**Bacopa monnieri** (Brahmi) Enhanced Cognitive Function and Prevented Cognitive Impairment by Increasing VGLUT2 Immunodensity in Prefrontal Cortex of Sub-Chronic Phencyclidine Rat Model of Schizophrenia

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**Background:** Glutamatergic hypofunction is affected in schizophrenia. The decrement of presynaptic glutamatergic marker, remarkably vesicular glutamate transporter type 1 (VGLUT1) indicates the deficit of glutamatergic and cognitive function in schizophrenic brain. However, there have been a few studies in VGLUT2. Brahmi, a traditional herbal medicine, might be a new frontier of cognitive deficit treatment and prevention in schizophrenia by changing cerebral VGLUT2 density.

**Objective:** To study cognitive enhancement- and neuroprotective-effects of Brahmi on novel object recognition task and cerebral VGLUT2 immunodensity in sub-chronic phencyclidine (PCP) rat model of schizophrenia.

**Material and Method:** Cognitive enhancement effect study; rats were assigned to three groups; Group-1: Control, Group-2: PCP administration and Group-3: PCP + Brahmi. Neuroprotective effect study; rats were assigned to three groups; Group-1: Control, Group-2: PCP administration and Group-3: Brahmi + PCP. Discrimination ratio (DR) representing cognitive ability was obtained from novel object recognition task. VGLUT2 immunodensity was measured in prefrontal cortex, striatum, cornu ammonis fields 1 (CA1) and 2/3 (CA2/3) of hippocampus using immunohistochemistry.

**Results:** DR was significantly reduced in PCP group compared with control. This occurred alongside VGLUT2 reduction in prefrontal cortex, but not in striatum, CA1 or CA2/3. Both PCP + Brahmi and Brahmi + PCP groups showed an increased DR score up to normal, which occurred alongside a significantly increased VGLUT2 immunodensity in the prefrontal cortex, compared with PCP group.

**Conclusion:** The decrement of VGLUT2 density in prefrontal cortex resulted in cognitive deficit in rats receiving PCP. Interestingly, receiving Brahmi after PCP administration can restore this cognitive deficit by increasing VGLUT2 density in prefrontal cortex. This investigation is defined as Brahmi’s cognitive enhancement effect. Additionally, receiving Brahmi before PCP administration can also prevent cognitive impairment by elevating VGLUT2 density in prefrontal cortex. This observation indicates neuroprotective effect of Brahmi. Therefore, Brahmi could be a new frontier of restoration and prevention of cognitive deficit in schizophrenia.

**Keywords:** Brahmi, Schizophrenia, Animal model, Novel object recognition, VGLUT2

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neuroprotectant due to its ability to prevent age-associated neurodegeneration in female Wistar rats\(^6\).

Schizophrenia is the most common psychotic illness found in approximately 1% of the world’s population. Lately, an incidence rate has been remarkably increasing. Schizophrenic patients express typical psychotic symptoms including positive symptom, negative symptom and cognitive dysfunction. Impairment of cognition is a significant problem leading to functional disability in schizophrenia. It eventually occurs in all patients. Neither typical nor atypical antipsychotics can prevent or ameliorate this impairment. To date, cognitive impairment is more likely to be the most critical problem of the patients.

There have been a few animal models of schizophrenia available such as rearing rats in isolation from weaning\(^7\) and administration of psychotomimetic drugs to animals\(^8\). The psychotomimetic administration has been widely accepted and utilized in this research area. Glutamatergic hypofunction has been implicated in the pathophysiology of schizophrenia. Therefore, phencyclidine (PCP), a non-competitive glutamate/N-methyl-D-aspartate (NMDA) receptor antagonist, can produce schizophrenia-like psychosis in normal subjects and aggravate psychotic symptoms in schizophrenic patients\(^9\).

Several studies have reported alterations in glutamatergic systems in schizophrenia as indicated by the abnormal expression of various synaptic markers including our previous studies, which identified NMDA up-regulation in prefrontal cortex and CA1-3\(^10\) and vesicular glutamate transporter I (VGLUT1) reduction in prefrontal cortex, striatum and CA1-3 in sub-chronic PCP rat model of schizophrenia\(^11\). VGLUT2 is one of three subtypes of VGLUT. It has been observed to share 82% amino acid homology with VGLUT1 and act as a VGLUT. VGLUT2 is predominantly expressed in the glutamatergic synaptic terminals in layer IV of the cerebral cortex, thalamus, hypothalamus and brain stem\(^12\). Therefore, VGLUT2 might be implicated in pathophysiology of schizophrenia and Brahmi could recover and prevent cognitive impairment in the disorder by alteration of cerebral VGLUT2 density.

The main aim of the present study was to assess whether the administration of Brahmi was able to ameliorate and prevent cognitive deficit (cognitive enhancement- and neuroprotective-effects, respectively) in sub-chronic PCP administered rats, assessed using the novel object recognition paradigm, and on the density of VGLUT2 in the prefrontal cortex, striatum and CA1 and CA2/3 of hippocampus.

Material and Method

Animals

Fifty-four adult male Wistar rats (200-220g; National Animal Center, Mahidol University, Thailand) were used in the present study. 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 experiment. All animal experiments were conducted in accordance with Mahidol University Code of Practice and the National Institutes of Health (USA) Guidelines for treatment of laboratory animals. The protocol for animal experiment in this study was approved by the Animal Research Committee of Thammasat University, Thailand. The project license number for animal experiment in the present study is AE 003/2012.

Drugs and drug administration

**Cognitive enhancement effect study**

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

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

2) Sub-chronic PCP group
   - Animals received 2 mg/kg of PCP i.p. bi-daily (08:00 and 16:00 h) for 7 days. They then orally received vehicle solution (distilled water) daily (08:00 h) for further 14 days.

3) PCP + Brahmi group
   - Animals received 2 mg/kg of PCP i.p. bi-daily (08:00 and 16:00 h) for 7 days. They then orally received vehicle solution (distilled water) daily (08:00 h) for further 14 days.

**Neuroprotective effect study**

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.

3) Brahmi + PCP group

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**

All animals receiving drugs or vehicles were acclimatized for one week before novel object recognition proceeded. The test was undertaken in a room with 360 lux lighting. The materials consisted of a solid black plastic box (63x63x45 cm) which was placed on the floor throughout the experiment. Animal behavior was recorded by a video recorder (Canon) which was positioned on a movable trolley above the plastic box. The objects to be discriminated were made of glass, plastic or ceramic and fixed by adhesive tape at the bottom in order to avoid displacing by the animals. For 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 was defined as 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)]. The data are expressed as mean ± SEM. One-way ANOVA was performed to determine the effect of treatment on DR value, followed by post hoc statistical comparison of treatment group. Independent t-test was used to compare DR value between PCP and PCP + Brahmi groups or 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).

After the novel object recognition test was undertaken, all rats were sacrificed and whole brains were removed. They then proceeded to immunohistochemistry.

**Analysis of VGLUT2 by immunohistochemistry**

After all brains were removed, they were fixed in 4% paraformaldehyde. All animal brain tissues were paraffin-embedded sections, which were sectioned coronally at a thickness of 5 μm then mounted onto 3-amino propyl triethoxysilane (APES) coated glass slides. For the sectioning, levels with respect to Bregma were determined with the use of a rat brain stereotaxic atlas. 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 posterior to Bregma 3.3 mm. All 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 antigen retrieval solution (1 mM EDTA in 0.1 M Tris-HCl, pH 8.0) and heated in a microwave oven on full power (850 W) for 3x5 min. The sections were left at room temperature for 30 min to cool down before washed in Tris-HCl buffer for 2x5 min, then incubated with endogenous peroxidase blocking solution (5% H2O2 in absolute methanol) for 30 min. The sections were washed in Tris-HCl buffer for 2x5 min before incubation for 45 min with protein blocking solution (2% normal goat serum in Tris-HCl buffer), followed by incubation at 4°C overnight with polyclonal antibody against VGLUT2, raised in guinea pig (Chemicon International Inc, Temecula, CA) at a dilution of 1:5,000 in protein blocking solution. The sections were washed for 2x5 min in Tris-HCl buffer before incubation for 1 hour with biotinylated secondary antibody (anti-guinea pig IgG) (Vector Laboratories, Burlingame, CA) at a dilution 1:200 then processed by using avidin-biotin-peroxidase complex (VECTASTAIN® Elite ABC-Peroxidase Kit) (Vector Laboratories, Burlingame, CA). The sections were washed for 2x5 min. Protein immunoreactivity was visualized by using the chromogen diaminobenzidine, intensified with nickel chloride (DAB) (Vector Laboratories, Burlingame, CA). The sections were dehydrated in 70%, 90%, 100% ethanol and xylene then cover slipped with DPX mounting medium for microscopy. Negative control sections processed as for VGLUT2 immunohistochemistry except that the primary antibody was omitted. No immunostaining could be detected under these conditions. All slides...
were analyzed blind to diagnosis. After staining for VGLUT2, the sections were scanned by Olympus microscope. VGLUT2 optical density of the regions of interest was measured using Scion Image Software based on NIH image (v. beta 3b; www.scioncorp.com; 1998). VGLUT2 optical density was made blind to the diagnostic category of the cases. Five regions of interest were measured in each of the subfields of prefrontal cortex, striatum and CA1 and CA2/3 of hippocampus of all sections. The value measured is the sum of the optical densities of all pixels in the region divided by the number of pixels. The average of values from five regions of interest in each brain subfield of each subject was used for statistical analysis. VGLUT2 optical density of each brain region was analyzed using one-way ANOVA with post hoc comparison of treatment group. Independent t-test was used to compare VGLUT2 optical density between PCP and PCP + Brahmi groups or between PCP and Brahmi + PCP groups.

Results

Cognitive enhancement effect study

Novel object recognition test

One-way ANOVA with Dunnett post hoc tests showed a significant decrease in DR score in sub-chronic administration of PCP \((p<0.001)\) and PCP + Brahmi \((p<0.01)\) compared with control. Independent t-test revealed a significant increase in DR score in PCP + Brahmi \((p<0.001)\) compared with PCP alone (Fig. 1).

VGLUT2 immunohistochemistry

Fig. 2 shows an expression of VGLUT2 immunoreactivity in hippocampus of control group. It demonstrates that VGLUT2 protein is present in glutamatergic terminals but not in cell bodies. Additionally, this protein is expressed in glutamatergic terminals of the prefrontal cortex but not in cell bodies (Fig. 3A) and in the matrix of striatum but not in striosomes (Fig. 3B).

One-way ANOVA with Dunnett post hoc tests revealed a significant decrease of VGLUT2 immunodensity in prefrontal cortex of sub-chronic PCP administration group compared with control \((p<0.01)\), however, it was not significantly different between PCP + Brahmi group and control. Independent t-test showed a significant increase of VGLUT2 immunodensity in prefrontal cortex of PCP + Brahmi group compared with PCP alone \((p<0.01)\) (Fig. 4). There was no significant difference of VGLUT2 immunodensity between any of the groups in striatum (Fig. 5), CA1 and CA2/3 (Fig. 6).

Neuroprotective effect study

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. DR was not significantly different between Brahmi + PCP and control. Independent t-test revealed a significant increase in DR score in Brahmi + PCP group \((p<0.001)\) compared with PCP alone (Fig. 7).

VGLUT2 immunohistochemistry

One-way ANOVA with Dunnett post hoc tests revealed a significant decrease of VGLUT2 immunodensity in prefrontal cortex of sub-chronic PCP administration group compared with control \((p<0.001)\); however, it was not significantly different between Brahmi + PCP group and control. Independent t-test showed a significant increase in VGLUT2 immunodensity in the prefrontal cortex of Brahmi + PCP group compared with PCP alone \((p<0.001)\) (Fig. 8). There was no significant difference in VGLUT2
immunodensity between all the groups in striatum (Fig. 9), CA1 and CA2/3 (Fig. 10).

According to the results of VGLUT2 optical density (Fig. 4-6 and 8-10), Fig. 11 summarizes the main findings of the present study. It shows that VGLUT2 immunodensity is reduced in the prefrontal cortex of PCP administered rats (Fig. 11B). Receiving Brahmi...
either after or before PCP administering can increase VGLUT2 immunodensity in this brain area up to normal (Fig. 11C and 11D).

**Discussion**

Reduced DR score represents cognitive impairment, which occurred alongside the deficit of VGLUT2 immunoreactivity in the prefrontal cortex in sub-chronic PCP administered rats. No significant differences of VGLUT2 immunoreactivity were found in striatum and hippocampus. These observations suggest glutamatergic synaptic pathology, which may reflect a loss of glutamatergic terminals in the prefrontal cortex in sub-chronic PCP administered rats. It has been reported that behavioral phenotyping of mice gene-targeted for either VGLUT1 or VGLUT2 play a role in behavior of putative relevance for psychiatric conditions. Mice heterozygous for the VGLUT1 allele expressed a depressive-like phenotype as well as anxiety- and schizophrenia-related behaviors\(^{13,14}\). Moreover, decrease in VGLUT2 during embryonic development led to neonatal lethality due to respiratory failure\(^{15,16}\). Additionally, decrease in VGLUT2 reduced problem-solving capacity in the mutant mice\(^{17}\) and also produced impairment in long-term retention of spatial memory (Morris water maze)\(^{18}\). Therefore, reduced VGLUT2 immunodensity in prefrontal cortex of PCP administrated rats observed in the present study led to cognitive impairment, which is related to pathophysiology of cognitive dysfunction in schizophrenia.

It has been reported that the prefrontal cortex is related to cognitive impairment in schizophrenia. The prefrontal cortex plays an important role in learning and memory. Additionally, it connects to the posterior areas of the brain and leads to normal perceptions in humans. There is evidence that patients with schizophrenia have a disconnection between the prefrontal and posterior areas, which could explain their characteristic misperceptions\(^{19}\). Therefore, reduced VGLUT2 immunodensity in the prefrontal cortex of PCP administered rats observed in the present study could be one explanation of misperceptions and to some extent, cognitive impairment in schizophrenia.

In the present study, the cognitive deficit found in novel object recognition task is attenuated to normal by administering Brahmi either after or before PCP receiving. These results were interpreted as cognitive enhancement- and neuroprotective-effects of Brahmi in schizophrenia-like psychosis. This recovery of cognitive deficit is together with an increase in VGLUT2 immunodensity in the prefrontal cortex. This interpreted that Brahmi can increase VGLUT2 density in the prefrontal cortex then raise glutamatergic neurotransmission at the presynaptic terminal in that the cognitive function is improved. Consistent with these findings, other animal studies have shown that
Brahmi could increase learning and memory task and also prevented age-associated neurodegeneration\(^{(20,21)}\). Recent studies in humans have suggested that Brahmi extract is a potential cognitive enhancer and neuroprotectant against Alzheimer’s disease\(^{(22)}\).

**Conclusion**

While sub-chronic administration of PCP produces cognitive deficits in novel object recognition task and VGLUT2 reduction in the prefrontal cortex, administering of Brahmi provides cognitive enhancement- and neuroprotective- effects against these behavioural deficit and VGLUT2 reduction. Therefore, Brahmi could be a valuable alternative medicine that attenuates and prevents 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.

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**Potential conflicts of interest**

None.

**References**


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Bacopa monnieri (พรหมณี) กระทุ่มการเรียนรู้ความจำและป้องกันความบาดเจ็บของการเรียนรู้ความจำโดยการเพิ่มปริมาณ VGLUT2 ใน prefrontal cortex ของหนูป่าลูกเห็บแนวให้เป็นโรคจิตหลอกวัย sub-chronic phencyclidine

ปรีดมาน สิทธิพันธ์, อิทธิกร เวชกลาง

คู่มือนี้: การทดลองของกลุ่มเกิดขึ้นในโรงจิตเวช การทดลองของตัวต่างๆ ได้แก่กลุ่มควบคุมโดยเฉพาะ vesicular glutamate transporter 1 (VGLUT1) ซึ่งเป็นส่วนหนึ่งของการเก็บข้อมูลและทำให้เกิดความทรงพลังของการเรียนรู้/ความจำในโรคจิตเวช อย่างไรก็ตามการศึกษาในมนุษย์ใน VGLUT2 พบว่ามีการเปลี่ยนแปลงที่หลากหลายในการรักษาและป้องกันความบาดเจ็บของการเรียนรู้/ความจำที่เกิดขึ้นในโรคจิตเวช โดยการเปลี่ยนแปลงปริมาณของ VGLUT2 ในสมอง

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ระดับการเรียนรู้ความจำและป้องกันความบาดเจ็บของการเรียนรู้ความจำของพรหมณีในการยกและรักษาโรคจิตเวช (novel object recognition) และดื่มน้ำมันของ VGLUT2 ในสมองของหนูป่าลูกเห็บแนวให้เป็นโรคจิตหลอกวัย sub-chronic phencyclidine (PCP)

วัสดุและวิธีการ: การศึกษานี้มีการแบ่งการเรียนรู้ความจำ ทำกับหนูป่าลูกเห็บแบบ 3 กลุ่ม: กลุ่ม-1: กลุ่มควบคุม, กลุ่ม-2: ได้รับ PCP และ กลุ่ม-3: PCP + พรหมณี การศึกษาฤทธิ์ระดับการเรียนรู้ความจำ; ทำกับหนูป่าลูกเห็บแบบ 3 กลุ่ม: กลุ่ม-1: กลุ่มควบคุม, กลุ่ม-2: ได้รับ PCP และ กลุ่ม-3: พรหมณี + PCP การวัดการเรียนรู้ความจำ (dr) แสดงความสามารถในการรวมความจำข้ามหน้าจากการทดสอบการแยกและวัดวิถีในอาการเรียนรู้ความจำของหนูป่าลูกเห็บแนว

การทดลองของ VGLUT2 ใน prefrontal cortex, striatum, cornu ammonis fields 1 (CA1) และ 2/3 (CA2/3) ของ hippocampus ใช้วิธี immunohistochemistry

ผลการศึกษา: DR ในกลุ่มที่ได้รับ PCP และกลุ่มที่ได้รับพรหมณี มีการแตกต่างกันกับไวภัยแล้วกลุ่มควบคุม การทดสอบของปริมาณ VGLUT2 ใน prefrontal cortex ได้ในหนูใน striatum, CA1 หรือ CA2/3 พบกลุ่ม PCP + Brahmi และ Brahmi + PCP มีการเพิ่มขึ้นของ DR โดยมีการแตกต่างกับกลุ่มควบคุม ซึ่งการเพิ่มขึ้นของ DR ในกลุ่มที่ได้รับพรหมณีและกลุ่มควบคุมกลับ การเพิ่มขึ้นของปริมาณ VGLUT2 ในสมองส่วน prefrontal cortex นั่นเองเป็นเหตุให้กลุ่มควบคุม(PCP) องค์เสีย

สรุป: การทดลองของ VGLUT2 ใน prefrontal cortex ทำให้การเรียนรู้ความจำแสดงในหนูป่าลูกเห็บได้รับ PCP เป็นต้นส่งผลต่อการเรียนรู้ความจำเข้าซึ่งเป็น PCP สามารถกระตุ้นการเรียนรู้ความจำอย่างชัดเจนได้โดยการเพิ่มปริมาณ VGLUT2 ใน prefrontal cortex ผลการศึกษาจะเรียกว่าการกระตุ้นการเรียนรู้ความจำของพรหมณี นอกเหนือจากทางการศึกษาระหว่างเพรียกเพิ่มปริมาณได้รับ PCP สามารถช่วยป้องกันความบาดเจ็บของการเรียนรู้/ความจำได้อย่างชัดเจน VGLUT2 ใน prefrontal cortex เช่นกัน ผลการศึกษาจะเรียกว่าลูกเห็บป้องกันความบาดเจ็บของการเรียนรู้/ความจำได้โดยเพิ่ม VGLUT2 ใน prefrontal cortex ดังนั้นพรหมณีเป็นแนวทางที่สามารถรักษาและป้องกันความบาดเจ็บของการเรียนรู้/ความจำในโรคจิตเวชได้