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WITHDRAWN: fMRI analysis of MCP-1 induced prefrontal cortex neuronal dysfunction in depressive cynomolgus monkeys

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Research

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EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Background Depression is a serious mental illness, which is one of the main causes of disability at present. The cause and location of depression are still unclear. The purpose of this study is to establish a stable and reliable model of non-human primate depression, and further confirm the significance of neuritis in the pathogenesis of depression by combining in vivo and in vitro experiments. Methods We simulated the environment of human depression and established a cynomolgus monkeys depression model by pro-depressive prodedure (PDP). The model was evaluated by behavioral test and neurotransmitter detection, and the important functional changes of brain area were detected by Functional magnetic resonance imaging (fMRI). Abnormal inflammatory factors in serum and cerebrospinal fluid (CSF) were determined by multi factor kit. In addition, the mechanism was further verified by stereotactic injection of inflammatory factor antagonists into mouse prefrontal cortex(PFC) and cell experiments. Results Here we found that a 12-week exposure to PDP can effectively induce the depressive behaviors of cynomolgus monkeys. PDP increases the time of depressive-like and anxious-like behaviors and decreases locomotor and exploratory behaviors, which were maintained after a 4-week recovery period. PDP lowers the serum serotonin (5-HT), brain-derived neurotrophic factor (BDNF) level at the end of the procedure. FMRI can reflect the state of brain function noninvasively based on the level of blood oxygen. The results demonstrate that fALFF signaling is downregulated in PFC. The downregulation of BDNF and NeuN(Neuronal nuclei antigen) in PFC are observed in depressive monkeys. At the same time, it was found that contents of the monocyte chemoattractant protein-1 (MCP-1) in serum, CSF and PFC are increased in cynomolgus monkeys receiving PDP treatment. Furthermore, we found that MCP-1 receptor antagonist (CCR2-RA-[R]) can significantly reduce the susceptibility of depression in mice and increase the expression of BDNF in serum and PFC of depressed mice and blocked the downregulation of MCP-1 on the expression of BDNF in SHSY-5Y cells. Conclusions In conclusion, PDP induces cynomolgus monkeys depression by secreting MCP-1 to impair the neurotrophic function of 5-HT in PFC. PDP is a satisfying method to establish inducible depressive model in cynomolgus monkeys.

INTRODUCTION

Major depressive disorder (MDD) is one of the most common and most burdensome mental disorders worldwide. The 12-month and lifetime prevalence of major depressive disorder in US adults were 10.4% and 20.6%, respectively ¹. MDD generated great disability for its high severity and a long illness duration in a substantial part of patients. It thus ranked in the top five leading causes of years lived with disability in 2016 globally ^{2, 3}.

Animal models are indispensable tools for preclinical research and drug screening in mood disorders ⁴. Rats has traditionally been the species of choice in researching the pathogenesis of depressive-like behaviors. An explosion in the use of genetically modified mice switches most researchers to model depression and anxiety in mice ⁵. However, solid evidence from twin studies suggested that the influence of genetic factors on MDD is around 30–40% ⁶. Importantly, no specific gene was identified that authentically associated with the risk of depression ⁷. Further, these rodent models have always been

vilified for the "emotions are fictional causes to which we attribute animal behavior" ⁸. Primatologist suggests that there is a striking continuum between humans and non-human primates when they are suffering from crippling depression ⁹. The emotional similarities between the human and non-human primates far outweighs the differences as a dependent chimpanzee might starves to death when he encountered the death of his mother ^{8, 9}. So, non-human primates are ideal animal tools to under 'grief' and 'depression' in a closely related species.

The brain structures involved in limbic regulation of emotion, such as the hippocampus and amygdala, are evolutionarily conserved from mouse to man ¹⁰. However, the brain volume of a man is 2,700 times greater than that of a mouse. The tremendous difference suggests that the behavioral and physiological response to a stress in mice only represents a tiny fraction of the humankind. Cynomolgus monkey, which is behaviorally similar to human depression, was introduced in our research. The use of the near akin to human might deepen our understanding stress induced-social behaviors under the given social context and the biological background in the evolution history ⁵. The spontaneous low-frequency fluctuations (LFF) in fMRI at rest were highly synchronous among the brain regions of the same functional component ¹¹. Thereafter, the LFF of fMRI signals at the resting-state were proposed to measure the spontaneous neuronal activities ¹²⁻¹⁵. Further, the amplitude of LFF (ALFF) was introduced to quantify the regional intensity of spontaneous fluctuations in fMRI signals ¹⁶, and was considered as a reliable index to reflect the neuronal activities in vivo.

Most basic researches of neurobiology agree that the most classical hypothesis of depression is the decrease of monoamine neuron function and the lack of neurotransmitter. However, there are many defects in clinic, such as delayed onset, low efficiency and many adverse reactions, which can not fully explain the whole phenomenon of depression. Therefore, in recent years, more and more attention has been paid to the hypothesis of inflammation. It is believed that stress triggers the inflammatory process, which eventually leads to the occurrence of depression. MCP-1 plays a key role in the occurrence and development of depression.

In the present study, the fractional ALFF (fALFF) was adopted to reflect the neuronal activities in cynomolgus monkey receiving a pro-depressive programme. The neurobiological basis for the change of fALFF signals was also mined to bridge the functional imaging and depressive-like behaviors in non-human primates, and to intervene the mouse depression model through antagonistic drugs. To further clarify the fMRI dysfunction of cynomolgus monkeys from the interaction of inflammatory response, neurotransmitters and brain functional images.

MATERIALS AND METHODS

All experiments were performed in accordance with the guideline and regulation by the Administration Office of Laboratory Animals at Guangdong, China. All experimental protocols were approved by Institutional Animal Care Unit Committee in Administration Office of Laboratory Animals of Nanfang Hospital (NFYY-2014-53).

Subjects - cynomolgus monkeys

Fifteen healthy adult male Macaca cynomolgus subjects (aged 6–7 years) were obtained directly from Guangdong Blooming-Spring Biological Technology Development Co., Ltd, Guangdong, China, License number, SCXK (Guangdong) 2009-0027. They were inoculated with measles vaccine, and TB(tubercle bacilli), Yersinia, Shigella negative, BV (B virus), SRV (Sinian Rabies virus), STLV (Sinian T-lymphotropic virus), SIV (simian immune virus) were negative. They were housed individually in adjacent standard single cages till Experiment beginning. The site of the study is located in the monkey room of the Southern Hospital-Experimental Animal Research Center, Guangzhou, China. They were fed twice a day at 12:00 AM and 17:00 PM, respectively, including composite monkey food (about 200 g/time), fresh fruits and clean drinking water and were maintained on a 7:00AM–18:00PM light–dark cycle. The breeders clean the monkey house daily and disinfect every week. If any individual abnormality was found, the breeder promptly reported to the veterinarian and took appropriate measures. The normal lighting time of monkey room is 7:00AM to 18:00PM, room temperature 25–27 °C, relative humidity 50–70 °C.

Subjects - mice

8-week-old male C57BL/6 mice (n = 40, Nanfang Hospital Animal Experimental Center, Guangzhou, China) were randomly assigned into 4 groups: Vehicle group, Chronic Unpredictable Mild Stress (CUMS) group, CCR2-RA-[R] (MCP-1 receptor antagonist) group, CUMS + CCR2-RA-[R] group. All the mice were fed adaptively for 1 week, CCR2-RA-[R] / normal saline(0.5 ul, 10 nM) were injected into bilateral prefrontal cortex for stereotactic injection with the aid of a pump at a rate of 0.2 μ L/min.(Supplementary Material) Normal saline (NS) was administered in Vehicle group and CUMS group; CCR2-RA-[R] was administered in CCR2-RA-[R] group and CUMS + CCR2-RA-[R] group. and then fed for 1 week. A 28-day chronic unpredictable mild stress (CUMS) procedures were performed. All operations start at 9:00 am.(Fig. 5a,b) All animals were bred in a temperature (21 ± 2 °C) and humidity (55% ± 5%) controlled room with a 12/12 h light/dark cycle, with food and water provided ad libitum. The body weight, food and water consumption of each mouse were recorded weekly.

Pro-Depressive Procedure

Fifteen healthy adult male Macaca cynomolgus were single-housed 2 months in standard size single cage (about 160*120*120 cm³) for experiment adaptation and weighed. 15 cynomolgus were randomly divided into two groups: normal control group (n = 5), pro-depressive procedure (PDP) group (n = 10). At the beginning of the experiment, normal control group were housed in the standard single cages without any intervention. PDP group were transferred to 15 stainless steel cages (42*30*50 cm³) controlled by a telescopic rod respectively from their standard single cages, small cage within the rod device, control rod fixed a cynomolgus in a limited smaller space in the modeling process, Its space is 2/5 of the original small cage space. Their activities are limited, barely able to turn around, does not affect the intake of water, urination, defecation and other needs. Small cage equipped with troughs and cups, placed side by side fixed. They cannot see and touch each other (Supplementary Material, Fig. S1a-c).

Briefly, the PDP involved 10 different stressors that were randomly arranged throughout the day and night over 90 consecutive days. The stressors were (1) food deprivation, (2) water deprivation, (3) overnight illumination, (4) all day light deprivation, (5) ice-water, (6) social isolation, (7) restraint, (8) stick stimulation, (9) stick cage and (10) cage dystopia. Details were conducted as described in the Supplementary Material, Table S1¹⁷. The behavioral tests were performed and scored by trained and experienced observers who were blinded to the animals' conditions.

During the day, the environment-friendly shading cloth was installed to make the surrounding environment dim and invisible, the lights were turned on at night and the daytime is upside down. Social vision isolation for a period of 90 days, to release the rod every 5 days and restore the original space of the cages for 1 day, but they were still in social visual isolation environment, and then were transferred into their single-cage, non-visual isolation environment, for 1 day. The main purpose is to avoid prolonged detention, restricted activities lead to muscle atrophy, etc., the size of the cage in and out of the alternating time between 7–8 a.m. and then continue into the small cage detain modeling. The experimental design of the study was depicted in Fig. 1a.

Behavioral Categories and Definitions, Observation, and Recording

Monkey behavior data were collected using 30 high-resolution Infrared cylinder type network camera(DS-2CD2010FD-I/R, Hikvision, China) fixed on two long Stainless steel racks, which were located at the sideby-side standard single cage front and rear 1.5 m far for detailed and clearly recording behavioral data of every cynomolgus. Datas were converted via network lines and Fast Ethernet switch (S1700-26R-2T-AC, HUAWEI,China) and saved in the hard disk video recorder (DS-7932N-E4, Hikvision,China). While the observation time was set up on separate time window, 10:00-10:30 a.m. Avoiding entering the room to prevent disturbing the monkeys. All data recordings were analyzed blindly and scored in 30-minute observational periods by three trained technicians; the three viewers reached a consensus to the behavioral classification, interrater reliability was maintained at \geq 85% and all their analyses were done on the computer. Observers used the focus tracking observation method to statistically summarize the various behaviors of cynomolgus monkeys during the observation period, and count them in units of seconds (s).

fMRI data acquisition

The brain fMRI of all cynomolgus monkeys were collected at 3 time points (0th week, 12th week, 16th week). Before scanning, each monkey was given Telazol (3–5 mg/kg) and atropine sulfate (0.05 mg/kg) via intramuscular injection to induce anesthesia and reduce salivary secretion. All MR images were scanned using a 3.0T HDXT scanner (GE, USA) equipped with an 8-channel animal head coil, (Supplementary Material, Figure S2) For the resting-state fMRI, a single-shot echo-planar imaging sequence (EPI) was adopted. See Supplementary Material for scanning parameters, data processing and analysis. ^{18,19}

Mice behavioral tests

The mice behavioral tests were performed in the order of tail suspension test (TST), force swimming test (FST) and sucrose preference test (SPT) after CUMS procedure and scored by 3 trained and experienced observers.(Supplementary Material)

Cell Culture and treatment

Human SHSY-5Y cells were cultured in DMEM contained 10% FBS at 37°C under 5% CO₂ atmosphere. Human SHSY-5Y cells were incubated with or without MCP-1 (PeproTech, USA) 100 ng/mL for 24 h. In some of these cultures, the medium was replaced for 5 h with new one without treatment or containing 5-HT (Solarbio, China) 10nM CCR2-RA-[R] (MCE, USA) 100 nM. Protein was isolated and BDNF levels determined by western blot.

Statistical Analysis

All Data were analyzed using the SPSS 20.0 and GraphPad Prism 8.0 software. Data were presented as means ± SEM. One-way ANOVA followed by Dunnett multiple comparison tests were used for the statistical analyses according to the experimental design. P-value < 0.05 was considered as the significant. The voxel-wise statistical comparison of fALFF images between groups was performed by two-sample t-test based on the framework of the general linear model. The voxel-level statistical threshold was P < 0.005 (uncorrected) and the cluster-extent threshold was 20 voxels.

Results

Promoted-depressive procedure (PDP) can successfully induce and maintain depressive-like behaviors in cynomolgus monkeys

To establish a non-primate model of major depression disorder, 10 cynomolgus monkeys (Macaca fascicularis) were subjected to a PDP that composed of social isolation, chronic unpredictable mild stress (CUMS) and restraint stress (Fig. 1a and Supplementary Material, Table S1). Briefly, each cynomolgus monkey was habituated in single cage for 8 weeks. Then singly-housed monkeys underwent the PDP for 12 weeks and 5 monkeys recovered for another 4 weeks. 5 monkeys in the control group were kept in big cages. The blood oxygenation level dependent (BOLD) fMRI data were collected in all cynomolgus monkeys at the 0th week, 12th week and 16th week, respectively (Fig. 1a).

Behaviors and body postures between 10:00am and 10:30am were recorded and analyzed from singlyhoused macaques. The behaviors and postures are divided into six categories: depressive-like behaviors, anxious-like behaviors, exploratory behaviors, locomotion, rest, and maintenance behaviors ^{20–27}.

Inactivity, huddle and rest in the morning with closing or closed eyes are defined as depressive-like behaviors. The control monkeys were active and vigorous during the observation windows (Supplementary Material, Figure S3a). On the contrary, the PDP treated monkeys preferred to keep the huddle posture and be inactive. So, the time of depression was markedly increased in the experimental monkeys (Supplementary Material, Figure S3b). During the recovery period, the experimental monkeys remained inactive and the frequency of depressive-like behavior did not improve significantly (Fig. 1b).

Self-scratching, self-clasping and body quivering are identified as anxious-like behaviors. These behaviors are exacerbated by PDP and body quivering or tremor could be induced by intense clasping in some experimental subjects. The anxious-like behaviors were significantly suppressed at the end of the recovery phase (Fig. 1c).

The behaviors of cage investigation, such as searching and sniffing wall or bars are regarded as exploratory behaviors. The low aspiration of cage searching can be observed in PDP-treated monkeys. Walk, jumping and circling are defined as locomotor behaviors. The locomotor frequency was also reduced in these experimental monkeys. However, the time of locomotion and exploration was markedly increased after the four-week's recovery (Fig. 1d-e).

Urinate, defecate, rubbing eyes, biting its nails, rubbing hands or self-grooming, are necessary for maintaining daily life. Rest behaviors are defined as seating awakened for more than 5 seconds or with shaking head that are different from depressive-like behaviors. No significant difference was observed in maintenance behaviors and rest behaviors between the control and the experimental monkey (Fig. 1f-g).

The depletion of monoamine neurotransmitters hypothesis has proved to be the most classic theory on pathogenesis of depression ⁷. The serum 5-HT, tryptophan (Trp) and and BDNF level were significantly decreased in depressive monkeys at the 12th week and recovered at the 16th week (Fig. 1h-i, k; Supplementary Material). So, the 5-HT/Trp ratio was decreased at the end of 12th week and maintained at the lower level even at the end of 16th week (Fig. 1j).

Collectively, PDP not only causes inactivity, anxious-like behaviors, the lacking of locomotor and exploratory behaviors but also reduces monoamine neurotransmitters, which further confirms that PDP is a reliable procedure to induce depression in cynomolgus monkeys ²⁸. More, the amelioration and maintenance of depressive-like, anxious-like, locomotor and exploratory behaviors at the end of the recovery phase consolidate that simian depression is a kind of inducible mood disorder.

The effect of PDP on cynomolgus monkeys brain fALFF by fMRI

To identify the PDP-induced brain functional changes, the fALFF imaging was analyzed by software (Supplementary Material). The fractional ALFF (fALFF) reflects lower spontaneous neuronal activity during resting state ¹⁸. Compared with normal controls at the end 12th week, PDP-treated cynomolgus monkeys showed significantly decreased fALFF in the prefrontal cortices (8Bs, 8Bm, 9d, 9 m, 10mc, 12 l, 12 m, 12o, PrCO, 13b, 13 l, 13 m, 14r, 25t, 32t, and 46d), visual cortex (area V1), premotor and motor cortices (F2_6DR_6DC, F3, F4, F5_6Va_6Vb, and F6), somatosensory cortices (area SII and 5_PEa), striatum, temporal cortices (PGa, area TEO, and area TPO), posterior cingulate cortex (area 23a), anterior cingulate cortex (area 24a), insula cortices (area Iai, area Ia, area Ig, and area Iapl), auditory cortices (CM

and R), parietal cortices (area 7op, area LIPd, and AIP), and gustatory cortex (G), cerebellum. while increased in the temporal cortices (area TEa, area TEad, area Tem, and area TEpd) (Fig. 2a and Table 1).

Table 1A comparative study of fALFF in each group under different conditions

PDP vs control (12th week)	12th week VS 0th week (PDP)	16th week VS 0th week (PDP)	16th week VS 12th week (PDP)
Prefrontal cortex	Prefrontal cortex	Prefrontal cortex	Prefrontal cortex/ Prefrontal cortex
Visual cortex	Visual cortex	Visual cortex	
Cerebellum	Cerebellum	Cerebellum	
Somatosensory cortex	Somatosensory cortex	Somatosensory cortex	
Striatum	Striatum	Striatum	Striatum
Temporal cortex/ Temporal cortex	Temporal cortex	Temporal cortex	
Insula cortex	Insula cortex	Insula cortex	
posterior Cingulate cortex	posterior Cingulate cortex	posterior Cingulate cortex	
anterior Cingulate cortex	anterior Cingulate cortex		
Premotor and motor cortex	Premotor and motor cortex		
Auditory cortex	Parahippocampus cortex	Parahippocampus cortex	
Parietal cortex	Hippocampus		Hippocampus
gustatory cortex	Claustrum	Claustrum	
	Substantia Nigra		
PDP vs control (12th week)	12th week VS 0th week (PDP)	16th week VS 0th week (PDP)	16th week VS 12th week (PDP)
Prefrontal cortex	Prefrontal cortex	Prefrontal cortex	Prefrontal cortex/ Prefrontal cortex
Visual cortex	Visual cortex	Visual cortex	
Cerebellum	Cerebellum	Cerebellum	

Text indicates that fALFF decreases brain area, while Bold text in italics indicates that fALFF increases brain area. The voxel-level height threshold was P < 0.005 (uncorrected) and the cluster-extent threshold > 20 voxel.

PDP vs control (12th week)	12th week VS 0th week (PDP)	16th week VS 0th week (PDP)	16th week VS 12th week (PDP)	
Somatosensory cortex	Somatosensory cortex	Somatosensory cortex		
Striatum	Striatum	Striatum	Striatum	
Temporal cortex/ Temporal cortex	Temporal cortex	Temporal cortex		
Insula cortex	Insula cortex	Insula cortex		
posterior Cingulate cortex	posterior Cingulate cortex	posterior Cingulate cortex		
anterior Cingulate cortex	anterior Cingulate cortex			
Premotor and motor cortex	Premotor and motor cortex			
Auditory cortex	Parahippocampus cortex	Parahippocampus cortex		
Parietal cortex	Hippocampus		Hippocampus	
gustatory cortex	Claustrum	Claustrum		
	Substantia Nigra			
Text indicates that fALFF decreases brain area, while Bold text in italics indicates that fALFF increases brain area. The voxel-level height threshold was P < 0.005 (uncorrected) and the cluster-extent threshold > 20 voxel.				

The longitudinal comparison before(0th week) and after(12th week) PDP-treated cynomolgus monkeys showed that fALFF signaling was downregulated in the hippocampus (area paraS, area preS), temporal cortices (area TEad, area TEav, area TEm, area TEa, and area TGsts), cerebellum, substantia nigra (SNr_c), claustrum, striatum, parahippocampus cortex (area TH), prefrontal cortices (10mc, 12o, 12 m, 13b, 13 l, 13 m, 14c, 25t, 32t, 44t, 46f, PrCO, 8Bs, 8Bd, 8Bm, and 9d), premotor and motor cortices (F1_4, F2_6DR_6DC, F3, F4, F5_6Va_6Vb, F6, and F7), visual cortices (area V1, area V3v, and area V4), somatosensory cortices (area SII), insula cortices (lai, lal, and lg), posterior cingulate cortex (area v23a) and anterior cingulate cortex (area 24c). However, no increased fALFF signaling was observed after the 12-week's PDP. (Fig. 2b and Table 1)

In summary, both the cross-sectional between-group comparison and the longitudinal within group comparison revealed a widespread cerebral fALFF decrease relating to the PDP-induced depression, and the overlapping brain areas are mainly concentrated in prefrontal cortices, cerebellum, striatum, premotor and motor cortices, visual cortices, somatosensory cortices, insula cortices, posterior cingulate cortex, temporal cortices.

The effect of PDP recovery periodon on the fALFF of brain fMRI in cynomolgus monkeys

We performed a 4-week recovery of the PDP-treated cynomolgus monkeys and further evaluated the areas of the fALFF changes in their brain fMRI. After the 4-week's recovery period (i.e., at the 16th week), 16th week-PDP-treated cynomolgus monkeys as compared with themselves at 0th week. Significant longitudinal decrease of fALFF was observed in the prefrontal cortex cortex (9m 10mr 10o 11l 11m 12o 12m 12r 13l 13m 45a 46v 46d 46f PrCO), visual cortex (area V1 area V3v area V4v), temporal cortex (area TEav area IPa area TEa area TGsts), cingulate cortex (area 23c), Insula cortex (lai Id), claustrum, striatum, somatosensory cortex (area SII), Cerebellum. (Fig. 3a and Table 1)

Compared with the 12th week-PDP-treated cynomolgus monkeys, significant longitudinal increase of fALFF was observed in the hippocampus (area CA1, area DG, and area proS), striatum, and prefrontal cortices (13 m, 13 l, 46f, and 46v), while significant decrease was found in the prefrontal cortices (12 l, 12r, 44t, 45a, and 46v) (Fig. 3b and Table 1). In brief, the 4-week's recovery period after PDP was possibly related to the fALFF increases in a series of brain regions which previously exhibited decreases during PDP.

Chemokine and cytokine profiles of depressive-like monkeys

Activation of the inflammasome and release of pro-inflammatory cytokines may facilitate the occurrence of depressive disorders ²⁹. A panel of cytokines & chemokines, including MCP-1, IL-6, MIP-1β, TGFa were measured by multiplex Luminex assays (Supplementary Material). Both the serum and cerebrospinal fluid (CSF) MCP-1 was increased upon receiving PDP treatment (Fig. 4a,e). However, the upregulated MCP-1 levels restored by the 4-week recovery period, respectively. No significant difference was observed in the other cytokines between the experimental cynomolgus monkeys and the controls (Fig. 4b-d,4f-h). The levels of IFN_γ, IL-1β, IL-10, MIP-1α, TNFα, and VEGF in serum and CSF of cynomolgus monkeys were lower than the detection limit, and not detected. The results showed that the level of MCP-1 in serum was positively correlated with the level of MCP-1 in CSF (R = 0.3193, P < 0.01, Fig. 4i), and negatively correlated with the level of BDNF and 5-HT in serum (R = 0.1796, P < 0.05, Fig. 4j; R = 0.2246, P < 0.01, Fig. 4k, respectively). The level of MCP-1 in CSF was negatively correlated with the level of 5-HT and Trp (R = 0.2163, P < 0.01, Fig. 4n; R = 0.3788, P < 0.001, Fig. 4o, respectively). The levels of BDNF in antidepressantfree MDD patients were significantly lower than those of healthy controls ^{30, 31}. We further found that PDP can not only reduce the level of BDNF in the serum of depressed cynomolgus monkeys, but also significantly decrease the expression of TrkB, NeuN and BDNF and significantly increase the expression of MCP-1 in mPFC. (Fig. 4q-r; Supplementary Material). The immunofluorescence staining of neuron marker NeuN indicated that the number of neurons decreased significantly and improved after 4 weeks (Fig. 4s; Supplementary Material). These results suggest that upregulated chemokine MCP-1 contributes to the pathogenesis of PDP-induced depression.

CCR2-RA-[R] can reduce the susceptibility of depressed mice and the downregulation of BDNF and SHSY-5Y cells injury by MCP-1 To further verify the important role of MCP-1 in the occurrence of depression, MCP-1 receptor antagonist (CCR2-RA-[R]) was injected into bilateral prefrontal cortex of mice to intervene the depression model induced by CUMS. (Fig. 5a-b; Supplementary Material) Mice behavior tests found that, compared with the vehicle group, the time of immobility of tail suspension test (TST) and the time of immobility in the forced swimming test (FST) of CUMS depression mice were prolonged(P < 0.001, Fig. 5c-d). Sucrose preference rate decreased significantly in sucrose preference test (SPT) of CUMS depression mice. (P < 0.001, Fig. 5c) The above behavior changes were inhibited by injecting CCR2-RA-[R] into the bilateral prefrontal cortex (P < 0.01, Fig. 5c; P < 0.05, Fig. 5d; P < 0.001, Fig. 5e, respectively). In addition, after CCR2-RA-[R] intervention, the high level of MCP-1 and the low level of BDNF in the serum (P < 0.001, Fig. 5f-g) and prefrontal cortex (P < 0.05, Fig. 5h-i) of CUMS depression mice were significantly improved.

BDNF and 5-HT are a dynamic duo in regulating synaptic plasticity and neurogenesis ³². Our results showed that 5-HT enhanced the expression of BDNF. However, the MCP-1 exposure downrelated its expression in SHSY-5Y cells. MCP-1 plays an inflammatory chemotaxis role on its receptor CCR2. The use of CCR2 inhibitor (CCR2-RA-[R]) blocked the effect of MCP-1 on the expression of BDNF in SHSY-5Y cells (Fig. 5j-k). The downregulation of neurotrophic factor by MCP-1 might be an important cause accounts for the occurrence of depressive-like behaviors in cynomolgus monkeys.

Discussion

It has been emphasized by Darwin that "the difference between the mind of the lowest man and that of the highest animal is immense" ³³. However, the homology of DNA sequence between chimps and human is more than 98%, indicates the conservation and continuity of traits across species. More strikingly, the emotion of primates are similar to that of human's, such as grief, reactive depression and severe depressive disorders. The genome homology between cynomolgus monkeys and human is 92.83 by a whole-genome shotgun sequencing approach ³⁴. Because of its relative small figure size, vulnerable to depression, and commercial availability ³⁵, cynomolgus monkeys were choose in our study. The prodepressive programme, which includes CUMS, social isolation and restraint, was satisfying in inducing depressive-like behaviors in cynomolgus monkeys through our continuous observation and careful discrimination. CUMS was of choice based on our previous experience in rodents ^{17, 36, 37}. Social isolation is destructive for the development of appropriate monkey behaviors ³⁸. Administration of cage-restraint has a further devastating effects for their mentality which drives the emotion to despair and distress ³⁹.

Inactivity is usually defined as the phenotype of depressive-like behavior in non-human primates ^{21, 27}. However, it is difficult to differentiate rest from depression if we only focused on "inactivity" itself. The posture of "huddle" was introduced as a sign of depression. The curling up position which means head lower than shoulders indicated mood of fear, unease and escape of communication. So, the inactivity with huddle gesture is identified as a kind of depressive mood which is distinguish from rest or sleep ²⁷. Monoamine neurotransmitter depletion hypothesis has been proved to be the most classical theory in the pathogenesis of depression. In order to further evaluate the stability and reliability of the PDP model of primates, we detected the monoamine neurotransmitter content in the blood of cynomolgus monkeys and found that the serum 5-HT and Trp levels in depressed monkeys decreased significantly after the end of modeling. More, the 5-HT / Trp ratio decreased at the 12th week and remained low even at the 16th week. Combined with the behavioral data of animals, the depressive-like behavior increased in the 16th week and remained in a crouching posture for a long time. It is more reasonable to believe that the model of depression caused by PDP is stable and lasting.

fMRI has been widely applied in exploring mood regulating circuit in human for more than 20 years. However, it is difficult to clarify the underlying anatomical structure and neurobiological basis for the inaccessibility of corresponding brain tissues from the patients. Previous neuroimaging has showed that human functional results are closely resemble to primate anatomy ⁴⁰. In this study, fMRI is a noninvasive technique performed to characterize the neurophysiological changes relating to the pro-depressive programme in cynomolgus monkeys. In vivo evidence suggested a widespread fALFF decreasing in the brain by the within-group longitudinal comparison and the cross-sectional comparison with the control subjects. The decreased fALFF distribution of major brain clusters were consistent with the findings of previous studies on depressed individuals ⁴¹⁻⁴³ and animal model ⁴⁴, such as prefrontal cortices, cerebellum, striatum, visual cortices, insula cortices, posterior cingulate cortex, temporal cortices, etc. Thus, our current results provided additional evidence that CUMS-based PDP successfully induces the depression-related impairment of neuronal activity.

More importantly, after the 4-week recovery period, compared with the 0th week' cynomolgus monkey, part of the brain area has been improved, such as the anterior cingulate gyrus, premotor and motor cortex, hippocampus, etc., the vast number of brain areas still maintain a decreasing trend level, such as prefrontal cortex, striatum, visual cortex, temporal cortex, posterior cingulate gyrus, cerebellum, etc. In Khan's study ⁴⁴, microstructure alteration in prefrontal cortex and hippocampus were observed in rats exposed to chronic mild stressors, and this alteration turned to normal after a period of recovery. Taken together, our results may well illustrated the in vivo neuronal activities changes corresponding to the recovery process from the stressful environment. Another interesting finding should be noted that, compared with the 12th week' PDP treatment, the fALFF signal of some frontal cortex, parietal cortex and striatum is increased, while the fALFF signal of some frontal cortex is still decreased. According to Yang ⁴⁵ and Cano ⁴⁶, significantly elevated functional connectivity of prefrontal cortex was found accompanied with the improvement of the depression during treatment. Therefore, we have reason to believe that the frontal cortex is the central brain area, and other brain areas are the auxiliary role to form a brain network structure, which provides an important basis for the occurrence and development of depression. And, we speculated that the elevated prefrontal activity may be an important neural indicator of recovery from depressive status.

We hypothesized that PDP is the key factor of depression. Our results indicated that PDP is due to the increase of inflammatory chemokine MCP-1 in central and peripheral blood and further shunt of Trp (5-HT precursor) to further reduce the level of 5-HT and BDNF, and induce depressive-like behavior of cynomolgus monkeys and behavior of depression mice. Our results suggested that serotonergic neurons

might play pivotal functions in PDP-induced mood depression ⁴⁷. It is consistent with our previous report that 5-HT synthesis was downregulated in the hippocampus of rats with CUMS-induced depressive-like behaviors ¹⁷. Moreover, we also found the elevated fALFF signalling at the end of recovery phase were correlated with the level of BDNF. MCP-1 exposure has been proven to induce dysfunction of synaptic transmission and plasticity in the hippocampus ^{48, 49}. Our results further confirmed that MCP-1 participate in the formation of depressive-like behaviors by causing chronic neuroinflammatory response to impair the expression of BDNF. MCP-1 antagonists (CCR2-RA-[R]) injected into the PFC of mice could not only significantly reduce the susceptibility of depressed mice and the level of MCP-1 in serum and PFC, but also increase the level of BDNF. In addition, it can also block the downregulation of BDNF expression of SHSY-5Y cells by MCP-1. The disadvantage is that the price of cynomolgus monkeys is expensive, so the number of experimental samples is small, and the mechanism of MCP-1 on neuroimmune cells deserves further study.

Conclusion

Our results establish a bridge between imaging and neurobiological functions: the downregulation of fALFF signal in limbic system centered on prefrontal cortex is closely related to the high expression of MCP-1 and the low expression of BDNF and 5-HT. It is a valuable attempt to elucidate the fMRI dysfunction of cynomolgus monkeys from the interaction among Neuroinflammatory responses, neurotransmitter and neurotrophic factors in the brain. As a 'depressed' cynomolgus monkey may really feel the same things as a depressive human ⁹, The depression model of cynomolgus monkey induced by PDP will contribute to deep excavating the neurobiological underpinnings of depressive-disorders, especially the gene-environment interactions in the pathogenesis of affective disease.

Abbreviations

PDP: Pro-depressive prodedure; 5-HT:Serotonin, 5-hydroxytryptamine; Trp:Tryptophan; BDNF:Brain-derived neurotrophic factor; fMRI:Functional magnetic resonance imaging; PFC:Prefrontal cortex; MCP-1 (CCL2):Monocyte chemoattractant protein-1, Chemokine (C-C motif) ligand 2; CSF:Cerebrospinal fluid; MDD:Major depressive disorder; LFF:Low-frequency fluctuations; ALFF:Amplitude of low-frequency fluctuations; fALFF:Fractional amplitude of low-frequency fluctuations; CUMS:Chronic unpredictable mild stress; TST:Tail suspension test; FST:Forced swimming test; SPT:Sucrose preference test; BOLD:Blood oxygenation level dependent; EPI:Echo-planar imaging sequence; GAPDH:Glyceraldehyde 3-phosphate dehydrogenase; TrkB:Tyrosine-protein kinase receptor B; NeuN:Neuronal nuclei antigen.

Declarations

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Authors' contributions

Weixin Yan[†] and Di Zhao[†] contributed equally to this work.

WXY, DZ, LPX, YQL, JWL, WLH, SWX and FRX conducted experiments. KL, YJd analyzed the image data. CM, TZ and CYZ provided help for image acquisition and sampling. LG, GW, XGS, LLJ and ZPL supervised the work. All authors contributed to experimental design and results interpretation. WXY, DZ and XGS wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figures



Figure 1

Promoted-depressive procedure(PDP), which is a new composite pattern induced depression in cynomolgus monkeys. (a) The experimental design and pattern diagrams of the study. (b-g) Behavioral evaluation of cynomolgus monkeys. (b) Depressive-like behavior total time. (c) Anxious behavior total time. (d) Exploratory behavior total time. (e) Locomotor behavior total time. (f) Rest behavior total time. (g) Maintain behavior total time. (h-i, k) Serum concentrations of 5-HT (h), Trp (i) and BDNF (k) in controls and PDP-treated cynomolgus monkeys at different time points. (j) The ratio of 5-HT/ Trp in serum concentration of cynomolgus monkeys The data are presented as means \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001. PDP-treated cynomolgus monkeys versus control group at 12th week; #P < 0.05, ###P < 0.001. 16th week versus 12th week at PDP-treated cynomolgus monkeys.See also Supplementary 1.



Figure 2

Effect of PDP on cynomolgus monkeys brain fALFF. (a) FALFF in PDP-treated cynomolgus monkeys as compared with healthy controls at 12th week. The reduced brain areas of fALFF are as follows (white arrows): prefrontal cortex, visual cortex, temporal cortex, cingulate cortex, insula cortex, premotor and motor cortex, etc. The increased brain area of fALFF is as follows: temporal cortex (red arrows). (b) FALFF in PDP-treated cynomolgus monkeys at 12th week as compared with themselves at 0th week. The

reduced brain areas of fALFF are as follows (white arrows): prefrontal cortex, visual cortex, temporal cortex, cingulate cortex, insula cortex, premotor and motor cortex, etc. The voxel-level height threshold was P< 0.005 (uncorrected) and the cluster-extent threshold > 20 voxels.



Figure 3

Effect of PDP on the persistence of brain fALFF in cynomolgus monkeys. (a) FALFF in PDP-treated cynomolgus monkeys at 16th week as compared with themselves at 0th week. The reduced brain area of fALFF are as follows (white arrows): prefrontal cortex, hippocampal, parahippocampal cortex, visual cortex, temporal cortex, cingulate cortex, claustrum, striatum, nsula cortex, premotor and motor cortex, etc. (b) FALFF in PDP-treated cynomolgus monkeys at 16th week as compared with themselves at 12th week. fALFF reduced brain area is prefrontal cortex (white arrows). The increased brain areas of fALFF are as follows (red arrows): prefrontal cortex, hippocampal, striatum. The voxel-level height threshold was P< 0.005 (uncorrected) and the cluster-extent threshold > 20 voxels.



Figure 4

Effects of PDP exposure on MCP-1 in serum, CSF and mPFC of cynomolgus monkeys. (a-d) Serum concentrations of MCP-1 (a), IL-6 (b), MIP-1 β (c) and TGF- α (d) in controls and PDP-treated cynomolgus monkeys at different time points. (e-h) CSF concentrations of MCP-1 (e), IL-6 (f), MIP-1 β (g) and TGF- α (h) in controls and PDP-treated cynomolgus monkeys at different time points. (i) Correlation analysis of MCP-1 in serum and CSF of cynomolgus monkeys. (j-l) Correlation analysis of serum MCP-1 and BDNF (j), 5-HT (k) and Trp (l) in cynomolgus monkeys. (m-o) Correlation analysis of MCP-1 level in CSF and BDNF (m), 5-

HT (n) and Trp (o) in serum of cynomolgus monkeys. (p)Structural location of medial prefrontal cortex(mPFC) in cynomolgus monkeys. (q-r) Western blot and semi quantitative results of TrkB, NeuN, MCP-1 and BDNF in mPFC of cynomolgus monkeys. (s) Immunofluorescence of NeuN in mPFC of cynomolgus monkeys. The data are presented as means \pm SEM. **P < 0.01, ***P < 0.001. PDP-treated cynomolgus monkeys versus control group at 12th week; #P < 0.05, ###P < 0.001. 16th week versus 12th week at PDP-treated cynomolgus monkeys; $\triangle P < 0.05$, $\triangle \triangle P < 0.001$. 12th week versus 0th week at PDP-treated cynomolgus monkeys; $\triangle P < 0.05$, $\triangle \triangle P < 0.01$. 16th week versus 0th week at PDP-treated cynomolgus monkeys; $\triangle P < 0.05$, $\triangle \triangle P < 0.01$. 16th week versus 0th week at PDP-treated cynomolgus monkeys; $\triangle P < 0.05$, $\triangle \triangle P < 0.01$. 16th week PDP cynomolgus monkeys versus control.



Figure 5

CCR2-RA-[R] reduce the susceptibility of depression in mice and the downregulation of BDNF. (a) Schematic overview of experimental design for (b)-(i) . After injection of CCR2-RA-[R] into prefrontal cortex, the 28 day cums was performed, and the 29 day behavioral test was started. (b) Schematic diagram of CCR2-RA-[R]/0.9% normal salin (NS) injected into prefrontal cortex of mice. (c-e) Mice behavior tests. (c) Tail suspension test (TST). (d) Force swimming test. (e) sucrose preference test (SPT) . (f-g) CCR2-RA-[R] can reduce the expression of MCP-1 (f) and increase the expression of BDNF (g) in serum of depressed mice. (h-i) CCR2-RA-[R] can decrease the expression of MCP-1 (h) and express more BDNF (i) in the prefrontal cortex of depressed mice. (j-k) CCR2-RA-[R] blocked the downregulation of MCP-1 on the expression of BDNF in SHSY-5Y cells. Data are means ± SEM of n = 8 replicates per group.*P < 0.05, **P < 0.01, *** P < 0.001. CUMS group VS Vehicle group; #P < 0.05, ##P < 0.01, ###P < 0.001. CUMS group VS CCR2-RA-[R] group; $\triangle P < 0.05$, $\triangle \triangle P < 0.001$. CUMS+CCR2-RA-[R] group VS CCR2-RA-[R] group. Cell data are means ± SEM of n = 3 replicates per group. $\square P$ <0.01, 5-HT group VS Control; P<0.01, MCP-1 group VS CCR2-RA-[R] group VS COntrol; P<0.01, MCP-1+5-HT group VS MCP-1 group; $\blacksquare P$ <0.01, 5-HT+CCR2-RA-[R] group VS CCR2-RA-[R] group VS MCP-1 group; VS MCP-1 group; P<0.01, 5-HT+MCP-1+CCR2-RA-[R] group VS MCP-1 group; VS MCP-1 group; P<0.01, 5-HT+MCP-1+CCR2-RA-[R] group VS MCP-1 group; VS MCP-1 group; P<0.01, 5-HT+MCP-1+CCR2-RA-[R] group VS MCP-1 group; P<0.01, 5-HT+MCP-1+CCR2-RA-[R] group VS MCP-1 group; P<0.01, 5-HT+MCP-1+CCR2-RA-[R] group VS MCP-1 group; P<0.01, 5-HT+MCP-1+CCR2-RA-[R] group; group;

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