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Radix *Puerariae* modulates glutamatergic synaptic architecture and potentiates functional synaptic plasticity in primary hippocampal neurons

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ABSTRACT

Ethnopharmacological relevance: Neurologic disorders are frequently characterized by synaptic pathology, including abnormal density and morphology of dendritic spines, synapse loss, and aberrant synaptic signaling and plasticity. Therefore, to promote and/or protect synapses by the use of natural molecules capable of modulating neurodevelopmental events, such as, spinogenesis and synaptic plasticity, could offer a preventive and curative strategy for nervous disorders associated with synaptic pathology. Radix *Puerariae*, the root of *Pueraria monatana* var. *lobata* (Willd.) Sanjappa & Pradeep, is a Chinese ethnomedicine, traditionally used for the treatment of memory-related nervous disorders including Alzheimer's disease. In the previous study, we showed that the ethanolic extracts of Radix *Puerariae* (RPE) and its prime constituent, puerarin induced neuritogenesis and synapse formation in cultured hippocampal neurons, and thus could improve memory functions.

Aims of the study: In the present study, we specifically investigated the abilities of RPE and puerarin to improve memory-related brain disorders through modulating synaptic maturation and functional potentiation.

Materials and methods: Rat embryonic (E19) brain neurons were cultured in the absence or presence of RPE or puerarin. At predetermined times, cells were live-stained with DiO or fixed and immunostained to visualize neuronal morphologies, or lysed for protein harvesting. Morphometric analyses of dendritic spines and synaptogenesis were performed using Image J software. Functional pre- and postsynaptic plasticity was measured by FM1-43 staining and whole-cell patch clamping, respectively. RPE or puerarin-mediated changes in actin-related protein 2 were assessed by Western blotting. Neuronal survivals were measured using propidium iodide exclusion assay.

Results: RPE and puerarin both: (1) promoted a significant increase in the numbers, and maturation, of dendritic spines; (2) modulated the formation of glutamatergic synapses; (3) potentiated synaptic transmission by increasing the sizes of reserve vesicle pools at presynaptic terminals; (4) enhanced NMDA receptor-mediated postsynaptic currents, and (5) increased cell viability against naturally occurring cell death. Moreover, upregulation of actin-related protein 2 (ARP2) in RPE and puerarin treated brain neurons suggest that RPE and puerarin induced synaptic plasticity might be associated, at least in part, with ARP2-mediated actin-dependent regulation of spinogenesis.

Conclusions: Our findings indicate that RPE and puerarin might play a substantial role in the morphological and functional maturation of brain neurons and suggest that RPE and puerarin are potentially valuable preventative therapeutics for memory-related nervous disorders.

Abbreviations: AP-5, (2R)-amino-5-phosphonovaleric acid; ARP2, actin-related protein 2; BDNF, brain-derived neurotrophic factor; DiO, 3,3-dioctadecyloxacarbocyanine perchlorate; DIV, day in vitro; DMSO, dimethyl sulfoxide; D-PBS, Dulbecco's phosphate buffered saline; FM1-43, N-(3-triethylammoniumpropyl)–4-(4-(dibutylamino)styryl) pyridinium dibromide; GluN2A and GluN2B, 2A and 2B subunits of the NMDAR; NMDAR, *N*-acetyl-*D*-glutamate receptor; PSD-95, postsynaptic density protein 95; RPE, Radix *Puerariae*; SNP, synaptophysisn; TBS, Tris-buffered saline; TTBS, Tween-20 in TBS

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1. Introduction

The brain regulates and/or coordinates our cognitive behavior and motor functions through effective communication between neurons, occurring at specialized junctions called synapses. Precise control of synaptic development and functionality is crucial for maintaining appropriate neuronal network activities and normal brain functions (Van Spronsen and Hoogenraad, 2010). It is therefore not surprising that a dysfunction or loss of synapses may lead to the disruption of neuronal circuits and to brain diseases. Synaptic malfunction is evident to be the major underlying cause of many psychiatric and neurological disorders, such as mental retardation (Pfeiffer et al., 2009), schizophrenia (Stephan et al., 2006), Parkinson's disease (Calabresi et al., 2006) and Alzheimer's disease (Dickson and Vickers, 2001; Selkoe, 2002). Indeed, synapse loss is the best current pathologic correlate of cognitive decline in Alzheimer's disease patients (Van Spronsen and Hoogenraad, 2010). Current pharmacological therapies are effective in managing symptoms but fail to halt or reverse disease syndromes. Therefore, the use of natural or synthetic molecules with neurotrophic potentials has emerged as an alternative therapeutic strategy for restoring and maintaining neuronal function during neurodegenerative disorders. Thus, researchers continue to search for natural agents with potential neurodevelopmental effects that could be used to develop new drug candidates for the effective treatment of neurodegenerative diseases.

Radix *Puerariae*, the root of *Pueraria monatana* var. *lobata* (Willd.) Sanjappa & Pradeep, has been used in traditional Chinese medicine for its clinical efficacy including neurological disorders (Xiao et al., 2016). For example, Gegen Qinlian decoction, which includes Radix *Puerariae* (Yege or Gegen) as a main constituent, has been used to treat cerebral infarction (Xu and Wu, 2009), and another widely used Radix *Puerariae* derived traditional Chinese medicine, Gegen decoction, has been successfully used clinically for the treatment of cervicogenic and functional headache, cervical vertigo, diabetic peripheral neuropathy and temporomandibular joint dysfunction syndrome (reviewed in Xiao et al. (2016)). Moreover, puerarin, the major active components of flavones in Radix *Puerariae*, is proven to be effective in cardiovascular protection and neuroprotection in Parkinson's and Alzheimer's diseases (Xiao et al., 2016).

We previously demonstrated that ethanolic extracts of traditional medicine Radix *Puerariae* (RPE) and its major active constituent, puerarin exerted neurotrophic supports through inducing neuritogenesis and synaptic protein expression along dendrites of rat primary hippocampal neurons (Bhuiyan et al., 2015b). In addition to morphological development, neurotrophic factors promote the maturation and stabilization of cellular and molecular components, and thus, enhance functional synapses (Gasparini et al., 2000; Renger et al., 2001). Moreover, neurotrophins also regulate molecular events necessary for efficient signal transmission at the postsynaptic membrane. For instance, brain-derived neurotrophic factor (BDNF) increases the phosphorylation of GluN2A and GluN2B subunits of the *N*-acetyl-*D*-glutamate receptor (NMDAR) (Lin et al., 1998), which may increase the open probability of the ion channel (Levine et al., 1998); BDNF also selectively enhances synaptically evoked NMDA receptor-mediated currents (Kolb et al., 2005). Therefore, exogenous factors capable of promoting synapse formation and functional maturation could have therapeutic significance in halting or even reverting synaptic neuropathy and thereby may ameliorate the pathogenesis of cognitive disorders associated with aging or Alzheimer's disease.

In the present study, we further investigated into the abilities of the ethanolic extracts of Radix *Puerariae* (RPE) and puerarin in the modulation of glutamatergic synapse formation/maturation, and functional plasticity both at the pre- and postsynaptic ends of cultured hippocampal neurons.

2. Materials and methods

2.1. Chemicals

All chemicals, reagents, and media used in this experiment were purchased from Invitrogen (Carlsbad, CA), unless otherwise stated.

2.2. Extracts preparation and standardization

Roots of Pueraria were collected at Mt. Yukbecksan in Samcheok, Kangwon-do, Republic of Korea, and authenticated by Dr. Chang-Ho Han at the Department of Internal Medicine, College of Korean Medicine, Dongguk University, Gyeongju, Republic of Korea. The RPE, which was prepared by extraction of Radix *Puerariae* with 95% (v/v) ethanol, filtration, freeze-drying and finally dissolving in dimethyl sulfoxide (DMSO), was standardized for puerarin content as described previously (Bhuiyan et al., 2015b). Voucher specimens (RP-VS-02) are deposited in the laboratory of Dr. I. S. Moon (Dongguk University, Graduate School of Medicine, Gyeongju, Republic of Korea).

2.3. Primary neuronal culture and extract treatment

Timed-pregnant Sprague-Dawley rats were purchased on gestation day 13, and reared in a temperature controlled environment under a 12 h light/dark cycle with ad libitum access to food and water. Animal experimental procedures were approved by the institutional animal care and use committee of the College of Medicine, Dongguk University (Gyeongju, Korea). Primary cultures of hippocampal neurons were performed as previously described (Bhuiyan et al., 2015b; Sharif et al., 2016). For harvesting proteins, cortical cells were seeded at 5.0×10^5 cells/cm^2 on 6-well culture plates coated with poly-DL-lysine. RPE (1 µg/ml), puerarin (5 µM), or vehicle [DMSO, < 0.2% (v/v)] was added to plating media prior to cell seeding. RPE or puerarin concentration was optimized based on their neuritogenic effects in our previous experiments (Bhuiyan et al., 2015b). To induce spinogenesis, neurons were first seeded in plating medium and 10% fetal bovine serum and incubated for 4 h, when the plating media was replaced completely with growth medium containing RPE or puerarin or vehicle. At least, three replicates were maintained for each concentration of the test substances in individual experiments.

2.4. Labeling neurons with DiO

DiO (3,3-dioctadecyloxacarbocyanine perchlorate) is a lipophilic dye that facilitates the visualization of entire neurons, including dendritic filopodia and spines by binding to the plasma membrane of neuronal cells. To investigate the effects of RPE or puerarin on dendritic filopodial growth and eventually spine maturation, neurons grown at 200 cells/mm^2 on coverslips in 24-well plates, were live-stained with Vybrant DiO (Molecular Probes, Inc., Eugene, OR), according to the manufacturer's instruction.

2.5. Immunocytochemistry

At the indicated time, cultured hippocampal neurons were fixed by treating them with a paraformaldehyde/methanol series, as previously described (Moon et al., 2007). Fixed neurons were then double-immunostained with primary antibodies, that is, synaptophysin (SNP, mouse MAb, 1:500, Sigma), GluN2A (rabbit polyclonal, 1:200, Moon, 2003; Moon et al., 1994), actin-related protein 2 (ARP2, rabbit polyclonal, 1:1000, Abcam, Eugene, OR) and postsynaptic density protein 95 (PSD-95, chicken polyclonal UCT-C1, 1:500, a kind gift from Dr. R S Walikonis, University of Connecticut, CT). After overnight incubation at 4 °C with primary antibodies, neurons were incubated with secondary antibodies for 1 h at room temperature and then mounted on slides, as previously described (Moon et al., 2007).

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