Pueraria Mirifica, Phytoestrogen-Induced Change in Synaptophysin Expression Via Estrogen Receptor in Rat Hippocampal Neuron

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Objective: *To examine Pueraria mirifica (Leguminosae) containing-phytoestrogen effect on synaptic density and involvement of estrogen receptor.*
Material and Method: *The level of synaptophysin, a presynaptic vesicle protein, was measured using Western blot analysis and immunocytochemistry in hippocampal primary cell cultures at 6 days in vitro.*
Results: *P. mirifica and 17β-estradiol (0.1 μM) treatment for 4 days, but not for 2 days, significantly increased synaptophysin immunoreactivity and level of synaptophysin. P. mirifica up to 60 μg/ml resulted in a dose related increase in the level of synaptophysin immunoreactivity. The classical estrogen receptor antagonist, ICI 182 780, significantly blocked P. mirifica-induced increase in synaptophysin.*
Conclusion: *P. mirifica-containing phytoestrogen affects synaptic density by inducing synaptophysin expression via estrogen receptor.*

Keywords: *Phytoestrogens, Pueraria mirifica, Hippocampal neuron, 17β-estradiol, Synaptophysin, Synaptic density*

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Hippocampal neurons, which carry both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), are a very useful model for investigating estrogenic effect on the central nervous system[1]. Dissociated cultures of hippocampal neurons, which have functional synapses[2] and relative sparseness of dendritic spines[3], offer a viable and convenient means for studying factors that regulate the development and function of dendritic spines or synapse formation[3,4]. A number of studies have suggested that synaptophysin, a 38-kDa calcium-binding glycoprotein, participates in synaptic vesicle formation, trafficking, release and recycling of vesicles containing neurotransmitters[5,6]. An increase in synaptophysin immunoreactivity has most often been interpreted as an increase in presynaptic terminals reflecting increase in synapse formation[7].

Estrogens have been reported to have cognitive enhancing and neuroprotective effects. They have been known to increase spine formation, synaptogenesis and synaptic proteins in hippocampal CA1 pyamidal neurons that mediate learning and memory[8]. The effects of estrogen replacement on memory and synaptophysin expression in aged post-estropausal female rodents suggest that it might be related to memory and synaptic plasticity as shown by increasing in synaptophysin expression[9].

Phytoestrogens, plant-derived estrogen-like substances, have been extensively studied for their potential beneficial effects against hormone-dependent and age-related disease[10-12]. However, limited data exists regarding the effects of phytoestrogens on the brain. *Pueraria mirifica (P. mirifica)*, is a Thai indigenous herb that belongs to the Family *Leguminosae*

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with a long history of domestic consumption as a rejuvenating herb to promote youthfulness in both women and men[13]. Active phytoestrogenic ingredients found in its roots are isoflavones (daidzin, daidzein, genistin, genistein, and puerarin), and miroestrol and its derivatives including β-sitosterol, stigmasterol and coumestrol[14,15]. *P. mirifica* has estrogenic effects on ovarian function and the menstrual cycle in rats[16] and monkeys[17].

In order to examine whether *P. mirifica*-containing phytoestrogens affect synapse formation, the authors examined the role of *P. mirifica* on synaptophysin expression in primary hippocampal neuron cultures. The involvement of estrogen receptors with *P. mirifica* effects was also investigated.

Material and Method

Animals

Nineteen-old pregnant Spraque-Dawley rats were obtained from the National Laboratory Animal Center, Thailand. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Primary cultures of prenatal hippocampal neurons

Primary cultures of prenatal hippocampal neurons were prepared from 19-day old Sprague-Dawley rats. Dissection of hippocampi, which were removed from anesthesized embryos, was done in dissociation media (0.08 M Na_2SO_4, 0.03 M K_2SO_4, 6 mM $MgCl_2$, 0.25 mM $CaCl_2$, 1 mM HEPES pH 7.4, 0.02 M glucose, phenol red). containing 1mM kynurenate, 10 mM $MgCl_2$ and 0.35 M NaOH. Hippocampi were digested with 1 mg/ml papain (Sigma, St. Louis, MO). Mechanical trituration was performed through a 5 ml pipette in L17 GM solution (0.2% $NaHCO_3$, 6.5% fetal calf serum, 35 mM glucose, 0.5 mM L-glutamine and 100 unit/ml pen/strep, 0.1 mM putrescine, 0.02M B 27 (GIBCO, Carlsbad, CA), 1X ITS (Sigma, St. Louis, MO), pH 7.4). About 10^5 suspended cells/ml were plated onto 12 mm glass coverslips precoated with 2 µg/ml of poly-L-lysine (Sigma, St. Louis, MO) and 2 µg/ml of laminin (GIBCO, Carlsbad, CA) in 35 mm dishes for immunocytochemistry or at about 10^6 cells/ml in 60 mm dishes for immunoblot. Cells were cultured at 37°C in 5% (v/v) CO_2/95% air atmosphere. During the first day of culture, 2.4 µM cytosine arabinoside (Sigma, St. Louis, MO) were added to the cultures to prevent glia proliferation. Medium was changed to fresh L17 GM supplemented with B-27 after plating for two and a half hours[18].

Preparation of P. mirifica

Tuberous roots of *P. mirifica* were collected in Chiang Mai province, Thailand. Plant materials were washed, dried, sliced into pieces, and ground to powder. Two g of plant powder were subsequently extracted with 250 ml of ethyl acetate for 4 h in a reflux apparatus. The eluted solution was evaporated to dryness with a rotary evaporator. Thirty mg of the dry brownish herb powder were dissolved in 1 ml of absolute ethanol overnight. The solution was filtered to remove undissolved powder and passed through a 0.22 µm Millipore filter. The sterile filtrate was kept at -20°C before use.

Cell dosing

Experimental solutions were prepared on the day of dosing by adding 17β-estradiol (Sigma, St. Louis, MO) and *P. mirifica* in growth media to give a final concentrations of 0.1 µM and 30 µg/ml respectively on the second and fourth day of culture. Cultured cells (treated and untreated) were collected on the 6th day *in vitro* and were then processed for immunocytochemistry or immunoblot. For treatment with the estrogen antagonist, 1 µM ICI 182 780 (Tocris, Bristol, England) was added prior to adding *P. mirifica*.

Immunocytochemistry

The 6-day *in vitro* treated and untreated hippocampal neurons on cover slips were washed with 0.01 M phosphate buffer saline (PBS) once, then fixed with 100% methanol at -20° C for 10-15 min. Autofluorescence of cells was quenched twice, 5 min each time, with 10 mM glycine in PBS and permeabilized with 0.5% NP40 in PBS for 5 min. After treating with blocking solution (10% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.5% Triton X-100 in PBS) for 1 h, cells were incubated for 1 h at room temperature with rabbit primary anti-human synaptophysin antibody (Zymed, South San Francisco) diluted 1:250 in primary antibody buffer (3% NGS, 1% BSA, 0.5% Triton X-100, 0.05% sodium azide in PBS). After washing to remove excess antibodies with PBS, cells were incubated at room temperature with secondary goat anti-rabbit IgG TRITC-conjugated antibody (Zymed, South San Francisco, CA) diluted 1:500 in secondary antibody buffer (3% NGS, 1% BSA, 0.5% Triton X-100 in PBS) for 1 h. Cells were washed three times with PBS, mounted with vectashield mounting media and examined under light and fluorescence microscopy with a 40x objective lens and using laser at excitation of 553 nm.

Western blot

Treated and untreated neurons were washed with PBS three times then lysed in 100 □l of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% DOC, 0.1% SDS, 5 mM EDTA, 30 mM Na_2HPO_4, 50 mM NaF, 0.5 mM $NaVO_4$, 2 mM PMSF, 1% aprotinin and 1% leupeptin). per 60 mm dish. Insoluble materials were removed by high-speed centrifuge at 12,000xg at 4°C for 10 min. The supernatants were carefully placed into clean tubes, and protein concentrations were determined by the Bradford protein assay. Equal amounts of protein (10 µg per lane) in the loading buffer were denatured at 95°C for 5 min and separated by 12.5% SDS-PAGE. Proteins in the gels were transferred to a nitrocellulose membrane, washed with TBST (1 M Tris pH 7.4, 3 M NaCl, 0.1% Tween 20) and incubated with 5% skim milk in TBST. Membranes were stored at 4°C overnight and then were incubated with primary antisynaptophysin antibody (1:1000 in TBST). After washing three times with TBST, the membranes were incubated with a horseradish peroxide-conjugated secondary antibody (1:5000 in TBST) (Zymed, South San Francisco, CA) and treated using a LumiGLO Chemiluminescent Substrate kit (KPL, Gaithersburg, MD).

Quantification

The number of radiant dots, shown in Fig. 1A, was used as a measure of the level of synaptophysin immunoreactivity. The number of dots was randomly counted per 50 □m of neuronal process. Manual counting was performed by a person unaware of the treatment protocol. Five to ten processes were counted per image. At least 3 cover slips per group were processed, at least 3 images were taken for each cover

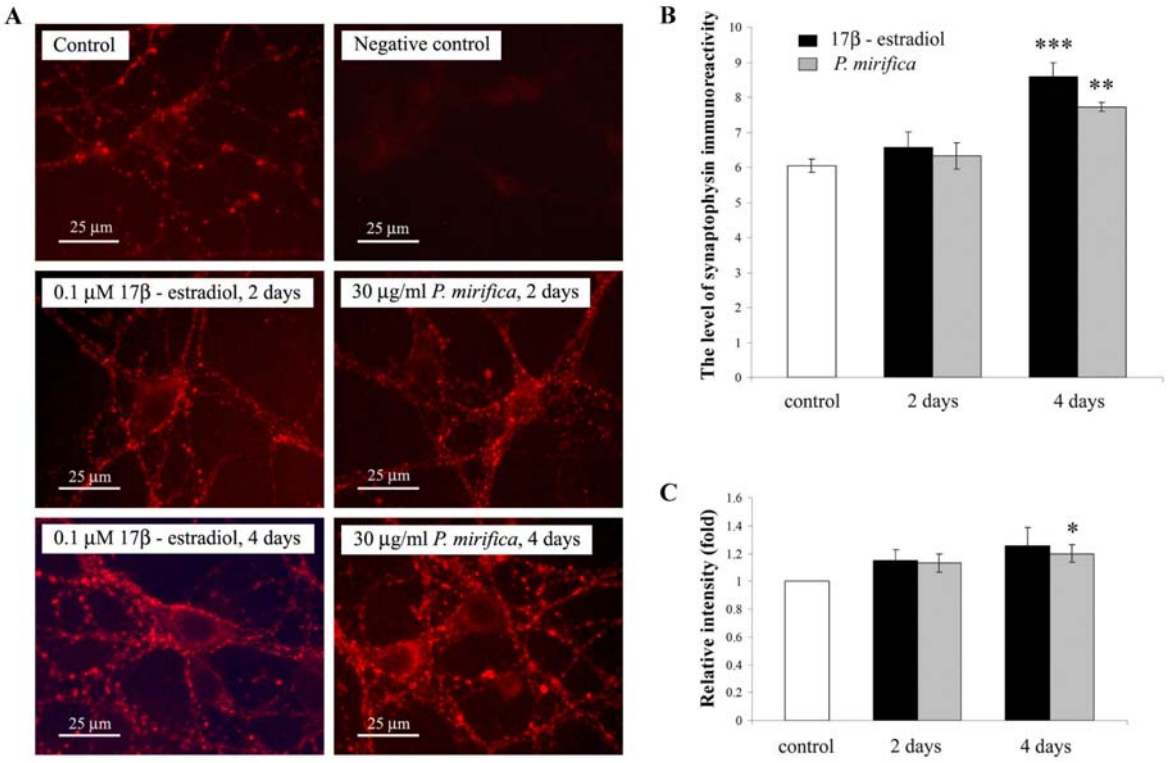

Fig. 1 Effect of *P. mirifica* on synaptophysin expression in rat hippocampal neurons
(A) Immunocytochemical detection of synaptophysin in 6-day cultured hippocampal neuron treated with 0.1 µM 17β-estradiol and 30 µg/ml *P. mirifica* for 2 and 4 days
(B) Level of synaptophysin immunoreactivity per 50 µm neuronal process after treatment of 0.1 µM 17β-estradiol and 30 µg/ml *P. mirifica* for 2 and 4 days
(C) Densitometric analysis of synaptophysin Western blot of relative intensity for individual experiments was calculated compared to control and averaged
Immunochemical data represent mean ± S.E.M. of 5 independent experiments
*** $p < 0.001$ vs. control, ** $p < 0.01$ vs. control, * $p < 0.05$ vs. control

slip and 3 independent experiments were performed. Western blot was scanned using Scion imaging software and the relative intensity (compared to control) of each independent experiment was evaluated. Data are presented as the mean ± S.E.M of 5 independent experiments (Western blotting was performed twice in each experiment).

Statistical analysis

All parameters were analyzed using mean ± standard error of mean (S.E.M.) and student t-test analysis and P value < 0.05 was considered significant.

Results

Effect of *P. mirifica* extract on synaptophysin expression, synaptophysin immunoreactivity in untreated neurons was prominent at puncta whereas they were located mostly along the process in *P. mirifica* and 17β-estradiol treated groups (Fig. 1A). Administration of *P. mirifica* (30 µg/ml) for 4 days significantly increased the level of synaptophysin immunoreactivity along the process in 6-day cultured rat hippocampal neurons ($p < 0.01$ vs control) (Fig. 1B). A similar effect was evident in 17β-estradiol (0.1 µM) treated group ($p < 0.001$). The quantification of synaptophysin immunoreactivity may not represent the actual protein expression of synaptophysin.

Immunoreactive synaptophysin protein levels quantified by Western blotting indicated that *P. mirifica* (30 µg/ml) induced a significant increase in synaptophysin levels after 4 days ($p < 0.05$) but not 2 days of treatment (Fig.1C). However treatment with 17β-estradiol (0.1 µM) for 2 and 4 days failed to demonstrate an increase in synaptophysin expression by Western blotting analysis.

The dose-dependency effect of *P. mirifica* on synaptophysin level was tested at 4 doses (3, 15, 30, and 60 µg/ml) and a significant stimulatory effect of *P. mirifica* on synaptophysin expression was observed at doses of 30 and 60 µg/ml ($p < 0.05$ and $p < 0.01$ respectively) (Fig. 2). Time-dependent effect of *P. mirifica* (30 µg/ml) was evaluated only on the 2nd and 4th day of treatment and synaptophysin was significantly increased on the 4th day after administration ($p < 0.05$) (Fig. 1C, 2, 3).

P. mirifica- induced synaptophysin activation through estrogen receptor

The simultaneous application of the highly specific nuclear estrogen receptor antagonist ICI 182 780 completely prevented the induction effect of *P. mirifica* (30 µg/ml) treatment for 4 days (Fig. 3). ICI 182 780 alone had no influence on synaptophysin level.

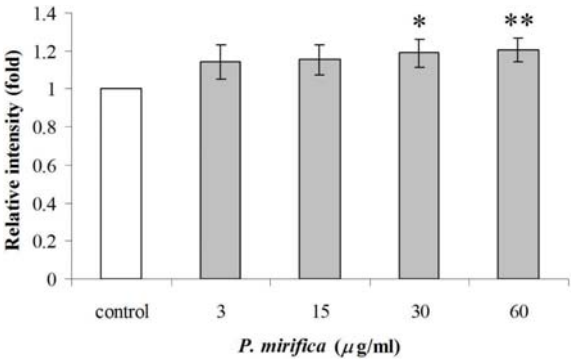

Fig. 2 Dose-dependent effect of *P. mirifica* on synaptophysin protein level in 6-day cultured rat hippocampal neurons
Densitometric analysis of synaptophysin Western blots were performed after treatment with 3, 15, 30 and 60 µg/ml of *P. mirifica* for 4 days
Data represent the mean ± S.E.M. of 5 independent experiments
** $p < 0.01$ vs. control, * $p < 0.05$ vs. control

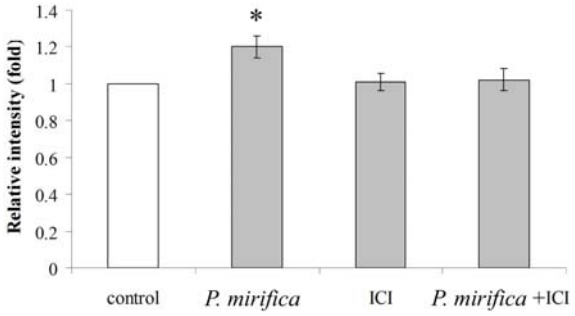

Fig. 3 Effect of ICI 182 780 on the *P. mirifica* induced synaptophysin expression in 6-day cultured rat hippocampal neurons
Densitometric analysis of synaptophysin Western blots were performed after treatment with *P. mirifica* alone (30 µg/ml) (*P. mirifica*), ICI 182 780 alone (1 µM) (ICI) and *P. mirifica* plus ICI 182 780 (*P. mirifica* + ICI) for 4 days
Data represent the mean ± S.E.M. of 5 independent experiments
* $p < 0.05$ vs. control

Discussion

This is the first report that *P. mirifica* has a significant effect on synaptophysin expression. The fact that *P. mirifica* induced a change in synaptophysin

expression could reflect its effect on synaptogenesis or synapse formation. Synaptophysin was reported to be one of the earliest synaptic proteins to accumulate at developing synapses in culture and its expression is high during synaptogenesis[2]. The expression of synaptophysin is compatible with its role in regulating synapse formation in cultured hippocampal neurons[19]. Also, loss of synaptic proteins, such as synaptophysin and syntaxin, in the frontal neocortex provides the best neurobiological correlates of cognitive impairment[20]. The effect of *P. mirifica* on synaptophysin expression could be interpreted that it has an indirect effect on the increase in synaptic density associated with neuronal plasticity.

Earlier studies have shown that the administration of estrogen to ovariectomized rats augments protein synthesizing machinery in neurons of the hypothalamic ventromedial nucleus[21]. Estradiol induces rise in spine density and increases dendritic branching in cultured rat hippocampal neurons[22]. Phytoestrogen also induces an increase in sexually dimorphic nuclei of preoptic area in male rats[23] and increases aggressive behavior including altered hormonal changes in the brains of male hamsters[24]. Genistein, a component of soy products, modulates morphological change of gonadotropin-releasing hormone neurons in ovarectomized ewes[25], increases the rate of neurite elongation in PC12 cells and produces a concentration-dependent effect on the growth of MCF-7 cells via estrogen receptor[26]. At a concentration of 50 nM genistein significantly reduces neuronal apoptosis in an estrogen receptor-dependent manner but at a concentration greater than 50 μM genistein is toxic in primary neurons[27]. The authors found no significant amount of neuronal death induced by *P. mirifica* (up to 60 μg/ml). Possible explanations are that the concentration of genistein in *P. mirifica* is lower than the apoptotic dose used in the other studies and *P. mirifica* has no apoptotic effect on hippocampal neurons.

Time course effects of estradiol (0.1 μg/ml) on spine density production after 4 days of treatment involving alteration of presynaptic and post-synaptic markers were reported[22]. This is consistent with our results that the level of synaptophysin after 4 days of treatment with 30 μg/ml of *P. mirifica* was significantly higher than the level after 2 days of treatment. The results are also in agreement with a recent study demonstrating the ability of estrogen to increase synaptophysin in the CA1 region of the hippocampus in female rhesus macaques (Macaca mulatta)[8].

The nuclear estrogen receptor antagonist, ICI 182 780, inhibited *P. mirifica*-induced increase in synaptophysin expression in rat hippocampal neurons. ICI 182 780 binds nuclear ER, alters receptor conformation and facilitates binding of co-regulatory proteins that could activate or repress transcriptional activity of estrogen target genes[28]. It has been reported that phytoestrogen exhibits greater affinity to ERβ relative to ERα[29]. The present studies are consistent with evidence showing that estrogen-induced increase in dendritic spine density in cultured rat hippocampal neuron is inhibited by ICI 182 780[28].

In summary, phytoestrogen-containing *P. mirifica* induced synaptophysin expression in dose- and time-dependent manner in cultured rat hippocampal neurons. Antagonism by ICI 182 780 suggests that this phenomenon may be mediated through estrogen receptor.

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ไฟโตเอสโตรเจนมีผลต่อการแสดงออกของโปรตีนไซแนปโตไฟซินผ่านทางเอสโตรเจนรีเซ็ปเตอร์ในเซลล์ประสาทฮิปโปแคมปัสของหนูแรท

รัตติยา จินเดหวา, สายธิดา ลาภอนันตสิน, ยุพิน สังวรินทะ, สุขุมาล จงธรรมคุณ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของกวาวเครือขาวซึ่งประกอบด้วยไฟโตเอสโตรเจนหลายชนิดต่อการเปลี่ยนแปลงของไซแนปโตไฟซิน ซึ่งเป็นโปรตีนที่บ่งชี้ถึงการเปลี่ยนแปลงที่ปลายประสาทขาออก และไซแนปซ์ในเซลล์ประสาทฮิปโปแคมปัสของหนูแรทในจานเพาะเลี้ยง ด้วยวิธีการวิเคราะห์โปรตีนและอิมมูโนไซโตเค็มมิสตรี ผลพบว่า การให้กวาวเครือขาว 30 ไมโครกรัม/มิลลิลิตรและเอสโตรเจน 0.1 ไมโครโมล เป็นเวลา 4 วัน กระตุ้นเซลล์ให้มีการเพิ่มปริมาณโปรตีนไซแนปโตไฟซินได้มากกว่า 2 วันอย่างมีนัยสำคัญ และกวาวเครือขาวที่ความ เข้มข้น 30 และ 60 ไมโครกรัม/มิลลิลิตร กระตุ้นการสร้างโปรตีนนี้ได้มากกว่าที่ความเข้มข้น 15 และ 3 ไมโครกรัม/มิลลิลิตร อย่างมีนัยสำคัญ เมื่อให้สาร ICI 182 780 ซึ่งเป็นสารยับยั้งการทำงานของเอสโตรเจนรีเซ็ปเตอร์ ก่อนกระตุ้นเซลล์ด้วยกวาวเครือขาว ผลไม่พบการเพิ่มของโปรตีนไซแนปโตไฟซิน สรุป กวาวเครือขาวในปริมาณที่เหมาะสมมีผลต่อการเปลี่ยนแปลงของไซแนปซ์ กระตุ้นให้มีการเพิ่มการสร้างโปรตีนไซแนปโตไฟซิน ผ่านทาง เอสโตรเจนรีเซ็ปเตอร์