Different oxytocin and corticotropin-releasing hormone system changes in bipolar disorder and major depressive disorder patients

Lei Guo,[a,b,1] Yang-Jian Qi,[a,b,1] Hong Tan,[a] Dan Dai,[a,b] Rawien Balesar,[c] Arja Sluiter,[c] Joop van Heerikhuize,[c] Shao-Hua Hu,[d] Dick F. Swaab,[a,b,c]* and Ai-Min Bao [a,b]**

[a]Department of Neurobiology and Department of Neurology of the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China
[b]NHC and CAMS key laboratory of Medical Neurobiology, MOE Frontier Science Center for Brain Research and Brain-Machine Integration, School of Brain Science and Brain Medicine, Zhejiang University, China
[c]Netherlands Institute for Neuroscience, an Institute of the Royal Netherlands Academy of Arts and Sciences, KNAW, Meibergdreef 47, 1105 BA Amsterdam, the Netherlands
[d]Department of Mental Health, Zhejiang Province Key Laboratory of Mental Disorder's Management, First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qing Chun Road, Hangzhou 310003, China

Summary

Background Oxytocin (OXT) and corticotropin-releasing hormone (CRH) are both produced in hypothalamic paraventricular nucleus (PVN). Central CRH may cause depression-like symptoms, while peripheral higher OXT plasma levels were proposed to be a trait marker for bipolar disorder (BD). We aimed to investigate differential OXT and CRH expression in the PVN and their receptors in prefrontal cortex of major depressive disorder (MDD) and BD patients. In addition, we investigated mood-related changes by stimulating PVN-OXT in mice.

Methods Quantitative immunocytochemistry and *in situ* hybridization were performed in the PVN for OXT and CRH on 6 BD and 6 BD-controls, 9 MDD and 9 MDD-controls. mRNA expressions of their receptors (OXTR, CRHR1 and CRHR2) were determined in anterior cingulate cortex and dorsolateral prefrontal cortex (DLPFC) of 30 BD and 34 BD-controls, and 24 MDD and 12 MDD-controls. PVN of 41 OXT-cre mice was short- or long-term activated by chemogenetics, and mood-related behavior was compared with 26 controls.

Findings Significantly increased OXT-immunoreactivity (ir), OXT-mRNA in PVN and increased OXTR-mRNA in DLPFC, together with increased ratios of OXT-ir/CRH-ir and OXTR-mRNA/CRHR-mRNA were observed in BD, at least in male BD patients, but not in MDD patients. PVN-OXT stimulation induced depression-like behaviors in male mice, and mixed depression/mania-like behaviors in female mice in a time-dependent way.

Interpretation Increased PVN-OXT and DLPFC-OXTR expression are characteristic for BD, at least for male BD patients. Stimulation of PVN-OXT neurons induced mood changes in mice, in a pattern different from BD.

*Corresponding author at: Prof. of Neurobiology, University of Amsterdam, Netherlands Institute for Neuroscience, KNAW, Meibergdreef 47, 1105 BA Amsterdam, the Netherlands.
**Corresponding author at: Prof. of Neurobiology, Department of Neurobiology and Department of Neurology of the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310058, China.
    E-mail addresses: d.swaab@nin.knaw.nl (D.F. Swaab), baoaimin@zju.edu.cn (A.-M. Bao).
[1] Contributed equally.

stress response and centrally released CRH is proposed to be responsible for symptoms of depression.[5] Intracerebroventricular CRH administration in the rat caused depression-like behaviors, and CRH receptor (CRHR) antagonist showed an anti-depressive effect in humans.[5,6] OXT, on the other hand, is involved in pro-social behavior and in reducing fear, anxiety and depression-like behavior.[7—9] Indeed, a negative correlation was found between plasma OXT levels and Hamilton Depression Rating Scale scores in MDD patients.[10] It is of interest to note that higher peripheral plasma OXT levels were found in BD patients in both depressive and remission periods, while the highest levels were observed in a manic episode.[11] In addition, peripheral plasma OXT levels were found to be much higher in BD II, i.e. in BD patients who had experienced at least one episode of hypomania (not mania as in BPI) and at least one episode of depression, than in MDD patients or in controls.[12] These data indicate that an activated central OXT system could be a trait marker for BD. However, plasma OXT levels do not necessarily reflect central OXT changes, and at present no information is available on the different central OXT and CRH system changes in BD and MDD.

Based upon the evidence mentioned above, we hypothesized that there may be different OXT and CRH system changes in BD and MDD patients, and that a hyperactive OXT system may be characteristic for BD. Therefore, in this study, we compared CRH and OXT expression in the PVN in MDD and BD patients with their respective controls, both, at the peptide and at the mRNA level. In addition, we compared the mRNA expression of their receptors (OXTR, CRHR1 and CRHR2) in the anterior cingulate cortex (ACC) and dorsolateral prefrontal cortex (DLPFC) of MDD and BD patients with their respective controls. DLPFC and ACC are terminal fields for the OXT and CRH systems,[13,14] and both crucially participate in the pathology of mood disorders[15] and are therapeutic targets for mood disorders, such as for deep brain stimulation[16,17] or for repetitive transcranial magnetic stimulation.[18,19] Finally, we activated PVN-OXT by chemogenetics in mice to validate whether mood-related behavior changes could be induced by specific PVN-OXT stimulation.

Introduction

Major depressive disorder (MDD) and bipolar disorder (BD), although showing distinct symptoms, are both characterized by hyperactive stress-related brain systems.[1—3] The different molecular mechanisms underlying these two pathologies have not yet been revealed. Neuropeptides of the hypothalamic paraventricular nucleus (PVN) play a significant role in the stress response, in mood regulation and in mood disorders.[1] Both animal models and human studies show that oxytocin (OXT) has anxiolytic effects while corticotropin-releasing hormone (CRH) causes depression-like symptoms including anxiety.[1,2,4] CRH is the motor of the

Methods

Part I: postmortem human brain studies

Postmortem hypothalami of MDD or BD patients and their respectively matched controls were obtained from the Netherlands Brain Bank (NBB). Postmortem prefrontal cortices (DLPFC, ACC) of MDD or BD patients and their respectively matched controls were obtained from Stanley Medical Research Institute (SMRI). Diagnoses of MDD or BD were made according to the Diagnostic and Statistical Manual of Mental Disorders

(DSM)-III-R/DSM-IV criteria. The absence of neuro-pathological changes, both in the mood disorders and in the controls, was confirmed by systematic neuropathological investigation.

The MDD patients and BD patients were matched with their respective controls for potential confounding factors including age, sex, postmortem delay (PMD), fixation time (for hypothalamus material), storage time in paraffin (for hypothalamus material), RNA integrity number (RIN) value (for prefrontal cortices), clock time of death, month of death, CSF-pH (a measure of agonal state), brain weight and Braak stages of Alzheimer's pathology.[20] The pH of either brain tissue or CSF, obtained at autopsy, is influenced more by agonal state rather than by postmortem delay. Since the pH of CSF indicates the agonal state of a donor, it has been introduced as a routine procedure in the NBB so that brain samples can be matched for agonal state. Exclusion criteria for control subjects were in the first place the use of corticosteroids, as they inhibit the CRH cells in the human hypothalamus.[21] Clinico-pathological details and p-values of matching are given in Tables 1, 2, Tables S1, S2. Information for sub-group matching is shown in Supplementary Material S1. It should be mentioned that most of the BD patients (n=6) were men (n=5). In addition, it should be noted that the medication use cannot be matched between the mood disorder group and its control group. The possible effect of medicine on our results is discussed in the Discussion section.

Immunocytochemistry, *in situ* hybridization and quantification on hypothalamic material

The hypothalami were dissected at autopsy and fixed in 0·1M phosphate buffered 4% w/v formaldehyde (pH 7·2) for 1−2 months, and were embedded in paraffin. Paraffin-embedded blocks were serially-sectioned at 6μm along the rostro-caudal direction. Thionin staining was performed on every 100th section throughout the hypothalamus to localize the PVN. In addition, every 100th section was stained for CRH or OXT. The rostral and caudal PVN borders were defined as the sections showing no CRH- or OXT-stained cells, respectively. Sections at the peak level of CRH-ir and OXT-ir were selected for CRH-mRNA and OXT-mRNA *in situ* hybridization, respectively. Moreover, sections around the central part of the PVN (according to the thionin staining) were used to test the possible presence of co-localization of CRH and OXT by immunocytochemistry.

Immunocytochemistry and quantification

Information and specificity of CRH and OXT antibodies, as well as key information of immunocytochemical staining and double staining are shown in Table S3 and Table S6. OXT-ir and CRH-ir was quantified by the image analysis procedure described in our previous studies.[22,23] In brief, the set-up consisted of an image analysis system (Image-Pro version 6.3, Media Cybernetics, Rockville, USA) connected to a black and white camera (SONY XC-77E) mounted on a microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany). Quantitative image analysis was carried out by one investigator (L. Guo) who was blind to the experimental conditions. For quantification, the PVN area covered by either CRH-ir or OXT-ir signal was outlined manually at 20x objective. The threshold for the positive signal was set at 2·5 times of the optical density (OD) of the background. The computer determined the OD of the pixels and the surface area covered by the signal (area mask, Fig. S1a-b). To get a measure of signal in a given surface area, the integrated optical density (IOD) was calculated by multiplying the OD by the area of the mask. Completeness of the CRH or OXT staining in the hypothalamus was confirmed by graphically presenting the IODs measured in every section from rostral to caudal, with a line drawn by the Excel trend-line option 'Moving Average'. The value under each curve represented the total IOD of the subject (Fig. S1c). For the analysis of CRH-OXT double staining, Nuance 3.0.1.2 software was used to build up spectral libraries of the chromogens 2. In brief, a single CRH-stained or OXT-stained slide was scanned using a 40x objective to define the respective spectral curves, which were then used to distinguish the two chromogens in the double-stained sections for signal recognition. To define the specific signal during masking, the OD threshold was set at 3 times the background value for both the red and blue chromogens (Table S3).

In situ hybridization and quantification

Chimeric 2′-O-methyl/LNA oligoribonucleotide probes were obtained from Eurogentec (Odense, Denmark). The sequence of the CRH-mRNA anti-sense probe was: 5′-FAM- TmUmG CmUmG TmGmA GmCmU TmGmC TmGmU G-3′ with "FAM" denoting a fluorescein tag, "LNA" denoting a locked nucleic acid at the first position of each triplet and "m" 2′-O-methyl modified ribonucleic acid. The sequence of the scrambled probe for CRH-mRNA was: 5′-FAM-CmGmA TmUmG GmUmG GmUmC TmGmU TmGmC T-3′ The sequence of the OXT-mRNA anti-sense probe was: 5′-FAM-AmGmC CmAmU CmAmA GmUmU TmCmA GmCmG C-3′. The sequence of the scrambled probe for oxytocin-mRNA was: 5′-FAM- CmAmU TmAmC CmGmA CmGmU GmCmA AmUmC G-3′. The protocol used for in situ hybridization was the same as in our previous study.[24] In brief, sections were rehydrated and boiled in 0·05M Tris-HCl (pH 9·0) for 10 min, followed by 20 min 0·2N HCl wash. After phosphate-buffered saline wash, sections were treated with proteinase K at 2μg/ml (37°C) for 15min, and the reaction was stopped by glycine buffer. The labeled probe was dissolved in

NBB	Group	Sex	Age at death (y)	PMD (hr:min)	FT (d)	CSF pH	BW (g)	Storage time (d)	CTD	MOD	Braak stage	Medication in the past	Medication in the last 3 months	Suicide attempt	Cause of death
92-003	MDD	F	55	6:45	52	6·4	1320	295	7:45	11	0	BZD, SSRI, TeCA	SSRI, BZD, bromocriptine,	Yes	Suspected urosepsis, heart failure
94-032	MDD	M	71	16:15	38	ND	975	267	16:15	2	0	ZUC, BZD, MAOI, clomipramine	None	Yes	Probably bronchopneumonia, next to cerebral ischemia
94-017	MDD	F	72	22:00	39	ND	1287	269	19:00	1	1	TeCA, BZD, prednisone	TeCA, BZD, prednisone	No	Bronchopneumonia, mesothelioma
12-097	MDD	F	73	5:45	61	6·7	1205	45	15:30	9	3	TeCA	TCA,TeCA, BZD, pan-curonium, Ba	No	Heart failure, legal euthanasia
95-036	MDD	M	74	62:55	35	ND	1444	255	17:05	3	0	SSRI, BZD, cisordinol	SSRI, BZD, cisordinol	Yes	Suicide by hanging
02-051	MDD	M	81	6:00	34	6·5	1280	168	15:30	6	3	None	Hal	No	Renal insufficiency
11-058[a]	MDD	M	83	10:40	57	6·5	1200	59	5:00	7	2	TCA, TeCA, Hal, Pipamperone, SSRI	BZD, gabapentin, Mo	No	PTSD with depression, acute heart failure
08-076	MDD	F	91	5:20	36	6·5	1163	94	9:05	8	3	Hal, SSRI, BZD, levothyroxine	SSRI, BZD, levothyroxine	No	Cachexia and dehydration by pneumonia and renal insufficiency
08-031	MDD	F	93	4:20	51	6·8	1023	99	4:55	3	2	SSRI, BZD	Mo, BZD, SSRI	Yes	Pneumonia
median			74	6:45	39	6·5	1205	168							
09-003	CTR	M	62	7:20	47	6·4	1520	89	3:30	1	1	None	Chemo and radio therapy	No	Pancreas carcinoma, metastases
97-042	CTR	F	65	12:50	28	6·9	910	230	2:00	4	1	None	Adrenalin, dopamine	No	Cardiac arrest, pneumonia, pulmonary edema
92-049	CTR	M	71	5:40	32	7·4	1250	290	0:00	4	0	None	None	No	Found dead
98-104	CTR	F	74	7:25	31	7	1167	215	9:50	7	2	BZD	BZD, βB	No	Necrosis of the intestines secondary to thrombosis
94-039	CTR	M	78	53	88	ND	1354	269	12:00	1	0	None	Nitroglycerine	No	Myocardial infarction
00-022	CTR	F	83	7:45	34	6·5	1072	196	21:00	2	2	Digoxin, methimazole	Digoxin, methimazole	No	Acute myocardial infarction
09-075	CTR	M	88	7:00	44	6·8	1230	80	2:25	10	3	Salbutamol, prednisolone,	Mo, Hal, gosereline	No	Cachexia and dehydration by rectum carcinoma and prostates carcinoma
97-068	CTR	F	61	10:15	33	7·2	1311	229	10:15	5	1	Homeopathy	None	No	Ovarian carcinoma with metastases, cachexia, pneumonia
08-105	CTR	F	89	3:52	58	7·3	1258	90	0:10	12	3	prednisolone	Hal, digoxin, Mo	No	Pneumonia
Median			74	7:25	34	6·9	1250	215							
p-Value				0·696	0·773	0·251	0·070	1·0	0·930	>0·05	>0·05				

***Table 1*: Clinico-pathological information of major depressive disorder patients and their controls for hypothalamus study.**

Note: Ba, barbiturate; Braak stage, progression of pathological changes for Alzheimer's disease according to Braak et al., 1991; BW, brain weight; BZD, benzodiazepine; CSF, cerebrospinal fluid; CTD, clock time at death; CTR, control; CVA, cerebrovascular accident; ECT, electroshock treatment; F, female; FT(d), fixation time in days; Hal, haloperidol; Li, lithium; M, male; MAOI, monoamine oxidase inhibitor; MDD, major depressive disorder; Mo, morphine; MOD, month of death; NBB, Netherlands Brain Bank; ND, no data; None, no medication; NSAIDs, nonsteroidal anti-inflammatory drugs; PMD, postmortem delay; PTSD, posttraumatic stress disorder; SSRI, selective serotonin reuptake inhibitor; Storage time: the time the tissue was stored in paraffin; TCA, tricyclic antidepressant; TeCA, tetracyclic antidepressant; ZUC, zuclopenthixol. a: Patient also diagnosed with PTSD. p-Value: differences between CTR and MDD group were tested using the Mann-Whitney U test.

NBB	Group	Sex	Age at death (y)	PMD (hr:min)	FT (d)	CSFpH	BW (g)	Storage time (m)	CTD	MOD	Braak stage	Medication in the past	Medication in the last 3 months	Suicide attempt	Cause of death
02-014	BD	M	68	12:00	30	6·6	1414	172	0:00	2	1	Li, Hal, ZUC, MAOI	MAOI	No	Subdural hematoma, after fall
99-118	BD	M	68	5:55	33	6·8	1174	200	23:15	10	1	Li, SSRI	Li	No	Cardiac ischemia
00-088[b]	BD	M	73	5:15	36	6·4	1145	191	9:30	7	2	Li, BZD, SSRI, Hal, ECT, MAOI, Methylphenidate	Li, SSRI, Hal, Methylphenidate	No	Cachexia, dehydration
98-010	BD	F	75	4:00	38	ND	1123	221	20:45	1	1	TeCA, TCA	BZD, TeCA, SSRI, Hal, Mo	No	Acute abdomen secondary to a perforation of stomach / intestines due to NSAIDs
12-048	BD	M	81	6:40	60	6/7	1283	49	20:00	5	1	Li, prednisolone	Li, BZD, amitriptyline, valproate	No	Legal euthanasia
12-110	BD	M	87	3:15	53	6·4	1285	44	23:00	10	3	BZD, valproate	BZD, Mo, valproate	No	CVA, pneumonia
Median			74	5:35	37	6·6	1228·5	181·5							
99-044	CTR	F	88	5:55	34	6·1	1115	206	7:00	4	1	BZD	BZD	No	Cardiac arrest
00-007	CTR	M	85	15:10	35	6·9	1328	197	22:30	1	2	None	None	No	Myocardial infarction
90-080	CTR	M	85	4:55	28	6·3	1035	307	11:50	11	3	None	None	No	Myocardial infarction
99-101	CTR	M	69	19:15	41	6·4	1352	202	3:30	8	1	None	βB, Hal, BZD	No	Pneumonia, small infarction in brainstem
09-001	CTR	M	88	4:43	51	6·2	1418	89	19:47	1	2	βB	βB	No	Gastro-intestinal bleeding
92-043	CTR	M	72	8:00	73	8·2	1150	290	0:00	4	0	None	None	No	Shock
Median			85	6:58	38	6·4	1239	204							
p-Value				0·198	0·297	1·0	0·584	1·0	0·109	>0·05	>0·05				

***Table 2*: Clinico-pathological information of bipolar disorder patients and their controls for hypothalamus study.**

Note: βB, beta-blocker; Ba, barbiturate; BD, bipolar disorder; Braak stage, progression of pathological changes for Alzheimer's disease according to Braak et al., 1991; BW, brain weight; BZD, benzodiazepine; CSF, cerebrospinal fluid; CTD, clock time at death; CTR, control; CVA, cerebrovascular accident; ECT, electroshock treatment; F, female; FT(d), fixation time in days; Hal, haloperidol; Li, lithium; M, male; MAOI, monoamine oxidase inhibitor; MDD, major depressive disorder; Mo, morphine; MOD, month of death; NBB, Netherlands Brain Bank; ND, no data; None, no medication; NSAIDs, nonsteroidal anti-inflammatory drugs; PMD, postmortem delay; PTSD, posttraumatic stress disorder; SSRI, selective serotonin reuptake inhibitor; Storage time: the time the tissue was stored in paraffin; TCA, tricyclic antidepressant; TeCA, tetracyclic antidepressant; ZUC, zuclopenthixol. b: Patient also diagnosed with old cerebrovascular accident and mild dementia. *p*-Value: differences between CTR and BD group were tested using the Mann-Whitney U test.

Articles

hybridization buffer (HEPES pH 7·5, 0·6M sodium chloride, 5 x Denhardt's solution, 1mM EDTA, 50% Formamide, 200µg/ml herring sperm DNA) to final concentrations of 5nM (CRH) and 0·5nM (OXT). Each section was first treated with 70µl of hybridization buffer, covered with Nescofilm and prehybridized at 55° C for 1h. Then the section was provided with 70µl of diluted CRH or OXT probe, covered with Nescofilm and hybridized overnight at 55°C. The next day, Nescofilms were removed and sections were washed in 2 x SSC, 0·5 x SSC, and 0·2 x SSC, each for 5min at 55°C. Signal detection was done through incubation with anti-FAM-alkaline phosphatase antibody (cat. no. 1426338, Roche Life Sciences, Germany), and NBT/BCIP to develop the color. The specificity of the CRH-mRNA or OXT-mRNA antisense probes was checked with their respective scrambled probes. The image analysis procedure for CRH-mRNA and OXT-mRNA quantification was the same as for CRH-ir and OXT-ir signals as mentioned above, except that the threshold for positive signal was set at 3·5 times of the background OD. The corrected (c) IOD of CRH-mRNA or OXT-mRNA was calculated by dividing the IOD of CRH-mRNA or OXT-mRNA by the total delineated area of PVN. The cIOD was used as the final density parameter of CRH-mRNA or OXT-mRNA in the PVN for each subject. The method was originally fully described in a previous study by our group.[25]

Quantitative PCR (qPCR) for mRNA expression of OXTR, CRHR1 and CRHR2 on DLPFC or ACC

RNA samples isolated from the grey matter of the ACC (Brodmann area 24) and DLPFC (Brodmann area 46) were obtained from SMRI. Complementary DNA (cDNA) synthesis was performed as described previously.[26] Details on genes and primers are shown in Table S4. The expression of target genes was normalized using the sets of stable reference genes, including tubulin alpha (TUBα), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ubiquitin-C (UBC, Table S4). cDNA template (equivalent to 5ng of total RNA) was amplified in a final volume of 20µl using a SYBR Green PCR master mix (Applied Biosystems, CA, USA) and a mixture of forward and reverse primers (each 2pmol/µl, Table S4). Data were acquired and processed automatically by the Applied Biosystems 7300 Real-time PCR System. The procedures and data analysis are same with a previous study of our group.[26] The relative amount of target genes were calculated by $10^{10} \times E^{-ct}(E = 10^{-(1/slope)})$.[27] The 'mRNA relative value' were interpreted in relation to housekeeping gene transcript levels. All analyses were performed by investigators unaware of the group information.

Part II: stimulation of mouse PVN-OXT via chemogenetics

OXT-cre mice (C57BL/6J background, Jackson's Lab, USA; RRID: IMSR_JAX:024234) were housed (12h

light-dark cycle) with food and water ad libitum. Male and female OXT-cre mice were bred in the facility and were 8-12 weeks of age (male: 25-35g, female: 20-30g) at the beginning of each experiment. The genotypes were determined using PCR analyses. Mice were randomly assigned to hM3Dq group or control group, and were anesthetized by isoflurane. Virus (AAV-hSyn-DIO-hM3Dq-eGFP or AAV-hSyn-DIO-eGFP; Taitool Bioscience, Shanghai, China) was injected into hypothalamic PVN at both sides of OXT-cre mice that were placed in a stereotaxic frame with bregma and lambda in a horizontal plane (A-P: -1·0mm, M-L: ±1·2mm, D-V: -5·1mm) with the angle of 10° (Figure 4a-b in Result). Injection speed was 60nl/min, and injection volume was 250nl. After injection, the mice received prophylactic penicillin.

Chemogenetic stimulation of PVN-OXT neurons and behavior tests

Mice which were injected with AAV-DIO-hM3DGq virus (male n=23; female n=18) or control virus (male n=12; female n=14) were randomly assigned to a 'short-term OXT stimulation' or a 'long-term OXT stimulation' group (details see Figure 4g-h in Results). The sample size was calculated by the resource equation approach,[28] and was considered to be sufficient.[29] After 5 weeks of recovery, animals were injected with clozapine-N-oxide (CNO, 5mg/kg, i.p.),[30] according to a short-term or a long-term schedule. Each behavior test took place at 15 min after injection. Behavior tests were conducted according to previous studies of our group[31,32] details are presented in the Supplementary Materials S2. Behavior tests were carried out and analyzed by investigators who were blind to the experimental conditions. CNO is a metabolite of clozapine and was reported to be possibly converted to clozapine in mice and rats.[33,34] So it might affect behaviors in Open Field test (OFT) and Elevated Plus Maze (EPM) test.[35] Therefore, we performed a pilot in C57BL/6J wild type mice and excluded the CNO dosage used in our study on mouse behaviors in OFT or EPM test (Supplementary material Fig.S3).

There is no animal model that can mimic the cycling depression and mania symptoms of human BD. So far, in the field of BD studies, some mood-related behaviors were regarded as mania-like or depression-like behaviors. We used five mood-related behavior tests, i.e. OFT, EPM, Light-Dark Box (LDB) exploration test, Social Interaction (SI) test and Forced Swim test (FST) to check mania-like behaviors, according to previous studies.[36−38] In these studies, mania-like behaviors included 'increase time spent in the light box' in LDB,[36,38] and 'more approaches' and 'spent more time approaching' in SI[38] while depression-like behaviors included 'less time in the interaction zone in SI',[39] and 'less time in the light compartment in LDB'.[40] In addition, the increased locomotor activity has been regarded as

mania-like behavior.[41] It should be noted that LDB and SI tests are also used for checking anxiety-like behaviors that mimic symptoms of depression in clinical populations: Less time in the lighted section in LDB and decreased social behavior in SI are considered to mimic increased anxiety in depression.[39,40] Details of behavior tests were presented in supplementary material S2.

Selective AAV-DIO-hM3DGq virus expression and activation of the OXT neurons

After behavior tests, mice were anesthetized and the whole brain tissues were first perfused with 4% paraformaldehyde for one day, then dehydrated with 30% sucrose overnight. The tissues were then sectioned into 20-μm coronal slices using a sliding microtome (Leica, Germany). PVN sections were used for immunocytochemical staining of OXT (H-051-01, Phoenix Pharmaceuticais, Germany; RRID: AB_2876858) and c-fos (226003, SYSY, Germany; RRID: AB_2231974), to make sure the AAV-DIO-hM3DGq virus infected PVN-OXT neuron specifically and the infected neuron were activated by CNO. After PBS washing and BSA blocking, the brain slices were incubated with OXT or c-fos antibody at 4°C for 48 hours, followed by TBS washes and secondary antibody incubation for 2 hours. Images were captured with a confocal microscope (ZEISS, Germany), and the fluorescent intensity was analyzed using Image J. We verified the expressing area and cell type specificity of virally labeled neurons. Injection of AAV-DIO-hM3DGq virus in PVN resulted in clear and selective expression in the OXT neurons (Figure 4f in Results). Most virally labeled neurons were OXT-ir neurons. In the AAV-DIO-hM3DGq virus group, CNO administration leads to more co-localization of AAV-DIO-hM3DGq virus signals with c-fos-ir signals when compared to control group (Figure 4c-e in Results).

Ethics statement

Postmortem hypothalami of mood disorder patients and their matched controls were obtained from the Netherlands Brain Bank (NBB) using well standardized protocols with written informed consent obtained by the NBB for a brain autopsy and for the use of the material and clinical information for research purposes. RNA samples isolated from the grey matter of the ACC and DLPFC were obtained from the Stanley Medical Research Institute using well standardized protocols, which included permission for the use of brain material that was provided by the next of kin. Use of human tissue for post mortem studies have been reviewed and approved by the institutional review board of the independent Ethics Committee of Zhejiang University School of Medicine (No.2018002), and by the Medisch Ethische Toetsingscommissie of VU medisch centrum (No. 2009148), and the study is in line with the

Declaration of Helsinki. Mice were humanely euthanized at defined end points and all experimental procedures had prior approval (No. 12267) from the Animal Care and Use committee at Zhejiang University.

Statistics

Kolmogorov-Smirnov test and Shapiro-Wilk test were used to analyze the normality of data. The postmortem human brain data were sometimes not normally distributed and nonparametric statistics were applied. The differences between 2 groups were evaluated by Mann-Whitney U test. Cohen's d analysis was applied to justify the group comparisons that showed trend of differences, and the trend would be excluded if Cohen's d <0.4. The Kruskal−Wallis H test was applied for multiple comparison, and if a significant difference was identified, Mann-Whitney U test with Bonferroni correction was performed for further comparison between two groups. Correlations were examined with the Spearman test. Differences in clock time and month of death were analyzed with the Mardia−Watson−Wheeler test. The OXT-cre mice data were normally distributed and t-test was applied to the comparisons between two groups. There was no exclusion of the samples or data. SPSS 25.0 (IBM Corp., Armonk, NY) was applied for Statistics. All tests were 2-tailed and $p \leq 0.05$ was considered to be significant, $0.05 < p < 0.1$ to be a trend.

Role of funder

The funders had no role in the study design, data collection, analysis, interpretation, decision to publish, or writing the manuscript. None of the authors have been paid to write this article by a pharmaceutical company or other agency.

Results

Part I: postmortem human brain studies

OXT and CRH expression in the hypothalamic PVN of BD and MDD patients. OXT-ir and OXT-mRNA, and CRH-ir and CRH-mRNA signals were clearly and widely distributed in the hypothalamic PVN (Figure 1). Increased OXT-ir ($p=0.037$, Mann-Whitney U test) and increased OXT-mRNA ($p=0.055$, Mann-Whitney U test, Cohen's d =1.40) were found in BD but not in MDD patients compared with their respective controls (Figure 2a-d). For CRH, a trend of lower CRH-ir ($p=0.078$, Mann-Whitney U test, Cohen's d =-0.83) but higher CRH-mRNA ($p=0.037$, Mann-Whitney U test) levels were found in BD but not in MDD patients compared with their respective controls (Figure 2e-h). In addition, a highly significant increased ratio of OXT-ir/CRH-ir was found in BD ($p=0.006$, Mann-Whitney U test, Figure 2i), but not in MDD, patients compared
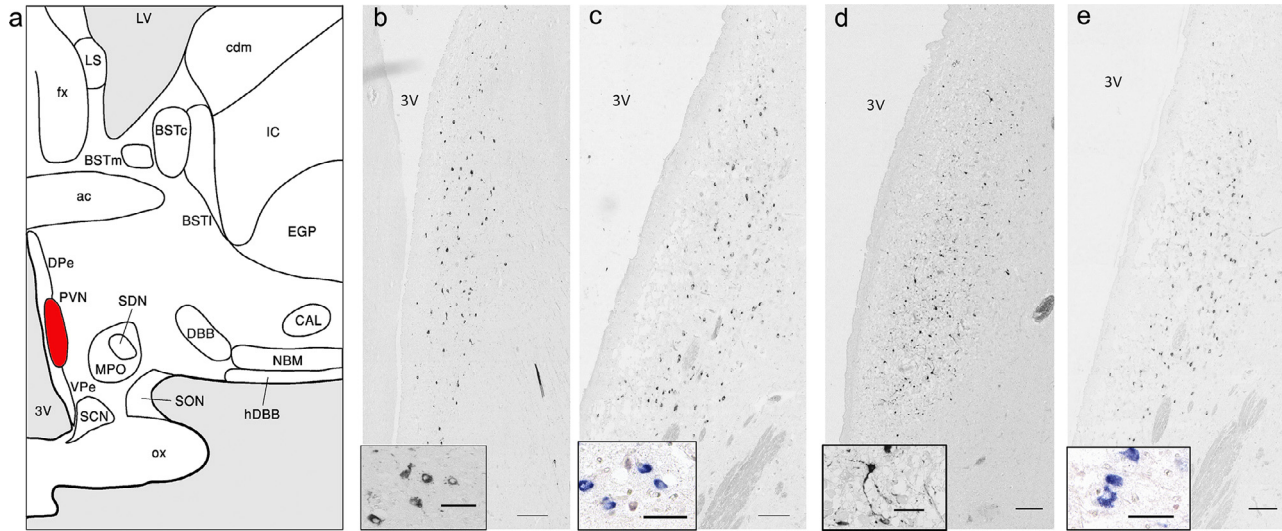
Figure 1. Immunocytochemistry and in situ hybridization images of oxytocin and corticotropin-releasing hormone in the human hypothalamic paraventricular nucleus. (a) Schematic representation of the nuclei of the human hypothalamus. Abbreviations: OX, optic chiasma, NBM: nucleus basalis of Meynert, hDBB: horizontal limb of the diagonal band of Broca, SDN: sexually dimorphic nucleus of the preoptic area, SCN: suprachiasmatic nucleus, BST: bed nucleus of the stria terminalis, (c = centralis; m = medialis; l = lateralis; p = posterior); PVN: paraventricular nucleus, SON: supraoptic nucleus; Dpe: periventricular nucleus dorsal zone, VPe: periventricular nucleus ventral zone, fx: fornix, 3V: third ventricle, ac: anterior commissure (From Swaab, Dick. (2003). The Human Hypothalamus. Basic and Clinical Aspects. Mycological Research. 79), please note that PVN is marked in red. (b-c) Overview and higher magnification images of oxytocin (OXT)-immunoreactivity (ir) (b) or OXT-mRNA (c) signal in the PVN. (d-e) Overview and higher magnification images of corticotropin-releasing hormone (CRH)-ir (d) or CRH-mRNA (e) signal in the PVN. Scale bar=200μm in the overviews of b-e; and scale bar=50μm in the higher magnification images of b-e.
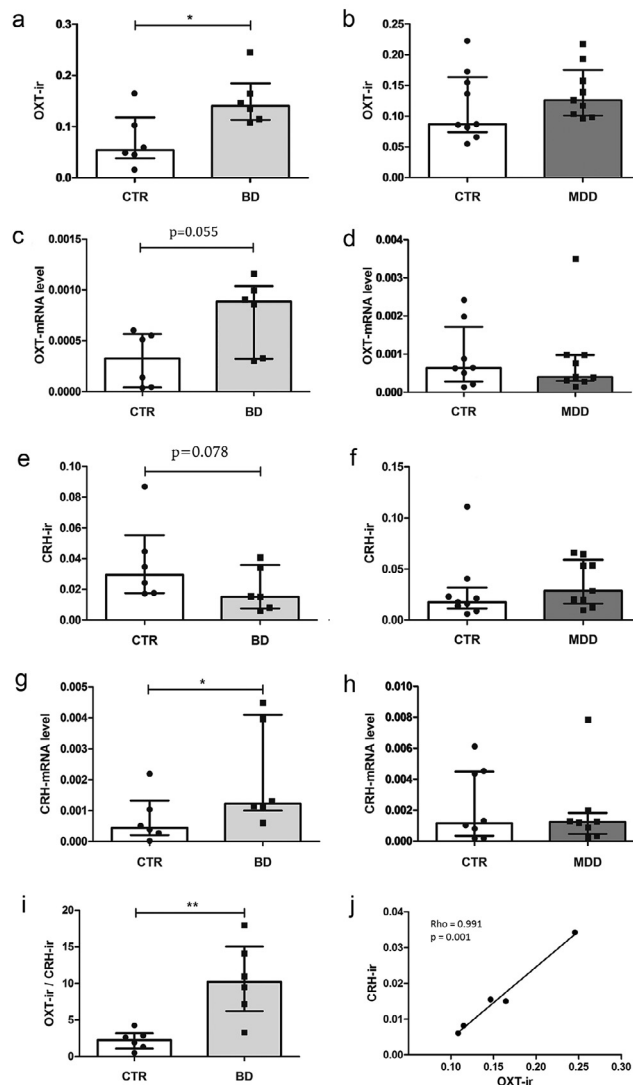
Figure 2. Analysis on the expression of oxytocin and corticotropin-releasing hormone in the hypothalamic paraventricular nucleus in mood disorder patients and their controls. (a-b) Oxytocin (OXT)-ir showed a significant increase in bipolar disorder (BD) patients compared with their controls ($p=0.037$, Mann-Whitney U test, a), while no significant difference was present between major depressive disorder (MDD) and their controls (b). (c-d) A trend of increased OXT-mRNA ($p=0.055$, Mann-Whitney U test, c) was found in BD patients compared with their controls, while no significant difference in OXT-mRNA (d) was found between MDD patients and their controls. (e-f) PVN CRH-ir showed a trend of lower expression in BD patients compared with their controls ($p=0.078$, Mann-Whitney U test, e), but no significant difference between MDD patients and their controls (f). (g-h) Significantly increased CRH-mRNA ($p=0.037$, Mann-Whitney U test, g) was found in BD patients compared with their controls, but no significant difference in CRH-mRNA (h) was found between MDD patients and their controls. (i) The ratio of OXT-ir/CRH-ir was significantly higher in BD patients compared with their controls ($p=0.006$, Mann-Whitney U test). (j) Significant positive correlation was found between OXT-ir and CRH-ir in male BD patients ($p=0.001$, Spearman test). Columns are shown as median with interquartile range. CTR: control. * $p < 0.05$.

with their respective controls. Moreover, a significant positive correlation was found between OXT-ir and CRH-ir in BD ($n=6$, Rho=0.900, $p=0.037$, Spearman test), the samples of which were mainly males ($n=5$, Rho=0.991, $p=0.001$, Spearman test, Figure 2j). This correlation was not seen in MDD patients, nor in controls. The double-labeling of CRH-ir (blue) and OXT-ir

(red) experiment in the PVN showed no co-localization (Supplementary material S3 and Fig. S2).

OXTR-mRNA and CRHR-mRNA expression in the DLPFC and ACC of BD and MDD patients. To identify the prefrontal downstream changes in the OXT and CRH

systems, we analyzed mRNA levels of OXTR and CRHR in the DLPFC and ACC of BD and MDD patients and their respective controls. It turned out that male BD, but not female BD nor MDD patients, showed significantly increased OXTR-mRNA levels in the DLPFC (p=0·001, Mann-Whitney U test) compared to their respective controls (Figure 3a-d, Table 3). In addition, male BD patients showed significantly increased ratios of OXTR-mRNA/CRHR1-mRNA (p=0·040, Mann-

Whitney U test) and OXTR-mRNA/CRHR2-mRNA (p=0·007, Mann-Whitney U test) in the DLPFC, as well as increased ratio of OXTR-mRNA/CRHR2-mRNA in the ACC (p=0·043, Mann-Whitney U test), as compared with their controls (Figure 3i-k).

On the other hand, MDD patients but not BD patients showed a trend of lower CRHR2-mRNA in the DLPFC (p=0·056, Mann-Whitney U test, Cohen's d=-0·71, Figure 3e-h, Table 3) compared with their controls.
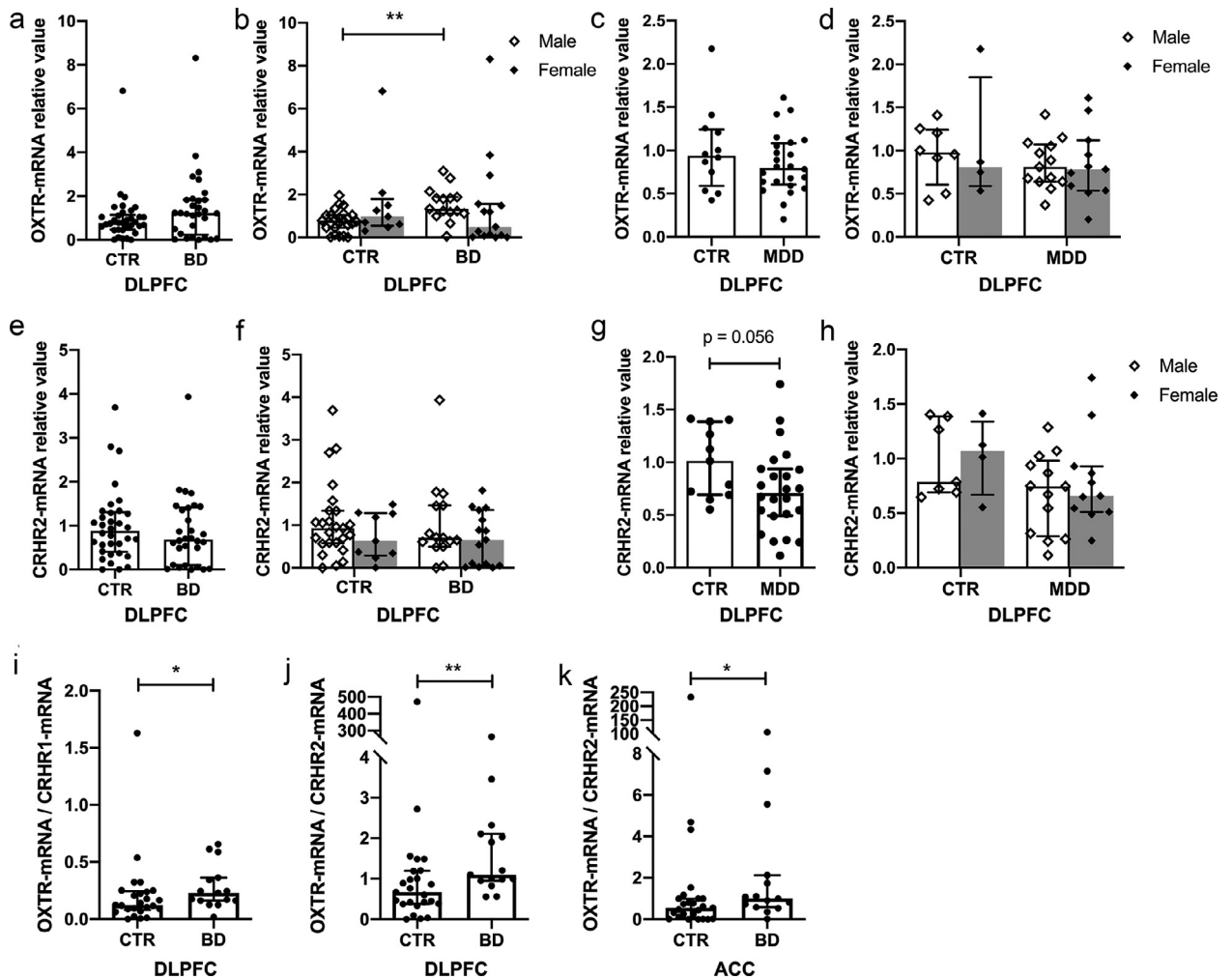
Figure 3. Analysis on the mRNA expression of oxytocin receptor and corticotropin-releasing hormone receptors in the prefrontal cortex of mood disorder patients and their controls. (a-b) oxytocin receptor (OXTR)-mRNA levels in the dorsolateral prefrontal cortex (DLPFC) in bipolar disorder (BD) patients showed no significant difference compared with their controls (a), while male BD patients showed significantly increased OXTR-mRNA in DLPFC compared with male controls (p<0·01, Mann-Whitney U test) (b). (c-d) No significant change was found within OXTR-mRNA levels in DLPFC of major depressive disorder (MDD) patients and their controls. Still, no significant difference was found after male patients were separated from female patients. (e-f) No significant change of CRHR2-mRNA was found in the DLPFC in BD patients compared with their controls, and no sex difference was found. (g-h) A trend (p=0·056, Mann-Whitney U test) of lower CRHR2-mRNA was found in the DLPFC in MDD patients compared with their controls, and no sex difference was found. (i) Significantly higher ratio of OXTR-/CRHR1-mRNA was found in the DLPFC of male BD patients compared with male controls. (j-k) Significantly higher ratio of OXTR-mRNA/CRHR2-mRNA was found in both the DLPFC (j) and anterior cingulate cortex (ACC, k) of male BD patients compared with male controls. Columns are shown as median with interquartile range. ** p<0·01; * 0·01< p <0·05.

			CRHR1	CRHR2	OXTR
MDD vs. CTR					
	ACC	Pooled			
		Male			
		Female			
	DLPFC	Pooled		▼ *p*=0·056, Cohen's d=-0·71	
		Male			
		Female			
BD VS. CTR	ACC	Pooled			▲*p*=0·056, Cohen's d<0·4
		Male			▲*p*=0·069, Cohen's d<0·4
		Female			
	DLPFC	Pooled			▲*p*=0·10, Cohen's d<0·4
		Male			▲*p*=0·001
		Female			

***Table 3*: The significant or trend of changes in CRHR1-, CRHR2- and OXT-mRNA expression in the ACC or DLPFC of MDD and BD patients.**
Note: ACC: anterior cingulate cortex; BD: bipolar disorder; CTR: control; CRHR: corticotropin-releasing hormone receptor; DLPFC: dorsolateral prefrontal cortex; MDD: major depressive disorder; OXTR: oxytocin receptor. ▼: decrease; ▲: increase.

As OXTR and CRHR were reported to be related to suicide and psychotic symptoms,[42−45] we subsequently analyzed whether suicidal behavior or psychotic symptoms of the BD or MDD patients might account for our results. OXTR-mRNA levels were not affected by suicide or psychotic symptoms in BD or MDD (Table S5), while a significant difference in CRHR2-mRNA expression was found in the DLPFC (*p*=0·027, Kruskal−Wallis H test) among suicide MDD, non-suicide MDD patients and the controls. Subsequent analysis showed that CRHR2-mRNA expression was significantly lower in suicide MDD patients than in the controls (*p*=0·011, Mann-Whitney U test with Bonferroni correction, Table S5).

Part II: stimulation of mouse PVN-OXT by chemogenetics

In the OXT-cre mice, bilateral PVN injection of AAV-DIO-hM3DGq virus but not control virus could activate PVN-OXT with the intraperitoneal injection of CNO (Figure 4c-e). In addition, the p values of all the significant or trends for changes in behaviors are presented in Table 4.

PVN-OXT stimulation induced anxiety/depression-like behaviors in male mice

Short-term stimulation of PVN-OXT neurons in male OXT-cre mice led to significantly less central time in OFT (*p*=0·039, Student t test, Figure 4i), implying anxiety/depression-like behavior, while long-term stimulation of PVN-OXT led to significantly less distance in the light zone (*p*=0·028, Student t test, Figure 5a) in LDB, and significantly more interactions with object in SI test (p≤0·021, Student t test, Figure 5b), implying also

anxiety/depression-like behaviors. Taken together, these results indicate that stimulation of PVN-OXT in male mice caused anxiety/depression-like behaviors. Some other behaviors which showed trends for changes also support this finding (Table 4).

PVN-OXT stimulation led to both mania-like and depression-like behaviors in female mice

In female OXT-cre mice, short-term stimulation of PVN-OXT neurons led to significantly more time contacting with the mouse and significantly more entries to all the compartments in SI test (p≤0·019, Student t test, Figure 4j), indicating increased social behavior and locomotoactivity, i.e. mania-like behaviors. On the other hand, long-term stimulation of PVN-OXT led to significantly more entries to the central area (*p*=0·006, Student t test) in the OFT (Figure 5c), significantly more immobility in FST (*p*=0·025, Student t test, Figure 5d), and significantly less time interacting with the mouse in SI (*p*=0·029, Student t test, Figure 5e), indicating mixed mania-like and depression-like behaviors. Taken together, these results indicate that stimulation of PVN-OXT in female mice caused both mania-like and depression-like behaviors depending on the time of stimulation. Some other behaviors which showed trends for changes also support this finding (Table 4).

Discussion

In the present study, we observed significantly increased OXT-ir, OXT-mRNA in the hypothalamic PVN and increased OXTR-mRNA in the DLPFC in BD, at least in male BD patients, but not in MDD patients. In addition, we found higher ratios of PVN OXT-ir/CRH-ir in BD and higher ratios of OXTR-mRNA/
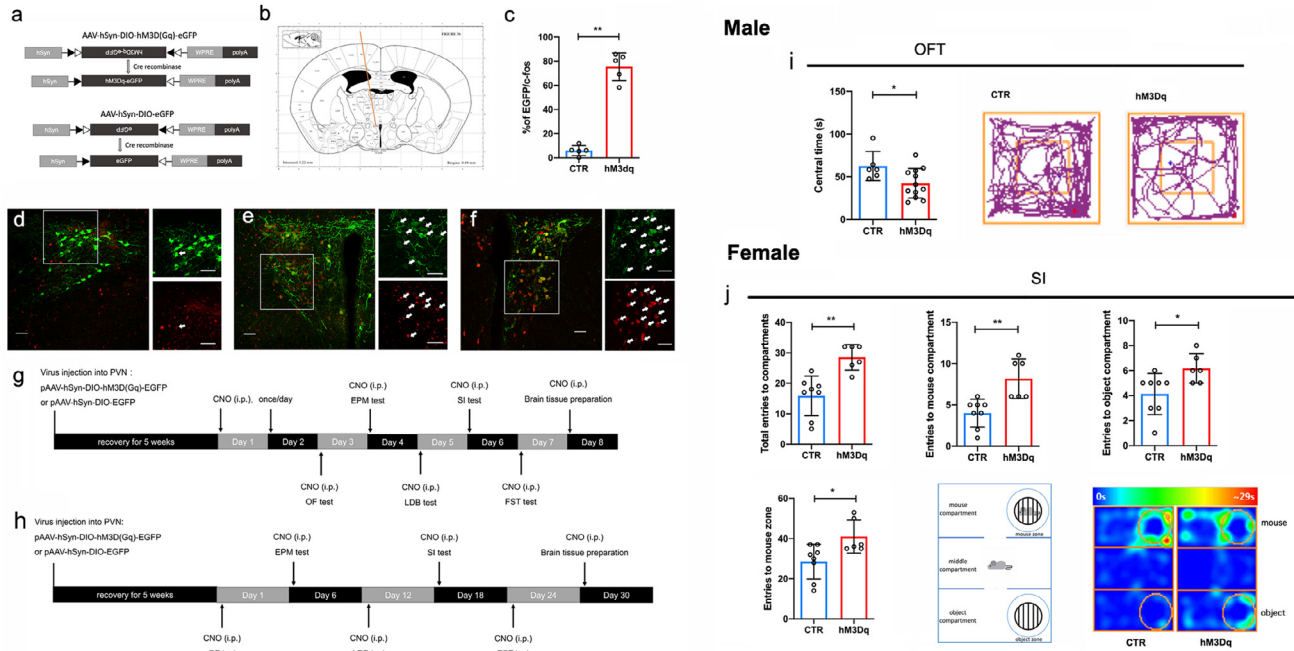
Figure 4. Significant mood-related behavior changes in mice with short-term oxytocin chemogenetic stimulation in hypothalamic paraventricular nucleus. (a-b) Virus (AAV-hSyn-DIO-hM3Dq-eGFP, activated group) or AAV-hSyn-DIO-eGFP (control group), were injected with the angle of 10° into hypothalamic paraventricular nucleus (PVN) (A-P: -1.0 mm, M-L: ±1.2mm, D-V: -5.1mm) at the speed of 60nl/min, 250nl/side. (c-e) Co-localization (yellow) of the virus signal (green) and c-fos-ir signal (red) in the control group (d) and chemogenetic activated group (e). Quantification analysis (c) showed that there was less co-localization in the control group than in the activated group. (f) Co-localization (yellow) of the AAV-DIO-hM3DGq virus signal (green) and oxytocin (OXT)-ir signal (red). The virus signal is fully co-existed with PVN-OXT signal as it shows in the highlight box, which indicates the virus expresses specifically in the OXT neurons and the injection place is correct. (g-h) Time schedule of short-term or long-term PVN-OXT stimulation and behavior tests. In the short term PVN-OXT stimulation group, behavior tests were conducted 15min after the first injection of clozapine N-oxide (CNO, *i.p.* g), while in the long term PVN-OXT stimulation schedule, behavior tests were conducted after at least 3 days of CNO injection (once a day, h), and behavior tests began at 15min after the last injection. (i-j) Significant behaviors changes observed in short term PVN-OXT stimulation. Male PVN-OXT-activated mice spent significantly less time in the central area in the open field test (OFT, $p=0.039$, Student t test) compared with male controls. The moving tracks of the median-level mouse of each group were presented (i). In the social interaction (SI) test, female PVN-OXT-activated mice showed more entries into the mouse chamber ($p<0.01$, Student t test) and more contact with the other mouse ($p=0.018$, Student t test), more entries into the object chamber ($p=0.019$, Student t test), and more total entries through three chambers ($p<0.01$, Student t test) compared with their female control mice. The heatmap showed the median level of the mouse movement in each group (j). Columns are shown as mean with SD. Scale bar=50μm in image d-f. ** $p<0.01$; * $0.01< p <0.05$.

| Behavior tests	Parameters	Short-term stimulation hM3DGq vs. CTR		Long-term stimulation hM3DGq vs. CTR	
		Male	Female	Male	Female
OFT	Distance				▲#
	Central entries				▲**
	Central time	▼*			▲#
EPM	Open arm entries				▲#
LDB	Light zone entries		▲#		
	Light zone time		▲#	▼#	
	Distance in light zone		▲#	▼*	
	Latency of the first entry to the light				▲#
FST	Immobile time	▲#			▲*
SI	Contact with mouse		▲*		
	Contact mouse time				▼*
	Mouse chamber entries		▲**		
	Mouse chamber time				
	Contact with object			▲*	
	Contact object time			▲**	
	Object chamber entries		▲*		
	Object chamber time		▼#	▲*	
	Total chamber entries		▲**		▲#

Table 4: The significant or trend of changes in mood-related behaviors of mice with hypothalamic paraventricular oxytocin stimulation.
Note: CTR: control, AAV-DIO virus group; EPM: elevated plus maze; FST: forced swim test; hM3Dq: AAV-DIO-hM3DGq virus group; LDB: light-dark box; OFT: open field test; SI: social interaction test. Data was shown as Mean $\pm$ SD. ▲ represents higher levels in hM3Dq mice compared with CTR group. ▼ represents lower levels in hM3DGq mice compared with CTR group. Short-term stimulation: In the short-term hypothalamic paraventricular oxytocin (PVN-OXT) stimulation group, behavior tests were conducted 15min after the first injection of CNO, which could activate PVN-OXT neurons; Long-term stimulation: In the long term PVN-OXT stimulation group, behavior tests were conducted after at least 3 days of CNO injection (once a day). ** $p < 0.01$; * $0.01 < p < 0.05$; # $0.05 < p < 0.1$.

CRHR1-mRNA and OXTR-mRNA/CRHR2-mRNA in male BD patients, but not in MDD patients. These findings show different OXT and CRH system changes in postmortem human brain of BD and MDD patients. We further found in male mice that stimulation of PVN-OXT led to anxiety/depression-like behaviors, and mixed mania-like and depression-like behaviors in female mice. Concluding, although stimulation of PVN-OXT caused mood-related changes in mice, the sex-specific changes in the depression-like and/or mania-like behaviors in mice are different from what we observed in the sex differences in OXT system activation in BD patients.

A previous study by our group showed higher PVN OXT-ir in depression in a mixed group of MDD patients and BD patients,[46] while in the present study we showed that higher OXT-ir is found only in BD but not MDD patients. Our findings of significantly increased OXT expression in the PVN at both protein and mRNA levels, and significantly increased OXTR-mRNA in the DLPFC, at least in male patients, are in accordance with previous findings that BD patients have higher peripheral plasma OXT levels,[11,12] and higher DLPFC OXTR levels.[13] We also found higher ratios of OXT-ir/CRH-ir in BD but not in MDD patients, compared with their respective well-matched controls. In addition, male BD patients showed increased ratios of OXT-mRNA/CRHR1-mRNA, OXT-mRNA/CRHR2-mRNA in the DLPFC, and OXTR-mRNA/CRHR2-mRNA in the ACC compared with their controls. These data imply that in BD, at least in male BD cases, increased expression of PVN-OXT and increased expression of OXTR in prefrontal cortex exceed those of CRH and its receptors. Moreover, a significant positive correlation between OXT-ir and CRH-ir was observed in BD, implying a close interaction between OXT and CRH systems in BD. Previous studies in rat found that after OXT application, changes in spontaneous inhibitory synaptic transmission of PVN CRH neurons was observed.[47] In addition, it was found that in female rats, intracerebroventricular OXT infusion may significantly attenuate the increase of CRH-mRNA induced by a 30 min restraint stress, and attenuate the release of adrenocorticotropic hormone (ACTH) and corticosterone.[48] These data indicate that the inhibiting role of OXT on HPA activity might take place at both the peripheral level, i.e. the pituitary and/or adrenal gland, and the central level, i.e. the local hypothalamic nuclei. While the decrease of ACTH and/or glucocorticoid may further increase CRH production via diminished negative feedback, the central OXT release inhibits CRH excitability. Indeed, we observed that BD patients showed a trend of decreased
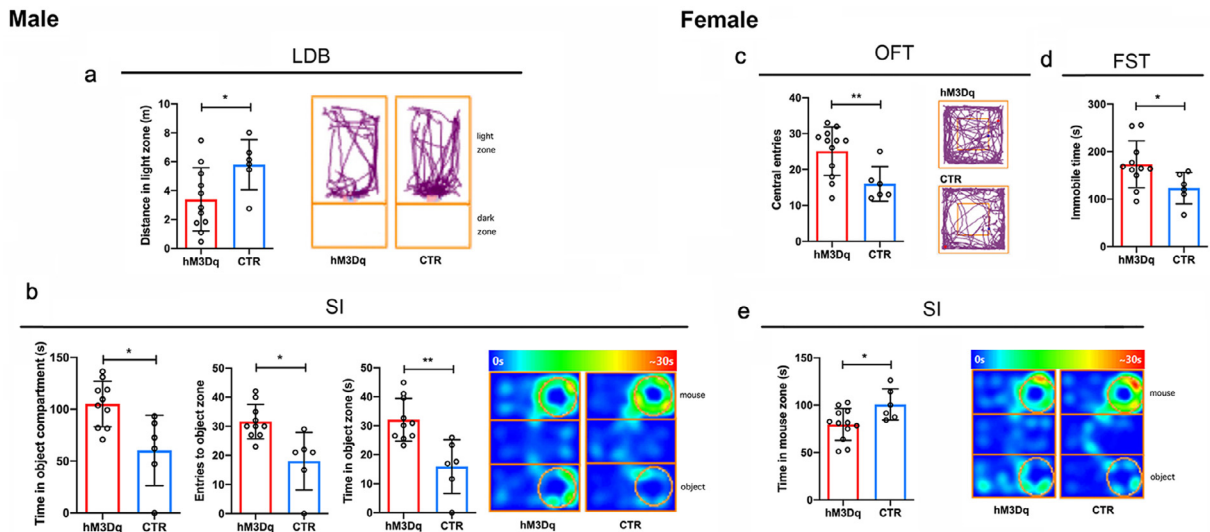
Figure 5. Significant mood-related behavior changes in mice with long-term oxytocin chemogenetic stimulation in hypothalamic paraventricular nucleus. (a) Male paraventricular nucleus (PVN)-oxytocin (OXT)-activated mice showed significantly less moving distance (p=0·028, Student t test) in the light zone in the light-dark box (LDB) compared with male control mice. The moving tracks of the median-level mouse of each group were presented. (b) In the social interaction (SI) test, male PVN-OXT-activated mice showed more time in the object chamber, more interactions with the object and more time interacting with the object ($p \leq 0 \cdot 021$, Student t test) compared with male control mice. The heatmap showed movements of the mouse that was at the median level in each group. (c) Female PVN-OXT-activated mice showed significantly more entries ($p < 0.01$, Student t test) to the central area in the open field test (OFT) compared with female control mice. The moving tracks of the median-level mouse of each group were presented. (d) Female PVN-OXT-activated mice showed significantly more immobile time (p=0·025, Student t test) in the forced swim test (FST). (e) Female PVN-OXT-activated mice showed less time interacting with the mouse (p=0·029, Student t test). The heatmap showed movements of the median-level mouse of each group. Time schedule of long-term OXT chemogenetic stimulation see Figure 4. hM3Dq: injected with AAV-hSyn-DIO-hM3Dq-eGFP virus; CTR: injected with AAV-hSyn-DIO-eGFP virus, control group, Columns are shown as mean with SD. ** $p < 0 \cdot 01$; * $0 \cdot 01 < p < 0 \cdot 05$.

CRH-ir (p=0·078, Mann-Whitney U test) but a significantly higher CRH-mRNA in the PVN compared with their controls, which is clearly different from MDD cases. The higher CRH-mRNA combined with a relatively lower CRH-ir in BD patients suggests a faster transport of CRH from the PVN. Whether these data may account, at least partly, for the distinct symptoms of BD and MDD warrants further study.

Although OXT was often reported to exhibit an anxiolytic effect,[49,50] its effect may be dose-dependent. Lower doses of OXT (1mg/kg mice i.p.; 0·8 IU mice intranasal; 24 IU human intranasal) not only led to decrease of plasma corticosterone in mice, together with more interaction with mice in SI test and less freezing time in the Resident-Intruder test, but also decrease of cortisol in humans. However, higher dose of OXT (10mg/kg mice i.p.; 8 IU mice intranasal; 48 IU human intranasal) showed no effect on these parameters.[51–53] It should be noted that a time-of-treatment-dependent effect of OXT on social behaviors was also shown in our study: short-term stimulation of PVN-OXT caused more interaction with mice, while long-term stimulation of PVN-OXT led to more interaction with objects in female mice in the SI test. Moreover, in

a previous study with a relatively lower CNO dose (1mg/kg) stimulation of PVN-OXT neurons promote social interaction in male mice,[54] while our study with a higher CNO dose (5mg/kg) stimulation of PVN-OXT neurons led male mice to show more contact with objects. Undoubtedly, such a dose/time-dependent effect of OXT on animal behavior changes strongly indicates its important role in the pathogenesis of BD. The possible mechanism, together with the potential sex differences, deserves further study.

Our study has some limitations. For example, medication is one of the potential confounding factors in postmortem case-control studies that cannot really be matched for, but there is no indication that our main conclusions were influenced by medication. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), was found in rats to decrease OXT levels in the hippocampus, while no effect was observed on the OXT levels in the medial preoptic area.[55] In addition, no obvious change in plasma OXT levels was observed during SSRI or electroconvulsive treatment of depressed patients.[56] SSRIs can therefore not explain the stimulation of the OXT system we found in BD patients. SSRIs also showed no clear effect on PVN CRH-mRNA expression

in rats.[57] Moreover, chlordiazepoxide, a benzodiazepine, inhibits OXT release in response to noxious stimuli,[58] but no study has shown its effect on OXT-mRNA and OXTR-mRNA exprssion. In our study, the numbers of BD patients and their controls who used benzodiazepines in the last 3 months before death were equal (n=3 vs n=3). Finally, although lithium was reported to increase OXT-mRNA expression in rat hypothalamic PVN,[59] the amount of OXT-ir and OXT-mRNA of the mood disorder patients who had used lithium in the present study (median IOD=0·146 and 0·0011) was not higher than those who did not use lithium (median IOD=0·134 and 0·0014). Taken together, we do not think that the antidepressants have affected our main conclusion, i.e. the increased expression of PVN-OXT and its receptor in DLPFC in BD, as drawn from post-mortem human brain study.

In summary, increased PVN-OXT and DLPFC-OXTR-mRNA, together with increased ratios of OXT-ir/CRH-ir, OXTR-mRNA/CRHR1-mRNA, OXTR-mRNA/CRHR2-mRNA in the DLPFC, and OXTR-mRNA/CRHR2-mRNA in the ACC are characteristic for BD, at least for male BD patients, and not for MDD patients. This indicates BD and MDD hold different molecular pathologies. Short-term or long-term stimulation of PVN-OXT neurons caused different sex-dependent, mood-related behavior changes in mice. The factor time should be taken into account in the development of OXT-targeted therapeutic strategies using animal models.

Contributors
Dick F. Swaab and Ai-Min Bao conceived and designed the study. Lei Guo, Yang-Jian Qi, Hong Tan and Dan Dai performed the experiments and collected the data. Rawien Balesar, Arja Sluiter and Joop van Heerikhuize offered crucial technique support. Lei Guo and Yang-Jian Qi analyzed and interpreted data. Lei Guo and Yang-Jian Qi wrote the manuscript, under the supervision of Shao-Hua Hu, Dick F. Swaab, and Ai-Min Bao. Lei Guo and Yang-Jian Qi, Hong Tan, Dick Swaab and Ai-Min Bao have verified the underlying data. All authors read and approved the final version of the manuscript.

Data sharing statement
The authors declare that the data supporting the findings of this study are available from the corresponding author upon request.

Declaration of interests
We declare no competing interests.

Supplementary materials
Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104266.

References
1 Bao AM, Swaab DF. The human hypothalamus in mood disorders: the HPA axis in the center. *IBRO Reports*. 2019;6:45–53.
2 Belvederi Murri M, Prestia D, Mondelli V, et al. The HPA axis in bipolar disorder: systematic review and meta-analysis. *Psychoneuroendocrinology*. 2016;63:327–342.
3 Sigitova E, Fišar Z, Hroudová J, Cikánková T, Raboch J. Biological hypotheses and biomarkers of bipolar disorder. *Psychiatry Clin Neurosci*. 2017;71(2):77–103.
4 Scantamburlo G, Ansseau M, Geenen V, Legros JJ. Oxytocin: from milk ejection to maladaptation in stress response and psychiatric disorders. A psychoneuroendocrine perspective. *Annales d'endocrinologie*. 2009;70(6):449–454.
5 Holsboer F, Ising M. Central CRH system in depression and anxiety–evidence from clinical studies with CRH1 receptor antagonists. *Eur J Pharmacol*. 2008;583(2-3):350–357.
6 Holsboer F. Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *J Affect Disord*. 2001;62(1-2):77–91.
7 Dölen G. Oxytocin: parallel processing in the social brain? *J Neuroendocrinol*. 2015;27(6):516–535.
8 Knobloch HS, Charlet A, Hoffmann LC, et al. Evoked axonal oxytocin release in the central amygdala attenuates fear response. *Neuron*. 2012;73(3):553–566.
9 Quintana DS, Rokicki J, van der Meer D, et al. Oxytocin pathway gene networks in the human brain. *Nat Commun*. 2019;10(1):668.
10 Scantamburlo G, Hansenne M, Fuchs S, et al. Plasma oxytocin levels and anxiety in patients with major depression. *Psychoneuroendocrinology*. 2007;32(4):407–410.
11 Turan T, Uysal C, Asdemir A, Kılıç E. May oxytocin be a trait marker for bipolar disorder? *Psychoneuroendocrinology*. 2013;38(12):2890–2896.
12 Lien YJ, Chang HH, Tsai HC, Kuang Yang Y, Lu RB, See Chen P. Plasma oxytocin levels in major depressive and bipolar II disorders. *Psychiatry Res*. 2017;258:402–406.
13 Lee MR, Sheskier MB, Farokhnia M, et al. Oxytocin receptor mRNA expression in dorsolateral prefrontal cortex in major psychiatric disorders: A human post-mortem study. *Psychoneuroendocrinology*. 2018;96:143–147.
14 Zhao J, Qi XR, Gao SF, et al. Different stress-related gene expression in depression and suicide. *J Psychiatr Res*. 2015;68:176–185.
15 Quidé Y, Witteveen AB, El-Hage W, Veltman DJ, Olff M. Differences between effects of psychological versus pharmacological treatments on functional and morphological brain alterations in anxiety disorders and major depressive disorder: a systematic review. *Neurosci Biobehav Rev*. 2012;36(1):626–644.
16 Berlim MT, McGirr A, Van den Eynde F, Fleck MP, Giacobbe P. Effectiveness and acceptability of deep brain stimulation (DBS) of the subgenual cingulate cortex for treatment-resistant depression: a systematic review and exploratory meta-analysis. *J Affect Disord*. 2014;159:31–38.
17 Drobisz D, Damborská A. Deep brain stimulation targets for treating depression. *Behav Brain Res*. 2019;359:266–273.
18 Gaynes BN, Lloyd SW, Lux L, et al. Repetitive transcranial magnetic stimulation for treatment-resistant depression: a systematic review and meta-analysis. *J Clin Psychiatry*. 2014;75(5):477–489. quiz 89.

19 Mutz J, Edgcumbe DR, Brunoni AR, Fu CHY. Efficacy and acceptability of non-invasive brain stimulation for the treatment of adult unipolar and bipolar depression: a systematic review and meta-analysis of randomised sham-controlled trials. *Neurosci Biobehav Rev*. 2018;92:291–303.

20 Bao AM, Swaab DF. The art of matching brain tissue from patients and controls for postmortem research. *Handbook Clin Neurol*. 2018;150:197–217.

21 Gabriella D, Klemens R, Xiao-Hui RF, Corinna B, Eva H. Effect of personality traits on the oral health-related quality of life in patients with oral lichen planus undergoing treatment. *Clin Oral Investig*. 2021;25(4):2381–2389.

22 Wu X, Balesar R, Lu J, et al. Increased glutamic acid decarboxylase expression in the hypothalamic suprachiasmatic nucleus in depression. *Brain Struct Funct*. 2017;222(9):4079–4088.

23 Lu J, Zhao J, Balesar R, et al. Sexually dimorphic changes of hypocretin (Orexin) in depression. *EBioMedicine*. 2017;18:311–319.

24 Zhu QB, Unmehopa U, Bossers K, et al. MicroRNA-132 and early growth response-1 in nucleus basalis of Meynert during the course of Alzheimer's disease. *Brain*. 2016;139(Pt 3):908–921.

25 Guo L, Stormmesand J, Fang Z, et al. Quantification of tyrosine hydroxylase and ErbB4 in the locus coeruleus of mood disorder patients using a multispectral method to prevent interference with immunocytochemical signals by neuromelanin. *Neurosci Bull*. 2019;35(2):205–215.

26 Wang SS, Kamphuis W, Huitinga I, Zhou JN, Swaab DF. Gene expression analysis in the human hypothalamus in depression by laser microdissection and real-time PCR: the presence of multiple receptor imbalances. *Mol Psychiatry*. 2008;13(8):786–799.

27 Kamphuis W, Schneemann A, van Beek LM, Smit AB, Hoyng PF, Koya E. Prostanoid receptor gene expression profile in human trabecular meshwork: a quantitative real-time PCR approach. *Invest Ophthalmol Vis Sci*. 2001;42(13):3209–3215.

28 Zou X, Zhu Z, Guo Y, et al. Neural excitatory rebound induced by valproic acid may predict its inadequate control of seizures. *EBioMedicine*. 2022;83:104218.

29 Charan J, Kantharia ND. How to calculate sample size in animal studies? *J Pharmacol Pharmacotherapeut*. 2013;4(4):303–306.

30 Milosavljevic N, Cehajic-Kapetanovic J, Procyk CA, Lucas RJ. Chemogenetic activation of melanopsin retinal ganglion cells induces signatures of arousal and/or anxiety in mice. *Curr Biol*. 2016;26(17):2358–2363.

31 Guo L, Chen YX, Hu YT, et al. Sex hormones affect acute and chronic stress responses in sexually dimorphic patterns: consequences for depression models. *Psychoneuroendocrinology*. 2018;95:34–42.

32 Toth I, Neumann ID. Animal models of social avoidance and social fear. *Cell Tissue Res*. 2013;354(1):107–118.

33 Chang WH, Lin SK, Lane HY, et al. Reversible metabolism of clozapine and clozapine N-oxide in schizophrenic patients. *Prog Neuropsychopharmacol Biol Psychiatry*. 1998;22(5):723–739.

34 Manvich DF, Webster KA, Foster SL, et al. The DREADD agonist clozapine N-oxide (CNO) is reverse-metabolized to clozapine and produces clozapine-like interoceptive stimulus effects in rats and mice. *Sci Rep*. 2018;8(1):3840.

35 Pınar N, Akillioglu K, Sefil F, Alp H, Sagir M, Acet A. Effect of clozapine on locomotor activity and anxiety-related behavior in the neonatal mice administered MK-801. *Bosn J Basic Med Sci*. 2015;15(3):74–79.

36 Logan RW, Ozburn AR, Arey RN, et al. Valproate reverses mania-like behaviors in mice via preferential targeting of HDAC2. *Mol Psychiatry*. 2021;26(8):4066–4084.

37 Engelhardt KA, Fuchs E, Schwarting RKW, Wöhr M. Effects of amphetamine on pro-social ultrasonic communication in juvenile rats: Implications for mania models. *Eur Neuropsychopharmacol*. 2017;27(3):261–273.

38 Andrabi M, Andrabi MM, Kunjunni R, et al. Lithium acts to modulate abnormalities at behavioral, cellular, and molecular levels in sleep deprivation-induced mania-like behavior. *Bipolar Disord*. 2020;22(3):266–280.

39 Iñiguez SD, Flores-Ramirez FJ, Riggs LM, et al. Vicarious social defeat stress induces depression-related outcomes in female mice. *Biol Psychiatry*. 2018;83(1):9–17.

40 Landgraf D, Long JE, Proulx CD, Barandas R, Malinow R, Welsh DK. Genetic disruption of circadian rhythms in the suprachiasmatic nucleus causes helplessness, behavioral despair, and anxiety-like behavior in mice. *Biol Psychiatry*. 2016;80(11):827–835.

41 Yu X, Ba W, Zhao G, et al. Dysfunction of ventral tegmental area GABA neurons causes mania-like behavior. *Mol Psychiatry*. 2021;26(9):5213–5228.

42 Schatzberg AF, Keller J, Tennakoon L, et al. HPA axis genetic variation, cortisol and psychosis in major depression. *Mol Psychiatry*. 2014;19(2):220–227.

43 De Luca V, Tharmalingam S, Kennedy JL. Association study between the corticotropin-releasing hormone receptor 2 gene and suicidality in bipolar disorder. *Eur Psychiatry*. 2007;22(5):282–287.

44 Parris MS, Grunebaum MF, Galfalvy HC, et al. Attempted suicide and oxytocin-related gene polymorphisms. *J Affect Disord*. 2018;238:62–68.

45 Montag C, Brockmann EM, Bayerl M, Rujescu D, Müller DJ, Gallinat J. Oxytocin and oxytocin receptor gene polymorphisms and risk for schizophrenia: a case-control study. *World J Biol Psychiatry*. 2013;14(7):500–508.

46 Dai D, Li QC, Zhu QB, et al. Direct involvement of androgen receptor in oxytocin gene expression: possible relevance for mood disorders. *Neuropsychopharmacology*. 2017;42(10):2064–2071.

47 Jamieson BB, Nair BB, Iremonger KJ. Regulation of hypothalamic corticotropin-releasing hormone neurone excitability by oxytocin. *J Neuroendocrinol*. 2017;29(11). https://doi.org/10.1111/jne.12532.

48 Windle RJ, Kershaw YM, Shanks N, Wood SA, Lightman SL, Ingram CD. Oxytocin attenuates stress-induced c-fos mRNA expression in specific forebrain regions associated with modulation of hypothalamo-pituitary-adrenal activity. *J Neurosci*. 2004;24(12):2974–2982.

49 Neumann ID, Wigger A, Torner L, Holsboer F, Landgraf R. Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. *J Neuroendocrinol*. 2000;12(3):235–243.

50 Slattery DA, Neumann ID. Chronic icv oxytocin attenuates the pathological high anxiety state of selectively bred Wistar rats. *Neuropharmacology*. 2010;58(1):56–61.

51 Cardoso C, Ellenbogen MA, Orlando MA, Bacon SL, Joober R. Intranasal oxytocin attenuates the cortisol response to physical stress: a dose-response study. *Psychoneuroendocrinology*. 2013;38(3):399–407.

52 Ring RH, Malberg JE, Potestio L, et al. Anxiolytic-like activity of oxytocin in male mice: behavioral and autonomic evidence, therapeutic implications. *Psychopharmacology (Berl)*. 2006;185(2):218–225.

53 Steinman MQ, Duque-Wilckens N, Greenberg GD, et al. Sex-specific effects of stress on oxytocin neurons correspond with responses to intranasal oxytocin. *Biol Psychiatry*. 2016;80(5):406–414.

54 Resendez SL, Namboodiri VMK, Otis JM, et al. Social stimuli induce activation of oxytocin neurons within the paraventricular nucleus of the hypothalamus to promote social behavior in male mice. *J Neurosci*. 2020;40(11):2282–2295.

55 Johns JM, Joyner PW, McMurray MS, et al. The effects of dopaminergic/serotonergic reuptake inhibition on maternal behavior, maternal aggression, and oxytocin in the rat. *Pharmacol, Biochem Behav*. 2005;81(4):769–785.

56 Keating C, Dawood T, Barton DA, Lambert GW, Tilbrook AJ. Effects of selective serotonin reuptake inhibitor treatment on plasma oxytocin and cortisol in major depressive disorder. *BMC Psychiatry*. 2013;13:124.

57 Gomez F, García-García L. Anxiogenic-like effects of fluoxetine render adult male rats vulnerable to the effects of a novel stress. *Pharmacol, Biochem, Behav*. 2017;153:32–44.

58 Yagi K, Onaka T. A benzodiazepine, chlordiazepoxide, blocks vasopressin and oxytocin release after footshocks but not osmotic stimulus in the rat. *Neurosci Lett*. 1996;203(1):49–52.

59 Cui SS, Bowen RC, Gu GB, Hannesson DK, Yu PH, Zhang X. Prevention of cannabinoid withdrawal syndrome by lithium: involvement of oxytocinergic neuronal activation. *J Neurosci*. 2001;21(24):9867–9876.