

Anxiety-Related Behaviours Associated with microRNA-206-3p and BDNF Expression in Pregnant Female Mice Following Psychological Social Stress

Zhuang Miao^{1,2} · Fengbiao Mao^{1,2} · Jialong Liang^{1,2} · Moshe Szyf³ · Yan Wang¹ · Zhong Sheng Sun^{1,2,4}

Received: 25 July 2016 / Accepted: 28 December 2016 / Published online: 14 January 2017
© Springer Science+Business Media New York 2017

Abstract Stress during pregnancy can induce various psychological disorders in women. However, the association linking psychological stress during pregnancy with abnormal behaviours in females remains largely unknown. We employed a novel psychological stress model by introducing pregnant mice to witness the defeat process of their mated partner (WDPMP) and examined the effects of WDPMP on depression-/anxiety-like behaviours and on the expression of brain-derived neurotrophic factor (BDNF) and miR-206-3p in the hippocampus, medial prefrontal cortex (mPFC) and amygdala. Compared to pregnant control (PC) mice, pregnant stressed (PS) mice showed decreased sucrose preference during the late period of gestation, and after lactation, they spent less time in the open arms of the elevated plus maze and in the light chamber of the light/dark box. After lactation, decreased BDNF expression in both the hippocampus and mPFC of PS mice was found to be associated with enhanced miR-206-3p levels; meanwhile, elevated BDNF associated with decreased

miR-206-3p expression was evident in the amygdala of the same PS mice. DNA methylation level in the *Bdnf* promoter did not show difference between PC and PS mice in the hippocampus. Transfection of miR-206-3p resulted in decreased BDNF levels in vitro. These results suggest that WDPMP stress during gestation can induce long-term mood alterations in pregnant mice, which may correlate with changes in miR-206-3p and BDNF expression in the hippocampus, mPFC and amygdala.

Keywords Social defeat · Psychological stress · Brain-derived neurotrophic factor · miR-206-3p · Anxiety

Introduction

Anxiety disorders affect one out of four people, with a higher incidence rate in women than in men [1]. In particular, pregnant women are susceptible to mental disorders such as anxiety and depression when exposed to adverse environments [2, 3]. These stress-induced psychological illnesses during pregnancy have been identified as a strong risk factor for postpartum depression and anxiety [4–6] and even cause negative consequences on their children [4, 7]. There are several female rodent models mimicking adverse environments during or around gestation, including models utilizing chronic restraint stress and chronic unpredictable mild stress [8, 9]. The above stressors lead to abnormal mood and psychological states in the pregnant dams, like in the pregnant women, and can seriously influence the development and health of their offspring under certain circumstances [8, 9]. However, these models mostly rely on physically stressing the individuals, with the dams being directly exposed to and physically experiencing the disturbed situations themselves.

Electronic supplementary material The online version of this article (doi:10.1007/s12035-016-0378-1) contains supplementary material, which is available to authorized users.

✉ Yan Wang
wangy@biols.ac.cn

✉ Zhong Sheng Sun
sunzs@biols.ac.cn

¹ Beijing Institutes of Life Science, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China

² University of the Chinese Academy of Sciences, Beijing 100049, China

³ Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec H3G 1Y6, Canada

⁴ Institute of Genomic Medicine, Wenzhou Medical College, Wenzhou 325000, China

The social defeat model is a validated model that mimics traumatic stress to induce related mood disorders [10–12]. However, this model suffers from the drawback of being unable to separate the influences of psychological and physical stress. To address this, Warren et al. have utilized a paradigm wherein witnessing social defeat triggers the emotional stress and successfully induces anxiety and depression-like behaviours in male mice [13]. In humans, the effects of psychological stressors, such as psychological reactions to terrorist attacks, on physiological and psychological function in individuals have also been investigated [14–16]. However, psychological violence, which may occur often during the gestational period, has been only minimally investigated [17, 18], and few animal models mimicking the influence of psychological shock in females during their gestation are established. Therefore, the influence of aversive factors, such as psychological fear related to “witness horror,” on the mood state of females during pregnancy requires investigation in both animal modelling and its molecular underpinnings.

It has been suggested that rodents are sensitive to the experiences of other members of their species. For instance, female rats that witnessed a cage-mate experiencing foot shock displayed vicarious freezing behaviour [19]. Similarly, male rodents witnessing other rodents undergoing presumably traumatic events related to social defeat displayed enhanced anxiety and depression-like behaviours [13, 20]. In this study, we have modified the social defeat witness model described above and created a novel psychological stress model by allowing pregnant mice to witness the defeat process of their mated partner (WDPMP) from the second day after mating for 17 consecutive days. The effects of WDPMP on mood states of pregnant mice were examined by a series of characteristic depression- and anxiety-like behavioural tests during gestation or after lactation. To uncover the molecular mechanisms underlying this model, transcriptomic alterations in the hippocampus of pregnant WDPMP mice were profiled by RNA sequencing. The mRNA and protein levels of brain-derived neurotrophic factor (BDNF), which is a member of the neurotrophin family and plays a key role in mood-related brain functions [21, 22], in behaviourally relevant brain regions were measured. The role of epigenetic regulations, including DNA methylation and microRNA (miRNA), in WDPMP-induced alterations of BDNF expression, were further examined in these brain regions.

Methods

Animals

Male and female 8-week-old C57BL/6J mice were purchased from the Chinese Academy of Medical Sciences. Eight-week-old CD1 male mice were ordered from the Animal Center of

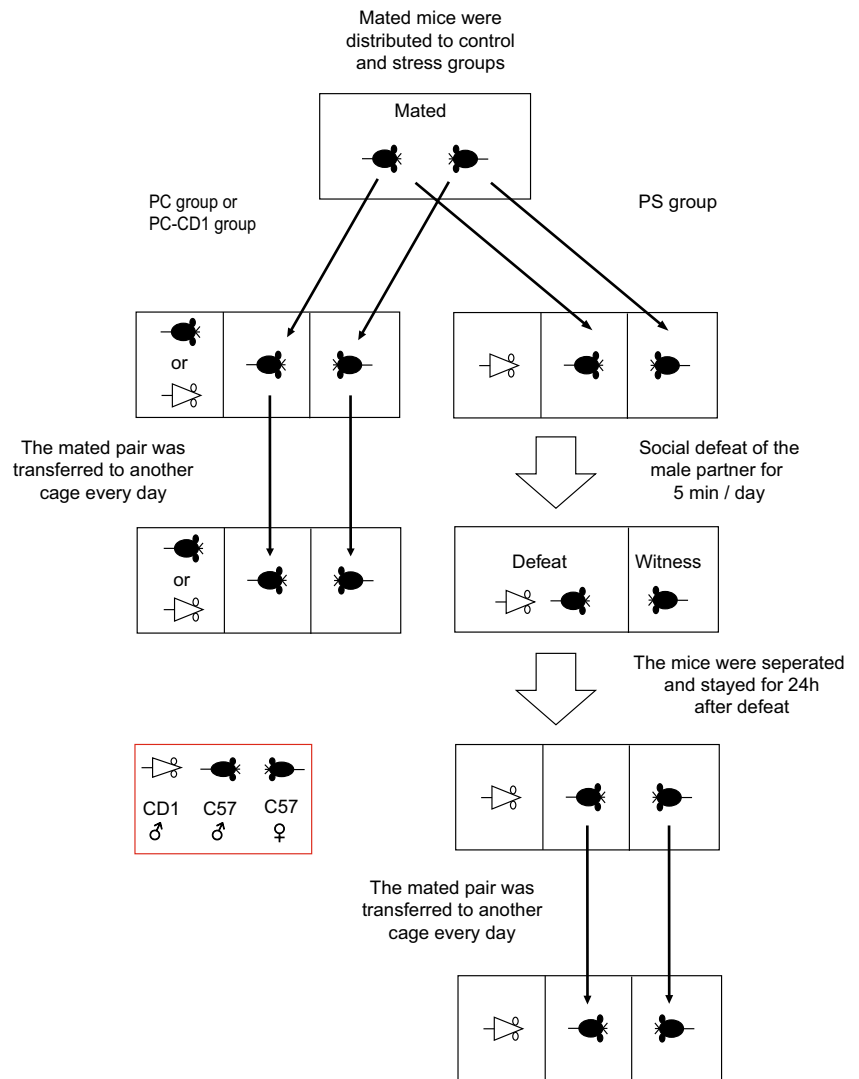
Vital River Laboratories (China). C57BL/6J mice were maintained in groups (no more than five mice per cage) in standard mouse cages, while CD1 male mice were housed individually. All the animals were housed within a temperature- (22–24 °C) and humidity (40–60 %) controlled environment with a 12/12-h light/dark cycle, and all had ad libitum access to food and water. All animal experiments were performed in accordance with the guidelines of the National Institutes of Health (NIH) and approved by the Animal Usage Committee at the Institute of Psychology, Chinese Academy of Sciences.

The Stress Model Witnessing the Defeat Process of a Mated Partner

Each stress cage (45 cm × 30 cm × 17.5 cm) for the WDPMP model was attached to an inner base plate (height 6.5 cm). The stress cage was divided into three equal rooms (45 cm × 10 cm × 11 cm) by two transparent boards with small air holes, designed so the experimental “witness” mice could see, smell and hear but not touch the mated partner. The distance between the base plate and the food/water supply was 7 cm. Aggressive CD-1 male mice were selected as described in detail elsewhere [10] and then transferred into stress cages to serve as social aggressors. The male and female C57BL/6J mice were paired and mated in one cage at 6:00–7:00 pm, and a vaginal plug, the known criteria for successful mating, was checked at 9:00–10:00 am on the second day. If a vaginal plug was not confirmed, the paired mice were separated and the female C57BL/6J mouse was introduced to the next round of mating with a different male C57BL/6J mouse. The mating procedure lasted for 3 days or until the vaginal plug was detected, after which the paired mice were left together in the mating cage for 24 h. They were then transferred to the stress cage, where the C57 mice were separately placed in adjacent but mutually inaccessible chambers. In the pregnant stressed (PS) and pregnant control-CD1 (PC-CD1) groups, the male CD1 mouse was in the chamber adjacent to the male C57 mouse, while in the pregnant control (PC) group, the male CD1 mouse was replaced by a male C57 mouse, which was kept in group and did not show obvious combat with other mice. Except for the social defeat process, there was no direct bodily contact among the three mice in one stress box (Fig. 1). The body weight of pregnant mice was recorded daily from (D)ay 0 to D7 of the WDPMP period and the gain of body weight was calculated.

The C57 mice were exposed to the social defeat process for 5 min/day at the fixed time point of 9:00–10:00 am, to avoid the effect of circadian rhythm, on 17 consecutive days (the period of WDPMP), throughout which the female C57 mouse stayed in the adjacent chamber. Paired mice in the PS group, but not the individual aggressor mice, were transferred to different experimental cages each day during the social stress period, to prevent the CD1 mice from growing familiar with

Fig. 1 Witness the defeat process of mated partner (WDPMP) stress modelling. The schematic description of WDPMP. In the pregnant stressed (PS) group, the female mouse witnessed its mate undergo social defeat for 5 min/day on 17 consecutive days. For pregnant control (PC) and control-CD1 (PC-CD1) groups, a C57BL/6J male mouse and a CD1 aggressive mouse were separately placed in the adjacent chamber of the mated C57BL/6J mouse without bodily contact. A given mated pair in all groups was transferred to another cage every day



(and less aggressive towards) the C57 mice. The paired mice in PC and PC-CD1 groups were also transferred to another cage every day without exposure to the individual resident mice. In the process of WDPMP, if the open wounds exceeded 1 cm, the male mice were removed and immediately killed.

After WDPMP stress induction, the behavioural tests were performed on 3-week postpartum (i.e., after lactation) dams. The behavioural tests were performed over the course of 2 weeks in the following sequence: locomotor activity, open field test, light/dark box test, elevated plus maze test, sucrose preference test and forced swimming test. During the 2 weeks of behavioural tests, mice were handled daily.

Sucrose Preference Test

The sucrose preference test was used to evaluate anhedonia, a typical feature of depression. Female mice were housed individually and habituated to sucrose water (1.5 %, w/v) for 24 h. To obtain a sucrose preference baseline, each mouse was

allowed to choose freely between two bottles containing 150 ml of either sucrose solution or water for 24 h. Then, the mated mice were randomly subjected to control and stress groups. During the period of WDPMP stress, sucrose preference was measured on D1, D4, D7, D10, D13 and D16 of the WDPMP period. After WDPMP stress, sucrose preference was measured once again for 24 h to assess the persistence of anhedonia. Positioning of sucrose and water bottles was exchanged during test periods to prevent position preference. The consumption of sucrose solution and water was quantified by subtracting the weights of the bottles from their initial weights. Sucrose preference was calculated as the ratio of sucrose intake over total fluid intake. A lower sucrose preference was representative of the anhedonic phenotype.

Open Field Test

An open field arena constructed with black PVC (30 cm × 30 cm × 40 cm) was used to assess the locomotor

activity and anxiety-like behaviour. The arena was divided into a central part (36 %, 18 cm × 18 cm) and a peripheral part (64 %). At the beginning of each test, the mouse was placed on the peripheral part of the arena and allowed to explore for 30 min. Total travelling distance covered during 30 min was recorded as the baseline locomotor activity. The travelling distance in the central part and in the whole arena (central part plus peripheral part) for the first 5 min was also recorded. The centre ratio of travelling was calculated as distance in the central part/distance in the whole part. The lower centre ratio was considered to reflect greater anxiety-like behaviour of mice.

Light/Dark Box Test

The apparatus consisted of two equal-sized chambers with one chamber illuminated and the other chamber dark. A test mouse was placed into the dark chamber and permitted to freely explore both chambers for 5 min. The time spent in the light chamber was recorded as an indicator of anxiety-like behaviour.

Elevated Plus Maze Test

The elevated plus maze test also represents a known paradigm examining anxiety-related behaviour in rodents. The apparatus consisted of two open arms, two closed arms (30 × 7 cm each arm) and a central part (7 × 7 cm). The platform was 1 m above the floor. At the beginning of each test, the test mouse was placed in the central part, facing one of the closed arms, and then allowed to explore for 5 min. Time spent in open arms reflected anxiety-like behaviour in mice.

Forced Swimming Test

The forced swimming test (FST) was performed according to a previously published protocol with some modifications [23]. Briefly, the protocol included a 15-min adaptive period (24 h before the formal test) and a 5-min testing period. Mice were individually placed into a glass cylinder (height 40 cm, diameter 20 cm) containing 30 cm of water (24–26 °C). The entire session was recorded by a camera located above the cylinder. Immobility time was recorded as the reflection of depression-like behaviour.

Tissue Preparation, DNA and RNA Extraction and Quantitative Real-Time PCR

Twenty-four hours after the last behavioural test, the mice were anaesthetised and sacrificed by decapitation. Hippocampal samples were directly dissected. For medial prefrontal cortex (mPFC) and amygdala samples, the brains were immediately frozen on dry ice and sliced coronally, and the samples were collected by needle punch and finally were

stored at –80 °C. Representative punch locations are shown in Fig. S1 [24]. Total DNA and RNA were extracted by using DNA/RNA/Protein Isolation kit (Omega, USA) or miRNeasy Mini Kit (Qiagen, USA). miRNAs were extracted by using miRNeasy Mini Kit and RNeasy® MinElute™ Cleanup Kit (Qiagen, USA). RNA of the HT22 cells was extracted by using TRIzol® Reagent (Tiangen, China) according to the manufacturer's instructions. Single-stranded cDNA of total RNA and miRNA was separately synthesized using the Reverse Transcription System (Promega, USA) and Mir-X™ miRNA First-Strand Synthesis kit (Clontech, USA). Real-time PCR was performed using Stratagene Mx3000P System with a two-step cycling program: 95 °C for 10 min, 40 cycles of 95 °C for 1 min and 60 °C for 30 s. GAPDH, U6 snRNA and miR-26b were used as endogenous controls for normalization. All PCR primers are listed in Tables S1 and S2. U6 primers and the reverse primer for q-PCR of miRNAs were provided in Mir-X™ miRNA First-Strand Synthesis kit (Clontech, USA). The relative expression level was calculated by the comparative Ct method, and all results were shown as fold change using the $2^{-\Delta\Delta Ct}$ approach.

RNA Sequencing and Target Gene Screening

Total RNA was extracted from the hippocampus of PC and PS mice, and the quality was checked on an Agilent Bioanalyzer 2100 RNA 6000 Nano Kit (Agilent Technologies, USA). Library generation and sequencing were performed using the TruSeq RNA Sample Prep Kit v2 and the HiSeq2000 Sequencing System (Illumina, USA) according to Illumina protocols. All reads were realigned to the UCSC mm10 version of the mouse genome assembly using the TopHat2 short read alignment program [25]. After removal of the adaptor sequences and trimming of low quality sequences with *cutadapt* [26], the reads were mapped to the genome using TopHat2 with parameter $-r$ 50. Only uniquely mapping reads were used in this analysis. The abundance of mRNAs for all annotated genes from the UCSC mm10 annotation of the mouse genome was calculated using the software package HTseq (version 0.6.1) (<http://sourceforge.net/projects/htseq/website>). Total count normalization and the fitting of the Poisson model were performed in R, and upper quartile normalization along with the negative binomial exact test was performed using the edgeR Bioconductor package [27]. The genes for which *P* values and FDR values were below 0.001, and for which fold change $\geq \pm 1.25$, were considered to be differently expressed between PC and PS groups. Different expression of isoforms was analysed by Cufflinks [28] with FDR values < 0.01 . The significantly differently expressed genes were analysed online using the Toppgene software (<https://toppgene.cchmc.org/>) [29]. This analysis could list the priority of genes based on several screening features,

including molecular function, biological process, cellular component, mouse phenotype, pathway and disease. Moreover, a training set of genes based on similar function was also required for screening target genes. In our study, the training set (Table S3) of anxiety-related genes was collected from previous studies [30–32].

Bisulfite Sequencing

Hippocampal DNA was bisulfite-treated by using the EZ DNA Methylation-Direct™ Kit (Zymo, USA). The converted DNA was employed to amplify two primary CpG islands (CGIs) at position chr2:109677173-109677637 and 109693467-109694207, according to mouse reference genome mm10 at <http://genome.ucsc.edu/>, in the promoter regions of *Bdnf*I, IIB, IIC, IV and VI (as shown in Fig. S2), using nest PCR. The two CGI regions were amplified in five sections: position 1, 109677173-109677457; position 2, 109677458-109677637; position 3, 109693467-109693769; position 4, 109693770-109694090; and position 5, 109694091-109694207. The PCR program for amplifying bisulfite-modified DNA was as follows: 95 °C for 10 min; 40 cycles of 95 °C for 1 min, 50 °C for 30 s and 72 °C for 50 s; and 72 °C for 10 min. PCR products were recycled and purified using a Zymoclean Gel DNA Recovery kit and DNA Clean & Concentrator-5 (Zymo, USA) and then ligated to the pGEM-T Vector (Promega, USA). After transformation and culture, 14–20 clones of each sample were sequenced at the Beijing Genomics Institute (BGI, China). The results were analysed at <http://quma.cdb.riken.jp/>, and the methylation levels of each single CpG site were subsequently compared between the PC and PS groups using Fisher's exact test.

Cell Culture and Transfection

Hippocampal HT22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with 10 % foetal bovine serum (Gibco, USA) and in an incubator with 5 % CO₂ at 37 °C. Twenty-four hours after seeding into six-well plates, the cultured cells were divided into two groups: a negative control (NC) and a miR-206 mimic group. For transient transfection, final concentrations of 50 nM microRNA mimicking negative control and 50 nM miR-206-3p mimic (Genepharma, China) were separately transfected into the cells with Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's recommendation. The serum-free medium was replaced with serum-containing DMEM 6 h after the operation of transfection. Cells were harvested by trypsin (Gibco, USA) digestion 48 h after transfection and washed twice by PBS. Then, mRNA was extracted from the cells for measurement of expression levels of *Bdnf* by q-PCR.

Western Blot

Hippocampal HT22 cells transfected with negative control and miR-206-3p mimic were cultured and harvested as described above. The cells and tissue samples were lysed in RIPA buffer with protease and phosphatase inhibitor and heated at 100 °C for 5 min. Protein samples were loaded and separated by 12 % SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked by 5 % BSA at 4 °C overnight and incubated with anti-BDNF primary antibody (Cat. no. sc-546, Santa Cruz (N-20), 1: 100, USA) and anti-GAPDH primary antibody (Cat. no. sc-32,233, Santa Cruz (6C5), 1: 1000, USA) at 4 °C overnight. After washing, the membranes were incubated with anti-rabbit (Cat. no. 7076s, Cell Signaling Technology, 1:5000, USA) and anti-mouse (Cat. no. 7074s, Cell Signaling Technology, 1:5000, USA) HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were visualized by using enhanced chemiluminescence detection reagents (Applygen Technologies Inc., China) and detected by the FluorChem E imaging system (Cell Biosciences, USA). The densitometric intensity was analysed using ImageJ software.

Data Analysis

Data were expressed as mean ± SEM. Changed body weight and sucrose preference during stress were analysed by using a repeated analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test. The normality assumption was evaluated using the Kolmogorov–Smirnov test. The differences of sucrose preference between three groups during different time points of WDPMP were analysed by one-way ANOVA followed by Tukey's test or Kruskal–Wallis test followed by the Dunn's multiple comparison. The differences of behavioural tests and expression levels of target genes between groups were analysed with Student's *t* test (with Welch's correction) or Mann–Whitney test. The methylation levels of CpG sites were analysed by Fisher's exact test. In all statistical results, significance was considered at *P* value <0.05.

Results

WDPMP During Pregnancy Reduces the Body Weight Gain in PS Mice

The schematic description of WDPMP and the time course of the experiments were shown in Figs. 1 and 2a. Given that the

weight of pregnant mice during the late gestation may be confounded by that of embryos to a large extent, the body weight gain of pregnant mice during the early gestation (days 0–7 of the WDPMP period) was calculated. Both the daily body weight gain (Fig. 2b, $F_{(1, 14)} = 6.333$, $P < 0.05$) and the overall body weight gain (Fig. 2c, $t_{14} = 2.517$, $P < 0.05$) during early WDPMP showed significant difference between the PC group and the PS group. These results suggest that the weight gain of the PS mice was reduced by WDPMP stress during the early period of gestation.

WDPMP Induces Anhedonia in PS Mice During the Late Period of Gestation

The sucrose preference baseline did not show significant differences among PC, PC-CD1 and PS groups (Figs. 2d and S3). Compared to PC group, PS group showed significantly decreased sucrose preference overall ($F_{(1, 43)} = 13.756$, $P < 0.01$), which was mainly confined to the late period of WDPMP (Fig. 2d, D10, $F_{(1, 43)} = 7.44$, $P < 0.01$; D13, $F_{(1, 43)} = 10.06$, $P < 0.01$; D16, $F_{(1, 43)} = 12.65$, $P < 0.01$; Fig. S3,

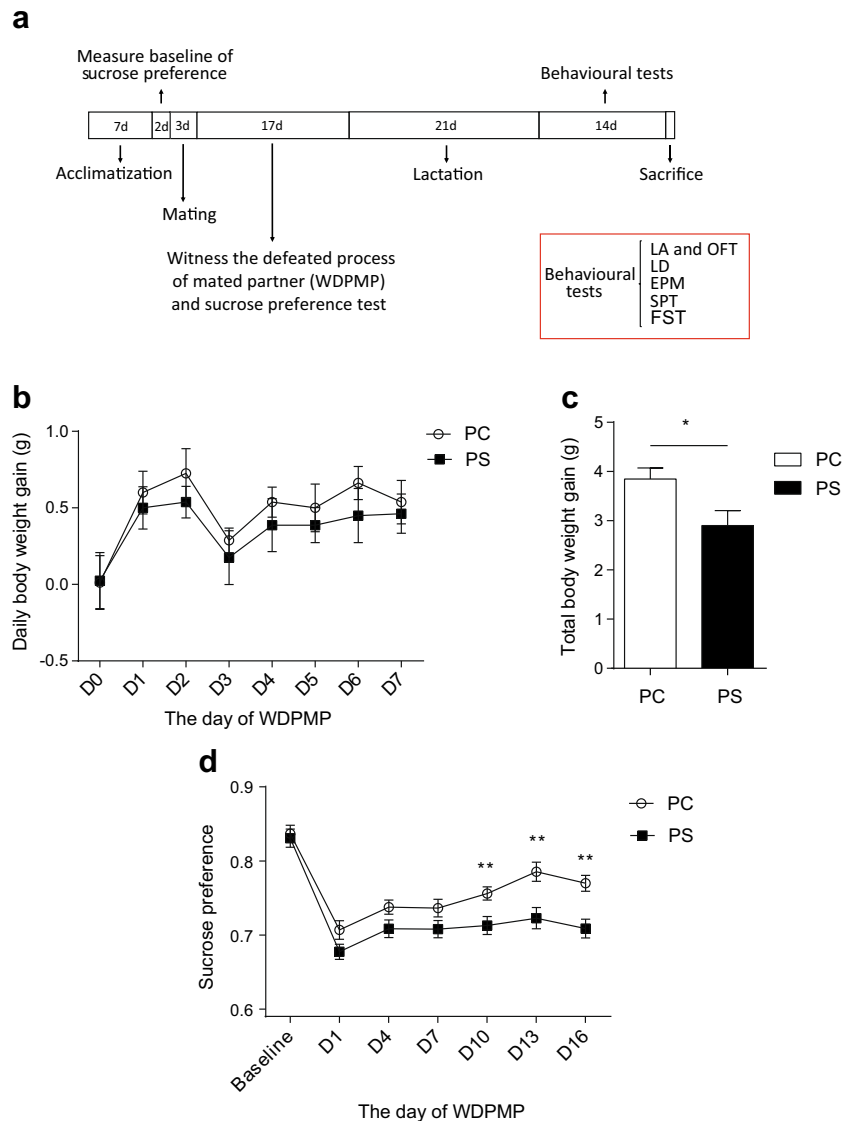


Fig. 2 Time schedule of the experiments, sucrose preference test and body weight gain during WDPMP. **a** The time course of the experimental design. Male and female C57BL/6J mice were habituated for 7 days, and then their baseline of sucrose preference was measured. After mating, WDPMP was performed on female mice in the stress group, and the sucrose preference of each mouse was measured on days (D) 1, 4, 7, 10, 13 and 16 of the WDPMP period. The open field test (OFT), locomotor activity (LA), light/dark test (LD), elevated plus maze test (EPM), sucrose preference test (SPT) and forced swimming test (FST) were performed on pregnant control

(PC) and pregnant stressed (PS) groups after lactation. Mice were sacrificed 24 h after the end of all behavioural tests, and specific brain tissues were dissected for nucleic acid extraction. **b**, **c** The daily and total gain of body weight and in PC and PS mice before (D0) and during the WDPMP period (D1–7) ($n = 8$ in both groups). **d** Sucrose preference in PC and PS mice before and during the WDPMP period ($n = 20$ in PC group, $n = 25$ in PS group). Repeated measures ANOVA was used for the comparisons of sucrose preference between control and stress groups. * $P < 0.05$, ** $P < 0.01$. Data are presented as mean \pm SEM

D13 and D16, $P < 0.05$). Compared to PC-CD1 mice, PS mice similarly displayed decreased sucrose preference during the late period of WDPMP (Fig. S3, D7, D13 and D16, $P < 0.05$). These results suggest that PS mice developed anhedonia, which worsened over the time course of application of WDPMP stress. Interestingly, the presence of CD1 mice during gestation (PC-CD1) without defeating the male C57 mice did not induce anhedonia in female mice, as there was no significant difference of sucrose preference between PC and PC-CD1 groups during gestation (Fig. S3). These results suggest that the simply existence of aggressive individuals may not induce anhedonia in pregnant mice.

WDPMP During Pregnancy Induces Anxiety-Like Behaviours in 3-Week Postpartum Dams

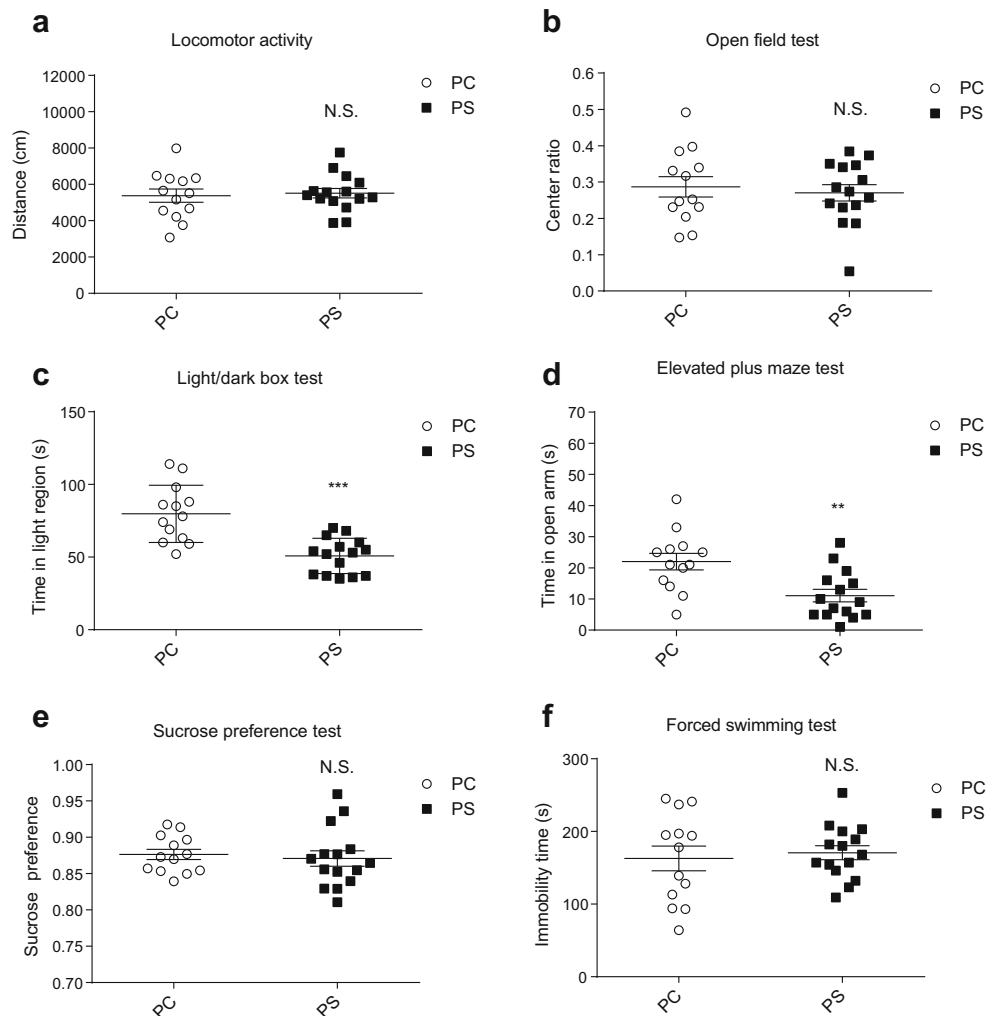
Considering the confounding influence of weaning and other related physiological conditions during lactation, we performed depression-/anxiety-related behavioural tests on the dams after lactation to examine the long-term effects of WDPMP (3 weeks postpartum; see Fig. 2a). No significant

differences were found in either the locomotor activity (Fig. 3a) or the open field test between the PC and PS groups (Fig. 3b). However, in the light/dark box test, PS mice spent less time in the light chamber than PC mice did (Fig. 3c, $t_{26} = 4.751$, $P < 0.001$). Similarly, PS mice exhibited significantly less time spent in the open arms of the elevated plus maze as relative to those in PC group (Fig. 3d, $t_{26} = 3.339$, $P < 0.01$). These results suggest that postpartum dams experienced WDPMP stress during pregnancy showed anxiety-like phenotypes even after lactation.

WDPMP Stress Does Not Induce Long-Term Depression-Like Behaviour in Postpartum Dams

To test whether the anhedonia in the stressed pregnant dams was persistent after pregnancy, mice from both PC and PS groups were assessed via the sucrose preference and forced swimming tests. Unlike the results obtained in the late gestational period, sucrose preference after lactation was not significantly different between PC and PS groups (Fig. 3e, $t_{26} = 0.423$, $P = 0.676$). In the forced swimming test, the PS

Fig. 3 Behavioural testing of psychologically stressed pregnant mice after lactation. The performance of pregnant control (PC) and pregnant stressed (PS) groups 3 week postpartum in the locomotor activity test (a), open field test (b), light/dark test (c), elevated plus maze test (d), sucrose preference test (e) and forced swimming test (f). $**P < 0.01$, $***P < 0.001$; N.S. represents not significant ($n = 13$ in PC group, $n = 15$ in PS group). Data are presented as mean \pm SEM and analysed via Student's t test



group was not significantly different from the PC group in immobility time (Fig. 3f). These results suggest that WDPMP stress may induce transient anhedonia in pregnant mice.

Abnormal Expression of BDNF in the Hippocampus, Medial Prefrontal Cortex and Amygdala of PS Mice

Hippocampus, mPFC and amygdala are the main brain regions associated with anxiety-like phenotypes [33]. In particular, the hippocampus is considered to be highly relative to the transiently appeared anhedonia phenotype [34–36]. In order to gain insight into the underlying mechanisms of the above phenotypic changes, we extracted mRNA from the hippocampus of postpartum dams from both PS and PC groups and used samples of the mRNA pooled from two to three individuals per group to perform gene expression profiling. Of all analysed genes, 434 were differentially expressed (fold change $\geq \pm 1.25$, P value and false discovery rate (FDR) < 0.001). Of these, 184 were upregulated and 250 were downregulated in the PS group vs. the PC group (Fig. 4a and Table S4). These differently expressed genes were further screened for functionality and/or protein–protein interactions online by the ToppGene analysis at <https://toppgene.cchmc.org/>. The 20 top-ranked genes were shown in Table S5. The expression levels of *Grin2a*, *Npy*, *Bdnf*, *Grm8*, *Cxcr4*, *Ptgds*, *Nr4a2*, *Cckbr*, *Enpp2*, *Igf2* and *Plagl1* were verified using quantitative PCR (Fig. S4a, b). We then selected genes with relative low individual variation within groups, including *Npy*, *Bdnf*, *Grm8* and *Igf2*, and performed q-PCR in a new batch of mRNA samples of the PC and PS groups. The results showed that the expression levels of total *Bdnf* ($t_{10} = 2.72$, $P < 0.05$) and *Npy* ($t_{10} = 3.10$, $P < 0.05$) were significantly different between the PC and PS groups (Fig. 4b). In mammalian brains, abnormal BDNF expression is associated with the pathogenesis of several mental disorders, including depression and anxiety [37, 38]. In view of different transcript variants of *Bdnf* that may function in distinct neurobiological processes such as depression [39], we analysed the expression levels of specific *Bdnf* transcripts in the hippocampus. Consistent with previous research [40], the results showed that the levels of *Bdnf* I, *Bdnf* IIB, *Bdnf* IIC, *Bdnf* IV and *Bdnf* VI were relatively higher than other variants in the hippocampus (Fig. 4c and Table S6). We then used q-PCR to quantify the levels of total *Bdnf* and its five primary transcripts in the hippocampus, mPFC and amygdala of PC and PS mice. The results showed that expression levels of total *Bdnf* and particular *Bdnf* transcripts changed significantly in the examined brain regions of the PS group relative to the PC group (Fig. 4d–f; hippocampus: *Bdnf* I, $t_{10} = 3.791$, $P < 0.01$; *Bdnf* IIB, $t_{10} = 2.815$, $P < 0.05$; *Bdnf* IIC, $t_{10} = 2.473$, $P < 0.05$; *Bdnf* IV, $t_{10} = 3.337$, $P < 0.05$; *Bdnf* VI, $t_{10} = 4.612$, $P < 0.01$; mPFC: total *Bdnf*, $t_8 = 2.416$, $P < 0.05$; *Bdnf* IV, $t_8 = 4.771$,

$P < 0.01$; *Bdnf* VI, $t_8 = 4.705$, $P < 0.01$; amygdala: total *Bdnf*, $t_8 = 2.482$, $P < 0.05$; *Bdnf* I, $t_8 = 3.990$, $P < 0.05$; *Bdnf* IIB, $P < 0.01$; *Bdnf* IIC, $t_8 = 3.067$, $P < 0.05$; *Bdnf* IV, $t_8 = 3.069$, $P < 0.05$). To further determine if these changes occurred at the protein level, Western blot was performed to detect BDNF in these three brain regions in PC and PS mice. Consistent with the results of mRNA expression, BDNF protein levels in PS mice were also significantly changed in the examined brain regions relative to the PC group (Fig. 4g, h; hippocampus, $t_4 = 3.419$, $P < 0.05$; mPFC, $t_4 = 2.990$, $P < 0.05$; amygdala, $t_4 = 6.448$, $P < 0.01$). These results suggest that BDNF expression levels were altered in the hippocampus, mPFC and amygdala following WDPMP stress.

DNA Methylation of CpG Islands in *Bdnf* Promoter Regions Remains Unchanged in the Hippocampus of PS Mice

DNA methylation is a key component of environmentally sensitive epigenetic regulation. To investigate the epigenetic regulation involved in decreased BDNF levels in the hippocampus of PS mice, we examined the DNA methylation levels at CGIs of the *Bdnf* promoter [41, 42]. CGIs have been reported to contain a high concentration of CpG sequences [43] that regulate transcription of genes by modulating their promoters [44]. We hypothesised that the decreased expression of *Bdnf* could be due to the hypermethylation of CGIs at its promoter. The methylation levels of two regions of the *Bdnf* promoter, chr2:109677173–109677637 and chr2:109693467–109694207 (compared to the mm10 reference genome at <http://quma.cdb.riken.jp/>), were analysed in PC and PS groups. However, none of the CpG sites in these two CGIs was significantly hypermethylated ($P > 0.05$, Fisher's exact test, Fig. 5a and Table S7), indicating that DNA methylation in these regions was not likely to be involved in the downregulation of different *Bdnf* transcripts in the hippocampus of PS mice.

Abnormal Levels of miR-206-3p in the Hippocampus, mPFC and Amygdala of PS Mice

miRNAs are a group of small (21–23 nucleotides), non-coding RNAs that can regulate gene expression by binding to the complementary sequences in the 3' untranslated region (UTR) [45]. It has been found that miR-206-3p are involved in the pathology of Alzheimer's disease and bipolar disorder through regulating BDNF expression in the hippocampus and mPFC [46, 47]. As miRNA binding on the 3'UTR of *Bdnf* is another potentially epigenetic mechanism regulating its expression, we then examined the levels of miR-206-3p in the above brain tissues of PC and PS mice. As shown in Figs. 5b and S5, miR-206-3p was significantly upregulated in both the hippocampus (miR-206-3p/U6, $t_{10} = 3.207$,

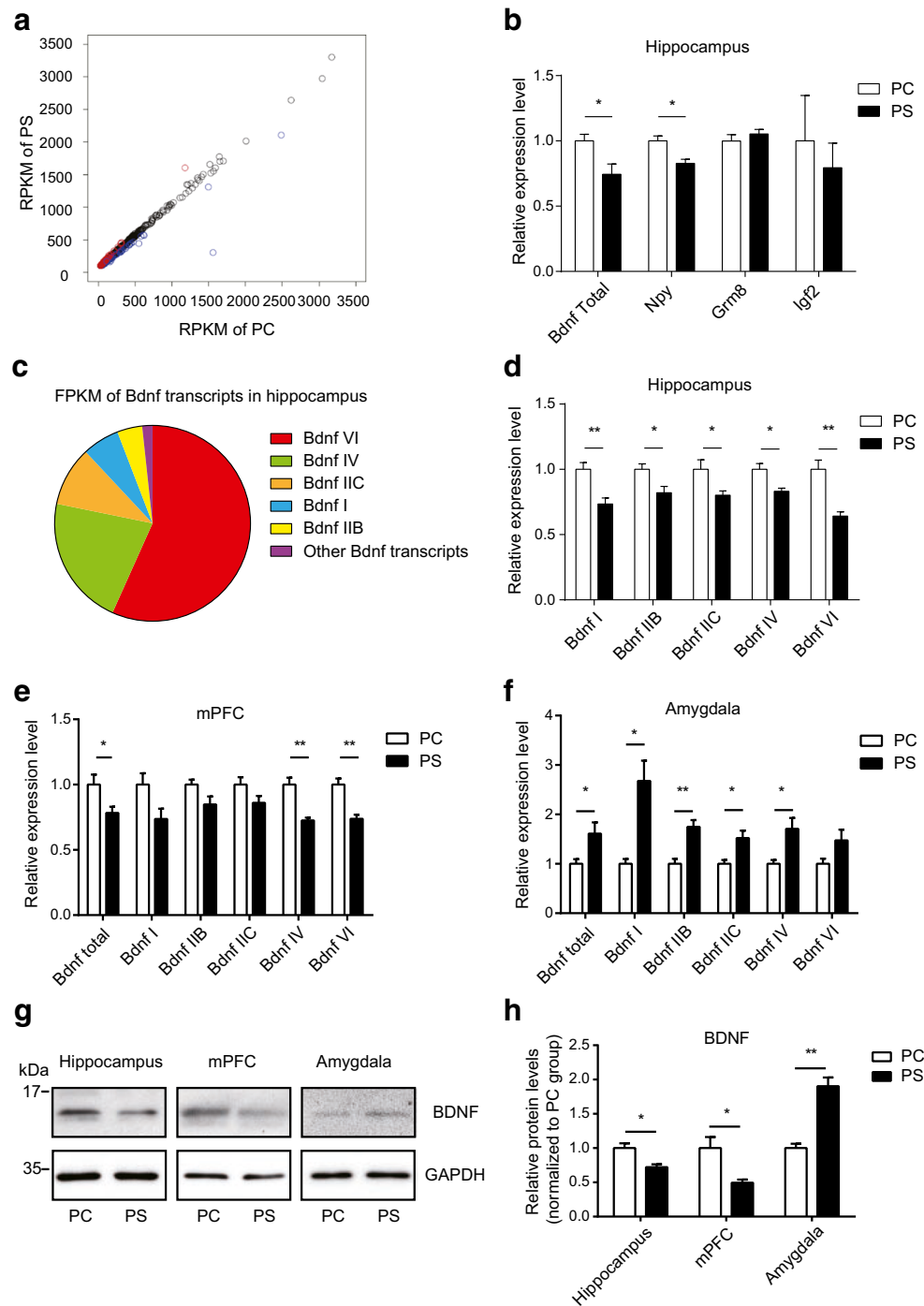
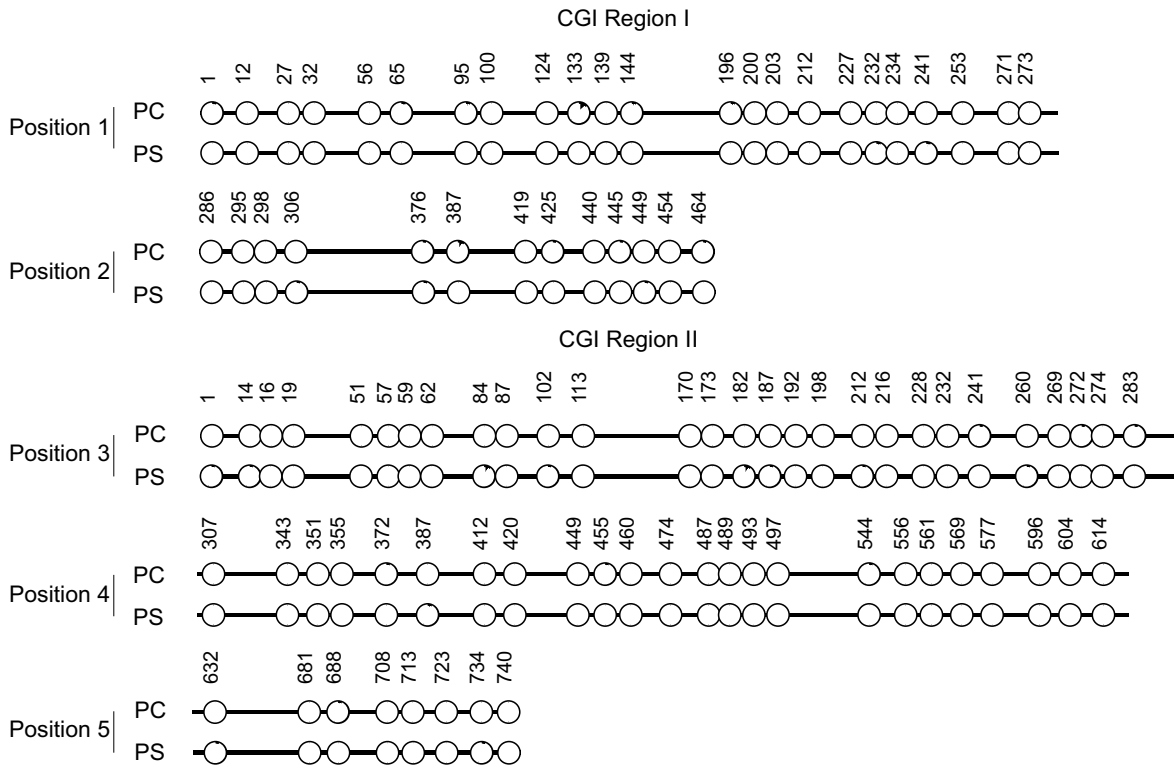


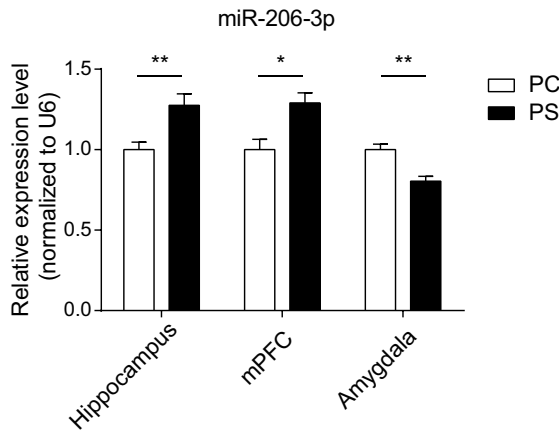
Fig. 4 Altered BDNF mRNA and protein levels in the hippocampus, medial prefrontal cortex (*mPFC*) and amygdala of pregnant stressed (*PS*) and pregnant control (*PC*) mice. **a** Comparison of reads per kilobase of exon model per million mapped reads (*RPKM*) in the hippocampus of the *PC* and *PS* groups as revealed by RNA sequencing data. Total RNA samples for RNA sequencing were pooled from two to three individuals per group. *Red and blue circles* respectively represent upregulated and downregulated genes (*PS* vs. *PC*, fold change $\geq \pm 1.25$, *P* value and false discovery rate (*FDR*) < 0.001), and *black circles* represent genes without significant changes in *PS* groups. **b** Quantitative PCR (*q-PCR*) verification of candidate genes in the hippocampus between *PC* and

PS groups ($n = 6$ in each group). **c** Fragments per kilobase of exon per million fragments mapped (*FPKM*) of *Bdnf* transcripts in the hippocampus as revealed by RNA sequencing data. The *FPKM* value of each *Bdnf* transcript was the sum of the relative values in the *PC* and *PS* groups. *q-PCR* verification of total *Bdnf* and primary *Bdnf* transcripts in the hippocampus (**d**) ($n = 6$ in *PC* and *PS* groups), *mPFC* (**e**) ($n = 5$ in *PC* and *PS* groups) and amygdala (**f**) ($n = 5$ in *PC* and *PS* groups). **g, h** The protein levels of BDNF in the hippocampus, *mPFC* and amygdala ($n = 3$ in *PC* and *PS* groups). $*P < 0.05$, $**P < 0.01$, Student's *t* test. Data are presented as mean \pm SEM

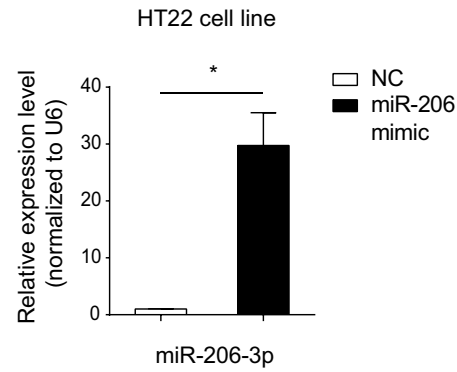
a



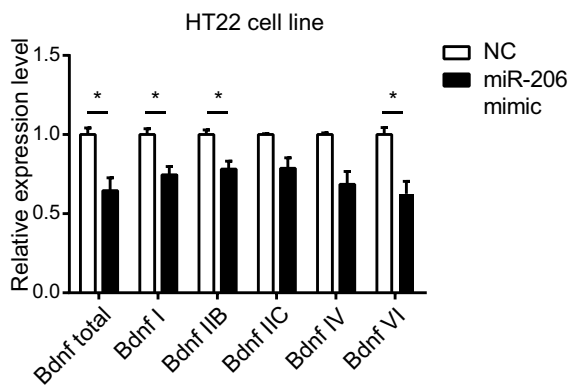
b



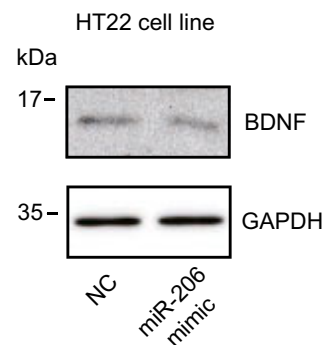
c



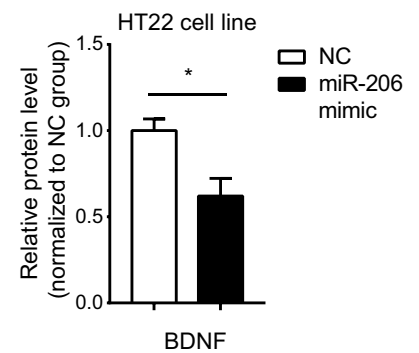
d



e



f



◀ **Fig. 5** Abnormal levels of miR-206-3p and BDNF in the hippocampus, medial prefrontal cortex (mPFC) and amygdala of pregnant stressed (PS) mice. **a** Comparison of the DNA methylation level of two CpG islands (CGIs) in *Bdnf* promoter regions of pregnant control (PC) and PS groups. CGI region I (position: chr2:109677173-109677637) includes two parts, and CGI region II (position: chr2:109693467-109694207) includes three parts, based on the mouse reference genome (mm10). Each circle represents one CpG site, and the black sector in circles represents the methylation level. The number of clones is 14–20, $P > 0.05$, Fisher's exact test. **b** Expression levels of miR-206-3p in hippocampus (U6 was used as an endogenous control) ($n = 6$ in each group), mPFC ($n = 5$ in each group) and amygdala ($n = 5$ in each group), of PC and PS groups. **c** q-PCR verification of miR-206-3p levels in a miR-206-3p transfected HT22 cell line (U6 was used as an endogenous control) ($n = 3$ in the negative control (NC) and miR-206 mimic group). **d** q-PCR verification of total *Bdnf* and five primary *Bdnf* transcripts in a miR-206-3p transfected HT22 cell line ($n = 3$ in the NC and miR-206 mimic groups). **e, f** Protein levels of BDNF in a miR-206-3p transfected HT22 cell line ($n = 3$ in the NC and miR-206 mimic groups). GAPDH was used as an internal control; * $P < 0.05$, ** $P < 0.01$, Student's *t* test. Data are presented as mean \pm SEM

$P < 0.01$; miR-206-3p/miR-26b, $t_{10} = 3.405$, $P < 0.01$) and mPFC (miR-206-3p/U6, $t_8 = 3.237$, $P < 0.05$; miR-206-3p/miR-26b, $t_8 = 4.027$, $P < 0.01$) but downregulated in the amygdala (miR-206-3p/U6, $t_8 = 4.272$, $P < 0.01$; miR-206-3p/miR-26b, $t_8 = 3.262$, $P < 0.05$) of PS mice. This result suggests a negative correlation between the regulation of *Bdnf* and miR-206-3p expression in the hippocampus, mPFC and amygdala of the PS mice.

Decreased BDNF Levels in HT22 Hippocampal Cells After the Transfection of miR-206-3p Mimics

To clarify the causal relationship between BDNF expression and miR-206-3p levels, miR-206-3p mimics, which can simulate the functions of endogenous miR-206-3p, were transfected into HT22 hippocampal cells. After the transfection, miR-206-3p was significantly increased in the miR-206 mimic group when compared to the NC group (Fig. 5c and S6, miR-206-3p/U6, $t_2 = 5.015$, $P < 0.05$; miR-206-3p/miR-26b, $t_2 = 5.729$, $P < 0.05$). Our data also revealed that the expression levels of particular *Bdnf* variants (Fig. 5d, total *Bdnf*, $t_{2.995} = 3.866$, $P < 0.05$; *Bdnf* I, $t_{3.607} = 3.989$, $P < 0.05$; *Bdnf* IIB, $t_{3.312} = 3.799$, $P < 0.05$; *Bdnf* VI, $t_{3.153} = 4.120$, $P < 0.05$) and BDNF protein (Fig. 5e, f, $t_4 = 3.098$, $P < 0.05$) were significantly decreased in the miR-206 mimic group when compared to the NC group. These results suggest that miR-206-3p negatively regulate BDNF expression.

Discussion

Conditioned aversive stress caused by direct physical manipulation of animals can induce abnormal physiological and emotional states; however, the effects of psychological stress devoid of contact, such as that from witnessing violent events,

are not well studied. More recently, by taking advantage of an indirect social defeat protocol, it was found that witnessing traumatic events could induce anxiety- and depression-like behaviours in male rodents [13, 20]. In consideration of gender differences and the importance of gestation on the health of mothers and their offspring, we focused on the effect of witnessing violent events in females during pregnancy by modifying the existing social defeat procedure. It has been suggested that female rats could be negatively affected by the cage-mate under aversive circumstances [19]; in addition, vicarious behaviour was found to be regulated by the degree of relatedness between the interacting individuals [48, 49]. Thus, the mated pairs were utilized in our WDPMP model, in view of that the female mice may suffer intense psychological stress on witnessing the defeat process of their mated partner than other unrelated male mice, due to the vicarious behaviour.

Similar to previous research [13], witness stress significantly reduced the gain of body weight in PS mice when compared to PC mice. Interestingly, we found that the PS mice were anhedonic during the late stage of pregnancy but not during the early stage. This might be due to that anhedonia became more severe with the accumulation of social witness stress. In addition, we cannot exclude the possibility that greater sensitivity to psychological stress in pregnant mice occurs at a late period of gestation. As the anhedonia and immobility time in the forced swimming test were not significantly different between PC and PS mice after lactation, we surmised that WDPMP stress-induced transient anhedonic behaviour in PS mice. In contrast, PS mice exhibiting anxiety-like behaviours after lactation suggest that the psychological stress in our model can also cause long-term disturbances in anxiety-related behaviours.

Warren et al. found that after witnessing social defeat process, male mice displayed social avoidance phenotype [13]. As a psychological stress model similar to that of Warren's, WDPMP stress may also impair the social interaction of female mice, which deserves further study. In addition, the sucrose preference decreased after the transfer of mice from mating cages to stress cages (as shown in Figs. 2d and S3), possibly due to the different tested sucrose preference levels between mating cages and stress cages or the additional stress of being transferred to new environment.

The hippocampus, mPFC and amygdala are the most important brain regions regulating anxiety and depression. Notably, these brain regions have been shown to be bidirectionally connected after stress exposure [33]. For example, chronic stress can impair synaptic plasticity in hippocampoprefrontal cortical pathways and strengthen amygdalo-hippocampal activity in rats [50, 51]. Previous studies in rodent models have shown that stress can regulate BDNF levels in these regions of the brain and that this regulation is related to psychiatric disorders such as anxiety [33, 52, 53]. In

addition, *Bdnf* is regulated by discrete promoters that generate multiple distinct transcripts [54]. These distinct transcripts are differentially expressed across brain regions, as well as in response to special stimulation [55, 56]. Interestingly, we found that the levels of total *Bdnf* and several of its primary transcript variants were decreased in the hippocampus and mPFC, and increased in the amygdala, in stressed postpartum mice. These results suggest that WDPMP stress may exert a general effect on the expression of distinct *Bdnf* isoforms through a shared mechanism. Furthermore, the BDNF protein levels showed the same changes as those of *Bdnf* mRNA in stressed postpartum mice. Together with the association of anxiety-like phenotypes and the changed levels of BDNF, our results suggest that BDNF play an important role in WDPMP-induced anxiety behaviours in pregnant mice, which would if confirmed be a novel function of neurotrophins.

Notably, we also observed decreased *Npy* levels in the hippocampus of PS mice. As *Npy* plays an important role in nervous system and interacts with BDNF [57, 58], this result implies that there are other genes triggered by WDPMP stress, which may also be involved in the anxiety-like behaviours of PS mice. In the study of male mice witnessing social defeat, Warren and colleagues have identified hundreds of overlapped genes between physical stress and emotional stress groups in ventral tegmental area [13]. However, *Bdnf* and *Npy*, which showed significant difference in the hippocampus between PC and PS groups, were not in their overlap list of genes. This suggests that psychological stress may exert sex-specific function through the specific pathways in different brain regions.

Although many studies have demonstrated that environmental stressors altered the DNA methylation level of *Bdnf* promoters, and ultimately its expression level in both humans and rodents [42, 59, 60], we did not detect significant methylation changes in the hippocampus between the PC and PS mice, suggesting that other epigenetic regulation is involved in the alterations of *Bdnf* expression in PS brains. miRNAs are relevant to the pathophysiology of several central nervous system diseases, including depression and anxiety [61, 62]. miRNAs are also critical epigenetic regulators that may modulate the effects of environmental cues acting on physiology and behaviours in rodents [63–65]. Remarkably, we found increased miR-206-3p levels in the hippocampus and mPFC as well as decreased miR-206-3p levels in the amygdala of PS mice. Considering the ability of miRNA to regulate the expression of distinct transcripts of a certain gene by binding to highly conserved sites of their shared 3'UTR, simultaneous alterations of total *Bdnf* and its primary variants were observed in the hippocampus, mPFC and amygdala of PS group. Similar to a previous study [66], we found that upregulation of miR-206-3p in vitro significantly downregulated the levels

of total *Bdnf*, specific *Bdnf* transcript variants and the BDNF protein itself. Our data therefore suggest that WDPMP stress resulted in anxiety-like behaviours in PS mice, which is concurrent with changed expression of BDNF and miR-206-3p levels in them.

Although we found a negative correlation of expression between BDNF and miR-206-3p, it should be noted that in addition to miR-206-3p tested here, other microRNAs (e.g. miR-1) have been reported to regulate expression of *Bdnf* by binding to the 3'UTR [67, 68]. Thus, it is possible that one or some of these other miRNAs, possibly in cooperation with miR-206-3p, induced the alterations of BDNF levels in this study. Moreover, the roles of other epigenetic mechanisms in BDNF regulation, such as DNA methylation in the mPFC and amygdala, histone modifications [69] and regulations of transcription factors and cofactors [70, 71], will need to be considered. Furthermore, the changes of BDNF in the hippocampus, mPFC and amygdala suggest that psychological stress may influence brain function in multiple regions and that the abnormal behaviours induced by this stress may depend on the interaction between different brain regions.

To sum up, our study showed that the psychological stress of witnessing social defeat of a mate during pregnancy induced transient anhedonia during the late period of pregnancy and persistent or recurrent anxiety-like behaviours up to 3 weeks postpartum in pregnant female mice. These behaviours were associated with decreased BDNF expression and increased miR-206-3p expression in the hippocampus and mPFC, as well as increased BDNF expression and decreased miR-206-3p expression in the amygdala of the stressed pregnant mice. The establishment and validation of this novel stress model may help to unveil the biological basis of mood-related behaviour on pregnant women and advance our understanding of the underlying mechanisms of depression and anxiety in general.

Acknowledgements This work was supported by a grant from the International S&T Cooperation Program of China (grant number 2011DFA30670), the State Key Laboratory of Integrated Management of Pest Insects and Rodents (grant number Chinese IPM1406) and the Major State Basic Research Development Program of China (grant numbers 2012CB517902 and 2012CB517904). We are grateful to Li Zhang for her care of the experimental animals and Jianghong Zhang for the experiments of transfection. We would like to thank Editage [<http://www.editage.cn/>] for English language editing, and the authors are entirely responsible for the scientific content of the paper.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

References

- Battle DE (2013) Diagnostic and statistical manual of mental disorders (DSM). *Codas* 25(2):191–192
- Marquesim NA, Cavassini AC, Morceli G, Magalhaes CG, Rudge MV, Calderon IM, Kron MR, Lima SA (2015) Depression and anxiety in pregnant women with diabetes or mild hyperglycemia. *Arch Gynecol Obstet*. doi:10.1007/s00404-015-3838-3
- Correia LL, Linhares MB (2007) Maternal anxiety in the pre- and postnatal period: a literature review. *Rev Lat Am Enfermagem* 15(4):677–683
- Evans J, Heron J, Francomb H, Oke S, Golding J (2001) Cohort study of depressed mood during pregnancy and after childbirth. *BMJ* 323(7307):257–260
- Leigh B, Milgrom J (2008) Risk factors for antenatal depression, postnatal depression and parenting stress. *BMC Psychiatry* 8:24. doi:10.1186/1471-244X-8-24
- Austin MP (2004) Antenatal screening and early intervention for "perinatal" distress, depression and anxiety: where to from here? *Arch Womens Ment Health* (1):1–6. doi:10.1007/s00737-003-0034-4
- Stein A, Pearson RM, Goodman SH, Rapa E, Rahman A, McCallum M, Howard LM, Pariante CM (2014) Effects of perinatal mental disorders on the fetus and child. *Lancet* 384(9956):1800–1819. doi:10.1016/S0140-6736(14)61277-0
- Mairesse J, Silletti V, Laloux C, Zuena AR, Giovine A, Consolazione M, van Camp G, Malagodi M et al (2013) Chronic agomelatine treatment corrects the abnormalities in the circadian rhythm of motor activity and sleep/wake cycle induced by prenatal restraint stress in adult rats. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 16(2):323–338. doi:10.1017/S1461145711001970
- Faron-Gorecka A, Kusmider M, Kolasa M, Zurawek D, Szafran-Pilch K, Gruca P, Pabian P, Solich J et al (2015) Chronic mild stress alters the somatostatin receptors in the rat brain. *Psychopharmacology*. doi:10.1007/s00213-015-4103-y
- Golden SA, Covington HE 3rd, Berton O, Russo SJ (2011) A standardized protocol for repeated social defeat stress in mice. *Nat Protoc* 6(8):1183–1191. doi:10.1038/nprot.2011.361
- Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM et al (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* 311(5762):864–868. doi:10.1126/science.1120972
- Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A et al (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 131(2):391–404. doi:10.1016/j.cell.2007.09.018
- Warren BL, Vialou VF, Iniguez SD, Alcantara LF, Wright KN, Feng J, Kennedy PJ, Laplant Q et al (2013) Neurobiological sequelae of witnessing stressful events in adult mice. *Biol Psychiatry* 73(1):7–14. doi:10.1016/j.biopsych.2012.06.006
- Blanchard EB, Kuhn E, Rowell DL, Hickling EJ, Wittrock D, Rogers RL, Johnson MR, Steckler DC (2004) Studies of the vicarious traumatization of college students by the September 11th attacks: effects of proximity, exposure and connectedness. *Behav Res Ther* 42(2):191–205. doi:10.1016/S0005-7967(03)00118-9
- Schlenger WE, Caddell JM, Ebert L, Jordan BK, Rourke KM, Wilson D, Thalji L, Dennis JM et al (2002) Psychological reactions to terrorist attacks: findings from the National Study of Americans' Reactions to September 11. *JAMA* 288(5):581–588
- van Wingen GA, Geuze E, Vermetten E, Fernandez G (2011) Perceived threat predicts the neural sequelae of combat stress. *Mol Psychiatry* 16(6):664–671. doi:10.1038/mp.2010.132
- Ribeiro MR, da Silva AA, EA MT, Batista RF, de Rocha LM, Schraiber LB, Medeiros NL, Costa DC et al (2014) Psychological violence against pregnant women in a prenatal care cohort: rates and associated factors in Sao Luis, Brazil. *BMC Pregnancy Childbirth* 14:66. doi:10.1186/1471-2393-14-66
- Raffo JE, Meghea CI, Zhu Q, Roman LA (2010) Psychological and physical abuse among pregnant women in a Medicaid-sponsored prenatal program. *Public Health Nurs* 27(5):385–398. doi:10.1111/j.1525-1446.2010.00871.x
- Atsak P, Orre M, Bakker P, Cerliani L, Roozendaal B, Gazzola V, Moita M, Keysers C (2011) Experience modulates vicarious freezing in rats: a model for empathy. *PLoS One* 6(7):e21855. doi:10.1371/journal.pone.0021855
- Patki G, Solanki N, Salim S (2014) Witnessing traumatic events causes severe behavioral impairments in rats. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 17(12):2017–2029. doi:10.1017/S1461145714000923
- Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B et al (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112(2):257–269
- Nakajo Y, Miyamoto S, Nakano Y, Xue JH, Hori T, Yanamoto H (2008) Genetic increase in brain-derived neurotrophic factor levels enhances learning and memory. *Brain Res* 1241:103–109. doi:10.1016/j.brainres.2008.08.080
- Nasca C, Bigio B, Zelli D, Nicoletti F, McEwen BS (2015) Mind the gap: glucocorticoids modulate hippocampal glutamate tone underlying individual differences in stress susceptibility. *Mol Psychiatry* 20(6):755–763. doi:10.1038/mp.2014.96
- Paxinos G, Franklin KBJ (2004) *The mouse brain in stereotaxic coordinates*, Compact 2nd edn. Elsevier Academic Press, Amsterdam; Boston
- Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25(9):1105–1111. doi:10.1093/bioinformatics/btp120
- Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. pp 10–12
- Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1):139–140. doi:10.1093/bioinformatics/btp616
- Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL et al (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7(3):562–578. doi:10.1038/nprot.2012.016
- Chen J, Bardes EE, Aronow BJ, Jegga AG (2009) ToppGene suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res* 37(Web Server issue):W305–W311. doi:10.1093/nar/gkp427
- Domschke K, Maron E (2013) Genetic factors in anxiety disorders. *Mod Trends Pharmacopsychiatri* 29:24–46. doi:10.1159/000351932
- Le-Niculescu H, Balaraman Y, Patel SD, Ayalew M, Gupta J, Kuczynski R, Shekhar A, Schork N et al (2011) Convergent functional genomics of anxiety disorders: translational identification of genes, biomarkers, pathways and mechanisms. *Transl Psychiatry* 1:e9. doi:10.1038/tp.2011.9
- Savage JE, McMichael O, Gorlin EI, Beadel JR, Teachman B, Vladimirov VI, Hettema JM, Roberson-Nay R (2015) Validation of candidate anxiety disorder genes using a carbon dioxide challenge task. *Biol Psychol* 109:61–66. doi:10.1016/j.biopsycho.2015.04.006
- Chattarji S, Tomar A, Suvrathan A, Ghosh S, Rahman MM (2015) Neighborhood matters: divergent patterns of stress-induced

- plasticity across the brain. *Nat Neurosci* 18(10):1364–1375. doi:10.1038/nn.4115
34. Tang M, Lei J, Sun X, Liu G, Zhao S (2013) Stress-induced anhedonia correlates with lower hippocampal serotonin transporter protein expression. *Brain Res* 1513:127–134. doi:10.1016/j.brainres.2013.03.042
 35. Elizalde N, Garcia-Garcia AL, Totterdell S, Gendive N, Venzala E, Ramirez MJ, Del Rio J, Tordera RM (2010) Sustained stress-induced changes in mice as a model for chronic depression. *Psychopharmacology* 210(3):393–406. doi:10.1007/s00213-010-1835-6
 36. Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature* 476(7361):458–461. doi:10.1038/nature10287
 37. Calabrese F, Molteni R, Maj PF, Cattaneo A, Gennarelli M, Racagni G, Riva MA (2007) Chronic duloxetine treatment induces specific changes in the expression of BDNF transcripts and in the subcellular localization of the neurotrophin protein. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology* 32(11):2351–2359. doi:10.1038/sj.npp.1301360
 38. Wray NR, James MR, Handoko HY, Dumenil T, Lind PA, Montgomery GW, Martin NG (2008) Association study of candidate variants from brain-derived neurotrophic factor and dystrobrevin-binding protein 1 with neuroticism, anxiety, and depression. *Psychiatr Genet* 18(5):219–225. doi:10.1097/YPG.0b013e3283050aee
 39. Hill RA, Klug M, Kiss Von Soly S, Binder MD, Hannan AJ, van den Buuse M (2014) Sex-specific disruptions in spatial memory and anhedonia in a "two hit" rat model correspond with alterations in hippocampal brain-derived neurotrophic factor expression and signaling. *Hippocampus* 24(10):1197–1211. doi:10.1002/hipo.22302
 40. Rousseaud A, Delepine C, Nectoux J, Billuart P, Bienvenu T (2015) Differential expression and regulation of brain-derived neurotrophic factor (BDNF) mRNA isoforms in brain cells from *Mecp2*(308/y) mouse model. *Journal of molecular neuroscience: MN* 56(4):758–767. doi:10.1007/s12031-014-0487-0
 41. Lubin FD, Roth TL, Sweatt JD (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 28(42):10576–10586. doi:10.1523/JNEUROSCI.1786-08.2008
 42. Roth TL, Lubin FD, Funk AJ, Sweatt JD (2009) Lasting epigenetic influence of early-life adversity on the BDNF gene. *Biol Psychiatry* 65(9):760–769. doi:10.1016/j.biopsych.2008.11.028
 43. Saxonov S, Berg P, Brutlag DL (2006) A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc Natl Acad Sci U S A* 103(5):1412–1417. doi:10.1073/pnas.0510310103
 44. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. *Genes Dev* 25(10):1010–1022. doi:10.1101/gad.2037511
 45. Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 10(2):126–139. doi:10.1038/nrm2632
 46. Lee ST, Chu K, Jung KH, Kim JH, Huh JY, Yoon H, Park DK, Lim JY et al (2012) miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model. *Ann Neurol* 72(2):269–277. doi:10.1002/ana.23588
 47. Wang Z, Zhang C, Huang J, Yuan C, Hong W, Chen J, Yu S, Xu L et al (2014) MiRNA-206 and BDNF genes interacted in bipolar I disorder. *J Affect Disord* 162:116–119. doi:10.1016/j.jad.2014.03.047
 48. Langford DJ, Crager SE, Shehzad Z, Smith SB, Sotocinal SG, Levenstadt JS, Chanda ML, Levitin DJ et al (2006) Social modulation of pain as evidence for empathy in mice. *Science* 312(5782):1967–1970. doi:10.1126/science.1128322
 49. Jeon D, Kim S, Chetana M, Jo D, Ruley HE, Lin SY, Rabah D, Kinet JP et al (2010) Observational fear learning involves affective pain system and Cav1.2 Ca²⁺ channels in ACC. *Nat Neurosci* 13(4):482–488. doi:10.1038/nn.2504
 50. Cerqueira JJ, Mailliet F, Almeida OF, Jay TM, Sousa N (2007) The prefrontal cortex as a key target of the maladaptive response to stress. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 27(11):2781–2787. doi:10.1523/JNEUROSCI.4372-06.2007
 51. Ghosh S, Laxmi TR, Chattarji S (2013) Functional connectivity from the amygdala to the hippocampus grows stronger after stress. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 33(17):7234–7244. doi:10.1523/JNEUROSCI.0638-13.2013
 52. Zhang LM, Zhou WW, Ji YJ, Li Y, Zhao N, Chen HX, Xue R, Mei XG et al (2015) Anxiolytic effects of ketamine in animal models of posttraumatic stress disorder. *Psychopharmacology* 232(4):663–672. doi:10.1007/s00213-014-3697-9
 53. Berman AK, Lott RB, Donaldson ST (2014) Periodic maternal deprivation may modulate offspring anxiety-like behavior through mechanisms involving neuroplasticity in the amygdala. *Brain Res Bull* 101:7–11. doi:10.1016/j.brainresbull.2013.12.005
 54. Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. *J Neurosci Res* 85(3):525–535. doi:10.1002/jnr.21139
 55. Hisaoka-Nakashima K, Kajitani N, Kaneko M, Shigetou T, Kasai M, Matsumoto C, Yokoe T, Azuma H et al (2016) Amitriptyline induces brain-derived neurotrophic factor (BDNF) mRNA expression through ERK-dependent modulation of multiple BDNF mRNA variants in primary cultured rat cortical astrocytes and microglia. *Brain Res* 1634:57–67. doi:10.1016/j.brainres.2015.12.057
 56. Lyons MR, West AE (2011) Mechanisms of specificity in neuronal activity-regulated gene transcription. *Prog Neurobiol* 94(3):259–295. doi:10.1016/j.pneurobio.2011.05.003
 57. Takei N, Sasaoka K, Higuchi H, Endo Y, Hatanaka H (1996) BDNF increases the expression of neuropeptide Y mRNA and promotes differentiation/maturation of neuropeptide Y-positive cultured cortical neurons from embryonic and postnatal rats. *Brain Res Mol Brain Res* 37(1–2):283–289
 58. Yoshimura R, Ito K, Endo Y (2009) Differentiation/maturation of neuropeptide Y neurons in the corpus callosum is promoted by brain-derived neurotrophic factor in mouse brain slice cultures. *Neurosci Lett* 450(3):262–265. doi:10.1016/j.neulet.2008.12.010
 59. Song Y, Miyaki K, Suzuki T, Sasaki Y, Tsutsumi A, Kawakami N, Shimazu A, Takahashi M et al (2014) Altered DNA methylation status of human brain derived neurotrophin factor gene could be useful as biomarker of depression. *American journal of medical genetics Part B, Neuropsychiatric genetics: the official publication of the International Society of Psychiatric Genetics* 165B(4):357–364. doi:10.1002/ajmg.b.32238
 60. Baker-Andresen D, Flavell CR, Li X, Bredy TW (2013) Activation of BDNF signaling prevents the return of fear in female mice. *Learn Mem* 20(5):237–240. doi:10.1101/lm.029520.112
 61. Dwivedi Y (2014) Emerging role of microRNAs in major depressive disorder: diagnosis and therapeutic implications. *Dialogues Clin Neurosci* 16(1):43–61
 62. Haramati S, Navon I, Issler O, Ezra-Nevo G, Gil S, Zwang R, Homstein E, Chen A (2011) MicroRNA as repressors of stress-induced anxiety: the case of amygdalar miR-34. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 31(40):14191–14203. doi:10.1523/JNEUROSCI.1673-11.2011
 63. Volk N, Paul ED, Haramati S, Eitan C, Fields BK, Zwang R, Gil S, Lowry CA et al (2014) MicroRNA-19b associates with Ago2 in the amygdala following chronic stress and regulates the adrenergic

- receptor beta 1. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 34(45):15070–15082. doi:[10.1523/JNEUROSCI.0855-14.2014](https://doi.org/10.1523/JNEUROSCI.0855-14.2014)
64. Uchida S, Nishida A, Hara K, Kamemoto T, Suetsugi M, Fujimoto M, Watanuki T, Wakabayashi Y et al (2008) Characterization of the vulnerability to repeated stress in Fischer 344 rats: possible involvement of microRNA-mediated down-regulation of the glucocorticoid receptor. *Eur J Neurosci* 27(9):2250–2261. doi:[10.1111/j.1460-9568.2008.06218.x](https://doi.org/10.1111/j.1460-9568.2008.06218.x)
65. Lin Q, Wei W, Coelho CM, Li X, Baker-Andresen D, Dudley K, Ratnu VS, Boskovic Z et al (2011) The brain-specific microRNA miR-128b regulates the formation of fear-extinction memory. *Nat Neurosci* 14(9):1115–1117. doi:[10.1038/nn.2891](https://doi.org/10.1038/nn.2891)
66. Tian N, Cao Z, Zhang Y (2014) MiR-206 decreases brain-derived neurotrophic factor levels in a transgenic mouse model of Alzheimer's disease. *Neurosci Bull* 30(2):191–197. doi:[10.1007/s12264-013-1419-7](https://doi.org/10.1007/s12264-013-1419-7)
67. Varendi K, Kumar A, Harma MA, Andressoo JO (2014) miR-1, miR-10b, miR-155, and miR-191 are novel regulators of BDNF. *Cell Mol Life Sci* 71(22):4443–4456. doi:[10.1007/s00018-014-1628-x](https://doi.org/10.1007/s00018-014-1628-x)
68. Ma JC, Duan MJ, Sun LL, Yan ML, Liu T, Wang Q, Liu CD, Wang X et al (2015) Cardiac over-expression of microRNA-1 induces impairment of cognition in mice. *Neuroscience* 299:66–78. doi:[10.1016/j.neuroscience.2015.04.061](https://doi.org/10.1016/j.neuroscience.2015.04.061)
69. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* 9(4):519–525. doi:[10.1038/nn1659](https://doi.org/10.1038/nn1659)
70. Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. *Neuron* 33(3):383–395
71. Pruunsild P, Sepp M, Orav E, Koppel I, Timmusk T (2011) Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. *J Neurosci* 31(9):3295–3308. doi:[10.1523/JNEUROSCI.4540-10.2011](https://doi.org/10.1523/JNEUROSCI.4540-10.2011)