# Neuroprotective Effects of *Bacopa monnieri* (Brahmi) on Novel Object Recognition and NMDAR1 Immunodensity in the Prefrontal Cortex, Striatum and Hippocampus of Sub-Chronic Phencyclidine Rat Model of Schizophrenia

Pritsana Piyabhan PhD*,
Thanitsara Wetchateng PhD*

** Department of Preclinical Science, Faculty of Medicine, Thammasat University, Rangsit Campus, Klong Luang, Pathumthani, Thailand*

***Background:*** *Cognitive impairment is a major problem, which eventually develops in schizophrenia. It contributes to the patients' functional disability and cannot be attenuated by antipsychotic drugs. Bacopa monnieri (Brahmi), a neuroprotective herbal medicine in the elderly, might be a novel neuroprotective agent for prevention of cognitive deficit in schizophrenia.*
***Objective:*** *To study neuroprotective effects of Brahmi on novel object recognition task and cerebral glutamate/N-methyl-D-aspartate receptor subtype 1 (NMDAR1) immunodensity in sub-chronic phencyclidine (PCP) rat model of schizophrenia.*
***Material and Method:*** *Rats were assigned to three groups; Group-A: Control, Group-B: PCP administration and Group-C: Brahmi + PCP. Discrimination ratio (DR) representing cognitive ability was obtained from novel object recognition task. NMDAR1 immunodensity was measured in prefrontal cortex, striatum, cornu ammonis fields 1 (CA1) and 2/3 (CA2/3) and dentate gyrus (DG) using immunohistochemistry.*
***Results:*** *DR was significantly reduced in PCP group compared with control. This occurred alongside NMDAR1 up-regulation in CA2/3 and DG, but not in prefrontal cortex, striatum or CA1. Brahmi + PCP group showed an increased DR score up to normal which occurred alongside a significantly decreased NMDAR1 immunodensity in CA2/3 and DG compared with PCP group.*
***Conclusion:*** *Cognitive deficit observed in rats receiving PCP was mediated by NMDAR1 up-regulation in CA2/3 and DG. Interestingly, receiving Brahmi before PCP administration can restore this cognitive deficit by decreasing NMDAR1 in these brain areas. Therefore, Brahmi could be a novel neuroprotective agent for the prevention of cognitive deficit in schizophrenia.*

***Keywords:*** *Brahmi, Schizophrenia, Animal model, Novel object recognition, NMDAR1*

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One of the most popular hypotheses in schizophrenia is the Glutamatergic hypofunction. It was first suggested as a mechanism involved in schizophrenia in 1980 when Kim et al investigated the reduction of glutamate concentrations in spinal fluid of schizophrenic patients[1]. Additionally, clinical research reported that phencyclidine (PCP), a non-competitive glutamate/N-methyl-D-aspartate (NMDA) receptor antagonist, can induce schizophrenia-like psychosis including both negative (e.g. emotional withdrawal, motor retardation) and positive (e.g. hallucinations, paranoia) symptoms[2]. Hence, PCP has been reported as a psychotomimetic, which can produce schizophrenia-like psychosis in normal subjects and aggravate psychotic symptoms in schizophrenic patients[3].

Lately, administration of PCP to animals has been accepted as a valuable method to produce an animal model of schizophrenia. It has been reported that PCP produces several behaviors similar to schizophrenia[4]. Low doses of PCP can produce disinhibition and a state of euphoria, paranoia and hallucinations whereas high doses can produce sedation, catalepsy, general anesthesia and seizures[5]. The acute dose-dependent PCP administration can increase locomotor activity in animals. Moreover, PCP can interrupt prepulse inhibition (PPI), a sensory gating

***Correspondence to:***
*Piyabhan P, Division of Physiology, Department of Preclinical Science, Faculty of Medicine, Thammasat University, Rangsit Campus, Klong Luang, Pathumthani 12120, Thailand.*
*Phone: 0-2926-9710, Mobile: 08-7063-1999, Fax: 0-2926-9711*
*E-mail: taipritsana@yahoo.com*

measurement that has been found to decrease in schizophrenia.

It has been investigated that cerebral NMDA receptor subunit 1 (NMDAR1) and NMDA receptor subunit 2 (NMDAR2) are up-regulated in rats receiving sub-chronic PCP administration[6]. The NMDA receptor up-regulation induced by PCP has been suggested to produce apoptosis in cerebral cortex[7] which may be implicated in cognitive deficits in schizophrenia. Post-mortem studies showed that NMDA receptor up-regulation has been observed in several brain areas such as putamen and temporal cortex in schizophrenic patients[8] which was interpreted as glutamatergic receptor up-regulation related to glutamatergic hypofunction.

Positive symptoms in schizophrenia could be attenuated by typical antipsychotic drugs; however, they can produce extrapyramidal movement disorder. Moreover, they could not fully ameliorate negative symptoms. For these reasons, atypical antipsychotic drugs have been investigated and proposed as more effective for negative symptom treatment without producing extrapyramidal side effects. However, it has been recently reported that atypical antipsychotics can produce weight gain and several disorders related to obesity[11].

Although positive and negative symptoms in schizophrenic patients could not be fully ameliorated, to date, antipsychotics seem to be the most helpful for sedating and making the patients sociable. Cognitive impairment is also a significant problem, which leads to functional disability in schizophrenia. It finally occurs in all schizophrenic patients. Neither typical nor atypical antipsychotics can prevent the patients from cognitive deficit. Therefore, cognitive deficit is more likely to be the most critical problem in schizophrenic patients.

*Bacopa monnieri* or Brahmi is a traditional Indian Ayurvedic medicinal plant, which has been suggested as an alternative medicine to boost memory, restore cognitive deficits and improve mental function[12]. Long-term orally administration of bacosides, the active saponins of Brahmi, can prevent neurodegeneration in aging female Wistar rats[13]. Moreover, it has been investigated that Brahmi can decrease an amount of cerebral beta-amyloid in a transgenic mouse model of Alzheimer's disease[14]. The studies in humans have shown that Brahmi extract could enhance cognitive performance in the aging[15]. Therefore, Brahmi might be a valuable herbal medicine to prevent the cognitive deficit, which could eventually develop in patients with schizophrenia.

The main aim of the present study was to assess whether administration of Brahmi was able to prevent the cognitive deficit, which could develop in sub-chronic PCP administration in rats, assessed using the novel object recognition paradigm, and on the density of the NMDAR1 in the prefrontal cortex, striatum and hippocampus.

## Material and Method

### *Animals*

Twenty-seven male Wistar rats weighing 200-220 g were obtained from the National Animal Center, Mahidol University, Thailand. The animals were housed one per cage and maintained at 21±2°C under a 12 h/12 h light/dark cycle with food and water available ad libitum in the home cage. All animals were acclimatized for 7 days before the experiment. All animal procedures were performed in accordance with Mahidol University Code of Practice and the National Institutes of Health (USA) Guidelines for treatment of laboratory animals. The protocol for the present study was approved by the Animal Research Committee of Thammasat University, Thailand. The number of the project license for animal experiment in the present study is AE 007/2552.

### *Drugs and drug administration*

Animals were assigned to three groups (n = 9/group);

***1. Control group***

Animals orally received vehicle solution (distilled water) daily (08:00 h) for 14 days. They then received vehicle solution (0.9% NaCl) i.p. bi-daily (08:00 and 16:00 h) for 7 days.

***2. Sub-chronic PCP group***

Animals orally received vehicle solution (distilled water) daily (08:00 h) for 14 days. They then received 2 mg/kg of PCP (Sigma, USA) i.p. bi-daily (08:00 and 16:00 h) for 7 days. PCP was dissolved in 0.9% NaCl.

***3. Brahmi group + PCP***

Animals orally received 40 mg/kg/day of Brahmi (Planetary™ Herbals) daily (08:00 h) for 14 days. They then received 2 mg/kg of PCP (Sigma, USA) i.p. bi-daily (08:00 and 16:00 h) for 7 days. PCP and Brahmi were dissolved in 0.9% NaCl and distilled water, respectively.

### *Novel object recognition test*

Novel object recognition test was performed in all groups of animals a week after drugs or vehicle

administration. The test was proceeded in a room with 360 lux lighting. The materials consisted of a solid black plastic box (63 cm x 63 cm x 45 cm) which was placed on the floor throughout the experiment. A video recorder (Canon) was positioned on a movable trolley above the plastic box in order to record behavior. The objects to be discriminated were made of glass, plastic or ceramic. During the task, the bottoms of objects were fixed by adhesive tape in order not to be displaced by the animals. In the 3 days prior to the novel object recognition test, all rats were initially habituated to the empty box for three sessions of 3 min daily. In the novel object recognition test each rat was placed in the box and exposed for 3 min to two identical objects placed approximately 10 cm apart in the center of the box. The rat was then returned to its home cage for an hour. The box and the objects were cleaned with 70% ethanol. Both objects in the box were replaced, one with an identical object and another with a novel object. Rats were then returned to the novel object recognition box and allowed to explore the objects for 3 min. All trials were recorded and behavioral analysis was carried out blind to treatment. Object exploring included rat sniffing, licking and touching the objects. The data were expressed as the discrimination ratio (DR) calculated from the following equation; DR = [(time exploring novel object-time exploring familiar object)/ total exploration time)].

***NMDAR1 immunohistochemistry***

After novel object recognition test was undertaken, rats were sacrificed and whole brains were removed and fixed in 4% paraformaldehyde. All animal tissues were paraffin-embedded sections, which were sectioned coronally at a thickness of 5 μm then mounted onto 3-aminopropyltriethoxysilane (APES) coated glass slides. For the sectioning, levels with respect to Bregma were determined with the use of a rat brain stereotaxic atlas[16]. The sections for prefrontal cortex were taken between Bregma 2.7 to 2.2 mm while those for striatum were taken from Bregma 0.7 mm. Sections for hippocampus were sectioned from Bregma -3.3 mm. The sections were dewaxed in xylene then rehydrated in 100%, 90% and 70% ethanol and washed for 5 min in distilled water. The sections were immersed in phosphate buffer saline (PBS; 0.01 M phosphate buffer, 0.9% NaCl, pH 7.4) and heated in a microwave oven on full power (650 W) for three periods of 5 min in order to aid antigen retrieval. The sections were left at room temperature for 30 mins to cool down then incubated in a solution of 5% $H_2O_2$ in 10% methanol and 0.1% Triton X-100 in PBS, pH 7.4 to inhibit endogenous peroxidase activity and then washed for 3x5 min in PBS. Non-specific binding was minimized by incubation for 1 h in 5% normal goat serum in PBS, followed by incubation at 4°C overnight with a polyclonal antibody against the glutamate/NMDA receptor subunit 1 (NMDAR1) (Sigma, USA) at a dilution of 1:1,000 in blocking solution. The sections were washed for 3x5 min in PBS before incubation for 2 h at room temperature with biotinylated secondary antibody (anti-goat IgG) diluted 1:200 in protein blocking solution. Sections were then processed by the avidin-biotin method using a Vectastain ABC kit (Vector Laboratories, UK) and peroxidase was visualized using 3',3'-diaminobenzadine (DAB) intensified with nickel chloride. The sections were dehydrated and mounted. Negative control sections were processed as for NMDAR1 immunohistochemistry except that the primary antibody was omitted. No immunostaining could be detected under these conditions. All slides were coded and analyzed blind to treatment.

***Image analysis***

NMDAR1 immunostained neuronal densities were measured in the prefrontal cortex and cornu ammonis fields 1 (CA1) and 2/3 (CA2/3) using Image ProPlus software. NMDAR1 immunoreactivity in the dentate gyrus (DG) and striatum were quantified by integrated optical density (IOD) using Scion Image Software based on NIH image (v. beta 3b; www.scioncorp.com; 1998). The value of IOD is the sum of the optical densities of all pixels in the region divided by the number of pixels and it was quantified by subtracting the background of the section. For IOD analysis, five regions of interest were measured in striatum and DG of all sections. The average of the values from the five regions of interest in each brain subfield of each rat was used for statistical analysis. Neuronal density and IOD were measured blind to the animal groups.

***Statistical analysis***

The data are expressed as mean $\pm$ SEM. One-way ANOVA was performed to determine the effect of Brahmi on the discrimination ratio, followed by post hoc statistical comparison of treatment group. NMDAR1 neuronal densities and IOD of each brain region were analyzed using one-way ANOVA with post hoc comparison of treatment group. Independent t-test was used to compare the discrimination ratio and

NMDAR1 immunodensity between PCP and Brahmi + PCP groups. Statistical significances were defined as $p<0.05$. All statistical analysis was performed using SPSS V13 for windows (SPSS Inc., Chicago, USA).

## Results

### *Novel object recognition test*

One-way ANOVA with Dunnett post hoc tests revealed a significant reduction in DR score in sub-chronic administration of PCP ($p<0.001$) compared with control. Independent t-test revealed a significant increase in DR score in Brahmi + PCP group ($p<0.001$) compared with PCP alone (Fig. 1).

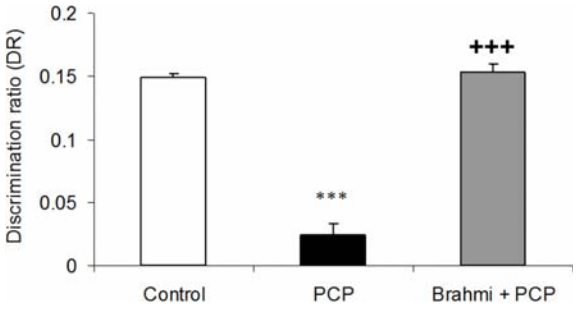


**Fig. 1** Discrimination ratio in control, PCP and Brahmi + PCP groups obtained from novel object recognition task. Data are mean ± SEM. ***$p<0.001$ vs. control, $^{+++}p<0.001$ vs. PCP.

### *NMDAR1 immunohistochemistry*

One-way ANOVA with Dunnett post hoc tests revealed no significant difference of NMDAR1 immunodensity in the prefrontal cortex (Fig. 2), CA1 (Fig. 3) and striatum (Fig. 5) between all groups of the experiment. However, NMDAR1 immunodensity was significantly increased above control in CA2/3 ($p<0.001$) (Fig. 4) and DG ($p<0.001$) (Fig. 5) in sub-chronic PCP administration group. Independent t-test showed a significant reduction of NMDAR1 immunodensity in CA2/3 in Brahmi + PCP group compared with PCP alone ($p<0.001$) and it was not significantly different from control (Fig. 4). NMDAR1 immunodensity in DG in Brahmi + PCP group was significantly decreased compared with PCP alone ($p<0.05$) but it was significantly increased above control ($p<0.001$) (Fig. 5).

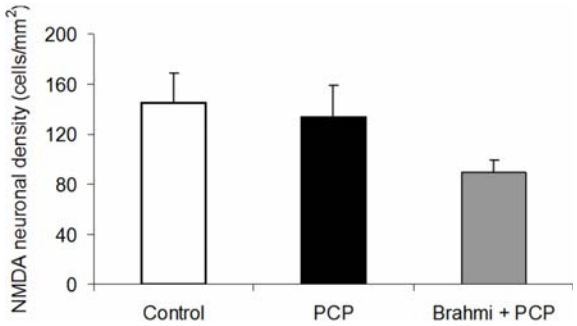


**Fig. 2** NMDAR1 immunodensity in the prefrontal cortex in control, PCP and Brahmi + PCP groups. Data are mean ± SEM.

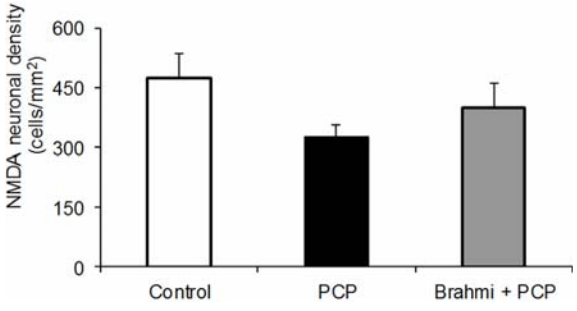


**Fig. 3** NMDAR1 immunodensity in the pyramidal layer of CA1 in control, PCP and Brahmi + PCP groups. Data are mean ± SEM.

## Discussion

The main finding of the present study was the deficits in novel object recognition (DR scores) in animals receiving sub-chronic PCP administration. This result is consistent with other studies in both male[17] and female rats[5] using other tasks such as reversal learning[18] and attentional set shifting[19]. A decrease in DR scores represents cognitive deficits. Therefore, sub-chronic PCP administration could be a valuable method to induce cognitive deficit in the schizophrenic rat model. The deficits in DR scores observed in rats receiving PCP occurred alongside increases in NMDAR1 immunodensity in CA2/3 and DG of the hippocampus. This NMDAR1 receptor up-regulation induced by PCP interpreted the production of apoptosis in CA2/3 and DG that thus produce cognitive deficits in sub-chronic PCP rat model of schizophrenia. However, the apoptosis is needed to be confirmed in the further study. The perforant path is the major input to the hippocampus including CA2/3 and DG. The axons of the perforant path arise principally in layers 2 and 3 of the entorhinal cortex, with minor contributions from the deeper layers 4 and 5. Axons from layers 2/4 project to the granule cells of the DG. Thus, the findings of the present study suggest NMDAR1 up-regulation in response to glutamatergic hypofunction in the perforant path of CA2/3 and DG in schizophrenia. These

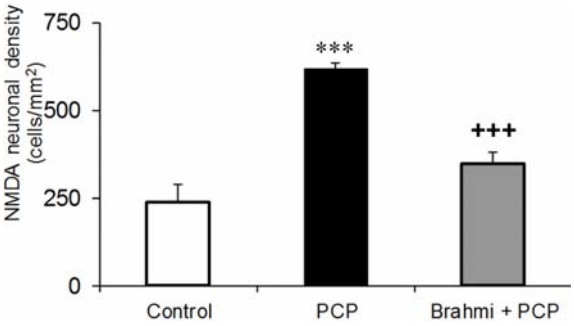


**Fig. 4** NMDAR1 immunodensity in the pyramidal layer of CA2/3 in control, PCP and Brahmi + PCP groups. Data are mean ± SEM. ***$p$<0.001 vs. control, $^{+++}p$<0.001 vs. PCP.

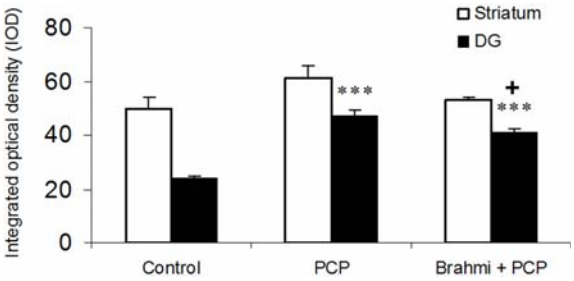


**Fig. 5** NMDAR1 immunodensity in the striatum and granule layer of DG in control, PCP and Brahmi + PCP groups. Data are mean ± SEM. ***$p$<0.001 vs. control, $^{+}p$<0.05 vs. PCP.

findings are consistent with the other animal studies that showed NMDA receptor up-regulation after PCP administration in the rat hippocampus[20]. Additionally, these results are also consistent with the studies in schizophrenic patients that investigated NMDA receptor up-regulation in post-mortem putamen and temporal cortex[8], which was interpreted as a receptor up-regulation in response to glutamatergic hypofunction in the disorder.

Receiving Brahmi before sub-chronic PCP administration, animals significantly improved object recognition memory. It showed no significant difference of DR score from control. Additionally, receiving Brahmi before sub-chronic PCP administration also revealed the reduction of NMDAR1 immunodensity in CA2/3 and DG. However, the reduction reached the normal level only in CA2/3 but not in DG. These results interpreted that receiving Brahmi before PCP administration can restore the cognitive deficit by decreasing NMDA density in CA2/3 and DG. The present study suggested that the neuroprotective effects of Brahmi might be due to down-regulation of NMDAR1 receptor in CA2/3 and DG. Consistent with these findings, other animal studies have shown that Brahmi can increase learning and memory task and also can prevent age-associated neurodegeneration[21,22]. Recent studies in human have suggested that Brahmi extract is a potential cognitive enhancer and neuroprotectant against Alzheimer's disease[23].

## Conclusion

While sub-chronic administration of PCP produces cognitive deficits in novel object recognition task and NMDAR1 up-regulation in CA2/3 and DG of hippocampus, administration of Brahmi provides neuroprotective effect against these behavioral deficit and NMDAR1 up-regulation. Therefore, Brahmi could be a valuable alternative medicine against cognitive impairment in the PCP administered rat model of schizophrenia and, to some extent, the patients with schizophrenia and other psychotic disorders. However, the investigation in the patients is needed to be confirmed in the further study.

## Acknowledgement

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## Potential conflicts of interest

None.

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# ฤทธิ์ป้องกันการเรียนรู้และความจำถดถอยของ Bacopa monnieri (พรมมิ) ต่อการแยกแยะวัตถุใหม่และต่อปริมาณของตัวรับชนิด NMDAR1 ใน prefrontal cortex, striatum และ hippocampus ของหนูที่ถูกเหนี่ยวนำให้เป็นโรคจิตเภทด้วย sub-chronic phencyclidine

ปริศนา ปียะพันธุ์, ธนิศรา เวชเตง

**ภูมิหลัง:** การเรียนรู้และความจำถดถอยเป็นปัญหาสำคัญซึ่งในที่สุดแล้วจะเกิดขึ้นกับผู้ป่วยโรคจิตเภททุกราย ส่งผลให้ผู้ป่วยสูญเสียประสิทธิภาพในการทำงานและการดำรงชีวิต การเรียนรู้และความจำถดถอยไม่สามารถบรรเทาได้โดยยารักษาโรคจิตเวช Bacopa monnieri (พรมมิ) เป็นสมุนไพรที่มีฤทธิ์ป้องกันความเสื่อมของสมองในผู้สูงอายุ อาจเป็นแนวทางใหม่ในการป้องกันการเกิดการเรียนรู้และความจำถดถอยในผู้ป่วยโรคจิตเภท

**วัตถุประสงค์:** เพื่อศึกษาฤทธิ์ป้องกันการเรียนรู้และความจำถดถอยของพรมมิต่อการแยกแยะวัตถุใหม่และต่อปริมาณของตัวรับชนิด NMDAR1 ในสมองของหนูที่ถูกเหนี่ยวนำให้เป็นโรคจิตเภทด้วย sub-chronic phencyclidine (PCP)

**วัสดุและวิธีการ:** หนูทดลองแบ่งเป็น 3 กลุ่ม; กลุ่ม A: กลุ่มควบคุม, กลุ่ม B: ได้รับ PCP, กลุ่ม C: พรมมิ + PCP ค่า discrimination ratio (DR) แสดงถึงความสามารถในการเรียนรู้และความจำได้มาจากการทดสอบการแยกแยะวัตถุใหม่ (novel object recognition) การวัดปริมาณของ NMDAR1 ใน prefrontal cortex, striatum, cornu ammonis fields 1 (CA1) และ 2/3 (CA2/3) ของ hippocampus และ dentate gyrus (DG) ใช้วิธี immunohistochemistry

**ผลการศึกษา:** DR ในหนูกลุ่มที่ได้รับ PCP มีค่าลดลงเมื่อเทียบกับหนูกลุ่มควบคุมการลดลงของ DR ในหนูที่ได้รับ PCP นี้เกิดขึ้นร่วมกับการเพิ่มขึ้นของปริมาณ NMDAR1 ใน CA2/3 และ DG แต่ไม่พบใน prefrontal cortex, striatum หรือ CA1 หนูกลุ่มพรมมิ + PCP มีการเพิ่มขึ้นของค่า DR โดยมีค่าเท่ากับหนูกลุ่มควบคุมซึ่งการเพิ่มขึ้นของค่า DR ในหนูกลุ่มนี้เกิดขึ้นร่วมกับการลดลงของปริมาณ NMDAR1 ในสมองส่วน CA2/3 และ DG เมื่อเปรียบเทียบกับหนูกลุ่มที่ได้รับ PCP อย่างเดียว

**สรุป:** การเรียนรู้และความจำถดถอยในหนูที่ได้รับ PCP เกิดขึ้นจากการเพิ่มขึ้นของปริมาณ NMDAR1 ใน CA2/3 และ DG เป็นที่น่าสนใจว่าการได้รับพรมมิก่อน PCP สามารถป้องกันการเรียนรู้และความจำถดถอยได้โดยการลดปริมาณของ NMDAR1 ใน CA2/3 และ DG ดังนั้นพรมมิน่าจะมีฤทธิ์ป้องกันการเสื่อมของสมองและการถดถอยของการเรียนรู้และความจำในโรคจิตเภท