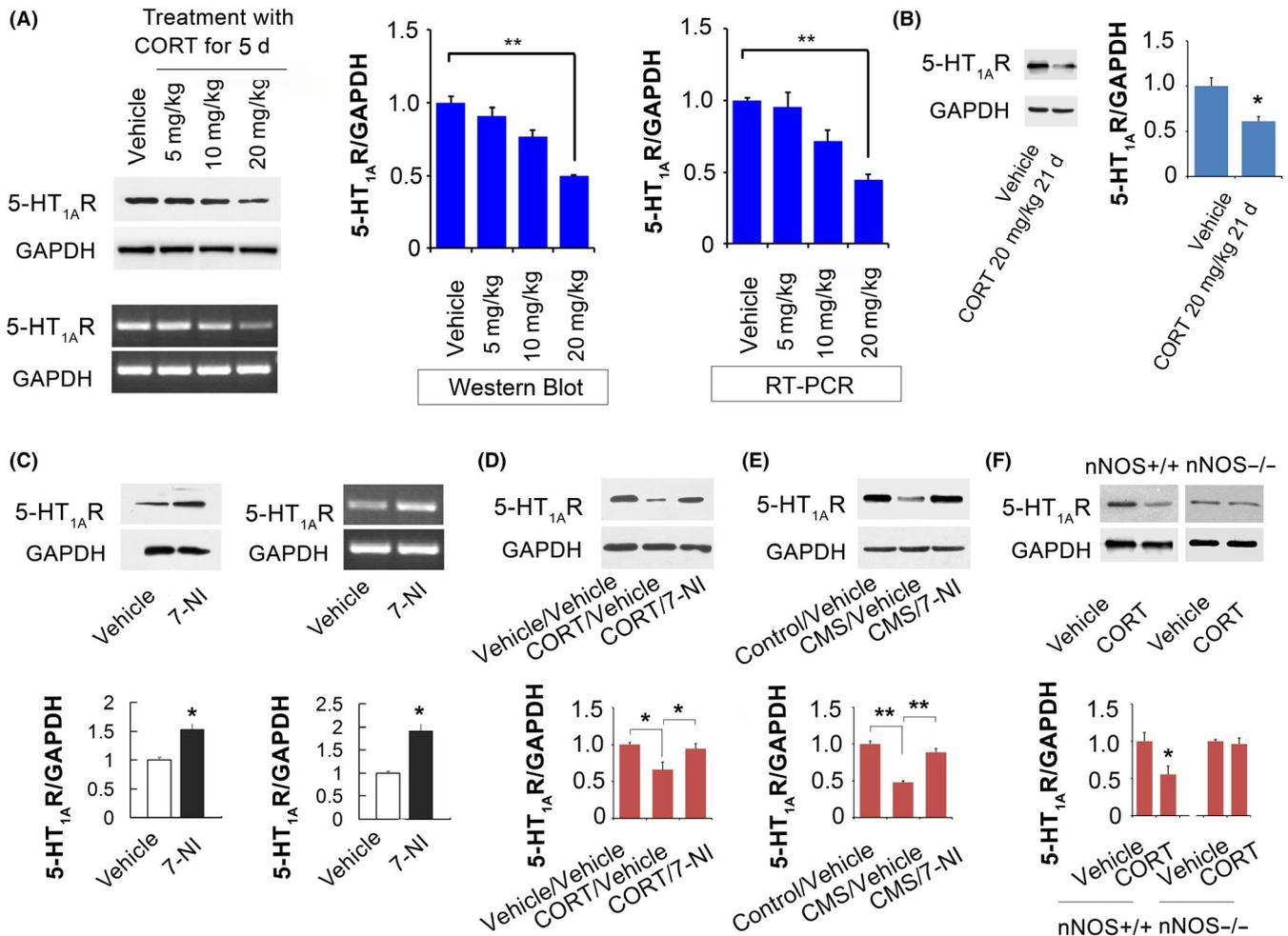
FIGURE 2 Dentate nNOS accounts for high concentration of glucocorticoids-induced 5-HT$_{1A}$ receptor expression decline. (A) Representative protein (upper) and mRNA level (lower) of 5-HT$_{1A}$ receptor and GAPDH in the DG of mice treated with different concentration of CORT (5, 10, and 20 mg/kg, s.c., 1 time per day) and vehicle for 5 d. The corresponding bar graphs are on the right side. (B) Representative Western blot of 5-HT$_{1A}$ receptor and GAPDH in the DG of mice treated with high concentration of CORT (20 mg/kg, s.c., 1 time per day) and vehicle for 21 d. Bar graph shows the analysis. (C) Representative Western blot (left) and RT-PCR (right) of 5-HT$_{1A}$ receptor and GAPDH in the DG of mice treated with 7-NI (10 mg/kg, i.p., 1 time per day) and vehicle for 5 d. Bar graphs show the analysis. (D) Representative Western blot of 5-HT$_{1A}$ receptor and GAPDH in the DG of mice treated with CORT (20 mg/kg, s.c., 1 time per day) and vehicle for 5 d with or without 7-NI infusion into the DG the day before the beginning of CORT administration. Bar graph shows the analysis. (E) Representative Western blot of 5-HT$_{1A}$ receptor and GAPDH in the DG of mice exposed to CUMS for 5 d with or without 7-NI infusion into the DG the day before the beginning of CUMS. Bar graph shows the analysis. (F) Representative Western blot and bar graph analysis of 5-HT$_{1A}$ receptor expression in the hippocampus of nNOS−/− and nNOS+/+ (WT) mice treated with CORT (20 mg/kg, s.c., 1 time per day) and vehicle for 21 d. Mean ± SEM, $*P < .05$, $**P < .01$. One-way ANOVA for A, D, and E. Two-way ANOVA for F. Student's t test for B and C

(WT) mice (Figure 2F, Student's t test, $P < .05$, n = 5) but not in nNOS−/− mice (Figure 2F, Student's t test, $P > .05$, n = 5). Together, nNOS plays a crucial role in the stress or glucocorticoids-induced 5-HT$_{1A}$ receptor expression decline in the DG of the hippocampus.

3.3 | nNOS downregulates 5-HT$_{1A}$ receptor expression via ONOO•

NO mediates the main biological function of nNOS.[22] To determine the role of NO in the regulation of 5-HT$_{1A}$ receptor by CORT, we cleared endogenous NO by cPTIO, a tool drug that directly

extinguishes NO generated by NO synthase (NOS) without affecting NOS activity.[17] The cPTIO was infused into the DGs via osmotic minipumps (Alzet, 20 μM, 0.25 μL/h) at day 1, followed by 5 days CORT administration (20 mg/kg, s.c., once per day). Western blots analysis showed that hippocampal 5-HT$_{1A}$ receptor expression remained unchanged after CORT exposure together with NO clearance compared with vehicle exposure, while CORT administration without NO clearance significantly reduced the expression of 5-HT$_{1A}$ receptor expression in the DG (Figure 3A, one-way ANOVA, $F_{2,6} = 9.34$, $P < .05$, n = 3). Next, the cPTIO was infused into the DGs via osmotic minipumps at day 1 (20 μM, 0.25 μL/h) followed by CORT administration (20 mg/kg, s.c., once per day, 21 days). Interestingly,
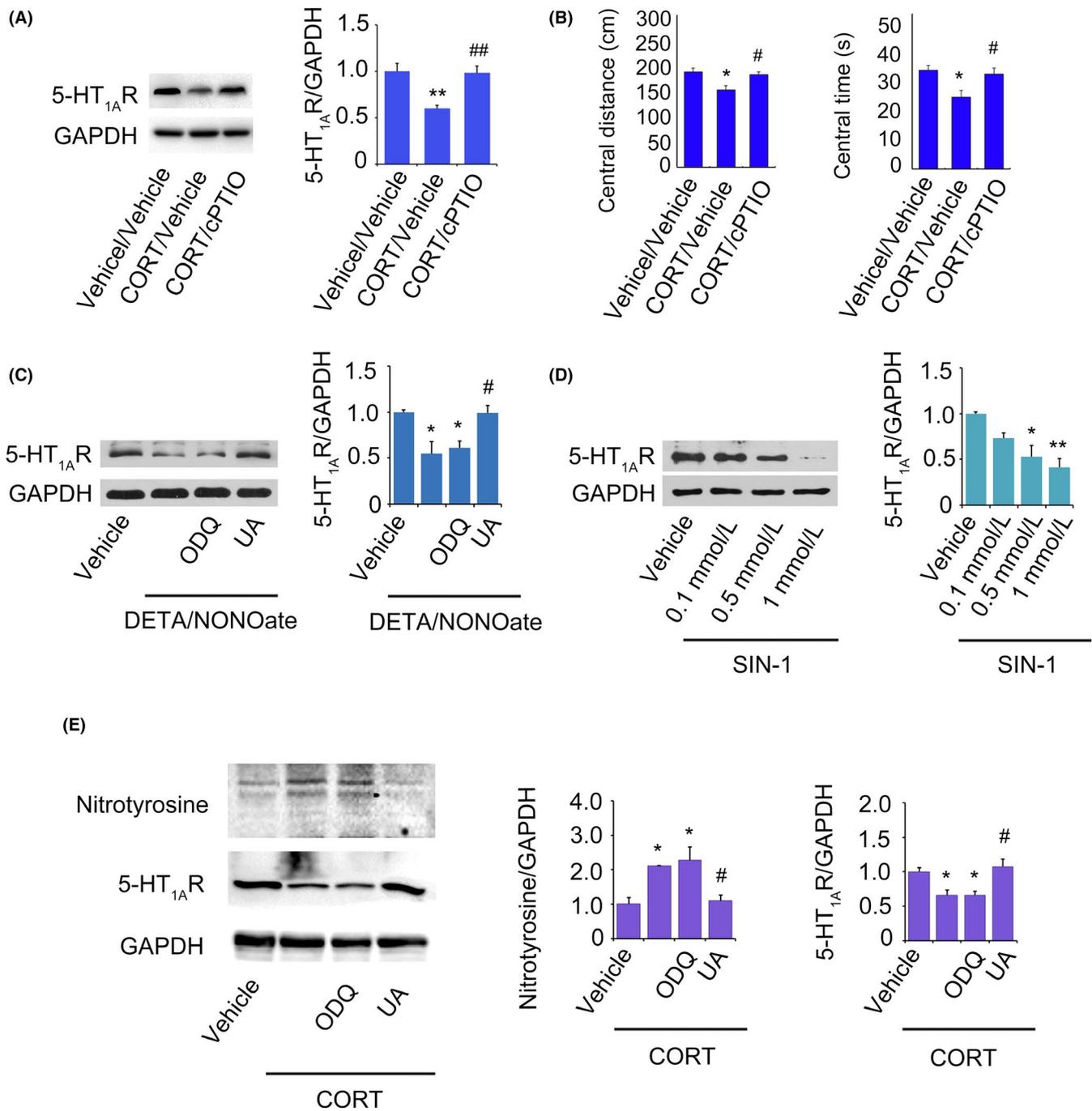
FIGURE 3 ONOO• pathway is implicated in the negative modulation of 5-HT$_{1A}$ receptor expression by nNOS. (A) Representative Western blot and bar graph analysis of 5-HT$_{1A}$ receptor in the hippocampus of mice administrated with CORT (20 mg/kg, s.c., 5 d, 1 time per day) and vehicle with or without cPTIO or vehicle infusion into the DGs. (B) Central distance (left) and central time (right) spent by mice in the open-field test after CORT (20 mg/kg, s.c., 21 d, 1 time per day) and vehicle administration with or without cPTIO or vehicle infusion. (C) Representative Western blot of 5-HT$_{1A}$ receptor and GAPDH in cultured hippocampal neurons incubated with DETA/NONOate and vehicle together with or without ODQ or UA or vehicle. Measurement is from 4 different samples. (D) Representative Western blot of 5-HT$_{1A}$ receptor in cultured hippocampal neurons incubated with different concentration of SIN-1 and vehicle. Measurement is from 3 different samples. (E) Representative Western blot of nitrotyrosine and 5-HT$_{1A}$ receptor in hippocampal neurons incubated with CORT and vehicle together with or without ODQ or UA or vehicle. Measurement is from 3 different samples. Mean ± SEM, *$P < .05$, **$P < .01$, compared with vehicle or vehicle/vehicle; #$P < .05$, ##$P < .01$, compared with CORT/Vehicle in A and B, DETA/NONOate in C, CORT in E, one-way ANOVA

consistently, chronic CORT exposure (20 mg/kg, s.c., 1 time per day) did not induce anxiety behaviors after hippocampal NO clearance by cPTIO (Figure 3B, one-way ANOVA, central distance: $F_{2,30} = 7.60$, $P < .01$; central time: $F_{2,30} = 5.53$, $P < .01$, n = 10-13).

The sGC-cGMP-PKG pathway and reactive peroxynitrite anion (ONOO•) are the two major physiological signaling mechanisms of NO bioactivity.[21] The question is how NO regulates 5-HT$_{1A}$ receptor expression? To know this, we incubated hippocampal neurons with high

concentration of DETA/NONOate (100 μM), a NO donor, in combination with ODQ (10 μM), an inhibitor of the sGC-cGMP-PKG pathway, or uric acid (UA, 1.0 mM), a natural scavenger of ONOO•,[17] at DIV14 for 24 hours. As shown in Figure 3C, DETA/NONOate markedly reduced the expression of 5-HT$_{1A}$ receptor, which was reversed by UA but not ODQ (one-way ANOVA, $F_{3,12}$ = 8.22, P < .01, n = 4, DETA/NONOate vs. DETA/NONOate & ODQ, P > .05; DETA/NONOate vs. DETA/NONOate & UA, P < .05), suggesting that the production of ONOO• accounts for the decrease in 5-HT$_{1A}$ receptor induced by DETA/NONOate. To further confirm the effect of ONOO• on 5-HT$_{1A}$ receptor, we applied SIN-1 (0.1, 0.5, and 1 mM), an "NO donor" that generates NO and superoxide and then react to produce a large amount of ONOO• in cultured hippocampal neurons at DIV 14 for 24 hours. As expected, the expression of 5-HT$_{1A}$ receptor was diminished by SIN-1 in a dose-dependent manner (Figure 3D, one-way ANOVA, $F_{3,8}$ = 8.92, P < .01, n = 3). More importantly, the high concentration of CORT exposure (10 μM) led to a high level of nitrotyrosine and reduced the content of 5-HT$_{1A}$ receptor in the cultured hippocampal neurons at DIV 14 for 24 hours, which were reversed by UA but not ODQ incubation for 24 hours (Figure 3E, n = 3). More importantly, we infused DETA/NONOate (100 μM) together with ODQ (10 μM) or UA (1.0 mM) into the DG via minipumps, and 5 days later, we collected the DGs for protein level measurement. Consistent with the results of drugs incubated in cultured hippocampal neurons, DETA/NONOate significantly decreased the expression of 5-HT$_{1A}$ receptor in vivo, which also was reversed by UA but not ODQ (data not shown, one-way ANOVA, $F_{3,8}$ = 10.14, P < .01, n = 3, DETA/NONOate vs. DETA/NONOate & ODQ, P > .05; DETA/NONOate vs. DETA/NONOate & UA, P < .05), suggesting that ONOO•·but not sGC-cGMP-PKG pathway accounts for the decrease in 5-HT$_{1A}$ receptor expression induced by excess NO in the DG. Taken together, these results suggest that the production of excessive ONOO• caused by high level of glucocorticoids results in 5-HT$_{1A}$ receptor expression diminish in the DG.

## 3.4 | Hippocampal nNOS mediates the glucocorticoids-induced anxiety behaviors

To know the role of nNOS in chronic stress-induced anxiety, we exposed adult mice to CUMS for 21 days with or without treatment with 7-NI (30 mg/kg, i.p., 21 days). Systematic inhibition of nNOS activity prevented anxiety behavior change (Figure 4 A-B, one-way ANOVA, central distance: $F_{2,33}$ = 7.45, P < .01; central time: $F_{2,33}$ = 29.00, P < .001, n = 10-12) after CUMS exposure without locomotor activity alteration as measured in the OPT (Figure 4C). Next, we infused 7-NI (10 μM, 2 μL) into bilateral DGs of the hippocampi followed by 3 weeks CUMS exposure. Behavior measurement in the OPT showed that selective inhibition of nNOS located in the DG blocked the induction of anxiety behavior by CUMS (Figure 4 D-E, one-way ANOVA, central distance: $F_{2,32}$ = 6.78, P < .01; central time: $F_{2,32}$ = 7.74, P < .01, n = 11-13). Meanwhile, the locomotor activity did not interrupt the anxiety behavior measurement (Figure 4F). To directly investigate whether the local nNOS in the DG account for glucocorticoids-induced anxiety behavior, we infused 7-NI (10 μM, 2 μL) into the DGs and administrated the mice with CORT (20 mg/kg, s.c.) for 21 days. Remarkably, nNOS activity inhibition in the DG blocked CORT-induced anxiety behavior in the OFT (Figure 4G-H, one-way ANOVA, central distance: $F_{2,29}$ = 4.47, P < .05; central time: $F_{2,29}$ = 8.80, P < .01, n = 10-11) without affecting the locomotors (Figure 4I). Consistently, 21 days CORT exposure (20 mg/kg, s.c.) caused anxiety behavior in WT mice but not in nNOS−/− mice (Figure 4J-K, two-way ANOVA, central distance: $F_{3,44}$ = 14.69, P < .001, WT vehicle vs. WT CORT, P < .05, nNOS−/− vehicle vs. nNOS−/− CORT, P > .05; central time: $F_{3,44}$ = 8.80, P < .01, WT vehicle vs. WT CORT, P < .05, nNOS−/− vehicle vs. nNOS−/− CORT, P > .05, n = 10-13). Meanwhile, the locomotor activity did not interrupt the anxiety behavior measurement (Figure 4L). These data suggest that nNOS in the hippocampal DG mediates the chronic stress and glucocorticoids-induced anxiety behavior change.

4 | DISCUSSION

Extensive evidences demonstrate that 5-HT$_{1A}$ receptor deficiency is implicated in the pathogenesis of anxiety and that agonists of 5-HT$_{1A}$ receptor have anxiolytic effect in clinical use.[8,11,29] However, it remains an important question unresolved that how 5-HT$_{1A}$ receptor become deficiency during the development of anxiety? Previous studies from our laboratory found that chronic stress and

FIGURE 4 Dentate nNOS inhibition rescues glucocorticoids-induced anxiety-related behaviors. (A) Central distance of the mice exposed to CUMS for 21 d and the mice treated with 7-NI accompanied with CUMS for 21 d in open-field test. (B) Central time of the mice exposed to CUMS for 21 d and the mice treated with 7-NI accompanied with CUMS for 21 d in open-field test. (C) CUMS and 7-NI have no effect on locomotor activity. (D) Central distance of the mice exposed to CUMS for 21 d and the mice treated with 7-NI infusion into hippocampus followed by CUMS for 21 d in open-field test. (E) Central time of the mice exposed to CUMS for 21 d and the mice treated with 7-NI infusion into hippocampus followed by CUMS for 21 d. (F) CUMS and 7-NI infused into hippocampus have no effect on locomotor activity. (G) Central distance of the mice with treatment of CORT for 21 d and the mice treated with 7-NI infusion into hippocampus followed by CORT treatment for 21 d in open-field test. (H) Central time of the mice with treatment of CORT for 21 d and the mice treated with 7-NI infusion into hippocampus followed by CORT treatment for 21 d. (I) CORT and 7-NI infused into hippocampus have no effect on locomotor activity. (J) Central distance of nNOS+/+ (WT) and nNOS−/− mice with treatment of CORT. (K) Central time of nNOS+/+ and nNOS−/− mice with treatment of CORT. (L) Locomotor activity of the nNOS−/− and nNOS+/+ mice with treatment of CORT. Parameters assessed were the number of square crossings (horizontal) and the times of standing (vertical). Mean ± SEM, *P < .05, compared with control or vehichel; #P < .05, compared with CUMS or CORT. One-way ANOVA for A-I, two-way ANOVA for J-L
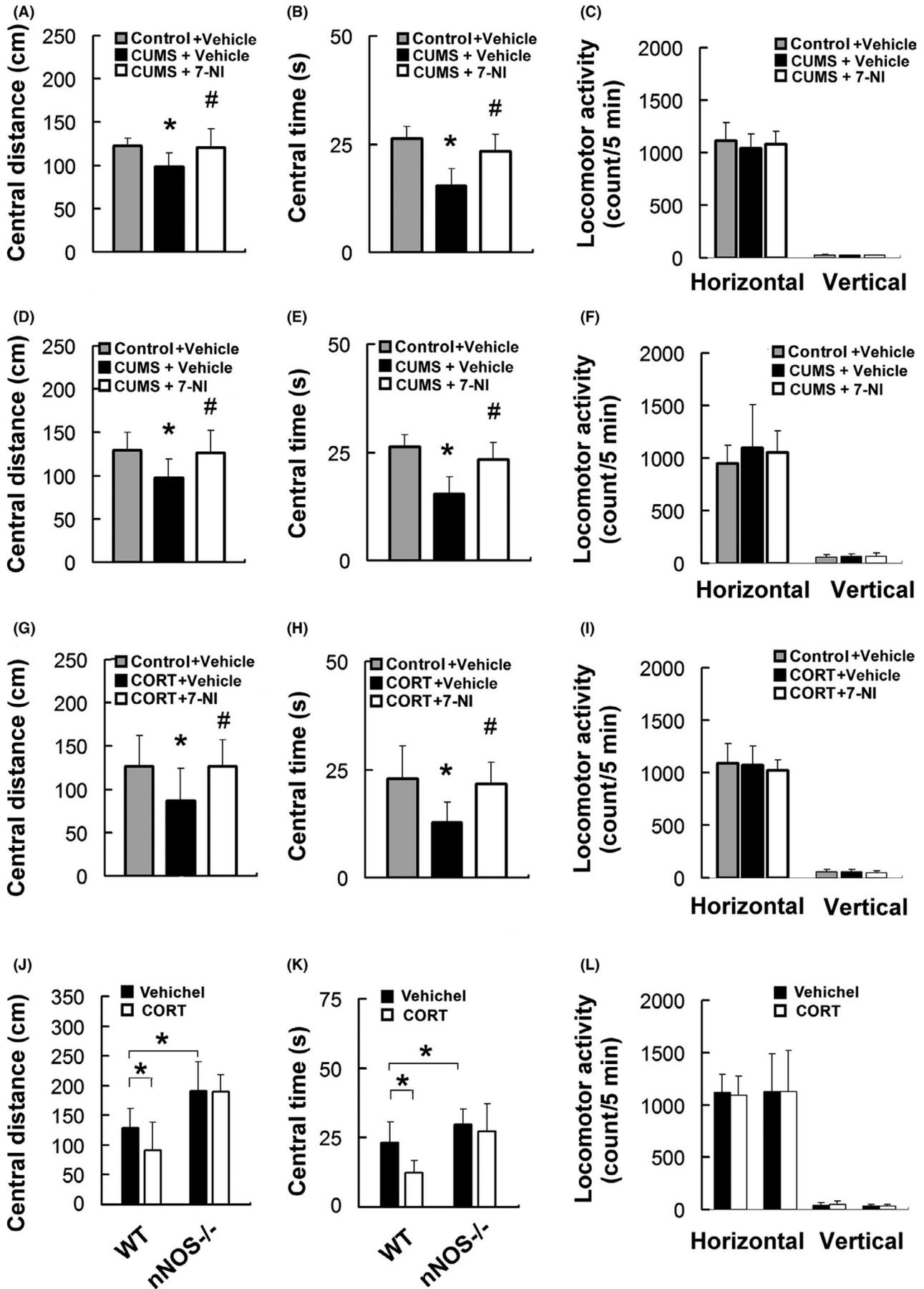
glucocorticoids exposure increased the expression of nNOS in the DG. Here, we report a causal link between the nNOS and the deficiency of the 5-HT$_{1A}$ receptor in the DG of the hippocampus after high dosage of glucocorticoids (corticosterone in rodent) exposure, which account for the generation of anxiety behavior after chronic stress. Interestingly, we found that ONOO• pathway but not cGMP pathway of NO was involved in the downregulation of 5-HT$_{1A}$ receptor expression.

The serotonergic system is a main pathway accounting for anxiety disease. Postsynaptic 5-HT$_{1A}$ receptor is expressed at a high density in the hippocampus.[34] Decreased content of 5-HT$_{1A}$ receptor is found in the hippocampus of patients with anxiety and depression disorder.[11] Re-expression of 5-HT$_{1A}$ receptor primarily in the hippocampus and cortex is sufficient to rescue the anxiety phenotype of the 5-HT$_{1A}$ receptor knockout mice.[7] The hippocampal CA1 and DG densely expresses 5-HT$_{1A}$ receptor.[35] In the DG, 5-HT$_{1A}$ receptor majorly expresses on dentate granular cells.[35,36] Both transgenic and pharmacological evidences showed that 5-HT1A receptor on dentate granular cells is engaged for depression and anxiety pathology, and antidepressant response.[29,33,37] However, the molecular mechanism responsible for the key pathophysiological process, the dysfunction of 5-HT$_{1A}$ receptor on dentate granular cells, remains obscure. It was reported that glucocorticoids have a profound influence on the function of the hippocampus via regulating several lines of genes.[38] Our previous study found that glucocorticoids modulate HPA activity through nNOS-GR (glucocorticoid receptors) pathway.[17] ONOO• produced by the diffusion-controlled reaction of NO with superoxide O_2^- radical can regulate the expression of translational factors and proteins.[22] Our data suggested that ONOO• mediated the repressive effect on 5-HT$_{1A}$ receptor expression after glucocorticoids action. Thus, it is possible that the enhanced concentration of NO in response to glucocorticoids exposure provides more NO diffused

into granule cells in the DG to produce ONOO•. Consistently, it was reported that a high dose of SNP, a type of NO donor causes an anxiogenic-like action in the elevated plus-maze test.[24] It has been demonstrated that 5-HT$_{1A}$ receptor on the hippocampal DG granule cells is crucial for anxiety-related behavior.[33] Thus, excessive ONOO• eliminates the content of 5-HT$_{1A}$ receptor in granule cells, which may finally induce anxiety phenotype. The OFT is a common measure of general locomotor activity levels and anxiety in rodent. Beside OFT, EPM and LDT are another two widely used behavior tests for assessment of anxiety. In this study, the anxiety behavior only assessed in OFT. Thus, more evidence for confirmation of the role of glucocorticoids-nNOS-Nitric oxide-ONOO•-5-HT1A receptor pathway in stress-related anxiety is needed.

Benzodiazepines, a type of typical anxiolytics, exert anxiolytic effects by regulating 5-HT release in the ventral hippocampus.[39] Recently, it is fully demonstrated that 5-HT$_{1A}$ receptor on mature dentate gyrus granule cells are critical for anxiety- and depression-related behaviors in rodents.[33] Stress potently modulates anxiety- and depression-related behaviors.[13,28,40] For instance, it has been demonstrated that foot shock stress as well as restraint stress can induce anxiety behavior in mice and rats.[41,42] Furthermore, 5-HT$_{1A}$ receptor expression decrease is strongly implicated in the pathology of anxiety and 5-HT$_{1A}$ receptor reactivation is critical for the anxiolytic effect of SSRIs.[43,44]

In recent years, the function of adult hippocampal neurogenesis in mediating the effects of antidepressants has been realized.[8,45] Treatment of antidepressants increased hippocampal neurogenesis in human and rodents.[46,47] Disruption of hippocampal neurogenesis by X-ray irradiation blocked the anxiolytic action of fluoxetine.[8] Long-term antidepressant treatment activated 5-HT$_{1A}$ receptor in the forebrain and 5-HT$_{1A}$ receptor activation in the hippocampus was required for the treatment of
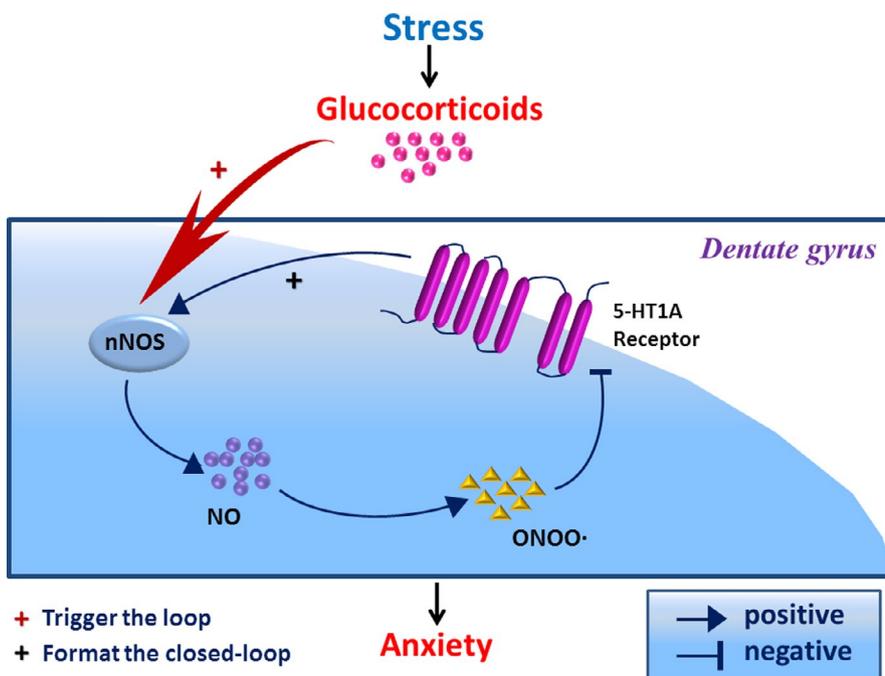
FIGURE 5 A proposed model of nNOS-ONOO•-5-HT$_{1A}$ receptor pathway in stress-induced anxiety. High level of glucocorticoids-induced dentate nNOS overexpression accounts for 5-HT$_{1A}$ receptor expression decline in the DG after stress. Our previous study demonstrated that hippocampal 5-HT$_{1A}$ receptor expression reduction results in nNOS overexpression. Accordingly, the reduction of hippocampal 5-HT$_{1A}$ receptor after stress, in turn, further increases the expression of nNOS, which is named as "nNOS-5-HT$_{1A}$ receptor closed-loop." ONOO• mediates the downregulation of 5-HT$_{1A}$ receptor by nNOS. Overall, the glucocorticoids-triggered "dentate nNOS-5-HT$_{1A}$ receptor closed-loop" plays a critical role in the pathology development of stress-induced anxiety

anxiety-associated behaviors.[29,48] Recently, we discovered that hippocampal neurogenesis is crucial for 5-HT$_{1A}$ receptor in modulating anxiety behaviors.[37,49] Here, we found that a single intrahippocampal injection of 7-NI into the DG counteracted the anxiety behavior of chronic stress or systemic corticosterone administration. Impaired hippocampal neurogenesis contributed to chronic stress and elevated glucocorticoids-induced depression and anxiety behavior.[27,28] Moreover, nNOS knockout and 7-NI significantly enhanced hippocampal neurogenesis.[28] Therefore, the 5-HT$_{1A}$ receptor reactivation-induced enhancement of hippocampal neurogenesis might contribute to the anxiety behavior rescue after a single infusion of 7-NI.

In our previous study, we found that blockade of 5-HT$_{1A}$ receptor increased nNOS expression [29] (Figure 5, demonstrated previously part). Downregulation of hippocampal nNOS expression mediated the anxiolytic effects of fluoxetine and 5-HT$_{1A}$ receptor agonists.[29] These findings suggest that nNOS works as a downstream molecule in the 5-HT$_{1A}$ receptor cascade, explaining the molecular basis of anxiolytic effects of selective serotonin reuptake inhibitor. However, the function of hippocampal nNOS in stress-induced anxiety remains unclear. Here, we went one more step to understand the pathology of stress-induced anxiety. We found that hippocampal nNOS accounted for chronic stress-induced decrease in 5-HT$_{1A}$ receptor expression via ONOO• pathway (Figure 5). Previously, we demonstrated that high concentration of glucocorticoids after stress upregulated nNOS via mineralocorticoid receptor (MR) in the DG of the hippocampus.[17] Altogether, these evidences support a novel hypothesis of anxiety that chronic stress-induced nNOS overexpression reduce the expression of 5-HT$_{1A}$ receptor in the DG, causing a higher level of nNOS, which in turn lead to more severe deficiency of 5-HT$_{1A}$ receptor in the DG. We, thus, name this undesirable interaction between nNOS and 5-HT$_{1A}$ receptor as "nNOS-5-HT$_{1A}$ receptor loop" (stress-glucocorticoids-nNOS-Nitric oxides-ONOO•-5-HT$_{1A}$ receptor -nNOS), which functions importantly in the pathology of stress-associated anxiety behavior (Figure 5). We also illustrated that ONOO• plays an important role in the "nNOS-5-HT$_{1A}$ receptor loop" under chronic stress state. Additionally, we clarified the mechanism by which 5-HT$_{1A}$ receptor modulated nNOS.[29] The coordinate used for microinjection in this study specially target the DG of the ventral hippocampus. Therefore, we suggested a glucocorticoids-nNOS-NO-ONOO•-5-HT$_{1A}$ receptor pathway, in the ventral hippocampal DG, implicated in the pathology of stress-related anxiety.

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REFERENCES

1. Martinowich K, Manji H, Lu B. New insights into BDNF function in depression and anxiety. *Nat Neurosci.* 2007;10:1089-1093.
2. Olivier B. Serotonin: a never-ending story. *Eur J Pharmacol.* 2015;753:2-18.
3. Marazziti D. Depression and serotonin: a never ending story. *Curr Drug Targets.* 2013;14:513.
4. Hoyer D, Clarke DE, Fozard JR, et al. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol Rev.* 1994;46:157-203.
5. Barnes NM, Sharp T. A review of central 5-HT receptors and their function. *Neuropharmacology.* 1999;38:1083-1152.
6. Kohler S, Cierpinsky K, Kronenberg G, Adli M. The serotonergic system in the neurobiology of depression: relevance for novel antidepressants. *J Psychopharmacol.* 2016;30:13-22.
7. Gross C, Zhuang X, Stark K, et al. Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature.* 2002;416:396-400.
8. Santarelli L, Saxe M, Gross C, et al. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science.* 2003;301:805-809.
9. Connors KA, Valenti TW, Lawless K, et al. Similar anxiolytic effects of agonists targeting serotonin 5-HT1A or cannabinoid CB receptors on zebrafish behavior in novel environments. *Aquat Toxicol.* 2014;151:105-113.
10. Celada P, Bortolozzi A, Artigas F. Serotonin 5-HT1A receptors as targets for agents to treat psychiatric disorders: rationale and current status of research. *CNS Drugs.* 2013;27:703-716.
11. Savitz J, Lucki I, Drevets WC. 5-HT(1A) receptor function in major depressive disorder. *Prog Neurogibol.* 2009;88:17-31.
12. Toth M. 5-HT1A receptor knockout mouse as a genetic model of anxiety. *Eur J Pharmacol.* 2003;463:177-184.
13. Ramsden EL, Taylor LJ. Stress and anxiety in the disabled patient. *Phys Ther.* 1988;68:992-996.
14. Koprdova R, Bogi E, Belovicova K, et al. Chronic unpredictable mild stress paradigm in male Wistar rats: effect on anxiety- and depressive-like behavior. *Neuro Endocrinol Lett.* 2016;37:103-110.
15. Wang SH, Zhang ZJ, Guo YJ, Teng GJ, Chen BA. Decreased expression of serotonin 1A receptor in the dentate gyrus in association with chronic mild stress: a rat model of post-stroke depression. *Psychiatry Res.* 2009;170:245-251.
16. Packer MA, Stasiv Y, Benraiss A, et al. Nitric oxide negatively regulates mammalian adult neurogenesis. *Proc Natl Acad Sci USA.* 2003;100:9566-9571.
17. Zhou QG, Zhu LJ, Chen C, et al. Hippocampal neuronal nitric oxide synthase mediates the stress-related depressive behaviors of glucocorticoids by downregulating glucocorticoid receptor. *J Neurosci.* 2011;31:7579-7590.
18. Dunn RW, Reed TA, Copeland PD, Frye CA. The nitric oxide synthase inhibitor 7-nitroindazole displays enhanced anxiolytic

efficacy without tolerance in rats following subchronic administration. *Neuropharmacology*. 1998;37:899-904.

19. Workman JL, Trainor BC, Finy MS, Nelson RJ. Inhibition of neuronal nitric oxide reduces anxiety-like responses to pair housing. *Behav Brain Res*. 2008;187:109-115.

20. Chiavegatto S, Dawson VL, Mamounas LA, Koliatsos VE, Dawson TM, Nelson RJ. Brain serotonin dysfunction accounts for aggression in male mice lacking neuronal nitric oxide synthase. *Proc Natl Acad Sci USA*. 2001;98:1277-1281.

21. Guix FX, Uribesalgo I, Coma M, Munoz FJ. The physiology and pathophysiology of nitric oxide in the brain. *Prog Neurogibol*. 2005;76:126-152.

22. Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nat Rev Neurosci*. 2007;8:766-775.

23. Foster MW, McMahon TJ, Stamler JS. S-nitrosylation in health and disease. *Trends Mol Med*. 2003;9:160-168.

24. Ferreira VM, Valenzuela CF, Morato GS. Role of nitric oxide-dependent pathways in ethanol-induced anxiolytic effects in rats. *Alcohol Clin Exp Res*. 1999;23:1898-1904.

25. Umathe SN, Bhutada PS, Jain NS, Mundhada YR, Borkar SS, Dhumal B. Role of nitric oxide in obsessive-compulsive behavior and its involvement in the anti-compulsive effect of paroxetine in mice. *Nitric Oxide*. 2009;21:140-147.

26. Calixto AV, Duarte FS, Duzzioni M, Nascimento Hackl LP, Faria MS, De Lima TC. Role of ventral hippocampal nitric oxide/cGMP pathway in anxiety-related behaviors in rats submitted to the elevated T-maze. *Behav Brain Res*. 2010;207:112-117.

27. David DJ, Samuels BA, Rainer Q, et al. Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. *Neuron*. 2009;62:479-493.

28. Zhou QG, Hu Y, Hua Y, et al. Neuronal nitric oxide synthase contributes to chronic stress-induced depression by suppressing hippocampal neurogenesis. *J Neurochem*. 2007;103:1843-1854.

29. Zhang J, Huang XY, Ye ML, et al. Neuronal nitric oxide synthase alteration accounts for the role of 5-HT1A receptor in modulating anxiety-related behaviors. *J Neurosci*. 2010;30:2433-2441.

30. Vyas A, Chattarji S. Modulation of different states of anxiety-like behavior by chronic stress. *Behav Neurosci*. 2004;118:1450-1454.

31. Vyas A, Pillai AG, Chattarji S. Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior. *Neuroscience*. 2004;128:667-673.

32. Griebel G, Simiand J, Serradeil-Le Gal C, et al. Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. *Proc Natl Acad Sci USA*. 2002;99:6370-6375.

33. Samuels BA, Anacker C, Hu A, et al. 5-HT1A receptors on mature dentate gyrus granule cells are critical for the antidepressant response. *Nat Neurosci*. 2015;18:1606-1616.

34. Pazos A, Palacios JM. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. *Brain Res*. 1985;346:205-230.

35. Klempin F, Babu H, De Pietri Tonelli D, Alarcon E, Fabel K, Kempermann G. Oppositional effects of serotonin receptors 5-HT1a, 2, and 2c in the regulation of adult hippocampal neurogenesis. *Front Mol Neurosci*. 2010;3:14.

36. Zhou QG, Lee D, Ro EJ, Suh H. Regional-specific effect of fluoxetine on rapidly dividing progenitors along the dorsoventral axis of the hippocampus. *Sci Rep*. 2016;6:35572.

37. Zhang J, Cai CY, Wu HY, Zhu LJ, Luo CX, Zhu DY. CREB-mediated synaptogenesis and neurogenesis is crucial for the role of 5-HT1a receptors in modulating anxiety behaviors. *Sci Rep*. 2016;6:29551.

38. Datson NA, van der Perk J, de Kloet ER, Vreugdenhil E. Identification of corticosteroid-responsive genes in rat hippocampus using serial analysis of gene expression. *Eur J Neurosci*. 2001;14:675-689.

39. Wright IK, Upton N, Marsden CA. Effect of established and putative anxiolytics on extracellular 5-HT and 5-HIAA in the ventral hippocampus of rats during behaviour on the elevated X-maze. *Psychopharmacology*. 1992;109:338-346.

40. Muller MB, Zimmermann S, Sillaber I, et al. Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. *Nat Neurosci*. 2003;6:1100-1107.

41. Smriga M, Kameishi M, Uneyama H, Torii K. Dietary L-lysine deficiency increases stress-induced anxiety and fecal excretion in rats. *J Nutr*. 2002;132:3744-3746.

42. Mitra R, Jadhav S, McEwen BS, Vyas A, Chattarji S. Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala. *Proc Natl Acad Sci USA*. 2005;102:9371-9376.

43. Persky H, Smith KD, Basu GK. Effect of corticosterone and hydrocortisone on some indicators of anxiety. *J Clin Endocrinol Metab*. 1971;33:467-473.

44. Lemonde S, Turecki G, Bakish D, et al. Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. *J Neurosci*. 2003;23:8788-8799.

45. Kheirbek MA, Klemenhagen KC, Sahay A, Hen R. Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. *Nat Neurosci*. 2012;15:1613-1620.

46. Boldrini M, Underwood MD, Hen R, et al. Antidepressants increase neural progenitor cells in the human hippocampus. *Neuropsychopharmacology*. 2009;34:2376-2389.

47. Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci*. 2000;20:9104-9110.

48. Haddjeri N, Blier P, de Montigny C. Long-term antidepressant treatments result in a tonic activation of forebrain 5-HT1A receptors. *J Neurosci*. 1998;18:10150-10156.

49. Cai CY, Wu HY, Luo CX, et al. Extracellular regulated protein kinaseis critical for the role of 5-HT1a receptor in modulating nNOS expression and anxiety-related behaviors. *Behav Brain Res*. 2019;357-358:88-97.