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Supplementary Information for

Involvement of Scratch2 in GalR1-mediated depression-like behaviors in the rat ventral periaqueductal gray

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SI Materials and Methods

Identification of the rat *GalR1* gene transcription start site. To determine the rat *GalR1* gene transcription start site, a 5′-RACE analysis was performed with a Smart Race cDNA Amplification Kit (Clontech, Palo Alto, CA). The first-strand cDNA was prepared by using 100 ng of total RNA isolated from the rat vPAG tissues. Then, the nested PCR was carried out by using two specific *GalR1* primers and universal primer (Clontech) to amplify the 5′-flanking sequence of the *GalR1* gene. The amplification products were purified, and then cloned into the pUC-57 vector (Clontech) for further sequence analysis. The two specific primer sequences for the rat *GalR1* gene were as follows: 5′-CAGAAGAGCAGGTAGGCCAGGT-3′, 5′-AGGCTGTTGCCCAGCAC GCCCA-3′.

Cell culture. Rat adrenal medulla tumour cells (PC12 cells), which are neuron-like cells, were cultured in Ham's F12K medium containing 5 % fetal bovine serum and 10 % horse serum (Gibco, Carlsbad, CA). Human embryonic kidney cell line (293T) cells were grown in DMEM medium containing 10% fetal bovine serum. Both above cells were incubated in a humidified incubator with a 5 % CO_2 atmosphere at 37 °C.

Plasmids. The 1,500-bp upstream regulatory sequence from the transcription start site of the rat *GalR1* gene was amplified by PCR from PC12 cell genomic DNA and inserted into pGL3-basic vector (Promega, Madison, WI). The resultant reporter plasmid was named pGL-1500. Then, a series of 5′-truncated deletion fragments were obtained by PCR using pGL-1500 as a template and inserted into pGL3-basic vector. The reverse primer sequence for the above deletion reporter plasmids was as follow:

5'-TACCCAAGCTTTAAAGAGGAGGCGGTGCTGCG-3' (restriction sites are underlined). The chimeric reporter plasmid containing the sequence between -250 and -220 of *GalR1* promoter was constructed by inserting the annealing R30 primers into the *Kpn* I and *Xho* I site of pGL3-promoter (Promega). The mutant reporter plasmid mE-box was constructed with Q5 Site-Directed Mutagenesis Kit (NEB, Ipswich, MA) by using pGL-250 as a template. The mammalian expression plasmids for Snail, Scratch2 and its truncated mutants were prepared by PCR, and the resulting fragments were cloned into the pcDNA3.1 vector (Clontech). Prokaryotic vector encoding GST-tagged fusion protein was obtained by PCR, and the resulting fragments were inserted in frame into pGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ). Other primer sequences used in this section were listed in TableS1-3.

Construction of the Scratch2 siRNA lentivirus. The optimal sequence of siRNAs against rat Scratch2 (5'- TGGCAAGGCCTACGTGTCTAT-3') was cloned into GV248 vector (GeneChem, Shanghai, China), which contains a multiple cloning site for the insertion of siRNA constructs to be driven by an upstream U6 promoter and a downstream cytomegalovirus promoter-GFP cassette. The scrambled siRNA control was constructed by a similar process (5'-GTCTTGATAGACGT ACCGGTC-3'). Then, HEK293T cells were co-transfected with above plasmids and lentiviral packaging plasmids to produce Scratch2 siRNA lentivirus or scramble siRNA lentivirus. The titers of both Scratch2 siRNA and scramble siRNA were 5×10^8 TU/ml.

Reporter gene assay. PC12 cells were seeded in 24-well plates and co-transfected with 100 ng of reporter plasmid and 20 ng of pRL-TK plasmid by Lipofectamine

2000 (Life Technologies, Carlsbad, CA). After transfection for 24 h, cells were collected to measure the luciferase activity which was done with a dual luciferase reporter assay system (Promega). The values are the mean ± SD and are normalized to Renilla luciferase activity.

DNA decoy assay. The decoy oligodeoxynucleotides (ODNs) were designed on the basis of the sequence from -250 to -220 of the rat *GalR1* promoter. The sequence between -250 and -220 of *GalR1* promoter was named as R30. The sense and antisense phosphorothioate ODNs were annealed to obtain the Decoy ODNs. The ODN sense sequences were as follows: R30 decoy: 5'-CAAAGCAACAGGTGCGACCTCAGGGCACTG-3'; Control decoy: 5'-ACCGAGCCGGACGCAATCGAAGATAGC-3'. PC12 cells were transfected with 100 ng of reporter plasmid together with a final concentration of 0.2 μM double-strand ODNs. After 48h of transfection, cells were collected and luciferase activities measured.

Purification of GST- Scratch2-ZF Protein and nuclear extract preparation. *Escherichia coli* BL21 cells were transformed with pGEX-4T-1-Scratch2-ZF plasmid, inoculated into 5 ml LB medium and induced by 0.2 mM IPTG at 30 °C for 4 h. Then, bacterial cells were harvested and lysed. The soluble glutathione-S-transferase (GST) fusion proteins were purified with MagneGST™ Protein Purification System (Promega) according to the manufacturer's protocol. Nuclear extracts were prepared according to the protocol provided by the NE-PER nuclear protein extraction kit (Thermo Scientific, Rockford IL).

Gel-shift assay. Double-stranded oligonucleotides were labeled with digoxigenin at the 5′-end and were then incubated with nuclear extracts or purified GST-fusion proteins in DNA binding buffer (10 mM Tris–HCl, pH 7.5, 1 mM MgCl2, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT) and 1 μg poly (dI-dC). After 40 min of incubation, the reaction mixtures were electrophoresed on a 6% non-denaturing PAGE and further analysed as described previously (1).

DNA pull-down assay. The biotinylated double-stranded oligonucleotides were immobilized to streptavidin beads (Sigma-Aldrich, St Louis, MO) and incubated with PC12 cell nuclear extract on a rotating shaker at 4°C overnight. After incubation, the supernatant was discarded. The beads were washed three times with cold PBS. Then the bound mixture was resuspended in distilled water at 70 °C for 5 min to break the bond between streptavidin and biotin. The eluted proteins from the beads were subjected to LC-MS/MS (Bruker Daltonics, San Diego, CA) for sequencing data analysis.

Chromatin immunoprecipitation (ChIP) assay. ChIP experiments were carried out according to the instructions provided with ChIP Assay kit (Millipore, Billerica, MA). Briefly, the vPAG tissues from 3 rats or 1×10^7 PC12 cells were cross-linked, lysed, and sonicated into DNA fragments of 200-1,000bp. The supernatants were collected and incubated with normal IgG serum (1:500, #2845; CST, Cambridge, MA) or anti-Scratch2 (1:50, #sc-85910; Santa Cruz, Santa Cruz, CA). Then, real-time PCR was performed with DNA extracted from the immunoprecipitated chromatin. The relative amount of immunoprecipitated DNA was analyzed by normalization to input. The

primer sequences used in this section were as follows: 5'-TGAAGGTGTGCATCCCTGGGCT-3', 5'-GTTCCAATCCCTGGCCTCTTGT-3'.

DNA affinity precipitation (DNAP) assay. 293T cells were transfected with Flag-Scratch2 for 24 h. Then, cells were lysed, and the lysates were precleared with streptavidin-agarose (Sigma-Aldrich). Next, the supernatant was incubated with 30 pmol of biotinylated double-stranded oligonucleotides together with poly·(dI-dC) for 3 h. DNA-bound proteins were precipitated with streptavidin-agarose for 30 min at 4 °C. After washing 3 times, DNA-bound proteins were detected with anti-Flag antibody (1:2,000, # SAB4301135; Sigma-Aldrich,).

Western blot analysis. Western blot was performed as described in our previous study with minor modifications (2). Briefly, protein samples were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. Then the membranes were blocked with 5 % non-fat milk in Tris-buffered saline containing 0.05 % Tween 20 (TBST) for 1 h and incubated with primary antibodies against Flag (1:2,000, #SAB4301135; Sigma), Scratch (1:1,000, #sc-85910; Santa Cruz), α-tubulin (1:5,000, #ABT170; Millipore) for 2 h at room temperature. Then, secondary antibodies (1:10,000, #7074; CST) were incubated for 1 h at room temperature. After 3 times washing, the bands on the membrane were visualized with an ECL kit (Thermo Scientific).

Real-time PCR assay. Total RNA was isolated by using RNeasy Lipid mini Kits (Qiagen, Hilden, Germany). cDNA was prepared by using SuperScript III first strand synthesis system (Life Technologies). Real-time PCR analysis was carried out using

SYBR Green PCR master Mix reagents (ABI, Foster city, CA). The primers used in this study are described in TableS5. Data were normalized to the expression of GAPDH for each experiment. All assays were run in triplicate, and the mean value was used for the analysis.

Animals. Male Sprague–Dawley rats (200 - 250g) were housed on a 12-hour-12-hour light-dark cycle with ad libitum access to food and water (except in restraint stress) and acclimated to the facility for 1 week before experiment. All experiments with animals were approved by the Animal Care Committee at Capital Medical University (Permit Number: AEEI-2015-035) and were carried out in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chronic mild stress (CMS) procedure. The CMS procedure was performed as previously described with slight modifications (2). Briefly, male rats were subjected to eight different stressors for 4 weeks in a random order, including foot shock, water deprivation, cold water swimming, food deprivation, restraint, clip tail, light/dark cycle reversal, and cage tilt. Control group rats were left undisturbed except for necessary procedures, including handling and routine cleaning. At the end of the CMS protocol, the OFT, the FST and the SPT were performed to evaluate the effects of CMS. The detailed procedures of OFT, FST, and SPT are presented in the supplementary materials.

Open field test (OFT)

Rat were allowed to explore freely for 5 min in the open field box (1m × 1 m × 40 cm) and the floor of the open field was divided into 25 rectangles (15 × 15 cm

each) by white lines. The time spent in the central area, and the total travelled distance was videotaped and quantified with EthoVision in each 5 min period (Noldus, VA, USA). The field was cleaned by 75% ethanol between tests.

Forced swimming test (FST)

The forced swimming test was carried out on rat as described in our previous study with minor modifications (3). Briefly, rats were put individually into a transparent glass beaker (50 cm in height and 30 cm in diameter) filled with water (25 ± 2 °C) to a height of 35 cm for 5 min. Every session was recorded on videotape and later scored by the EthoVision (Noldus). The total duration of immobility was recorded during the last 4 min of the 5-min session.

Sucrose preference test (SPT)

The evaluation of sucrose preference comprises acclimatization and testing portions. In the adaptation period, rats were trained to consume a sucrose solution (1%) or tap water in their cage for 24h. After the adaptation period, rats were deprived of food and water for 24h, and then given free to access to bottles containing sucrose solution and tap water for 1h. The sucrose preference was calculated as described in our earlier study (2).

Laser capture microdissection. Laser capture microdissection was performed by using Leica LMD-7000 system (Leica, Benshein, Germany). The rat brains were rapidly removed and snap frozen in powered dry ice. Serial coronal sections (40 μm) containing the vPAG tissues were cut at -20°C. The sections were then mounted on slides covered with a polyethylene naphthalate (PEN) membrane (Life Technologies).

Under the 6×objective, the vPAG region was identified and marked with the laser capture microdissection software. The UV laser was then turned on and the tissues were cut and collected. Total RNA from these samples was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. All steps were performed under RNase-free conditions.

Immunofluorescence. Coronal sections (30 μm) of frozen rat brain sections were blocked with 10 % goat serum and 0.3% Triton X-100 for 2h. Then, sections were incubated with anti-Scratch2 (1:200, #sc-85910; Santa Cruz,) and anti-NeuN (1:200, #MAB377; Millipore) overnight at 4 °C. Brain sections were washed with 0.01M PBS containing 0.01% Triton-X-100 for 3 times and further incubated in corresponding fluorescence-conjugated secondary antibodies (1:500, #CA11008S, #CA11005S; Invitrogen, Carlsbad, CA) for 2h. Finally, brain sections were mounted on slides using Prolong Gold antifade reagent (with DAPI) (Invitrogen). Immunostaining images were acquired using the Pannoramic SCAN II system (3DHistech, Budapest, Hungary).

***In situ* hybridization (RNAscope).** Adult male Sprague–Dawley rats (200–250 g) (n=3) were deeply anesthetized with sodium pentobarbital (50 mg/kg b.w.) and transcardially perfused with 50 ml of warm (37°C) 0.9% saline, 50 ml of warm fixative (4% paraformaldehyde, 0.4% picric acid, 0.1% glutaraldehyde in PBS) and 50 ml of the same ice-cold fixative. Brains were dissected out and post-fixed in the same fixative for 24h at 4°C. The tissues were then washed 3 times with PBS and cryoprotected in 10% [over night (O/N) at 4°C), 20% (O/N at 4°C) and 30% (O/N at

4°C) sucrose in PBS. Tissues were embedded in OCT compound, sectioned at 20 μm, and mounted onto Superfrost plus slides (Thermo Fischer Scientific, Waltham, MA). For *in situ* hybridization (RNAscope), the manufacturer's protocol was followed (Advanced Cell Diagnostics, San Francisco, CA). All experiments were replicated in three animals. The probes were designed by the manufacture and available from Advanced Cell Diagnostics. The following probes were used in this study: Rn-Galr1-C1 (#439791), Rn-Slc17a6-C2 (VLUT2) (#317011-C2), Rn-Slc32a1-C3 (GAD) (#424548-C3), Rn-Scratch2-C4 (#1039241-C4).

Stereotaxic surgery and viral injection. The rats were anesthetized and positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL). Injections were carried out with micropipette connected to a Nanoliter Injector (NANOLITER 2010, WPI, Sarasota, FL) and its controller (Micro4, WPI) at the dose of 4×10^5 TU per rat. For details, see *SI Appendix*.

Statistical analysis. Statistical analysis in this study was performed using GraphPad Prism 6.0 (GraphPad, San Diego, CA). The student's t test was used to evaluate significant differences between two sets of data. An ANOVA followed post-hoc Tukey's multiple comparison tests was carried out for group comparisons. Differences with $P < 0.05$ were considered statistically significant.

Table. S1

Primers used to construct deletion reporter constructs

Plasmid name	Primer sequence (5′-3′)	Fragment position
pGL-1500	TACCGCTCGAGGGGTAAGCCAGCTCCCTCTCC	-1500 to +1
pGL-1000	TACCGCTCGAGGCGGTTCATAGTTGGCGTCCC	-1000 to +1
pGL-500	CCGCTCGAGGCACAGAGGGGACGCAGTTGA	-500 to +1
pGL-300	TACCGCTCGAGTGAAGGTGTGCATCCCTGGGC	-300 to +1
pGL-250	TACTGCTCGAGCAAAGCAACAGGTGCGACCTC	-250 to +1
pGL-243	TATTGCTCGAGACAGGTGCGACCTCAGGGCA	-243 to +1
pGL-234	TACTGCTCGAGGACCTCAGGGCACTGAGAGC	-234 to +1
pGL-230	ATATCGGTACCCAGGGCACTGAGAGCAAGGGGA	-230 to +1
pGL-220	ACCGCTCGAGAGAGCAAGGGGACGCAGCTCA	-220 to +1
pGL-200	TACCGCTCGAGACAAGAGGCCAGGGATTGGAA	-200 to +1

Restriction sites are underlined, and the transcription start site are designated as "+1".

Table. S2

Primers used for construct chimeric reporter construct pGL3-p-30

Prime name	Primer sequence (5′-3′)
R30-Forward	GTACCCAAAGCAACAGGTGCGACCTCAGGGCACTGCTCGA
R30-Reverse	GCAGTGCCCTGAGGTCGCACCTGTTGCTTTGG

The pGL3-p-30 contains the 30 bp sequence between -250 and -220 of the *GalR1* promoter.

Table. S3

Primers used to construct expression constructs

Plasmid Name	Primer sequence (5′-3′)
pcDNA3.1-Scratch2	TACCGCTCGAGATGCCGCGTTCCTTCCTGGTG
	TACCGGAATTCTCAGCTGGCCGGGCCGGCGGA
pcDNA3.1-Scratch-ZF	TACCGCTCGAGCGCTACTTCCGCGGGGAGGCG
	ACCGGAATTCTCAGCTGGCCGGGCCGGCGGA
pcDNA3.1-ZF	TACCGCTCGAGGGGGAGCGCGCGGGGCGCTCG
	TACCGGAATTCTCAGCTGGCCGGGCCGGCGGA
pGEX-4T-1-Scratch2-ZF	ACCGGAATTCGGGGAGCGCGCGGGGCGCTCG
	TACCGCTCGAGTCAGCTGGCCGGGCCGGCGGA
pcDNA3.0-Flag-Scratch2	TACCGGAATTCTGATGCCGCGTTCCTTCCTG
	TACCGCTCGAGTCAGCTGGCCGGGCC
pcDNA3.1-Snail	TACCGCTCGAGATGCCGCGCTCCTTCCTGGTC
	ACCGGAATTCTCAGCGAGGGCCTCCGGAGCA

The pcDNA3.1-Scratch-ZF contains the Scratch domain and zinc finger domain of Scratch2. The pcDNA3.1-ZF contains the zinc finger domain of Scratch2. The pGEX-4T-1-Scratch2-ZF contains the zinc finger domain of Scratch2. Restriction sites are underlined.

Table. S4

Mass Spectrometry Analysis of the bound Scratch2 Protein

Identified protein	Peptides
Scratch2	AAEPPPSAGPAS
	GPPGDNGYVAH
	PWLLQGH
	ETAYVLPGTR
	SLDSQLAR
	TYATSSNLSR
	PESPQSSLSAR

Table. S5

Primers used for real-time PCR

Gene name	Primer sequence (5′-3′)
Scratch2	GCGAGAAGCCCTTTGGT
	GCTTGTGGAGGTAGGACTTG
GalR1	TCGGGACAGCAACCAAAC
	TGCAGATGATTGAGAACCTTGG
GAPDH	GACCACCCAGCCCAGCAAGG
	TCCCCAGGCCCCTCCTGTTG

SI Figures and legends

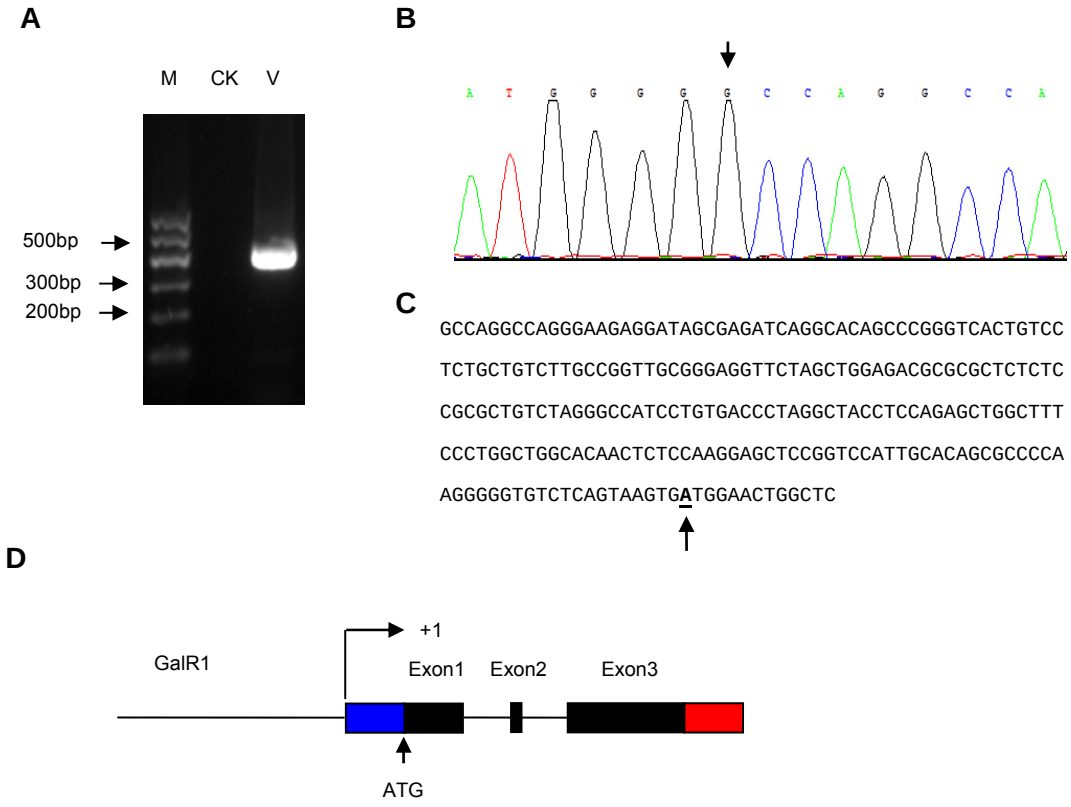

Fig. S1. 5′-RACE analysis of the transcription start site of the rat *GalR1* gene. (*A*) 5′-RACE products from nested PCR were analyzed by agarose gel electrophoresis from vPAG tissues. M: DNA marker, CK: Control, V: vPAG tissues. (*B*) The partial sequence diagrams of the 5′-RACE products. Arrow indicates the transcription start site. (*C*) Nucleotide sequence of the 5′-UTR of the *GalR1* gene. Arrow indicates the translation start site. (*D*) Schematic representation of the rat *GalR1* gene structure. The transcription start site is designated as "+1", and the translation start site is marked with arrow. The 5′-UTR is marked with blue, and the 3′-UTR is marked with red.

Fig.S2. Sequence of the rat *GalR1* promoter and partial downstream region. The nucleotides are numbered on the left, with the transcription start site designated as "+1".The transcription start site is underlined and marked with arrow, and the translation start site is boxed. The E-box is marked with red color and boxed.

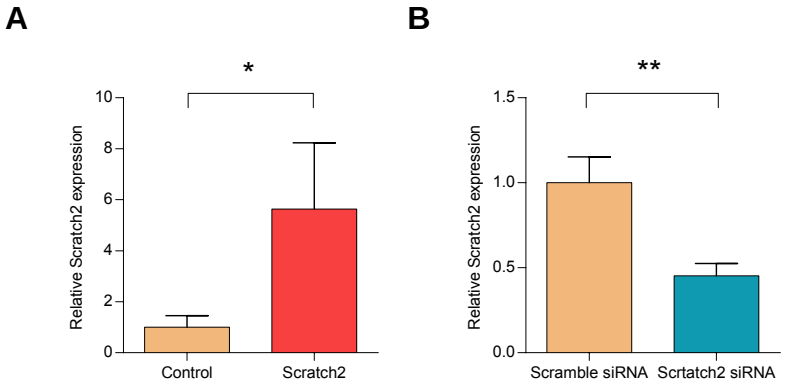

Fig.S3. Relative quantitative analysis of Scratch2 protein level in PC12 cells after overexpression of Scratch2 (*A*) or knockdown of Scratch2 (*B*). In (*A*), T_4=3.042, $p<0.05$. In (*B*), T_4=5.655, $p<0.001$. Data are presented as mean ± S.D. (n=3 per group). (*) indicates a p value <0.05. (**) indicates a p value <0.01.

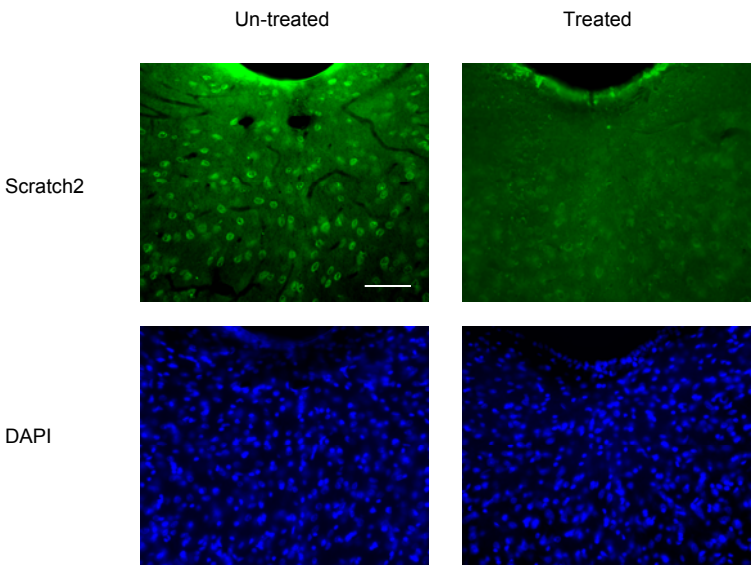

Fig.S4. Validation of Scratch2 antibody in immunofluorescence staining. Scratch2 antibodies were diluted into PBST solution and incubated with synthetic peptide derived from the region from 1 to 130 of rat Scratch 2 protein. After absorption treatment, the subsequent solutions were applied to perform immunofluorescence staining. Left panel: Untreated Scratch2 antibody; Right panel: the treated Scratch2 antibody. Scale Bar: 100 μm.

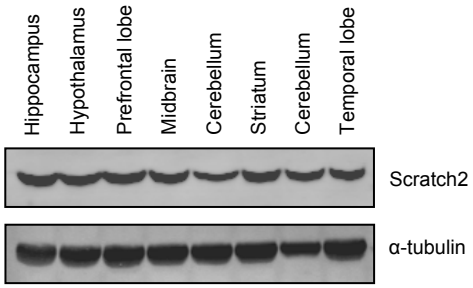

Fig.S5. The expression level of Scratch2 in rat brain. The total proteins of different rat brain regions were detected by western blot using an anti-Scratch2 antibody.

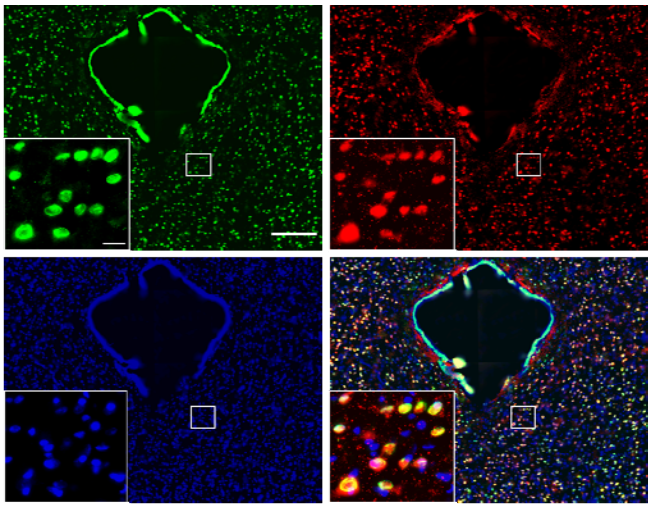

Fig.S6. Immunofluorescence staining images of Scratch2 in the rat vPAG region. The Scrath2 are shown with green, NeuN with red, and the nuclei with blue (DAPI). Scale bar: low-magnification images, 200 μm; High-magnification images, 20 μm.

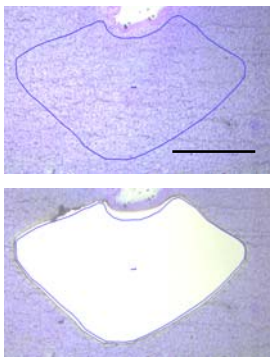

Fig.S7. Laser microdissection of the vPAG tissues from rat brain. Scale bar: 500μm.

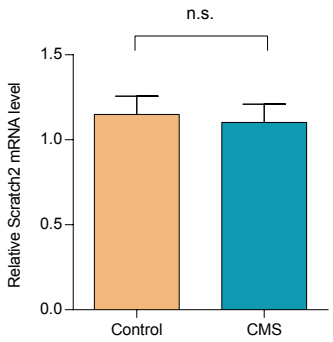

Fig.S8. CMS does not influence the expression of Scratch2 in the CA3 of dorsal hippocampus. The expression of Scratch2 was detected with real-time PCR in rat CA3 of dorsal hippocampus tissues after CMS treatment. Data are presented as mean ± S.E. (n=7 per group). T_{12}=0.3028, $p>0.05$.

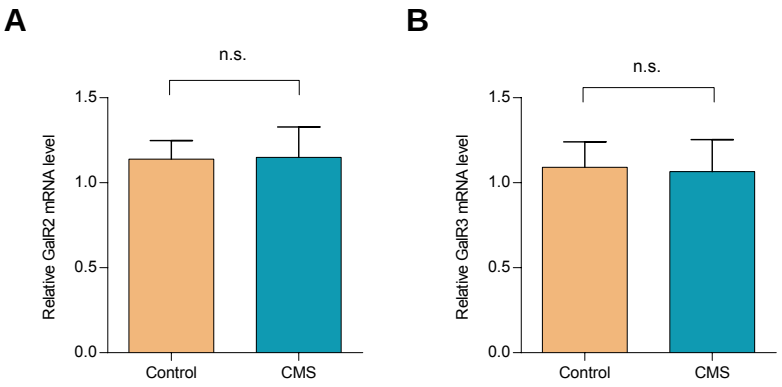

Fig.S9. Knockdown of Scratch2 does not influence the expression of GalR2 and GalR3 in vPAG tissues. (*A*-*B*) The mRNA level of GalR2 (*A*) or GalR3 (*B*) was determined by real-time PCR in the vPAG after the injection of Lenti-Scratch2 siRNA virus. Data are presented as mean ± S.D. (n=3 per group). In (*A*), T_4=0.04876, p>0.05. In (*B*), T_4=0.1046, p>0.05.

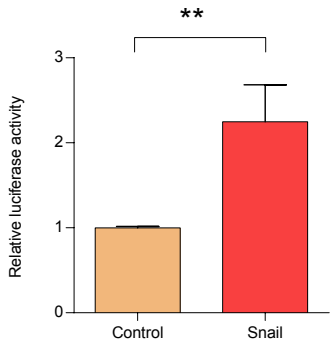

Fig.S10. Snail increased the activity of *GalR1* promoter. PC12 cells were co-transfected with pGL-250 and pcDNA3.1-Snail for 24h, and then luciferase activity was measured. Data are presented as mean ± S.D. (n=3 per group). T_4=4.948, $p<0.01$.

SI Reference

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3. P. Wang, *et al*., Depression-like behavior in rat: Involvement of galanin receptor subtype 1 in the ventral periaqueductal gray. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E4726-4735 (2016).