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Doctoral Dissertation

五味子醇與大豆甙元對於促進神經細胞胞突生長的作用與其機轉之探討

Study on the Effect and Mechanism of Schisandrin and Daidzein for Neurite Outgrowth in Primarily Cultured Neuronal Cells

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國立臺灣大學博士學位論文
口試委員會審定書

五味子醇與大豆甙元對於促進神經細胞胞突生長的作用與其機轉之探討

Study on the effect and signal transduction pathways of schisandrin and daidzein for neurite outgrowth in primarily cultured rat neuronal cells

本論文係楊士弘（D91446003）在國立臺灣大學醫學院解剖學暨細胞生物學研究所完成之博士學位論文，於民國100年1月26日承下列考試委員審查通過與口試及格，特此證明。

口試委員：＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿（簽名）

（指導教授）

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誌　謝

時值建國百年，普天同慶。對我而言，更多了個值得高興的理由：終於從研究所畢業，拿到博士學位了！

一路走來，真的要謝謝許多人的幫忙。最要感謝的，就是我的指導教授，王淑美老師。從準備投考研究所開始，包括計畫的擬定、研究方向的規劃、實驗結果的分析、論文的撰寫等，給予我許多的建議與指導，讓我能披荊斬棘，完成研究所的學程，順利畢業。而所上的老師們，包括吳建春老師、謝松蒼老師、呂俊宏老師、陳玉怜老師、錢宗良老師、盧國賢老師等，在實驗方法、實驗技術、學術討論上給予的幫助，亦讓我受益良多。而陽明大學的鄭瓊娟與王懷詩老師，在博士資格考試與博士學位考試擔任口試委員，給予許多寶貴的意見，也非常的謝謝她們。

另外，也要向這幾年來一同在實驗室打拼的學長與工作夥伴們致意。陳建行學長與怡珍是培養神經細胞的啓蒙老師，他們熱心的傳授實驗的細節與方法，至今仍令我銘記在心。王霈與鍾敦輝兩位學長也讓我分享了實驗的祕訣與技巧。永佳的跑膠技術令人讚歎，同時也感謝慷慨的她多次適時的將實驗動物與我互通有無。陳瀅是實驗室的智多星，實驗操作上的許多問題找她總能迎刃而解，同時也是歡樂星，有她在之處總是笑聲不斷、鮮有冷場。實驗室的助理子齊、佩靜、枳榛，在實驗操作上給予許多的協助，讓研究能夠順利的進行。所裡的張瓊容小姐與邵文昌先生，分別在行政手續與解剖課程上的諸多幫忙，也要一併致謝。

最後要與母親與妻子分享這份榮耀。父親雖在兩年多前瘁逝，但相信他若知道，一定也會十分欣慰。希望獨子秉錕也向老爸來看齊，未來能夠百尺竿頭，出人頭地。

摘　要

神經細胞依賴神經軸突（axon）與樹突（dendrite）來達成彼此間的聯繫。神經系統的疾病，包括中風、外傷等，常會造成軸突與樹突的傷害與萎縮，導致運動、感覺、語言等神經功能障礙。如何能夠促進神經胞突的生長，一直是基礎與臨床神經醫學的重要課題。我們的研究係以體外培養的大鼠神經元細胞為實驗對象，尋找能夠促進神經胞突生長的分子，與探討其訊息傳遞機轉。研究的第一部份，使用中藥草常用的五味子（*Schisandra chinensis*）所含的一個主要成分：五味子醇（schisandrin），觀察其對背根神經節神經元細胞（dorsal root ganglion neuron, DRG neurons）與海馬迴神經元細胞（hippocampal neurons）的效果。我們發現五味子醇作用24小時後，能夠促進 DRG neurons 的胞突延長伸展與分枝，且在濃度為 3 μg/ml 時的效果最好。而 3 μg/ml 的五味子醇對於大型或小型的 DRG neurons 皆有增加胞突生長的效果。因此進一步分析五味子醇促進 DRG neurons 胞突增生的機轉，在加入五味子醇前使用 KT5720（PKA 抑制劑）、PD98059（MEK 抑制劑）、或 LY294002（PI3K抑制劑）等激酶抑制劑，發現僅有 LY294002 能夠抑制胞突增生。而以西方點墨法分析激酶活化情形，發現五味子醇作用 15 分鐘後能促進 PI3K 與 Akt 的磷酸化；LY294002 預先處理則會抑制磷酸化 Akt 激酶的增加。五味子醇也增加 CREB 蛋白的磷酸化，且同樣會受到 LY294002 預先處理的抑制。綜合以上的結果，我們推論五味子醇係透過 PI3K-Akt-CREB 的訊息傳遞路徑來促進 DRG neuron 的胞突增生。另一方面，我們亦發現五味子醇能夠促進 hippocampal neurons 的胞突伸展與分枝。進一步分析五味子醇促進 hippocampal neurons 胞突增生的機轉，在加入五味子醇前使用 KN93（CaMKII 抑制劑）、

εV1-2（PKCε抑制劑）或 PD98059（MEK 抑制劑）均能夠抑制神經胞突增生。使用 Fluo-3 AM 染色，顯示加入 五味子醇十分鐘後會使細胞內鈣離子濃度上升。以西方點墨法分析，發現五味子醇作用 15 分鐘後能促進 CaMKII, PKCε, MEK 與 CREB 蛋白的磷酸化（活化）；KN93 會抑制因五味子醇引起的 PKCε 激酶活化、εV1-2 會抑制 MEK 激酶的活化、PD98059 則會抑制磷酸化 CREB 蛋白的增加。綜合以上的結果，我們推論五味子醇會使鈣離子進入 hippocampal neuron，然後啓動 CaMKII-PKCε-MEK-CREB 的訊息傳遞路徑來促進神經胞突增生。另外，五味子醇也會使海馬迴神經細胞的 post-synaptic protein 95 (PSD-95) 表達增加，與促進細胞攝入 FM1-43，顯示五味子醇亦具有促進海馬迴神經細胞突觸增生（synaptogenesis）的效果。

研究的第二部份，針對大豆異黃酮（isoflavone）的一個主要成分：大豆甙元（daidzein），分析它對於背根神經節神經元細胞（dorsal root ganglion neuron, DRG neurons）的效果。我們發現大豆甙元作用24小時後，能夠促進 DRG neurons 的胞突延長伸展與分枝，且在濃度為 30 μM 時的效果最好。而 30 μM 的大豆甙元對於大型或小型的 DRG neurons 皆有增加胞突生長的效果。進一步分析大豆甙元促進 DRG neurons 胞突增生的機轉，在加入大豆甙元前使用 ICI 182780（estrogen receptor α/β 拮抗劑）或 G15（membrane estrogen receptor GPR-30 拮抗劑），皆無抑制胞突增生的效果，顯示大豆甙元雖為植物雌激素的一種，但並非透過雌激素接受器使 DRG neuron 的胞突增生。而以 PP2（Src 抑制劑）、staurosporin（泛 PKC 抑制劑）、rottlerin（PKCδ 抑制劑）、U0126（MEK 抑制劑）等激酶抑制劑預先處理，則能夠抑制大豆甙元引起的胞突增生。而以西方點墨法分析激酶活化

情形，發現大豆甙元作用後能促進 Src, PKCδ, ERK 的磷酸化（活化）；PP2 會抑制因大豆甙元引起 PKCδ 激酶的磷酸化，rottlerin 則會抑制磷酸化 ERK 激酶的增加。歸納以上的結果，我們推論大豆甙元係透過 Src-PKCδ-MEK/ERK 的訊息傳遞路徑來促進 DRG neuron 的胞突增生。

總結我們的研究結果，顯示五味子醇與大豆甙元均有促進神經細胞胞突生長的作用，並透過不同的訊息路徑來完成反應。而五味子醇對於背根神經元與海馬神經元細胞皆能促進胞突增生，但亦經由不同的訊息傳遞方式來達成。

關鍵字：五味子醇、大豆甙元、背根神經節神經細胞、海馬迴神經細胞、神經胞突增生、神經突觸增生、訊息傳遞

Abstract

Neurons in different location require axons and dendrites to communicate with each other. Neurological diseases, e.g. stroke, head trauma, and spinal cord injury, often damage axons and dendrites and result in deficits of motor, sensory and language functions. How to promote growth of neuronal processes (neurites) is a major issue in the field of basic and clinical neuroscience. Our researches utilized in vitro cultured rat neurons to search for potential neuritogenic molecules and its signaling mechanisms. The first part of the research focused on schisandrin, a major ingredient of the Chinese herb *Schisandra chinensis*, and studied its effect on dorsal root ganglion (DRG) and hippocampal neurons. After treatment with schisandrin for 24 hours, DRG neurons showed increased lengthening and branching of neurites, and maximal effect was seen at a concentration of 3 μg/ml. Both large and small DRG neurons responded to schisandrin. To study the signaling pathway, KT5720 (PKA inhibitor), PD98059 (MEK inhibitor), or LY294002 (PI3K inhibitor) were applied before schisandrin treatment in DRG neurons, and only LY294002 blocked the neuritogenic effect of schisandrin. Western blot analysis showed that schisandrin enhanced phosphorylation of PI3K and Akt, which were blocked by pretreatment of LY294002. Schisandrin also increased phosphorylation of CREB, which was also inhibited by pretreatment of LY294002. Therefore, schisandrin activated PI3K-Akt-CREB pathway to enhance neurite outgrowth. Besides neuritogenic effect on DRG neurons, schisandrin also increased neurite length and branching complexity in hippocampal neurons. This effect was reversed by pretreatment with KN93 (CaMKII inhibitor), εV1-2 (PKCε inhibitor), or PD98059 (MEK inhibitor). Schisandrin also induced calcium inflow into hippocampal neurons in 10 minutes.

Western blot showed that schisandrin activated CaMKII, PKCε, MEK and CREB, and the activation of schisandrin-induced PKCε, MEK, and CREB were blocked by pretreatment with KN93, εV1-2, and PD98059, respectively. The result indicated schisandrin caused calcium inflow and activated CaMKII-PKCε-MEK-CREB pathway to increase neuritogenesis. In addition, schisandrin increased expression of post-synaptic protein 95 and uptake of FM1-43, suggesting its role in synaptogenesis of hippocampal neurons.

The second part of this study focused on daidzein, a major component of isoflavone. Following treatment of daidzein for 24 hours, both small and large DRG neurons demonstrated increased lengthening and branching of neurites, and maximal effect occurred at 30 μM. Despite structural similarity of daidzein to estrogen, ICI 182780 (estrogen α/β receptor inhibitor) and G15 (membrane estrogen receptor GPR-30) failed to inhibit the neuritogenic effect of daidzein, indicating that daidzein did not stimulate neurite outgrowth via the estrogen receptors. On the other hand, PP2 (Src inhibitor), staurosporin (pan-PKC inhibitor), rottlerin (PKCδ inhibitor), and U0126 (MEK inhibitor) pretreatment abolished the neuritogenic response of DRG neurons to daidzein. Increased phosphorylation of Src, PKCδ, and MEK occurred after daidzein treatment, and activation of the latter two kinases were blocked by PP2 and rottlerin respectively. Therefore, daidzein activated Src-PKCδ-ERK pathway, which led to neuritogenesis in DRG neurons.

In conclusion, the results of our research demonstrate that both schisandrin and daidzein have neuritogenic effect, and act by different signaling mechanisms. Schisandrin

enhanced neurite outgrowth of both DRG and hippocampal neurons, also via different pathways of signal transduction.

Keywords: schisandrin, daidzein, dorsal root ganglion neuron, hippocampal neuron, neurite outgrowth, synaptogenesis, signal transduction

目　錄

圖 目 錄

Chapter 1. Review of literature

The nervous system is the most sophisticated organ in the human body. It consists of 100 billion neurons that control our locomotion, sensorium, speech, thinking, etc. To properly execute these tasks requires communication and coordination of neurons in different locations via neuritic processes, i.e. axons and dendrites. These nerve fibers are often damaged in various neurological disorders, e.g. stroke, head injury, spinal cord injury, and demyelinating disorder, which could result in loss of neurological function and poor quality of life. For example, following injury of the axon, the distal segment degenerates and the proximal segment retracts first then tries to regrow toward the target. However, the regenerative process is not often successful due to the long distance and hostile environment to travel through. Therefore, how to improve neural regeneration remains a major issue in the field of basic and clinical neuroscience.

Using modern methodology of cell biology, biochemistry, and molecular biology, neuroscientists have discovered strategies to enhance neural regeneration. For example, growth factors (nerve growth factor, brain derived neurotrophic factor, neurotrophin-3) are capable of stimulating neurite outgrowth (Bradbury et al., 1999, Gao et al., 2003, Sjogreen et al., 2000). By blocking of inhibitory molecules in the CNS myelin and scar, e.g. Nogo, myelin associated glycoprotein, oligodendrocyte-myelin glycoprotein, chondroitin sulfate proteoglycan, the regenerating nerve fiber is able to extend through the scar tissue and hostile CNS environment (He and Koprivica, 2004, Laabs et al., 2005, Spencer et al., 2008, Wang et al., 2002). Transplantation of peripheral nerve, Schwann cells, olfactory ensheathing cell, and stem cells also shows great promise in promoting neural regeneration (Runyan and Phelps, 2009, Li et al., 2009, Thompson

and Buettner, 2006, Spector et al., 2000). However, beneficial effects of these agents are yet to be seen clinically, as some did not reproduce the favorable response in human subject or had unexpected side effects, while others are still under investigation. The goal of our study is to search for potential neurotrophic agents, and its underlying signaling mechanism.

Schisandrin is a major ingredient of the traditional medicinal Chinese herb, *Schisandra chinensis*, which have diverse biological functions in brain, heart, liver, and many other vital organs. For the nervous system, it is postulated to enhance intelligence and elevate spirit. Active ingredients of *Schisandra chinensis*, e.g. schisandrin, schisandrin A, schisandrin B, schisandrin C, Gomisin A, etc, share a common structure of dibenzocyclooctadiene, and are well documented to protect neurons and brain tissue against neurotoxins like tert-butyl hydroperoxide, gluatamate, and A-β amyloid protein (Cheng et al., 2008, Ko and Lam, 2002, Wang and Wang, 2009). Besides neuroprotective effects of these molecules, several studies suggested their potential roles in promoting neural regeneration and memory function. Shengmai-san, a herbal prescription containing schisandrin, facilitated neurite elongation of cultured dorsal root ganglion sensory neurons, and increase regeneration of rat corticospinal tract after spinal cord injury (Seo et al., 2009). *Schisandra chinensis* containing herb and schisandrin improved memory impairment caused by β-adrenergic blocker and cycloheximide in rat (Egashira et al., 2008, Kang et al., 2005b). Therefore, whether schisandrin could enhance neurite outgrowth deserves further investigation. In order to analyze the signaling pathway, the first part of our study utilized in vitro culture of dorsal root ganglion and hippocampal neurons, of which neuritic processes are major

components of the sensory tracts in spinal cord and neuronal circuits in memory system respectively, to study the effect and mechanism of schisandrin on neurite outgrowth.

Daidzein is one of the isoflavone products that are present in a variety of herb plants as well as food products like soybeans. Isoflavones are powerful antioxidant, and also able to interact with estrogen receptors to modulate endocrine, cardiovascular, musculoskeletal, and neurological functions. Regarding its action on the nervous system, daidzein and other isoflavones could improve stroke outcome, decreased anxiety, and increased memory and learning ability (Burguete et al., 2006, Thorp et al., 2009, Zeng et al., 2010). At the cellular level, daidzein was shown to have both neuroprotective and neurotrophic properties. In cultured hippocampal neurons, it provided protection against hypoxia induced apoptosis, and stimulated neuritogenesis (Schreihofer, 2005, Wang et al., 2008). These favorable results stem from both antioxidant mechanism and estrogen receptor pathway. However, toxic effect of daidzein on neuronal cells has also been reported, presumably at high concentrations (Jin et al., 2007). Thus, the controversial response of neuronal cells to daidzein treatment needs further verification. A recent publication defines the optimal concentration of daidzein to achieve maximal effect on neurite outgrowth in different neuronal populations, including the dorsal root ganglion and hippocampal neurons (Ma et al., 2010). Previously our lab reported that daidzein increased neurite outgrowth in hippocampal neurons through the estrogen receptor β-PKCα-GAP43 pathway (Wang et al., 2008). In the second part of our study we aimed to examine the effect of daidzein on DRG neurons and the underlying signaling transduction pathway.

Chapter 2. Study on the effect and mechanism of schisandrin for neurite outgrowth in primarily cultured rat dorsal root ganglion neurons

INTRODUCTION

Schisandra chinensis, also known as the "five flavor berry", is a widely used herbal medicine in the East countries. It is best known as a hepatoprotective agent (Pao et al., 1977). More than 30 molecules have been extracted from *Schisandra chinensis* (Ikeya et al., 1979), and found to have a wide variety of biological functions, e.g. anti-inflammatory, anti-tumoral, anti-viral, and anti-oxidant properties. In recent years, researchers have begun to explore the effect of these compounds on the nervous system. In rodents, memory impairment induced by scopolamine can be reversed by schisandrin, one of the lignan of *Schisandra chinensis* (Egashira et al., 2008). Schisandrin can also protect cultured rat cortical neurons against the neurotoxic effects of glutamate (Cheng et al., 2008).

The mechanism of the neuroprotective effects of schisandrin is still unclear. Shengmai-san, a Chinese herbal prescription containing *Schisandra chinensis*, was shown to enhance axonal regrowth in a rat spinal cord injury model (Seo et al., 2009). Thus, the potential beneficial effect of schisandrin may lies on its modulation of neurite outgrowth. Neurite outgrowth is a fundamental step in the establishment of synaptic connections and the formation of neural networks during development and following injury. The quest for potential neurotrophic agents and the elucidation of the underlying

signaling pathways have important implications for the potential facilitation of neural regeneration (Davies, 2000, Yiu and He, 2006).

DRG cultures provide a useful model system to study the mechanisms that regulate neuritogenesis. For example, neurotrophic factors have been shown to stimulate neurite outgrowth of cultured DRG neurons via signaling through the Raf-MEK-ERK, cAMP-PKA, and PI3K-Akt pathways (Gao et al., 2003, Markus et al., 2002a). Studies on DRG neurons have shed light on the regeneration of the spinal cord and peripheral nerves after injuries and other diseases (Neumann and Woolf, 1999). In this report, we show that schisandrin is a neuritogenic agent in dissociated DRG cultures. We further explore the signaling mechanism of schisandrin-induced neuritogenesis and demonstrate that schisandrin induces neurite outgrowth through activation of PI3K-Akt pathway. However, we measured the regeneration of corticospinal tract following rat spinal cord injury by schisandrin treatment, which did not increase in vivo neurite regeneration.

MATERIALS and METHODS

Cell culture

Postnatal day 2 (P2) Wistar rat pups were purchased from the Facility for Animal Research of the National Taiwan University. Animal care and procedures were performed according to the standards set out in the "Guide for the Care and the Use of Laboratory Animals", published by the U.S. National Institutes of Health (NIH publication no 85-23, revised 1985). P2 rat pups were placed on ice and decapitated to harvest the DRG, which were dissected out under a microscope and dissociated by incubation for 30 min at 37 °C with 0.25% trypsin (Gibco, Grand Island, NY, USA) and 0.05% collagenase (Sigma-Aldrich, St. Louis, MO, USA) in HBSS (Sigma-Aldrich) and mechanical trituration with a glass pipette. The pellet obtained by low-speed centrifugation was resuspended in L-15 Leibovitz medium (Gibco) supplemented with 1.2 g/L of $NaHCO_3$, 5% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), and 100 IU/ml of penicillin (Gibco) and streptomycin (Gibco). Cells were plated on coverslips for immunocytochemistry and 35 mm culture dishes for protein quantification by Western blotting, at a density of 100 cells/mm^2 and 400 cells/mm^2 respectively. The cell cultures were maintained at 37 °C in an atmosphere of 95% air and 5% CO2.

Drug treatment

Schisandrin (Wako Pure Chemical Industries Ltd., Osaka, Japan) was dissolved in DMSO. On day 3 in vitro (DIV 3), cultured DRG cells were treated with schisandrin (Fig 1A) at various concentrations from 0.3 μg/ml to 10 μg/ml, NGF (R&D Systems, Minneapolis, MN, USA) at 100 ng/ml, or an equal volume of vehicle solution DMSO (1

μl), and assessed for neurite outgrowth. For assays using inhibitors, cultured DRG cells were treated with the MEK inhibitor PD98059 (Biomol Research Laboratories, Plymouth meeting, PA, USA) at a concentration of 50 μM, the PKA inhibitor KT5720 (Biomol) at 5 μM, or the PI3K inhibitor LY294002 (Biomol) at 10 μM for 60 min before, and during, incubation with schisandrin.

Immunocytochemistry

After schisandrin or DMSO treatment for 24 h, DRG neurons on coverslips were fixed for 10 min in 10% formalin in PBS. After three PBS washes, the cells were permeabilized and blocked for 1 h at room temperature with blocking buffer 1 (0.15% Triton X-100 and 5% non-fat milk in PBS). The neurons were then incubated overnight at 4 °C with primary antibody [mouse anti-NF-L antibody (Sigma-Aldrich), 1:100 dilution in blocking buffer 1]. After three PBS washes, the cells were incubated for 1 h at room temperature with biotin-conjugated goat anti-mouse IgG antibodies (Vector lab, Burlingame, CA, USA; 1:50 dilution in blocking buffer 1), washed with PBS, and reacted for 1 h at room temperature with avidin-biotinylated enzyme complex. Following PBS washes, staining was performed using the peroxidase-chromogen reaction (SG substrate kit, Vector lab), which was stopped using Tris-buffered saline (TBS; 50 mM Tris-base, 150 mM NaCl, pH8.2). The coverslips were then dehydrated with ethanol and xylene and mounted with Permount. Images were taken on a light microscope, equipped with a Nikon DIX digital camera (Nikon, Tokyo).

Western blotting

Untreated and treated DRG neurons were homogenized in ice-cold lysis buffer solution (10 mM EGTA, 2 mM $MgCl_2$, 0.15% Triton X-100, 60 mM PIPES, 25 mM HEPES, pH 6.9, and 1 μM phenylmethylsulfonyl fluoride, 1 μM NaF, 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin) and sonicated. A 3-fold volume of 4X reducing SDS sample buffer was added to each lysate and the mixture heated at 95 °C for 5 min. Fifty micrograms of protein from each sample (protein concentration determined using a Bio-Rad protein Kit, Bio-Rad Life Sciences, Hercules, CA, USA) was separated by 10% polyacrylamide-SDS gel electrophoresis and electrotransferred to a nitrocellulose filter (Schleicher and Schuell, Inc., Keene, NH, USA), which was then blocked for 1 h at room temperature with blocking buffer 2 (TBS containing 5% non-fat milk and 0.1% Tween-20) and incubated overnight at 4 °C with one of the following primary antibodies diluted in blocking buffer 2: anti-pSer473-Akt (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pSer133-CREB (1:500 dilution, Epitomics, Burlingame, CA, USA), anti-total CREB (1:500 dilution, Cell Signaling, Beverly, MA, USA) or anti-total Akt (1:500 dilution, Santa Cruz). Following washes with TBS containing 0.1% Tween-20, alkaline phosphatase-conjugated secondary antibodies (1:7500 dilution, Promega Corp., Madison, WI, USA) in blocking buffer 2 were added for 1 h at room temperature and bound antibodies visualized using an enzyme-substrate reaction (substrate: 3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-base, pH 9.5).

Intracellular Calcium Levels Measured Using Fluo-3/AM

DRG neurons on dishes were washed once with PBS and loaded for 30 min at room temperature with 10 mM fluo-3/AM (Invitrogen, Carlsbad, CA, USA) in PBS. After a

PBS wash, either 0.1 % DMSO or schisandrin was added to the culture dish and calcium images were recorded 5 and 10 minutes later on a fluorescence microscope.

Quantification

The neuritogenic effect of various treatments on DRG neurons was analyzed based on the scanned images of anti-NF-L antibody-stained cells. The total neurite length and total tip number of neurite branches per cell were measured from the somata using PC-based image analyzer software, Image Pro 3.0 Plus (Media Cybernetics, Bethesda, MD, U.S.A.). For Western blotting, the intensity of the kinase protein bands was quantified using Gel Pro 3.1 (Media Cybernetics). The Western blots used for statistical analysis were repeated at least three times for each group. Student's t test was used to evaluate statistical differences between the means for different groups, a p value less than 0.05 being considered significant.

Surgery and drug administration

For spinal cord injury, adult female Wistar rats of 250-300 gm are anesthetized with Ketamine (60 mg/kg) and Xylazine (10 mg/kg). A T8 laminectomy is performed. After opening of the dura mater, a pair of Dumont No. 5 forceps are inserted into the spinal cord for 1 mm and held together for 10 seconds before being removed, thus crushing the dorsal columns with blunt forceps leaving the large midline dorsal blood vessel intact while entirely severing axons in the dorsal column projection. The muscle and skin layers are closed. An osmotic drug pump (Alzet pump model 2001, Duret Corporation, CA, USA) will be implanted under the back skin, containing either schisandrin

(delivering 0.5 mg/kg/day of schisandrin subcutaneously for seven days) or vehicle solution (50% DMSO).

To trace the corticospinal tracts, a small craniotomy window overlying cerebral cortex on the right side was made in the skull 14 days after spinal cord injury. Biotinylated dextran amine (BDA, MW 10,000, 10% in PBS, Molecular Probes, Eugene, OR, USA) was applied into 9 injection sites (0.4 μl/site) at a depth of 1.5 mm from the cortical surface (coordinates: 0-3 mm posterior to bregma, 0-2.5 mm lateral to bregma. The wound was closed in layers.

All animals received antibiotics (ampicillin, 50 mg/kg) and analgesics (ketorolac 5 mg/kg) for 1 day postoperatively after each procedure. Manual expression of the urinary bladder was performed at least twice daily until full recovery of bladder function.

Behavior test

The Basso, Beattie, Bresnahan (BBB) scoring system, shown below, was used to score the locomotor recovery after SCI, with 21 as normal and 0 as complete hind limb paralysis. The rat was monitored in the open field for 4 minutes at 2 days after injury, then every week by observers who were blind to the treatment each rat received.

Basso, Beattie, and Bresnahan Locomotor Rating Scale

0	No observable hindlimb (HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint *and* slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints *and* extensive movement of the third
6	Extensive movement of two joints *and* slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no FL–HL coordination
11	Frequent to consistent weight-supported plantar steps *and* no FL–HL coordination
12	Frequent to consistent weight-supported plantar steps *and* occasional FL–HL coordination
13	Frequent to consistent weight-supported plantar steps *and* frequent FL–HL coordination
14	Consistent weight-supported plantar steps, consistent FL–HL coordination, *and* predominant paw position during locomotion is rotated (internally or externally) when it makes *initial contact* with the surface as well as just before it is *lifted off* at the end of stance; or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL–HL coordination *and* no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL–HL coordination during gait *and* toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
17	Consistent plantar stepping and consistent FL–HL coordination during gait *and* toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact *and* lift off
18	Consistent plantar stepping and consistent FL–HL coordination during gait *and* toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent FL–HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact *and* lift off, and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, *and* trunk instability; tail consistently up
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

Tissue processing and immunohistochemistry

Animals will survive for 4 weeks after spinal cord lesioning. Each animal is deeply anesthetized with ketamine (100 mg/kg) and Xylazine (15 mg/kg), and perfused transcardially with 200 ml of saline, followed by 200 ml of paraformaldehyde (PFA, 4% in PBS, PH = 7.4). Spinal cord is dissected out from the animals, fixed in PFA overnight, and then embedded in 10% gelatin. Longitudinal sections of spinal cord (50 μm thickness), 7.5 mm rostral and 7.5 mm caudal to the lesion (a 15 mm segment), were made on a vibrotome. The sections were incubated at room temperature with

avidin-biotin-peroxidase complex for 60 minutes, followed by stained with peroxidase-chromagen reaction (SG substrate kit, Vector), dehydrated and then mounted. The sections were mounted, covered with mounting medium, and imaged for BDA-labeled CST fibers under light microscopy. The number of CST fibers that travelled through the injury site was recorded for each section, then sum up for statistical analysis described in previous paragraphs.

RESULTS

Enhanced neuritogenesis by schisandrin treatment in cultured DRG neurons

The chemical structure of schisandrin is shown in Fig. 1A. The effect of schisandrin on neurite outgrowth was studied in primary rat DRG neuronal cultures. To investigate the optimal concentration for promoting neurite outgrowth, DRG neurons were treated with schisandrin, NGF, or DMSO for 24 hrs. NGF treatment served as a positive control. The average length and tip number per neuron were calculated. As shown in figure 1, treatment of DRG neurons with schisandrin led to a concentration-dependent increase in total neurite length and tip number per neuron. Compared with DMSO-treated neurons, 1 μg/ml schisandrin significantly increased the total neurite length. At 3 μg/ml, the neuritogenic effect of schisandrin was comparable to that for NGF (Fig. 1C-D). MTT assay showed that schisandrin at 3 μg/ml had no effect on cell viability (data not shown). Therefore, 3 μg/ml was chosen as a working concentration to study the neuritogenic effect of schisandrin.

Blocking of the neuritogenetic effect of schisandrin by a PI3K inhibitor

To investigate the signal transduction pathway involved in the neuritogenic effect of schisandrin, various kinase inhibitors were applied to DRG cultures before and during schisandrin treatment. Since previous studies had indicated that the MEK-ERK, cAMP-PKA, and PI3K-Akt pathways mediated the signaling pathways of neurite outgrowth in DRG neurons (Gao et al., 2003, Markus et al., 2002a), inhibitors of these kinases were tested in our system. Neither the MEK inhibitor PD98059 nor the PKA inhibitor KT5720 had any inhibitory effect on schisandrin-induced neurite outgrowth (data not shown). In contrast, pretreatment of LY294002, a potent PI3K inhibitor, strongly

abolished neurite outgrowth and branching of DRG neurons in response to schisandrin treatment (Figs 2A and 2B). Meanwhile, LY294002 treatment alone had no effect on neurite outgrowth and branching, suggesting that the PI3K inhibitor specifically abolished the neuritogenic effect of schisandrin.

Induction of calcium inflow into DRG neurons following schisandrin treatment

The fluorescent calcium dye, fluo-3/AM, was applied to monitor the effect of schidandrin treatment on intracellular calcium levels. Compared to control cells , a significant increase in intracellular calcium levels was observed after 5 and 10 min of schisandrin treatment (Fig 3).

Schisandrin treatment resulting in activation of PI3K, Akt, and CREB; blocking of the schisandrin-induced kinase activation by a PI3K inhibitor

Since the PI3K inhibitor LY294002 suppressed the neuritogenic effect of schisandrin, we further examined whether schisandrin could activate the PI3K signaling pathway using Western blotting, with activation of PI3K and Akt being evaluated using antibodies against phospho-Tyrosine 458-PI3K p85 subunit (pPI3K) and phospho-Serine 493-Akt (pAkt). Phosphorylated form of PI3K increased to 168.6 ± 46.4%, 141.1 ± 14.5%, or 99.0 ± 14.8% of baseline levels after 15, 30, or 60 min of schisandrin treatment, and phosphorylated form of Akt kinase increased to 139.7 ± 13.1%, 106.8 ± 19.7%, or 101.2 ± 18.8% of baseline levels after 15, 30, or 60 min of schisandrin treatment, respectively. Both pPI3K and pAkt levels were significantly increased after 15 min of schisandrin treatment (Fig 4).

We then examined whether a PI3K inhibitor could block the kinase activation caused by schisandrin treatment. Phosphorylation of CREB, one of the downstream effector of the PI3K-Akt pathway (Markus et al., 2002a, Fernyhough et al., 2003, Read and Gorman, 2009, Finkbeiner, 2000), was also measured. The pAkt and pCREB levels were increased, respectively, to 141.2 ± 22.6% and 139.7 ± 12.7% of baseline levels after 15 min of schisandrin treatment and the values were decreased to 55.4 ± 23.7% and 94.1 ± 12.9% of baseline levels in cells pretreated for 1 h with LY294002 before schisandrin treatment (Fig 5). Thus, LY294002 treatment significantly inhibited the activation of Akt and CREB induced by schisandrin.

Schisandrin treatment showing no functional recovery from spinal cord injury or regeneration or corticospinal tract

Finally we tested the efficacy of schisandrin on neural regeneration after rat spinal cord injury. The DMSO and schisandrin groups showed similar pattern of recovery on BBB scores. The score was around 2 at two days after injury, and increased to 9 and 12 at the first and second week after injury. No further significant recovery occurred later. There was no statistical difference at each time point after injury between the two treatment groups (Fig 6A).

The regeneration of corticospinal tract was also examined on sagittal sections of spinal cord tissue. The BDA labeled corticospinal fibers failed to regrow through the injury site in all animals studied (Fig 6B).

DISCUSSION

This study demonstrated that schisandrin, also known as schisandrol A or schizandrin, has a neuritogenic effect on primary rat DRG neuronal cultures. Our result showed that schisandrin significantly enhances neurite outgrowth and branching. The optimal neuritogenic effect of schisandrin was comparable to that of NGF. We also demonstrated that schisandrin induced neuritogenesis was accompanied by increased phosphorylation of the Akt kinase. Furthermore, the PI3K inhibitor LY294002 abolished both the neuritogenesis and Akt activation induced by schisandrin. These results indicated that schisandrin stimulated neurite outgrowth in DRG neuronal cultures via the PI3K-Akt pathway. However, in vivo study did not demonstrate behavioral recovery and regeneration of corticospinal tract from rat spinal cord injury by schisandrin treatment (Fig 6). This is in contrast to the study using Shengmai-san, which enhanced corticospinal tract regeneration (Seo et al., 2009). The discrepancy may result from difference in experimental methodology, drug dosage, route of delivery, etc. Future study using different experimental design is indicated.

Several signaling pathways are involved in neurite outgrowth of DRG neurons. The MEK-ERK and PI3K-Akt pathways are activated by NGF and play key roles in neurite extension and branching of DRG neurons (Markus et al., 2002a). The cAMP-PKA pathway also contributes to neuritogenesis in DRG cultures (Chen et al., 2007a). The present study highlights the importance of the PI3K-Akt pathway in the signaling mechanism of schisandrin-induced neuritogenesis. *Schisandra chinenesis* is one of the major components in Shengmai-san (SMS) extracts which have been shown to enhance neurite outgrowth in cultured DRG neurons and promote axonal regeneration in both

injured sciatic nerves and spinal cord axons by activating cell division cycle 2 (cdc2) proteins and pErk1/2 pathways (Seo et al., 2009). Schisandrin B, a lignan of *Schisandra chinensis*, has recently been shown to modify the activity of one PI3K family kinases, ATR, in a cancer cell line (Nishida et al., 2009). Likewise, *schisandra chinensis* and several of its constituents were suggested to activate an orphan nuclear receptor in primary hepatocyte cultures (Mu et al., 2006). Traditionally, PI3K is activated by ligands acting on cell membrane receptors like tyrosine kinase or G-protein receptor (Vanhaesebroeck et al., 2010). However, recent evidence suggested that PI3K was also subjected to regulation by certain nuclear receptor through protein-protein interaction (Furuya et al., 2007). Because the hydrophobic structure of schisandrin (Fig 1A) predicts its ready penetration through cell membrane, it is possible that schisandrin directly activates PI3K or indirectly through intracellular messengers. On the other hand, we also showed that calcium ion influx into DRG neuronal cells at 5 to 10 minutes following schisandrin treatment. Since calcium ion was reported to mediate PI3K activation in stretched osteoblast cells (Danciu et al., 2003), it remains a possibility that calcium plays a role of the signal cascade of schisandrin-PI3K-Akt-CREB pathway (Fig 3). Further researches are required to address the interaction between schisandrin and PI3K.

Akt, also known as protein kinase B, is an important regulator of cell growth, metabolism, and survival. In neuronal cells, Akt activation by various ligands has been shown to prevent neuronal cell death (Datta et al., 1997), and to enhance elongation, branching and caliber size of neuritic processes (Markus et al., 2002b). Akt can modulate many downstream proteins that are associated with neurite outgrowth, e.g. mammalian target of rapamycin (mTOR), glycogen synthase kinase 3β (GSK3β), and

CREB (Read and Gorman, 2009). Our results showed that, following schisandrin treatment, the activated kinase Akt phosphorylated CREB protein, resulting in enhanced neuritogenesis of DRG neurons. In addition to PI3K-Akt, several other signaling pathways, e.g. cAMP-PKA and MEK-ERK, can also activate CREB and induce neurite outgrowth (Shaywitz and Greenberg, 1999). However, the current study did not support the role of either cAMP or MEK-ERK pathway in schisandrin-induced neuritogenesis. CREB, a transcription factor, can activate the expression of specific genes with CREB binding sites in their promoter region. Many of these gene products, e.g. neurofilament, fibronectin, fibroblast growth factor, BDNF, and TrkB, are relevant to neurite outgrowth (Lonze and Ginty, 2002b).

Schisandra chinenesis and its lignans have been shown to render protection against tissue damages by toxic substances in various organ systems, e.g. liver, heart, brain, etc. For the nervous system, Schisandrin B ameliorated cerebral toxicity from tert-butylhydroperoxide in mice (Ko and Lam, 2002), and protected rat cortical neurons against Abeta1-42-induced neurotoxicity (Wang and Wang, 2009). ESP-102, a herb mixture containing *Schisandra chinenesis*, protected cultured rat cortical neuronal cells against glutamate excitotoxicity (Ma et al., 2009). Several lignans of *Schisandra chinensis*, including schisandrin, were also able to counteract the toxic effect of glutamate by reducing oxidative stress and apoptotic cell death (Kim et al., 2004b, Cheng et al., 2008). Though schisandrin offered protection against glutamate-induced excitotoxicity in cultured rat cortical neurons at a concentration of 10 μM (4.33 μg/ml) or higher, we found the viability of rat DRG cells in our non-toxic culture condition measured by the MTT test was not affected by schisandrin treatment at a concentration

of 3 μg/ml. Thus, enhanced neuritogenesis by schisandrin in the current study is not confounded by a neuroprotective effect.

In traditional Chinese medicine, *Schisandra chinensis* has been proposed to be capable of improving memory and intelligence. Herbal prescription containing *Schisandra chinensis* could reverse memory deficit induced by anticholinergic agents in rats (Kang et al., 2005b). Interestingly, schisandrin and several other lignans were also found to inhibit the enzyme activity of acetylcholinesterase (Hung et al., 2007). Furthermore, deoxyschisandrin, also named as schisandrin A, modulated cellular calcium influx and synaptic neurotransmitter release in hippocampal neurons (Fu et al., 2008). In addition to changes of neurochemical substrate, learning and memory also involve structural alterations in neuronal circuits, e.g. dendritic branching and synapse formation. Our result showed that schisandrin significantly enhances neurite lengthening and branching in DRG neurons. It would be interesting to study whether schisandrin can promote neuritogenesis in hippocampal neurons, which could serve as fundamental steps to facilitate long-term memory formation.

The hallmark of spinal cord and peripheral nerve injuries is axonal loss. Failure of axonal regeneration results in significant morbidity, e.g. paralysis and sensory deficits. Numerous studies have been engaged in the search for neuritogenic agents and the potential of Chinese herbs is now receiving attention. Previously schisandrin has been shown to possess neuroprotective property in neuronal cultures (Cheng et al., 2008). Our results demonstrated that schisandrin was capable of inducing neurite outgrowth. Since *Schisandra chinensis*, from which schisandrin is extracted, has long been used as

an herbal medicine in human beings, further studies are still warranted to investigate the use of schisandrin for neural regeneration after injuries and diseases.

FIGURES and LEGENDS

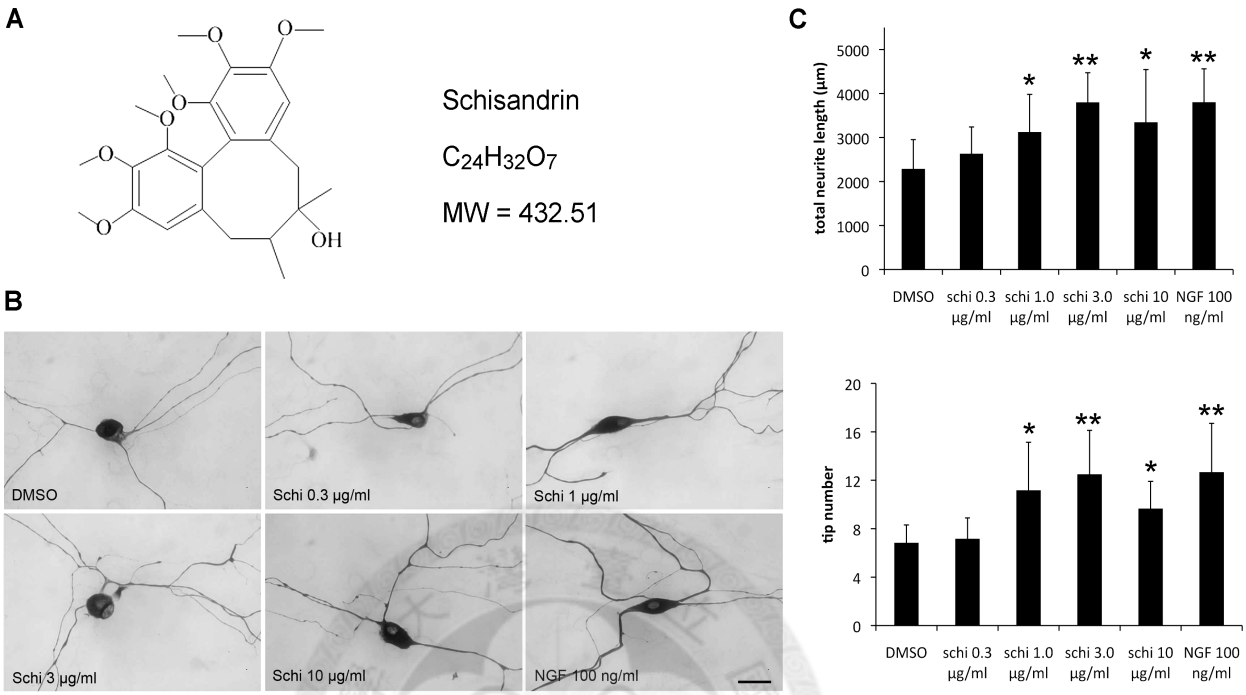

Fig 1. Effect of schidandrin on DRG neuronal profile. (A) Chemical structure of schisandrin. (B) Induction of neurite outgrowth of rat DRG neurons by schisandrin and NGF. DRG neuronal cultures were treated for 24 h with vehicle solution DMSO, various concentration of schisandrin (Schi), or 100 ng/ml NGF, then immunostained for NF-L. Scale Bar = 30 μm. (C) Quantitative analysis of total neurite length and tip number of neurite branches per neuron following various treatments. Three independent experiments were performed. Six neurons were chosen from each group in one representative experiment for analysis. *, $p < 0.05$; **, $p < 0.01$.

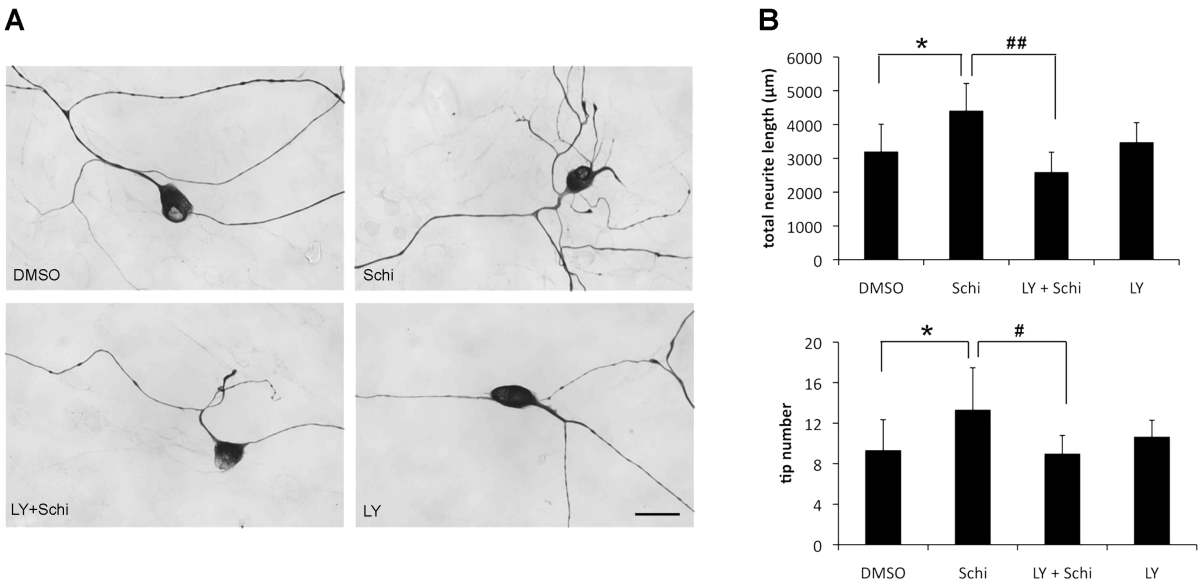

Fig 2. Effect of LY294000 on shicandrin-induced neurite outgrowth of DRG. (A) Inhibition of LY294000 on neurite outgrowth. DRG neuronal cultures were treated for 24 h with DMSO, 3 μg/ml of schisandrin (Schi), 10 μM of LY294002 for 60 min before 3 μg/ml of schisandrin treatment (LY + Schi), or 10 μM of LY294002 (LY) only, then immunostained for NF-L. Scale Bar = 30 μm. (B) Quantitative analysis of total neurite length and tip number of neurite branches per neuron following various treatments. Three independent experiments were performed. Six neurons were chosen from each group in one representative experiment for analysis. * and $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$.

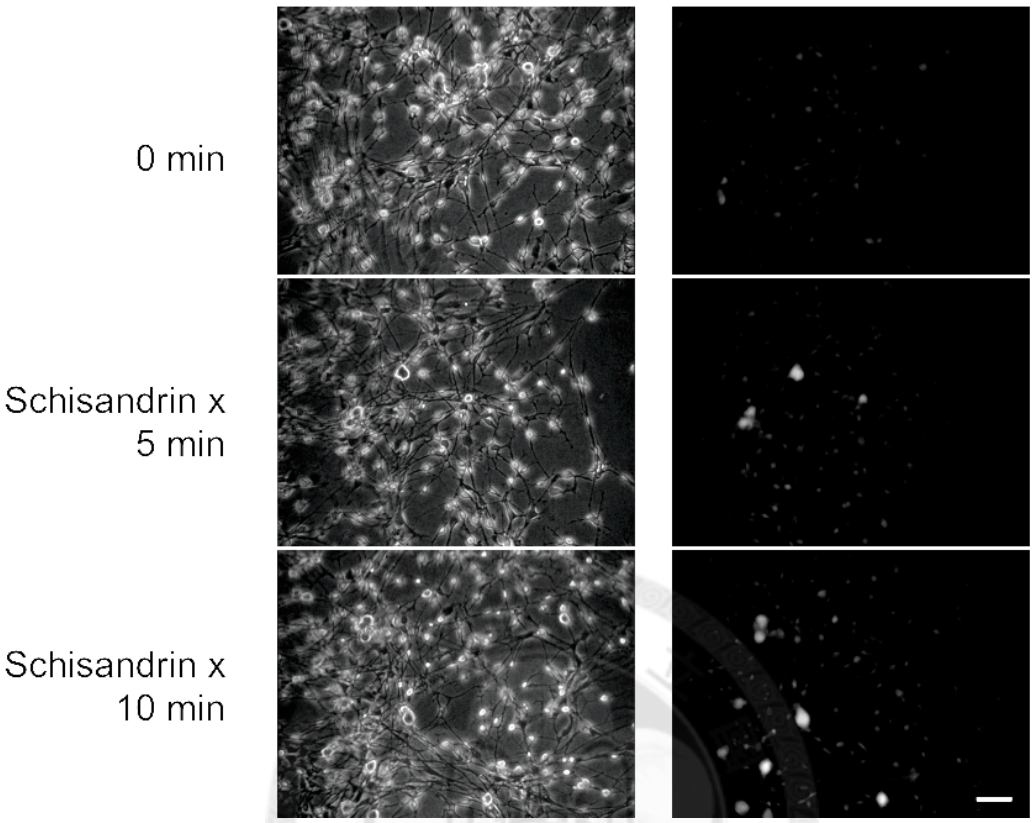

Fig 3. Schisandrin increases intracellular calcium levels. DRG neurons were loaded with fluo-3/AM, and then treated for 0, 5, and 10 min with 3 μg/ml schisandrin. Left column: phase images; right column: fluorescent images. Bar = 50 μm.

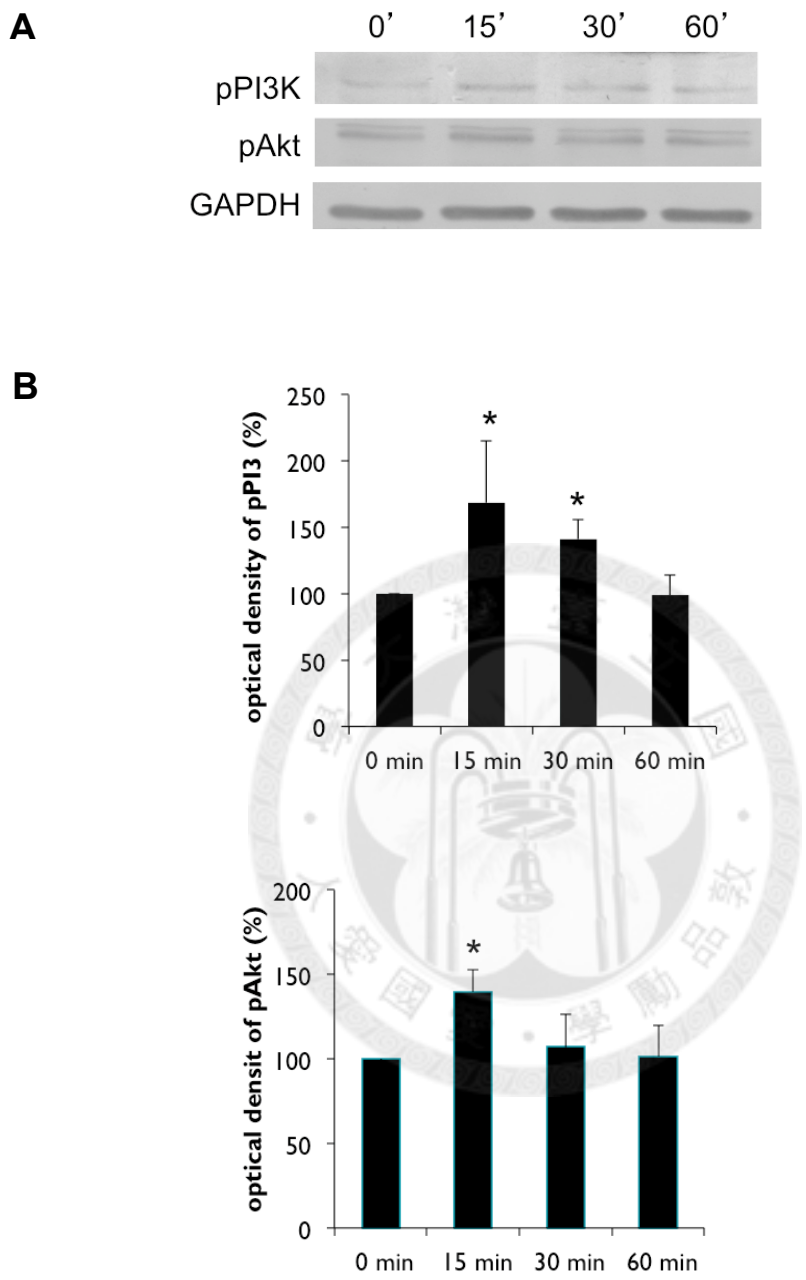

Fig 4. Time course analysis of PI3K and Akt phosphorylation induced by schisandrin. (A) Western blot analysis. DRG neuronal cultures were treated with 3 μg/ml of schisandrin for 0, 15, 30, or 60 min, then the cell homogenate was analyzed for phosphorylated PI3K (pPI3K), phosphorylated Akt (pAkt). GAPDH was run as a loading control. A representative blot from one experiment is shown. (B) Optical densities of the densitometric scan of the pPI3K and pAkt bands. *, $p < 0.05$. $n = 4$.

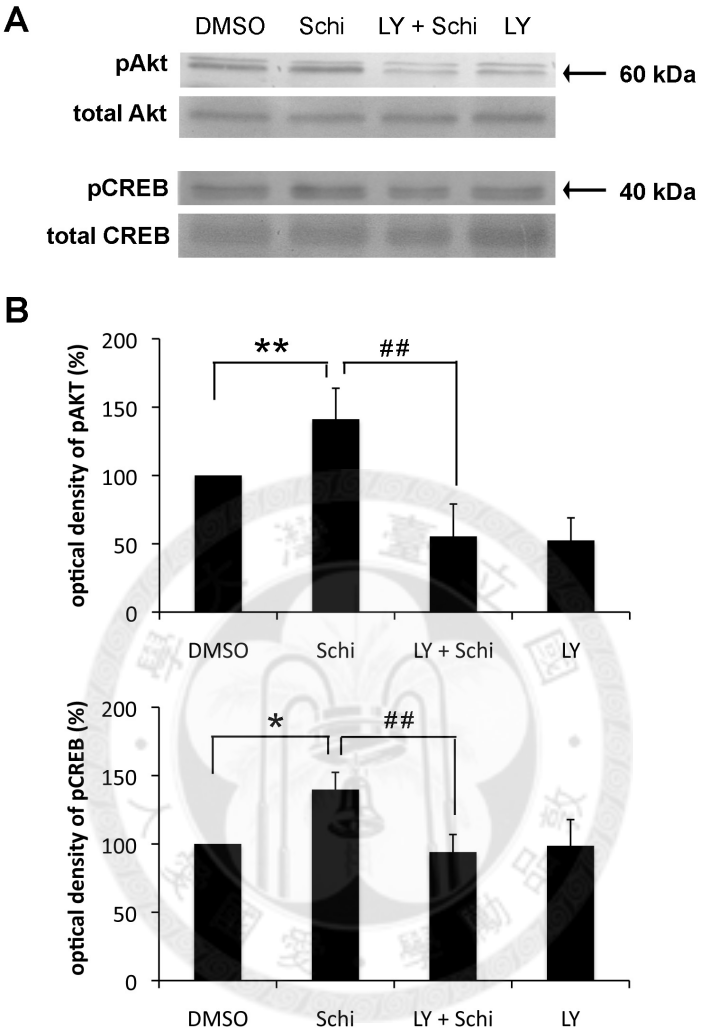

Fig 5. Effects of LY294002 on schisandrin-induced phosphorylation of Akt and CREB. DRG neuronal cultures were treated for 15 min with DMSO (DMSO), 15 min with schisandrin (Schi), 60 min with LY294002 then 15 min with schisandrin (LY + Schi), or for 75 min with LY294002 alone (LY). (A) Western blot analysis. The cell homogenate was analyzed for phosphorylated Akt (pAkt), total Akt, phosphorylated CREB (pCREB), or total CREB. A representative blot from one experiment is shown. (B) Optical densities of the densitometric scan of the pAkt and pCREB bands. *, $p < 0.05$; ** and $^{\#\#}$, $p < 0.01$. $n = 4$.

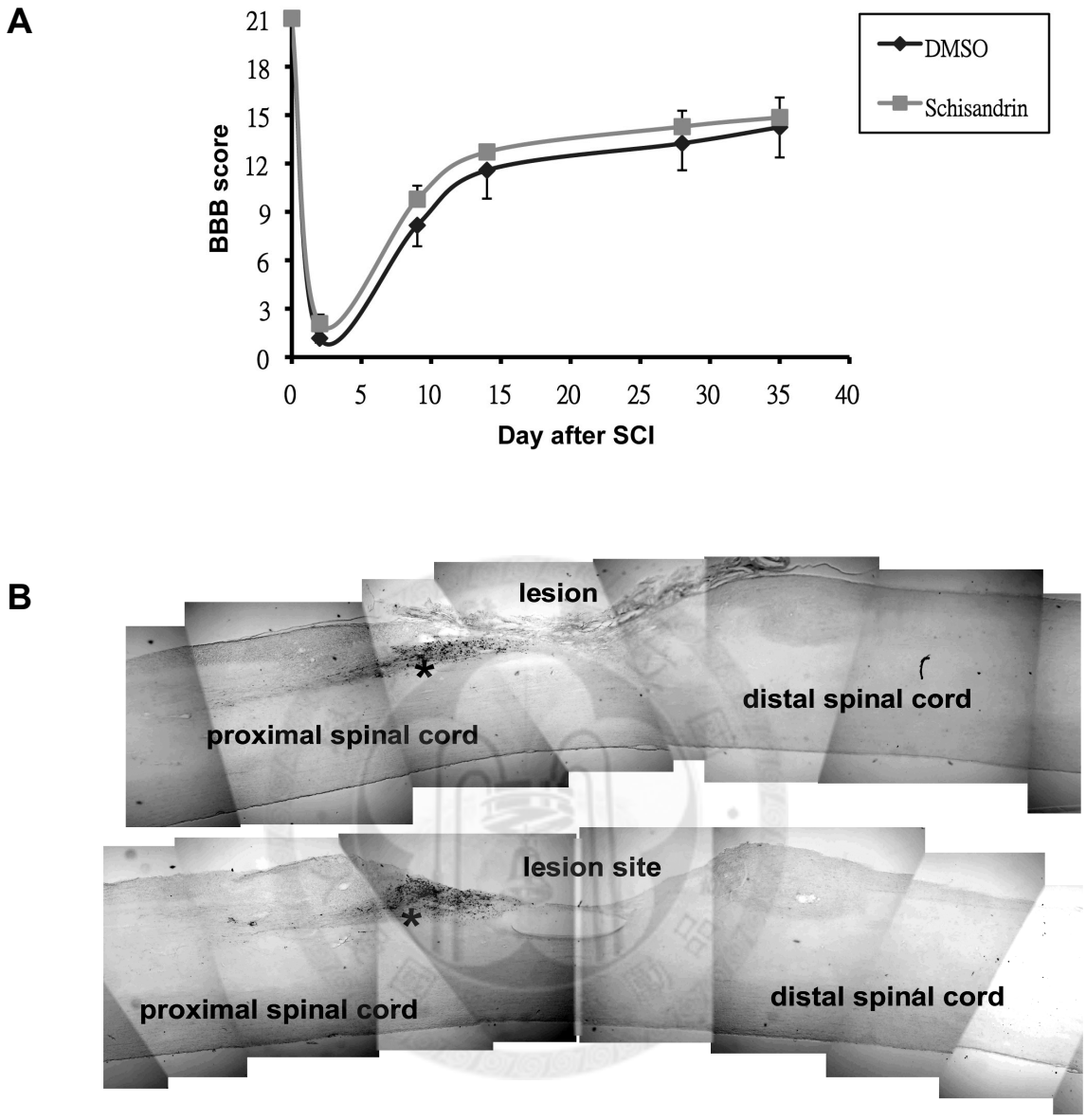

Fig 6. (A) BBB scores of rats after spinal cord injuries (SCI). No difference of BBB score between DMSO and schisandrin groups was found at all time points after injury. n = 7. (B) Sagittal sections across the injured spinal cord at T8 level. The upper diagram was from a schisandrin treated rat, and the lower diagram from a DMSO treated rat. * denoted BDA labeled corticospinal tract, which were unable to regenerate through the injury site in all animals.

Chapter 3. Study on the effect and mechanism of schisandrin for neuritogenesis and synaptogenesis in primarily cultured rat hippocampal neurons

INTRODUCTION

The berries of *Schisandra chinensis* are widely used in traditional Chinese medicine. In Korea, a special tea is made from the berries (Kim et al., 2009). Several in vitro studies have reported that lignan isolated from *Schisandra chinensis* could protect neurons from oxidative toxicity induced by glutamate, amyloid-β peptide, or hydrogen peroxide (Kim et al., 2004a, Liu et al., 2006a, Jung et al., 2007). In vivo studies showed that application of herbal prescriptions consisting of *Schisandra chinensis* to mice improved scopolamine-induced memory impairment and ameliorated electroconvulsive shock-induced memory deficits (Nishiyama et al., 1995, Kang et al., 2005a). Hsieh *et al.* showed that water extracts of *Schisandra chinensis* prevented cycloheximide-induced amnesia in rats via a decrease in the serotonergic neuronal activity, and an increase in the cholinergic and gamma-amino butyric acid systems (Hsieh et al., 1999, Hsieh et al., 2001). However, no previous studies have elucidated the underlying signaling mechanism of learning and memory-enhancement effects by *Schisandra chinensis*.

Several signaling molecules, including calcium/ calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and extracellular signal-related kinase 1 and 2 (ERK1/2), have been implicated as playing crucial roles in the regulation of neurite growth (Prekeris et al., 1996, Menegon et al., 2002, Vaillant et al., 2002, Lee et al., 2004, Kolkova et al., 2005). CaMKII is concentrated in the postsynaptic density (PSD),

with a broad range of substrates including cytoskeletal proteins and glutamate channel proteins (Wu and Cline, 1998, Cheng et al., 2006, Colbran and Brown, 2004). It can transduce input activity into coordinated changes in both neurite growth and synaptic plasticity (Wu and Cline, 1998). CaMKII controls the activity-dependent dendritic growth in neurons both in vitro and in vivo (Wu and Cline, 1998, Vaillant et al., 2002). In addition, Vaillant *et al.* (Vaillant et al., 2002) also showed that CaMKII promotes neurite outgrowth via activation of ERK, which belongs to the family of the mitogen-activated protein kinase (MAPK) in neurons. Phosphorylation of ERK1/2 is mediated via the MAPK kinase (MEK) and the MEK/ERK signaling pathway has been implicated as an important regulator of activity-dependent structural changes in hippocampal neurons (Goldin and Segal, 2003). PKC has been reported to play a key role in neuritogenesis and regulate the extension of microtubles in *Aplysia* growth cones (Nakhost et al., 2002). Among various PKC subtypes, PKCe is specifically involved in neurite outgrowth via activation of the ERK pathway in PC12 and neuroblastoma cells (Fagerstrom et al., 1996, Brodie et al., 1999, Tsuji et al., 2001). Together, these studies reveal the close relationship between neurite growth and the activation of CaMKII, PKCe, and ERK signaling molecules.

Hippocampal neurons can make extensive synaptic connection with one another while growing in dissociated cell cultures, forming a valuable model for studying events occurring in vivo. In the present study, we used the cultured hippocampal neurons as a model to investigate the neuritogenesis and associated molecular signaling pathways of schisandrin. Our results showed that schisandrin enhanced dendrite development and synapse formation in cultured hippocampal neurons. Furthermore, we identified that the

schisandrin-induced neurite outgrowth is mediated by the molecules CaMKII, PKCε, and MEK.

MATERIALS and METHODS

Cell culture

Pregnant female Wistar rats were purchased from the Facility for Animal Research of the National Taiwan University. The date of the presence of vagina plug was considered as embryonic day 0 (E0). The E17 embryos were used in this study. Animal care and procedures were performed according to the standards set forth by the "Guide for the Care and the Use of Laboratory Animals", published by the U.S. National Institutes of Health (NIH publication N0. 80-23, revised 1996). Primary cultures of hippocampal neurons were prepared from the E17 embryos of the Wistar rats. The hippocampus was dissected out under a dissection microscope and collected in prewarmed Hank's balanced salt solution (Gibco, Grand Island, NY). Collected hippocampi were minced and incubated in 0.25 % trypsin for 15 min at 37 °C. The digested fragments were triturated 5-10 times with a fine-pored Pasteur pipette. The dissociated cells were collected and plated on 12-mm coverslips or 35-mm dishes coated with poly-D-lysine (25 mg/ml, Sigma-Aldrich) and laminin (30 mg/ml, Sigma-Aldrich) at a density of 100 cells/mm^2 and 400 cells/mm^2, respectively. The hippocampal neurons were grown in neurobasal media (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM glutamine, and 12.5 mM glutamate at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Hippocampal cultures grown for 3 to 5 days in vitro (DIV) were used for experiments.

Drug treatment

Schisandrin was applied into the cultured medium at a final concentration of 1.3 μg/ml (3 mM). Cells were also pretreated with inhibitors, including CaMKII inhibitor KN93,

MEK inhibitor PD98059 or U0126 (Calbiochem, San Diego, CA, USA), PKCε inhibitor $\varepsilon V_{1\text{-}2}$ (Biomol), 30 min before applying schisandrin.

Immunocytochemistry

DIV-3 hippocampal neurons on coverslips were rinsed with phosphate buffer saline (PBS) and fixed with -20 ^{o}C methanol for 10 min according to the procedures of Fong (Fong et al., 2002). After PBS wash, cells were permeabilized and blocked with PBS containing 0.15% Triton X-100 and 5% non-fat milk in PBS for 1 h. For neuritogenesis studies, appropriate dilutions of primary antibodies were applied in blocking buffer overnight at 4^{o}C as following: 1:100 dilution for mouse anti-microtubule associated protein 2 (MAP2, Sigma-Aldrich), 1:100 dilution for mouse anti-tau (Chemicon, Temecula, CA, USA), 1:100 dilution for mouse anti-PSD95 (Sigma-Aldrich), and 1:50 dilution for rabbit anti-phosphorylated cyclic AMP responsive-element binding protein (CREB, Upstate Biotechnology Inc., Lake Placid, NY, USA). After washing with PBS, cells were incubated with biotinylated-goat anti-mouse IgG (Vector lab) at room temperature for 1 h, followed by reacting with the SG substrate solution (Vector lab), and then mounted on slides with aqueous mounting medium (Sigma-Aldrich). Images were taken on a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Oberkochen, Germany), equipped with a Nikon DIX digital camera (Nikon, Tokyo, Japan). For the immunostaining of phosphorylated CREB, 100-200 cells were calculated per experiment.

Western blot analysis

Cultured hippocampal neurons were homogenized in ice-cold lysis buffer solution (10 mM EGTA, 2 mM $MgCl_2$, 0.15% Triton X-100, 60 mM PIPES, 25 mM HEPES, pH 6.9, and 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 μg/ml of leupeptin and 1 μg/ml pepstatin A, and then sonicated for twenty 10 sec pulses. A 3-fold volume of 4X reducing SDS sample buffer was added to the lysates and boiled at 100 ^{o}C for 5 min. Protein concentrations were measured using a protein assay kit, and samples were then stored at -20 °C until further analysis. Samples (50 mg per lane) were separated by a 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane as described by Fritz *et al*. (Fritz et al., 1989). Membrane strips were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris base, pH 8.2) containing 0.1% Tween-20, and then incubated overnight at 4 ^{o}C with a series of antibodies as indicated: anti-phosphorylated MEK1/2 (1:500 dilution, BioVision Inc., Mountain View, CA, USA), anti-phosphorylated CaMKII, anti-phosphorylated CREB (1:500 dilution, Cell Signaling), anti-phosphorylated PKCε (Upstate). Mouse antibodies specific for anti-β-actin (1:10000, Sigma-Aldrich) and anti-GAPDH (1:10000, Abcam, Cambridge, UK) were used for internal controls. After wash, membrane strips were incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:7,500 dilution) and bound antibodies were detected using a substrate solution (3.3 mg/ml of nitro blue tetrazolium and 1.65 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris base, pH 9.5). For Western blotting analysis, positive bands were quantified with Gel pro 3.1 (Media Cybernetics). The Western blots used for statistical analysis were repeated at least three times for each group. The density of the DMSO

control group was defined as 100 and the densities of the other groups were expressed relatively.

Quantification of dendrite outgrowth

Immunostained neurons were photographed at 40X and 100X magnification, and the images were converted into 256 grayscale images. Each experimental group consisted of 30 neurons, and at least four coverslips of the cultured cells were examined. Only dendritic processes connected to cell bodies were collected for analysis. The total dendritic length and numbers, and dendritic branching tip numbers were assessed by using an image analyzer software (Image Pro 3.0 Plus, Media Cybernetics).

N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM 1-43) dye staining

Schisandrin was applied into the culture medium at a concentration of 1.3 μg/ml (3 mM) for 24 h. To initiate depolarization, the neurons were treated with 50 mM KCl in Hank's Buffered Salt Solution containing 15 mM FM 1-43 (Molecular Probes, Eugene, OR, USA) for 60 s. Cells were then washed with PBS five times. The active presynaptic axon terminals were shown by the uptake of FM 1-43 dye.

Intracellular Calcium Levels Measured Using Fluo-3/AM

Hippocampal neurons on dishes were washed once with PBS and loaded for 30 min at room temperature with 10 mM fluo-3/AM (Invitrogen) in PBS. After a PBS wash, 0.1% DMSO, or 1.3 μg/ml schisandrin was added to the culture dish and calcium images were recorded immediately on a fluorescence microscope.

Statistic analysis

All values in experiments are expressed as the means ± S.D. Statistical comparisons between groups were determined by the Student's *t* test, and a *p* value of less then 0.05 was considered a statistically significant difference.

RESULTS

Schisandrin enhances dendrite development of 3 DIV neurons

Dendritic elongation and branching of cultured hippocampal neurons take place during 3 to 5 DIV(Dotti et al., 1988); therefore, we examined the effect of schisandrin on neurite outgrowth and differentiation by treating hippocampal cultures with schisandrin at DIV 3. To examine the morphogenesis of developing dendrites and axons, we performed immunocytochemistry by using antibodies against MAP2 and tau, specific markers of dendrites and axons, respectively. The chemical structure of schisandrin was illustrated in Figure. 1A. Compared with control group (Figure. 1B-a, DMSO-treated), neurons treated with schisandrin displayed a significant increase in the outgrowth and arborization of dendrites (Figure. 1, B-b). Quantitative analyses indicated that schisandrin treatment raised total dendritic length, branch-tip numbers, and the numbers of the primary and secondary dendrites (Figure. 1, C-E). On the other hand, there was no detectable effect of schisandrin on the axonal arbor (Figure. 1, B-d; control, B-c), as confirmed by the quantitative data on total axonal length (Figure. 1F).

CaMKII, PKCε, and MEK1/2 regulate schisandrin-induced dendritic development

In order to identify the signaling molecules involved in the schisandrin-induced dendritic development, we applied specific inhibitors of CaMKII (KN93), MEK (PD98059), and PKCε ($\varepsilon V_{1\text{-}2}$). Pretreatment of hippocampal neurons with any of these inhibitors significantly decreased the effect of schisandrin on dendritic growth (Figure. 2, A-C). Similarly, quantitative analyses confirmed that pretreatment with the forgoing kinase inhibitors also prevented schisandrin on the augmentation of total dendritic length, total dendritic branch tip number, and second-order dendritic branching (Figure.

2, D-F). These data suggest that CaMKII, PKCε and MEK are involved in schisandrin-induced dendrite growth. We hypothesized that schisandrin induced an elevation of intracellular calcium level, which then caused CaMKII activation. Thus, we applied the fluorescent calcium dye, fluo-3/AM, to monitor the effect of schidandrin treatment on intracellular calcium levels. Compared to control cells (Figure. 3, A-B), a significant increase in intracellular calcium levels was observed after 10 min of schisandrin treatment (Figure. 3, C-D).

We also monitored the activation of these three kinases with antibodies specifically recognized pCaMKII, pMEK1/2, and pPKCε. Schisandrin significantly induced a rapid phosphorylation of CaMKII, PKCε, and MEK1/2 at 15 min after drug treatment (Figure. 4, A-D). Inhibition of CaMKII by KN93 attenuated schisandrin-increased pPKCε and pMEK1/2 (Figure. 4E), indicating that CaMKII acted upstream of PKCε and MEK1/2 phosphorylation. Moreover, inhibition of PKCε with εV_{1-2} reduced schisandrin-induced MEK1/2 phosphorylation (Figure. 4F).

CaMKII and MEK mediates the phosphorylation of transcription factor CREB

Phosphorylation of CREB at Ser-133 by several intracellular protein kinases (CaMKII, ERK, and PKA) resulted in the activation of CREB-mediated gene transcription.(Lonze and Ginty, 2002a) Activation of CREB was assessed by a specific antibody for CREB phosphorylated at Ser-133 (pCREB). The level of CREB phosphorylation was significantly increased after treatment of schisandrin for 1 h (Figure. 5A). Concomitantly, schisandrin treatment also increased the nuclear translocation of pCREB (Figure. 5C, a-b; 5D). Inhibition of MEK/ERK by PD98059 only partially prevented the CREB phosphorylation induced by schisandrin (Figure. 5A). Pretreatment

of hippocampal neurons with KN93 greatly abolished the schisandrin-induced CREB phosphorylation (Figure. 5B) and blocked the nuclear translocation of pCREB (Figure. 5C, c; 5D).

Schisandrin increased the expression of PSD-95 and FM 1-43

Schisandra chinenesis can improve learning and memory in memory-impaired mice and rats.(Kang et al., 2005a, Nishiyama et al., 1995, Hsieh et al., 1999) Since synapse formation plays an important role in neuronal development and synaptic plasticity, we further examine the synaptogenic effect of schisandrin on hippocampal neurons. Hippocampal neurons cultured from embryonic day 18 rat pups begin to form functional synaptic connections by 6 to 7 DIV.(Dotti et al., 1988) Thus, we performed immunocytochemistry with 5 DIV hippocampal neurons using antibody against PSD-95, a marker for postsynaptic specialization,(Sheng, 2001) to examine the effect of schisandrin on synaptogenesis. After 24 h treatment with schisandrin, the number of PSD-95 puncta per unit neurite length was significantly increased (Figure. 6, B, E). In contrast, no immunostaining signal of PSD-95 was detected in the DMSO-treated neurons (Figure. 6A). The schisandrin-induced maturation of presynaptic terminals was examined using the FM 1-43, a dye that is taken up into recycling synaptic vesicles in active presynaptic boutons. FM 1-43 labeling was performed by depolarizing the neurons for 60 s in a high potassium buffer. Compared with DMSO-treated neurons (Figure. 6C), the number of FM 1-43 fluorescent puncta were significantly increased in schisandrin-treated neurons (Figure. 6, D, F).

DISCUSSION

Previous animal studies have shown that the extract of *Schisandra chinensis* improves cognitive functions (Nishiyama et al., 1995, Hsieh et al., 1999, Kang et al., 2005a), the mechanism of which, however, remains unknown. To the best of our knowledge, we have presented the first evidence demonstrating that in cultured hippocampal neurons, schisandrin enhances dendritogenesis via the CaMKII-PKCε-MEK1/2 pathway. No similar effects, however, were observed for axons. In addition, our data indicate that schisandrin can promote PSD formation and presynaptic vesicle recycling, suggesting that not only does schisandrin increase dendritic arborization, but it also enhances synaptogenesis. Moreover, CaMKII activation induced by schisandrin leads to an increased CREB Ser-133 phosphorylation, which might in turn activate CREB-mediated gene transcription for dendrite growth and synapse formation.

Our data suggest that schisandrin induces dendritic, but not axonal, outgrowths in cultured hippocampal neurons. Schisandrin is one of the major components in shengmai-san (SMS) extract, however, SMS extracts have been shown to enhance axonal regeneration in both injured sciatic nerves and spinal cord axons (Seo et al., 2009). The discrepancy might be explained by the fact that there are many other ingredients in the SMS extracts that could have the axonal elongation effects. In addition, given the many potential differences between spinal/cortical and hippocampal neurons, it is also possible that hippocampal neurons could respond to schisandrin differently. Therefore, it remains to be determined what components in SMS extracts are directly responsible for the observed axonal arborization effect.

It is interesting that schisandrin displays a dendrite-specific growth-promoting effect. Dendrite and axon growths have been demonstrated to be differentially regulated by neuronal activities (Konur and Ghosh, 2005), intracellular signaling molecules (Fink et al., 2003), and environmental cues (Barnes and Polleux, 2009, Goldberg, 2003, McAllister, 2000). For example, in cortical neurons semaphorin A acts as a chemorepellant for axons, but a chemoattractant for dendrites (Polleux et al., 1998, Polleux et al., 2000). In cultured hippocampal neurons, RhoA GTPase activation is required for axonal growth and arborization, but not for dendritic growth (Ahnert-Hilger et al., 2004). By contrast, in hippocampal neurons protein kinase D is essential for the maintenance of dendritic arborization only (Czondor et al., 2009). Altogether, these findings support the notion that dendritic and axonal growth processes are subjected to distinct modulations mediated by a diverse variety of different signaling cascades.

Dendrite outgrowth and synapse maturation are two essential processes of dendrite development. Neuronal dendrites are active participants of synapse formation (Cline, 2001). For example, in optic tectal cells and hippocampal neurons, dendritic branches extending toward axons can be observed during synaptogenesis (Ziv and Smith, 1996, Wu and Cline, 1998). Small protrusions on the surface of dendrites are called dendritic spines, which are the major postsynaptic partners for the presynaptic boutons in excitatory synapses and the major localization of PSD proteins, such as PSD-95 (Harris and Kater, 1994, Kennedy, 2000). It has been shown that motile dendritic filopodia, the precursors of dendritic spines, are the predominantly active partner in synapse formation (Dailey and Smith, 1996). Our data showed that the fluorescent puncta of both FM 1-43 (indicator of synaptic maturation) and PSD-95 were increased after 24 h treatment of schisandrin, suggesting that schisandrin can enhance synapse formation. It

is thus possible that schisandrin not only promotes dendritic growth but also increases the motility of dendritic filopodia, thereby facilitating the synapse formation. The detailed molecular mechanism remains to be investigated.

CaMKII, PKCε, and MAPK have been previously shown to modulate neurite growth and synapse formation in several types of neurons, including hippocampal neurons, PC12 cells, and neuroblastoma cells (Fagerstrom et al., 1996, Prekeris et al., 1996, Kolkova et al., 2005). Similarly, here we found that these signaling molecules also participated in schisandrin-induced dendritic arborization, such as dendritic ougrowth and branching in hippocampal neurons. CaMKII and PKCe have been reported to be the upstream kinase of the MEK-ERK signaling cascade in neurite outgrowth (Brodie et al., 1999, Lee et al., 2004, Kolkova et al., 2005). When activated, CaMKII, PKCe, and MEK1/2 can directly affect dendritic stability by altering cytoskeletal dynamics in dendrites (Prekeris et al., 1996, Wu et al., 2001, Vaillant et al., 2002), thus influencing outgrowth and arborization of developing dendrites. The activation of these three kinases has also been implicated in mediating synapse formation by phosphorylating PSD proteins (Saito et al., 1993, Redmond and Ghosh, 2005); however, the functional consequences of most of these phosphorylation events are unclear. Thus, the exact mechanism of schisandrin-increased PSD formation deserves further investigation.

Phosphorylation of CREB at Ser-133 is critical for the development of long-lasting, activity-dependent synaptic plasticity, and in the consolidation of long-term memory (Kida et al., 2002, Lonze and Ginty, 2002a). CREB phosphorylation can be triggered by several kinases, such as PKA, CaMKII, CaMKIV, and MEK/ERK, and may lead to CREB-dependent gene expression, which is important for the maintenance of neuronal

cell population and synaptogenesis during neuronal development (Bito and Takemoto-Kimura, 2003, Lee et al., 2004, Redmond and Ghosh, 2005, Chen et al., 2007b). It has also been shown that activation-dependent arborization required sequential activation of CaMKK, CaMKI, and MEK/ERK to enhance CREB-mediated transcription of Wnt-2 (Wayman et al., 2006). Our data indicated that schisandrin-induced CREB phosphorylation was blocked by the CaMKII inhibitor, implying that CaMKII may serve as an upstream molecule for the signaling cascade leading to CREB phosphorylation. The activation of CaMKII is probably triggered by an increase in intracellular calcium, as evidenced by Fluo-3/AM calcium staining in the present study. An increase in intracellular calcium is often accompanied by a rise in nuclear calcium, which in turn can also activate CREB (Hardingham et al., 1997). Moreover, we found that schisandrin-induced CREB phosphorylation was partially inhibited by the MEK inhibitor PD98059. It is therefore possible that in response to schisandrin treatment, CREB phosphorylation can be stimulated by nuclear calcium surge, by direct CaMKII activation, or by the CaMKII-PKCε-MEK signaling cascade.

We have demonstrated that schisandrin stimulates dendritogenesis as well as synaptogenesis in hippocampal neurons. The signaling cascade involved is the CaMKII-PKCε-MEK pathway. Since the hippocampal formation is highly related to learning and memory in mammals, the current study highlights the potential therapeutic value of schisandrin in the modulation of memory and other cognitive functions.

FIGURES and LEGENDS

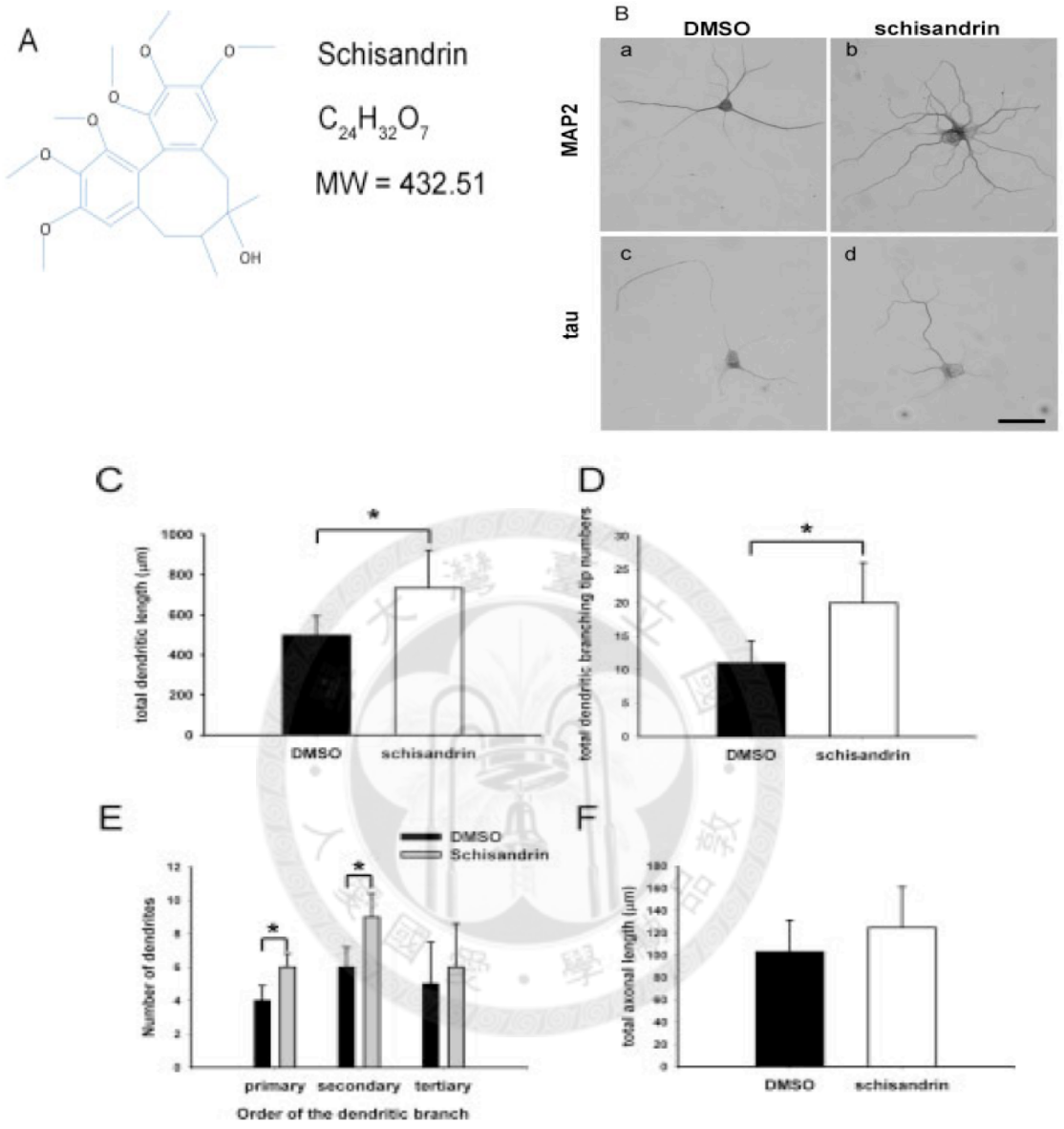

Fig 1. Effects of schisandrin on the growth of dendrites and axons in cultured hippocampal neurons. A, the chemical structure of schisandrin. B, 3-DIV hippocampal neurons were treated with 0.1% DMSO (vehicle control) alone (a, c), or 1.3 μg/ml (3 μM) schisandrin (b, d) for 24 h, and then immunostained for MAP2, a protein marker for dendrites, (a, b) and tau, a protein marker for axons (c, d). Scale bar: 30 μm. B-E, Quantitative analyses of the effects of schisandrin on dendritic length (C), dendritic branching tip numbers (D), numbers of different orders of dendritic branching (E), and axonal length (F). n = 30. * $p < 0.05$, compared to the DMSO-treated group.

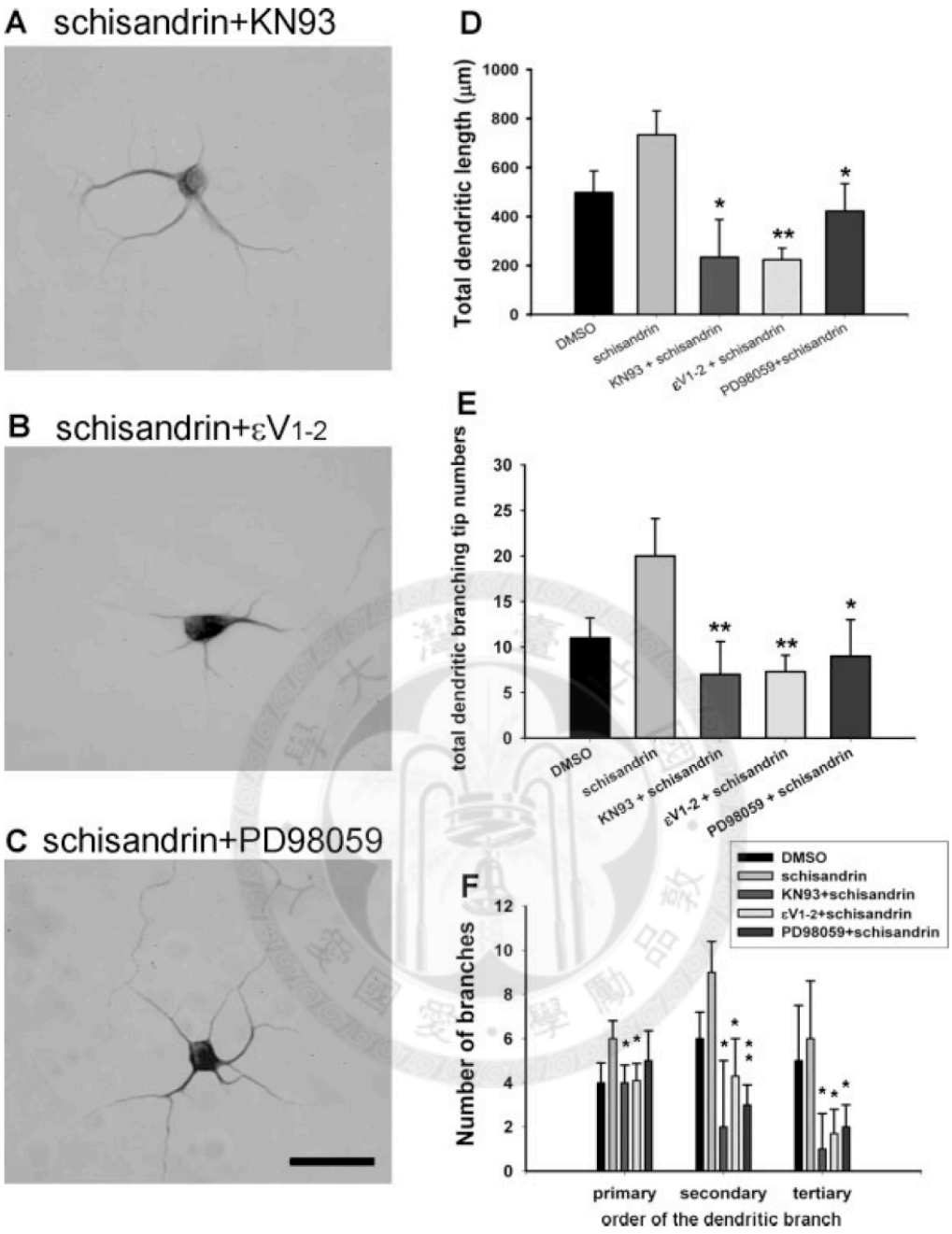

Fig 2. Selective inhibition of schisandrin-induced dendrite outgrowth of hippocampal neurons. 3-DIV hippocampal neurons were pretreated with 10 μM KN93 (A), 3 μM $\varepsilon V_{1\text{-}2}$ (B) or 30 μM PD98059 (C) for 30 min before incubation with 1.3 μg/ml (3 μM) schisandrin for 24 h and then immunostained for MAP2. Scale bar: 30 μm. D-F, Quantitative analyses of the effects of different kinase inhibitors on schisandrin-induced dendritic length (D), dendritic branching tip numbers (E), and numbers of different orders of dendritic branching (F). n = 30. * $p < 0.05$; ** $p < 0.01$, compared to the schisandrin-treated group.

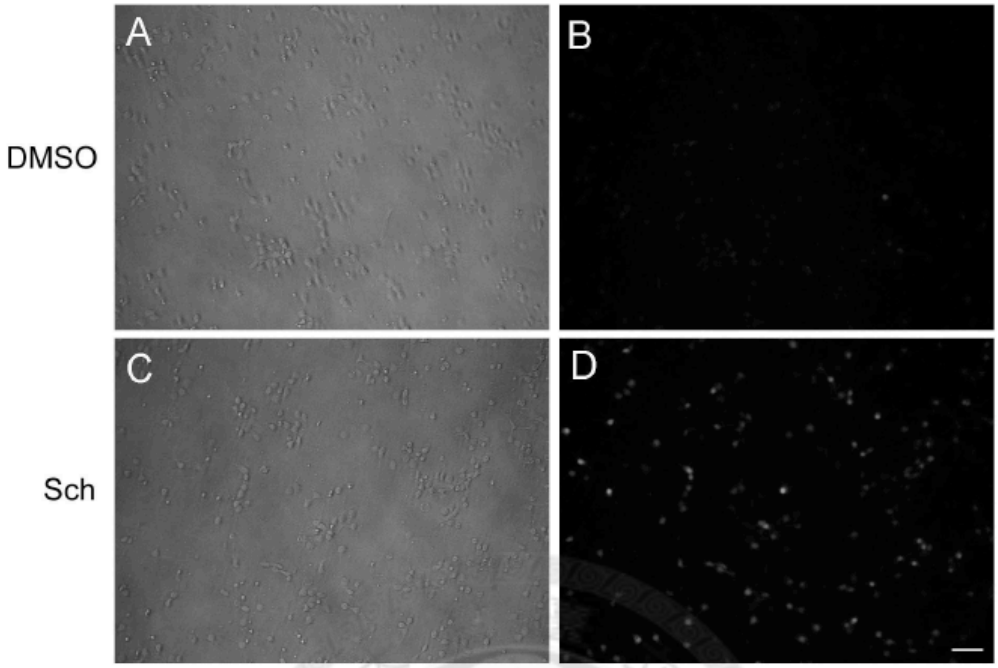

Fig 3. Schisandrin increases intracellular calcium levels. Hippocampal neurons were loaded with fluo-3/AM, then treated for 0 and 10 min with 0.1% DMSO (A, B) or 1.3 μg/ml (3 μM) schisandrin (C, D). A, C: Phase images of B and D, respectively. **B, D**: Fluorescent images. Bar = 50 μm.

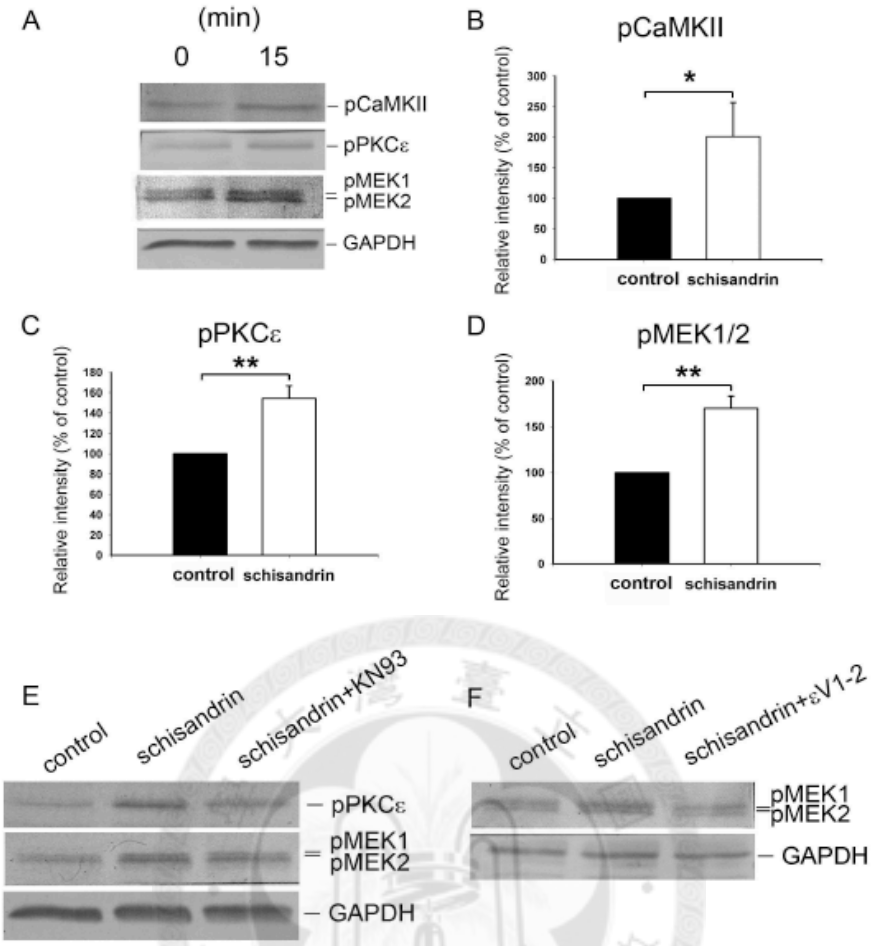

Fig 4. Activation of CaMKII, PKCε, and MEK1/2 by schisandrin and the effects of selective inhibitors on schisandrin-induced PKCε and MEK1/2 phosphorylation. A, 3-DIV hippocampal neurons were treated with 1.3 μg/ml (3 μM) schisandrin for 0 or 15 min, and then total cell lysates were collected and analyzed by Western blotting for pCaMKII, pPKCε, pMEK 1/2, and GAPDH (an internal control). Representative blots of three independent experiments are shown. The bar graphs represent the densitometric scans of triplicate blots for pCaMKII (B), pPKCε (C), and pMEK 1/2 (D). * $p < 0.05$; ** $p < 0.01$, compared to the DMSO-treated group. (E), Neurons were treated with 1.3 μg/ml (3 μM) schisandrin in the absence or presence of 10 μM KN93. Total cell lysates were analyzed by Western blotting for MEK 1/2, PKCε, and GAPDH. (F), Neurons were treated with schisandrin in the absence or presence of 3 μM $\varepsilon V_{1\text{-}2}$ for 15 min, and then total cell lysates were analyzed by Western blotting for MEK1/2 and GAPDH. Representative blots of three independent experiments are shown.

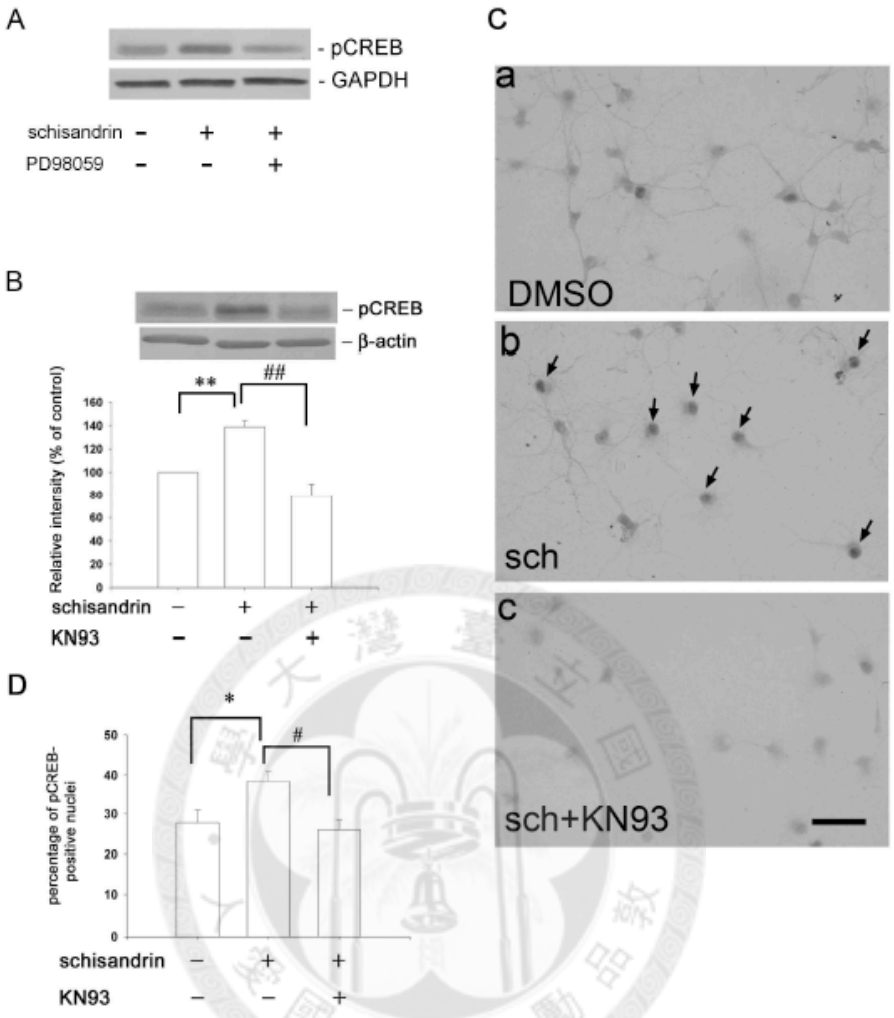

Fig 5. Schisandrin-induced CREB phosphorylation is mediated by CaMKII and MEK. 3-DIV neurons were treated with 1.3 μg/ml (3 μM) schisandrin in the absence or presence of 30 μM PD98059 (A) or 10 μM KN93 (B) for 1 h, and then total cell lysates were analyzed by Western blotting for phosphorylated CREB and β-actin (an internal control). Representative blots of three independent experiments are shown. The bar graphs represent the densitometric scans of triplicate blots for pCREB. ** $p < 0.01$, compared to the DMSO-treated group, $^{\#\#}$ $p < 0.01$, compared to the schisandrin-treated group. (C) Neurons were treated with 1.3 μg/ml (3 μM) schisandrin in the absence or presence of 10 μM KN93 for 1 h, and then immunostained for pCREB. Scale bar: 10 mm. Arrows indicate cells with pCREB-positive nuclei. (D) Quantitative analyses of cells containing pCREB-positive nuclei. n = 3. * $p < 0.01$, compared to the DMSO-treated group, # $p < 0.01$, compared to the schisandrin-treated group.

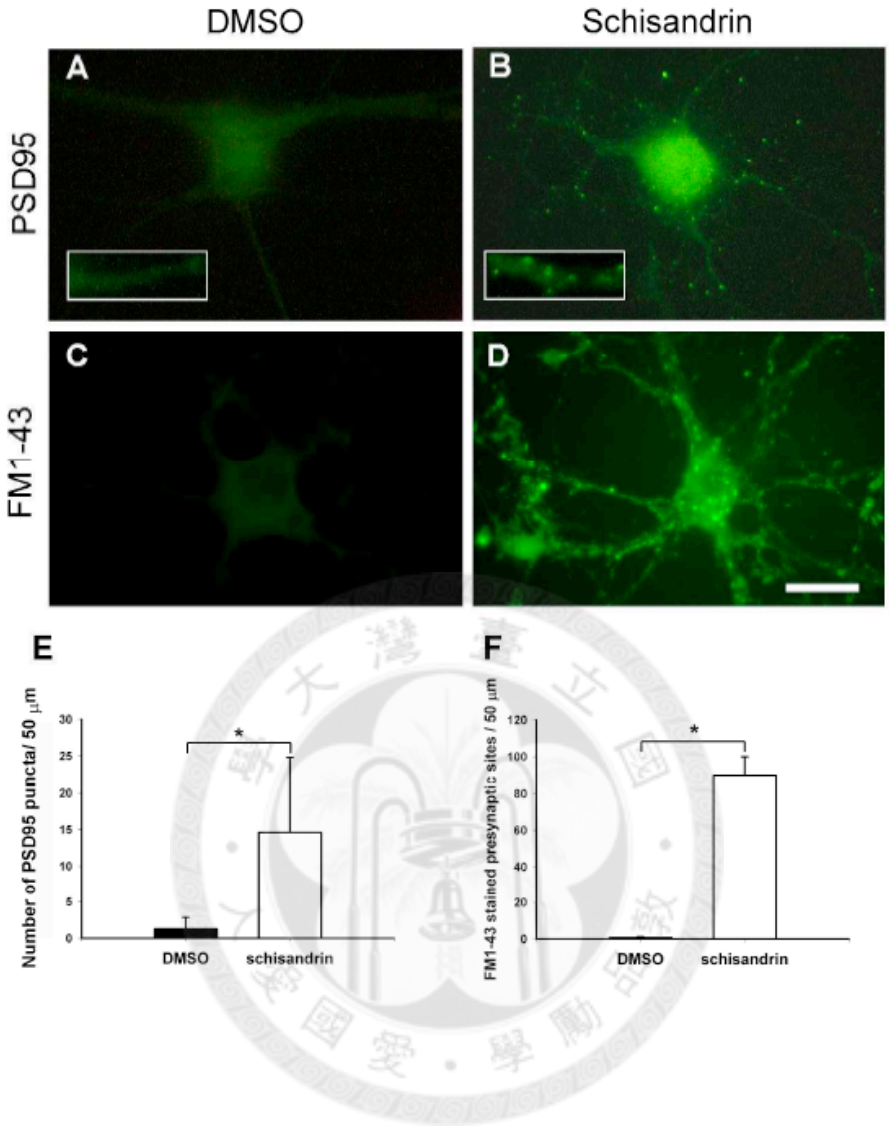

Fig 6. Effect of schisandrin on the numbers of PSD-95-positive and FM1-43-positive puncta. 5-DIV neurons were treated with 0.1% DMSO (A, C) or 1.3 μg/ml (3 μM) schisandrin (B, D) and then immunostained for PSD-95 (A, B) or processed for FM1-43 uptake (C, D), respectively. Scale bar: 30 mm. G, H: Quantitative analyses of PSD95-postive and FM1-43-positive puncta (E, F). n=3. * $p < 0.01$, compared to the DMSO-treated group.

Chapter 4. Study on the effect and mechanism of daidzein for neuritogenesis in primarily cultured rat dorsal root ganglion neurons

INTRODUCTION

Daidzein is one of the major ingredients of isoflavone, which is a compound of diverse nurtrient interest. Vegetables like soy and red clover contain high level of daidzein, genistein, biochanin A, and formononetin. These molecules are categorized as phytoestrogen due to high similarity with the mammalian endogenous hormone, estrogen, in chemical structure as well as biological activity (Ososki and Kennelly, 2003). Estrogen not only plays a vital role in reproductive and sexual organs, but also mediates diverse biochemical reactions in many other organ systems, e.g. skeletal, cardiovascular, and nervous systems. According to many clinical studies, the incidence of dementia, stroke, Parkinson's disease, and other nervous systems diseases is lower in woman than in man; the severity of these disorders is also less in the female gender. Therefore, it has been postulated that estrogen hormone is able to protect and repair neural tissue in various disease conditions (Behl, 2002, Brann et al., 2007, Dhandapani and Brann, 2002, Lephart et al., 2004). Furthermore, researches using animal models suggest that estrogen can enhance anti-oxidative activity of neuronal cells (Behl et al., 1995), improve neurocognitive function and memory deficit, and reduce volume of infarcted brain tissue following ischemic stroke (Ping et al., 2008). However, clinical use of estrogen for the prevention and treatment of post-menopausal syndrome increases the risk of breast cancer and cardiovascular disease in the woman population. On the other hand, phytoestrogen acts mainly as an estrogen agonist but may function as an estrogen antagonist in certain tissues and organs (Hwang et al., 2006). This explains the

fact that phytoestrogen does not increase the risk of breast cancer, and actually could prevent its occurrence (Gikas and Mokbel, 2005, Hedelin et al., 2008, Verheus et al., 2007). If phytoestrogen also possesses neurotrophic and neuroprotective functions, it would be more suitable for clinical use in the management of nervous system illness than estrogen. Previous studies on this subject have showed that phytoestrogen could ameliorate the neurotoxitiy of amyloid beta proteins in cortical neurons, and MPP (1-methyl-4-phenyl pyridium) in PC-12 cells (Gikas and Mokbel, 2005, Wang et al., 2001). Hypoxia induced apoptosis of cultured hippocampal neuron was prevented by the treatment of genisterin and daidzein (Schreihofer, 2005), which were also capable of stimulating neuritic outgrowth in PC-12 cells and hippocampal neurons (Miller et al., 1993, Wang et al., 2008). However, other researches suggest that daidzein and genistein had only modest neuroprotective function but little neuritogenic effect (Zhao et al., 2002). High concentrations of these molecules might paradoxically cause cell damage (Jin et al., 2007). These conflicting results very likely stem from the difference of experimental conditions, e.g. molecular structure and concentration of used phytoestrogens, neuronal cell types, cultural conditions, etc. Therefore, whether daidzein and other phytoestrogen could enhance neurite outgrowth and improve neuronal survival awaits further investigation.

Estrogen, daidzein and other phytoestrogens belong to the family of steroid hormone. Among the various estrogen molecules, 17-β estradiol is the most biologically active (Kuiper et al., 1998). Estrogen and phytoestrogen bind to intracellular estrogen receptors (ER) to trigger downstream signal transduction pathways and achieve various biological functions (Cheskis et al., 2007). Many parts of the nervous system have ER, including cerebral cortex, hippocampus, amygdala, hypothalamus, brainstem, spinal

cord, dorsal root ganglion, etc (Behl, 2002, Patrone et al., 1999). In comparison with 17-β estradiol, daidzein has one five hundredth affinity for estrogen recptor alpha, and a hundredth affinity for estrogen receptor beta (Kuiper et al., 1998). Since the optimal concentration of 17 β-estradiol for neurotprotective and neuritogenic effect lies in the range from 10 nM to 100 nM, the desired concentration of daidzein is estimated to be around 10 μM. Human beings who had food preference for soy and other vegetables enriched with isoflavones can have daidzein concentration up to 1200 to 2800 ng/ml (5 to 10 μM) in their blood and cerebral cortex tissues (Lephart et al., 2004). Therefore, effective amount of daidzein might be achievable by food intake only. If daidzein could be proved to provide neuroprotective or neuritogenic activity, its use in the treatment nervous system would have a major impact in clinical medicine.

The signaling pathways of estrogen have been well elucidated. As a steroid hormone, estrogen binds with intracellular ER-α and ER-β receptors, which when activated function as transcription factors by binding with estrogen responsive element in DNA molecules (Heldring et al., 2007). This so called genomic pathway will control the expression of many genes related to neurotrophism and neuroprotection, including neurotrophic factors (brain derived neurotrophic factor, neurotrophic-3, neurotrophic-4) and their receptors, apoptotic proteins (Bcl-2, Bcl-x), cell adhesion molecules, cytoskeleton proteins, synapse related proteins, stress response proteins, etc (Manthey and Behl, 2006). Besides the genomic pathway, estrogen can act via a non-genomic pathway by interacting with intracellular ER-α and β receptors, and cell membrane G-protein coupled receptor 30 (GPR-30) (Coleman and Smith, 2001, Lu et al., 2009, Mhyre and Dorsa, 2006). The signaling transduction pathways include protein kinase C (PKC), phosphoinositol 3-kinase (PI3K), and mitogen-activated protein

kinase/extracellular signal-regulated kinase kinase (MEK)-extracellular signal-related kinase (ERK)- cyclic AMP-response element binding protein (CREB), and activate related kinases, cytoskeletal proteins (Segars and Driggers, 2002). Finally, the molecular structure of estrogen molecule consists of benzene rings, which is associated with antioxidant activity by acting as a free radical scavenger (Dhandapani and Brann, 2002, Prokai and Simpkins, 2007). Increased expression of antioxidant proteins by estrogen also contribute to its neuroprotective effect (Behl, 2002). It is intriguing to see if daidzein acts through the same or similar pathways.

A few studies have addressed the issue of daidzein's signal transduction in various cell types. In cultured osteoblastic cells, daidzein could bind cell membrane ER-beta receptor and activate G-protein subunit β, which further activate phospholipase C β2 (PLC-β2)/PKC and PI3K/cSrc pathways (Hwang et al., 2006). This resulted in phosphorylation of c-Raf-1/MEK and actin filament, and also Elk-1 and CREB which in turn regulate expression of several groups of genes for cell differentiation, proliferation, and migration. In macrophage, daidzein inhibited the activation of STAT-1 and NF-kB proteins, decreased the expression of iNOS and production of NO (Hamalainen et al., 2007). In hippocampal neurons, daidzein interacted with ER-beta receptors to increase phosphorylation of PKC-alpha and growth associated protein 43 (GAP-43), leading to axonal growth (Wang et al., 2008). In the current study, we aim to study whether daidzein could stimulate neurite outgrowth in cultured dorsal root ganglion neurons, and the underlying signaling transduction pathways.

MATERIALS and METHODS

Cell culture

Postnatal day 2 Wistar rat pups were purchased from the Facility for Animal Research of the National Taiwan University. Animal care and procedures were performed according to the standard set forth by the "Guide for the Care and the Use of Laboratory Animals", published by U.S. National Institutes of Health (NIH publication no 85-23, revised 1985). P2 rat pups was put on ice and then decapitated for harvest of the dorsal root ganglia (DRG). DRG were then dissected out under microscope and dissociated with 0.25% trypsin, 0.05% collagenase (Sigma-Aldrich) in HBSS solution, for 30 min at 37°C. These ganglia were then dispersed by mechanically trituration with glass pipettes. A pellete was obtained by low-speed centrifugation, and resuspended in phenol-red free L-15 Leibovitz media (Gibco), supplemented with 1.2 g/L of $NaHCO_3$, 5% fetal bovine serum (FBS), 100 IU/ml of penicillin, and streptomycin. Cells were plated on coverslips for immunocytochemistry, and on 35 mm culture dishes for protein quantification by Western blot. The culture medium was changed to serum free L-15 for cultured DRG cells day 2 in vitro. The cell cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO2.

Drug treatment

Daidzein (Fig. 1) was purchased from the Pharmaceutical Industry Technology and Development Center, Taiwan. Day 3 cultured DRG cells received either daidzein at a concentration of 5 μM, 10 μM, 30 μM, 50 μM, or 100 μM, or vehicle solution DMSO (0.1%), in order to study the effect of daidzein on neurite outgrowth.

For inhibitors assay, cultured DRG cells were pretreated with either estrogen receptor antagonist ICI 182780 (Tocris, Ellisville, MO, USA) at 1 μM; GPR 30 inhibitor G15 at 100 nM (Tocris); or Src kinase inhibitor PP2 (Biomol) at 10 μM; or ERK1/2 inhibitor U0126 (Biomol) at 10 μM; or PI3K inhibitor LY294002 at 10 μM (Calbiochem); or PKC inhibitor Staurosporin at 0.1μM; or PKC α/β inhibitor Go6976 at 1 μM; or PKCε inhibitor εV1-2 at 2 μM; or PKCδ inhibitor rottlerin at 2 μM, 30 minutes before the addition of daidzein.

Apoptosis and Necrosis Assays

PI vital stain was used to detect plasma membrane disruption in necrotic cells. After various treatments, cells were washed with serum-free medium, and incubated for 30 min with PI (50 μg/mL in serum-free medium) in a CO_2 incubator. After a brief wash with PBS, the cells were fixed in 5% formalin and 0.5% Triton X-100 for 10 min at room temperature, then, after three washes with PBS, were stained for 15 min with 1 μg/mL of DAPI in 0.9% NaCl and mounted in fluorescence mounting medium (70% glycerol and 2% propyl gallate in PBS). All experiments were performed on triplicate dishes and more than one hundred cells were examined per dish.

Immunocytochemistry

DRG neurons on cover glasses were fixed for 10 min with 10% formalin in phosphate buffered saline (PBS) after DMSO or daidzein treatment for 24 hours. After washed in PBS, cells were then permeablized and blocked with 0.15% Triton X-100 and 5% non-fat milk in PBS for 1hour. The neurons are then incubated in mouse anti-NF-L (Sigma-Aldrich), 1:100 dilution overnight at 4°C. After PBS wash, the cells were incubated in

biotin-conjugated goat anti-mouse IgG (Vector) at 1:50 dilution for 1 hour at room temperature, washed with PBS, then reacted with avidin-biotinylated enzyme complex (Vector) for one hour at room temperature. Following PBS wash, staining was done with peroxidase-chromagen reaction (SG substrate kit, Vector), which was stopped by Tris-buffered saline (TBS). The coverslips were then dehydrated by ethanol and xylene, and mounted with Permount. Images were taken on a light microscope, equipped with a Nikon DIX digital camera (Nikon, Tokyo, Japan).

Western blotting

After various treatment, the cultured DRG neurons were homogenized in ice-cold lysis buffer solution (10 mM EGTA, 2 mM $MgCl_2$, 0.15% Triton X-100, 60 mM PIPES, 25 mM HEPES, pH 6.9, containing 1 μM phenylmethylshlfonyl fluoride, 1 μM NaF, 1 μg/ml of leupepsin and 1 μg/ml pepstatin) and sonicated. A 3-fold volume of 4X reducing SDS sample buffer was added to each lysate and boiled at 95°C for 5min. Fifty microgram of protein from each sample (protein concentration determined by Bio-Rad protein Kit, Bio-Rad Lab, CA, USA) were separated by 10% polyacrylamide-SDS gel electrophoresis, electrotransferred to nitrocellulose filter (Schleicher and Schuell, NH, USA), blocked by Tris-buffered saline (TBS: 50 mM Tris-Base, 150 mM NaCl, pH8.2) containing 5% non-fat milk and 0.1% Tween-20, and then incubated overnight at 4°C with the following primary antibodies: anti-pERK (Sigma-Aldrich) at 1:1000 dilution; anti-pPKCδ (Epitomics) at 1:500 dilution; anti-pCREB (Cell Signaling) at 1:500 dilution; anti-pSrc (Cell Signaling) at 1: 500 dilution; anti-GAPDH (Sigma-Aldrich) at 1:1000 dilution. Following washes with TBST (TBS containing 0.1% Tween-20), alkaline phosphatase conjugated secondary antibodies at 1:7500 dilution (Promega,

Madison, WI, USA) were added for an hour at room temperature, and the bound antibodies visualized using enzyme-substrate reaction (substrate: 3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-base, pH 9.5).

Quantification

Immunostained neurons were photographed at 10X magnification, and the images transformed into 256 gray scale images. The total neurite length was then measured from the somata using a PC-based image analyzer software Image Pro 3.0 Plus (Media Cybernetics, MD, USA). The signal intensity of bands stained on immunoblot was quantified with Gel pro 3.1 (Media Cybernetics). Student's t-test was used for evaluating statistical differences between the means of different groups, with p value of less than 0.05 considered significant.

RESULTS

Enhanced neuritogenesis by daidzein treatment in cultured DRG neurons

We first studied the effect of daidzein on neurite outgrowth in primary rat DRG neuronal cultures. DIV 3 Neurons were incubated with different concentrations of daidzein, DMSO (negative control group), or NGF (positive control groups) for 24 hours. Qualitatively, the extension and branching of DRG neurons was enhanced by daidzein treatment at high concentration, as well as NGF treatment, when compared with DMSO treatment (Fig. 2). The average length and tip numbers per neuron were calculated quantitatively, and was significantly better following treatment with 30 μM of daidzein and NGF 100 ng/ml (Fig.3). However, daidzein concentration higher than 30 μM was not as effective in promoting neurite outgrowth. Double staining with DAPI and PI was performed to identify apoptotic and necrotic cells, respectively. No apparent cell death was seen under our cultural condition with DMSO or daidzein 30 μM (Fig. 4).

DRG neurons can be classified into small and large neurons. Small neurons have a diameter less than 13.8 μm (area < 600 μm2) and carry unmyelinated fibers for pain sensation, and large nerons a diameter larger than 13.8 μm (area greater than 600 μm2) and send myelinated axons for vibration and joint position sense (Obata et al., 2004). Both types of neurons responded favorably to 30 μM of daidzein (Fig. 4). Further study for the neuritogenic effect of daidzein was conducted at a concentration of 30 μM.

Blocking of the neuritogenetic effect of daidzein by Src and ERK inhibitors, but not estrogen receptor (ER) antagonists

To investigate the signaling mechanism involved in the neuritogenic effect of daidzein, various inhibitors were applied to DRG neuronal cultures before and during daidzein treatment. Though daidzein is structurally similar to estrogen and previous reports of activation of ER by daidzein in other cell culture systems, the ER α/β inhibitor ICI 182780 and membrane ER G-protein 30 receptor (GPR-30) did not block the neurite lengthening and branching by daidzein in our cultured DRG neurons (Fig. 5). On the other hand, inhibition of the Src by PP2 and MEK by U0126 resulted in significant decrease of the increased lengthening and branching of neurites by daidzein in our system (Fig. 6).

Blocking of the neuritogenetic effect of daidzein by PKCδ inhibitor

To further investigate the signaling mechanism involved in the neuritogenic effect of daidzein, general and subtype PKC inhibitors were applied to DRG neuronal cultures before and during daidzein treatment. The pan-PKC inhibitor, staurosporin, blocked the neurite lengthening and branching of our cultured DRG neurons by daidzein. While inhibition of the PKC subtypes α, β, ε did not affect enhanced neurite outgrowth by daidzein, the PKCδ inhibitor rottlerin significantly blocked daidzein's neuritogenic effect (Figs. 7 and 8).

This suggested potential roles of Src, PKCδ, and ERK in the sigaling casade of daidzein on DRG neurons, and prompted further studies using kinase assay by immunoblotting.

Activation of Src kinase by daidzein treatment

We next examined whether daidzein could activate the Src signaling pathway using Western blotting with activation of Src being evaluated using antibodies against

phospho-Tyrosine 416-Src . Phosphorylated form of Src kinase increased to 146.6 ± 11.0 %, 129.9 ± 26.3%, or 117 ± 23.7% of baseline levels after 15, 30, or 60 min of daidzein treatment, respectively. Level of pSrc increased after 15 min and 30 min significantly after daidzein treatment (Fig. 9).

Blocking of the daidzein-induced PKCδ activation by a Src kinase inhibitor, and daidzein-induced ERK activation by Src and PKCδ inhibitors

Finally, we examined whether Src inhibitor could block the ERK kinase activation caused by daidzein treatment. The pERK-1 and pERK-2 levels were increased, respectively, to 121.5 ± 12.1% and 121.3 ± 26.5% of baseline levels after 60 min of daidzein treatment, and the values were decreased to 78.2 ± 17.1% and 64.4 ± 18.6% of baseline levels in cells pretreated for 30 min with PP2 before daidzein treatment (Fig. 11). Therefore, the Src kinase inhibitor PP2 significantly inhibited the activation of ERK by daidzein.

We next examined whether the PKCδ inhibitor could block the ERK kinase activation caused by daidzein treatment. The pERK-1 and pERK-2 levels were increased, respectively, to 125.3 ± 15.2% and 111.8 ± 14.7% of baseline levels after 60 min of daidzein treatment, and the values were decreased to 53.8 ± 22.4% and 45.2 ± 18.4% of baseline levels in cells pretreated for 30 min with rottlerin before daidzein treatment (Fig. 11). Therefore, the PKCδ inhibitor rottlerin also significantly inhibited the activation of ERK by daidzein.

DISCUSSION

The current study demonstrated that daidzein was able to enhance neurite lengthening and branching in rat DRG neuronal cultures. While higher concentration of daidzein conferred greater effect to promote neurite outgrowth, the maximal effect occurred at 30 μM of daidzein, beyond which showed no further benefit. Regarding the signaling transducion pathway, we discovered that daidzein mediated through Src-PKCδ-ERK to achieve its neuriotgenic effect. Interestingly, daidzein didn't act through the estrogen recetpor despite its structural similarity to estrogen molecules.

Daidzein and many other phytoestrogen molecules have been intensively investigated for their biochemical effects and molecular mechanisms on broad categories of tissues and organ systems, including the nervous system. For neuronal cells, estrogen is able to provide neuroprotective and neurotrophic functions, so phytoestrogen are expected to have similar effects. One study investigated the effect of estrogen, daidzein and several other phytoestrogens on cultured rat hippocampal neurons (Zhao et al., 2002). To avoid potential toxic effect, the concentration of daidzein was limited only up to 10 times the concentration of estrogen. Thus, the maximal concentration of daidzein tested was 1 μg/ml (3.9 μM) for neuroprotection, and 100 ng/ml (0.39 μM) for neurotrophism. While 17β-estradiol stimulated neurite outgrowth and protected neurons from neurotoxins, daidzein did not promote neuritogenesis or enhance neuronal survival. Using relative binding assay of cellular extracts, the affinity of daidzein for estrogen receptor was estimated to be several hundred times lower than estrogen (Kuiper et al., 1998). Therefore, adequate amount of daidzein would be required to achieve biological activities through the estrogen receptor pathways. Studies using daidzein at a much

higher concentration demonstrated daidzein promoted neurite extension and protected neuronal death for hippocampal and other neuronal cell types. The optimal concentration of daidzein was 30 μM for hippocampal neurons, 30 to 40 μM for DRG neurons, 20 μM for cerebellar granular neurons, and 10 μM for cortical neurons (Ma et al., 2010, Wang et al., 2008). Our result showed that daidzein increased neurite lengthening and branching for DRG neurons in a dose dependent manner up to 30 μM, which was in accordance with the results of previously published reports. We also examined the neuritogenic effect of daidzein and NGF, and found the neuritogenic effect of 30 μM of daidzein was comparable to that of daidzein. This indicates a true robust neuritogenic property of daidzein. Meanwhile, daidzein did not affect DRG neuronal cell survival at 30 μM, since no neurotoxins were used in our culture system. When daidzein concentration was higher than 30 μM, decreased neuritogenesis was observed (Jin et al., 2007). This is likely due to toxic effect of daidzein at concentrations equal to or higher than 50 μM. How to achieve an adequate concentration of daidzein in nervous tissues is a major issue. Though daidzein seemed to be able to cross the blood brain barrier, it can only reach the range of micromolar concentration in serum for healthy human subjects by high oral intake of soymilk or related foods rich in isoflavones, instead of the optimal tens of micromolar concentration required for beneficial effects in neurons (Gu et al., 2005, Nielsen and Williamson, 2007). It remains to be determined if daidzein can reach higher concentration by oral ingestion of isoflavones containing food in patients suffering acute neurological diseases like stroke or spinal cord injury. These conditions often associate with disruption of blood brain barrier and may facilitate further accumulation of daidzein. Delivery by venous or CSF

route is another alternative to achieve adequate amount of daidzein, but will need further safety tests before clinical application.

Daidzein has been shown to promote neurite outgrowth in hippocampal neurons and induce rapid signaling in osteoclast cells via estrogen receptor β (de Wilde et al., 2006, Wang et al., 2008). Estrogen receptors are widely expressed in the central and peripheral nervous systems, including DRG neurons. Researches using imunocytochemistry and in situ hybridization demonstrated that both the estrogen receptor α and β were present in DRG of both adult and newborn rats, as well as chick embryo as early as day 8.5 (Cui and Goldstein, 2000, Papka and Storey-Workley, 2002). Traditionally ER-α and ER-β receptors were found to locate mainly in the nucleus, inside which the binding of these receptors with estrogen further activated genomic pathways. This resulted in expression of a magnitude of genes including neurofilaments, NGF receptor TrkA, and antiapoptotic molecules, which further enhance neuronal survival and neuritic outgrowth for DRG neurons (Patrone et al., 1999, Scoville et al., 1997, Sohrabji et al., 1994). A recently discovered, third kind of estrogen receptor, g-protein receptor 30 (GPR-30), was also expressed in DRG neurons (Takanami et al., 2010). In contrast to previous studies, our result revealed that promotion of neurite outgrowth by daidzein did not mediate through ER α/β or GPR-30, as pretreatment of DRG neurons with ICI 182780 and G-15 did not block the neuritogenesis induced by daidzein. In one study, daidzein induced arginase 1 (Arg1) expression to counteract inhibition of neurite outgrowth by myelin associated protein (MAG) in DRG neurons, and the effect was blocked by ER antagonist fulvestrant (i.e. ICI 182780) and tamoxifen. However, 17β-estradiol did not activate gene expression of Arg1 (Ma et al., 2010). It was concluded that estrogen receptor activity was necessary but insufficient to activate

the Arg1 gene promotor. Therefore, signaling pathway other than ER is present for neurite outgrowth in DRG neurons. Since the neuronal culture system in the present study differs from other studies in animal age and species, coating materials of coverslips, and ingredients of growth medium, it is possible that ER activation was not required for neuritogenesis by daidzein in our system.

Cortical and cerebellar granular neurons growing on non-permissive substrate that contained myelin associated glycoprotein (MAG), daidzein enhanced neurite outgrowth by estrogen receptor activation. The effect required expression of arginase 1 (Arg1), a protein that has been shown to reverse neurite outgrowth inhibition by myelin proteins in DRG neurons. Interestingly, previous report showed that Arg1 was upregulated cAMP-PKA-CREB pathway (Gao et al., 2004), but daidzein did not activate through this way to increase Arg1. Among other signaling pathways involved in the process of DRG neurite outgrowth, Src kinase were activated by various molecules like NGF, laminin, artemin, and anti-Thy-1 antibody (Jeong et al., 2008, Tucker et al., 2008, Yang et al., 2008). Downstream signaling of Src included MEK/ERK and PI3K/Akt pathway. In our cultured DRG neurons growing on a permissive substrate of collagen, daidzein increased phosphorylation of Src kinase and ERK1/ERK2. While inhibition of Src kinase by pp2 and ERK1/2 by U0126 abolished the neuritogenic effect of daidzein, inhibition of Akt by LY294002 had no effect. The increased phosphorylation of ERK was blocked by the Src kinase inhibitor, PP2. This result indicated that promotion of neurite outgrowth by daidzein required signal transduction via the Src-ERK pathway. As phosphorylated ERK could activate CREB, Cdk5, GAP-43 and other neuritogenic related genes, the Src-ERK pathway activated by daidzein could lead to neurite outgrowth (Minano et al., 2008, Harada et al., 2001, Gundimeda et al., 2010). In

addition, we found that PKCδ also played a role in the signaling cascade, as daidzein increased phosphorylation of PKCδ and blocking of PKCδ by rottlerin resulted in blockage of neurite outgrowth and PKCδ activation by daidzein. Different PKC subtype isozymes, including PKCδ, have been found to be activated by neurotrophic agents. In diabetic rats, overexpression of PKCδ could ameliorate the retarded neurite outgrowth of DRG neurons (Sakaue et al., 2003). For PC12 cells, the activation of ERK by neuritogenic agents fibroblast growth factor (FGF) and NGF was dependent on PKCδ (Corbit et al., 1999). Besides, the activity of PKCδ phosphorylation could be activated by Src kinases in salivary and PC12 cells (Benes and Soltoff, 2001). In our cultured DRG neurons, the activation of PKCδ and ERK were inhibited by Src kinase inhibitor PP2 and PKCδ inhibitior rottlerin, respectively. The result implicated that daidzein increased neurite outgrowth by signaling transduction through Src-PKCδ-ERK pathway. Additional works will be needed to elucidate how these signaling pathways in daidzein induced neuritogenesis.

In conclusion, daidzein enhanced neurite outgrowth of cultured rat DRG neurons by activating src-PKCδ-ERK pathways. Since daidzein is one major constitute of soy and other isoflavone rich food products, treatment of neurological injuries and diseases with this compound would be highly desired. Preliminary study using daidzein for the treatment of optic nerve injury in rats have shown promising result (Ma et al., 2010). Further studies of daidzein should be conducted in animal models and aimed at treatment of disabling neurological disorders including CNS and PNS injuries, stroke, and motor neuron diseases, how to achieve and maintain adequate tissue levels by different methods of drug delivery, and potential toxicity and side effect.

FIGURES and LEGENDS

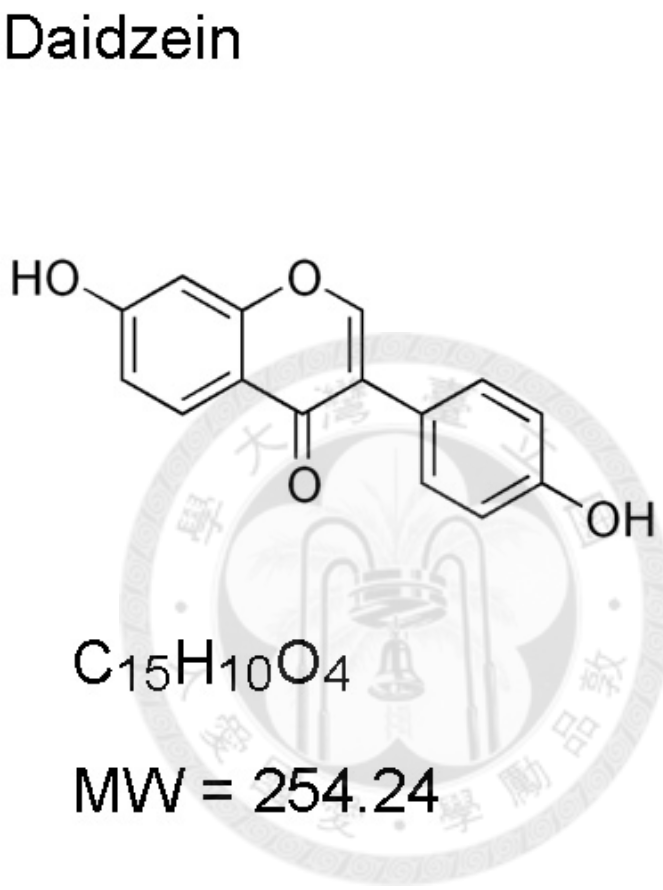

Fig 1. Molecular structure of daidzein.

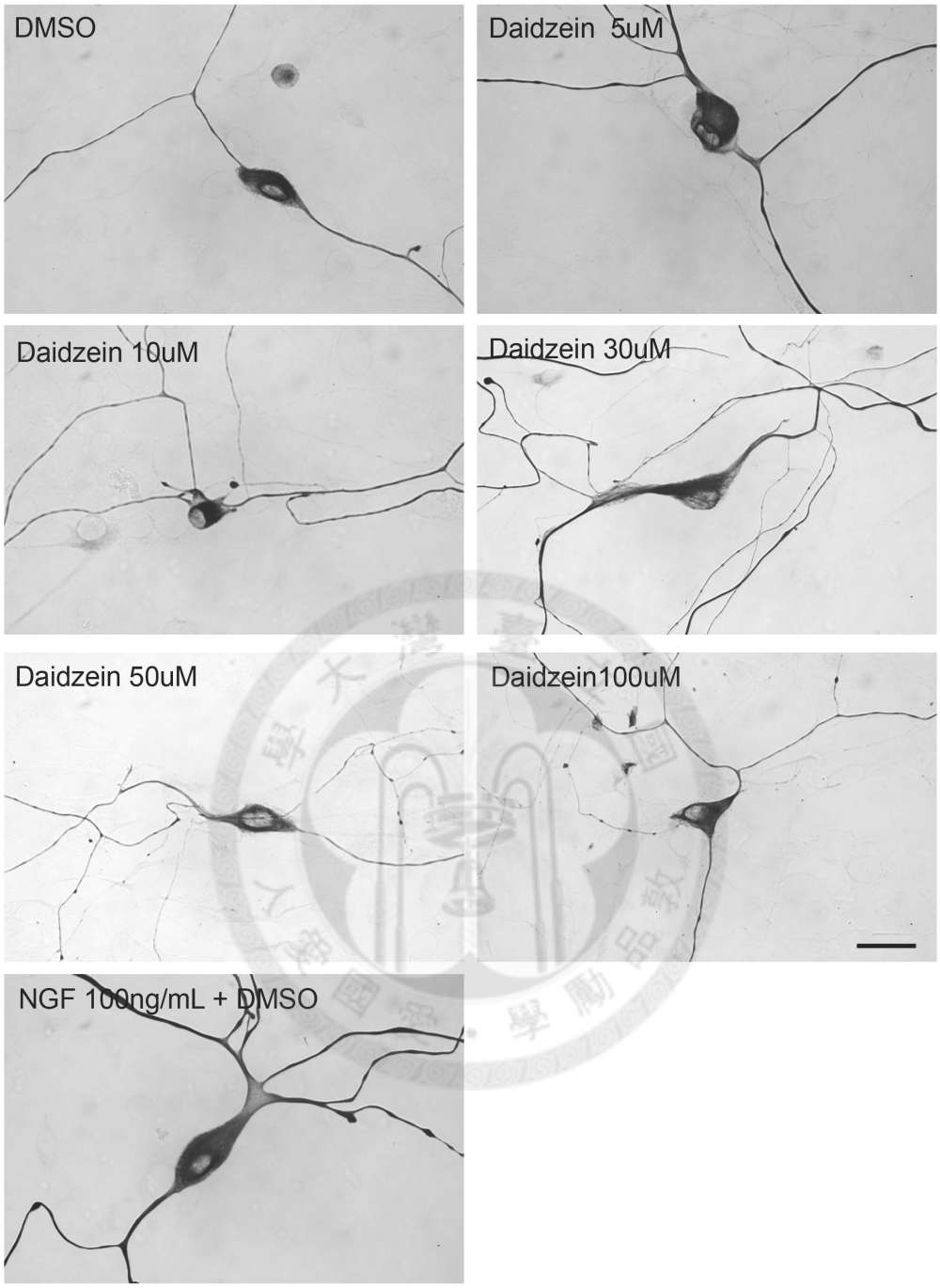

Fig 2. Induction of neurite outgrowth of rat DRG neurons by daidzein and NGF. DRG neuronal cultures were treated for 24 h with vehicle solution DMSO, various concentration of daidzein, and NGF 100 ng/ml, then immunostained for NF-L. Scale Bar = 30 μm.

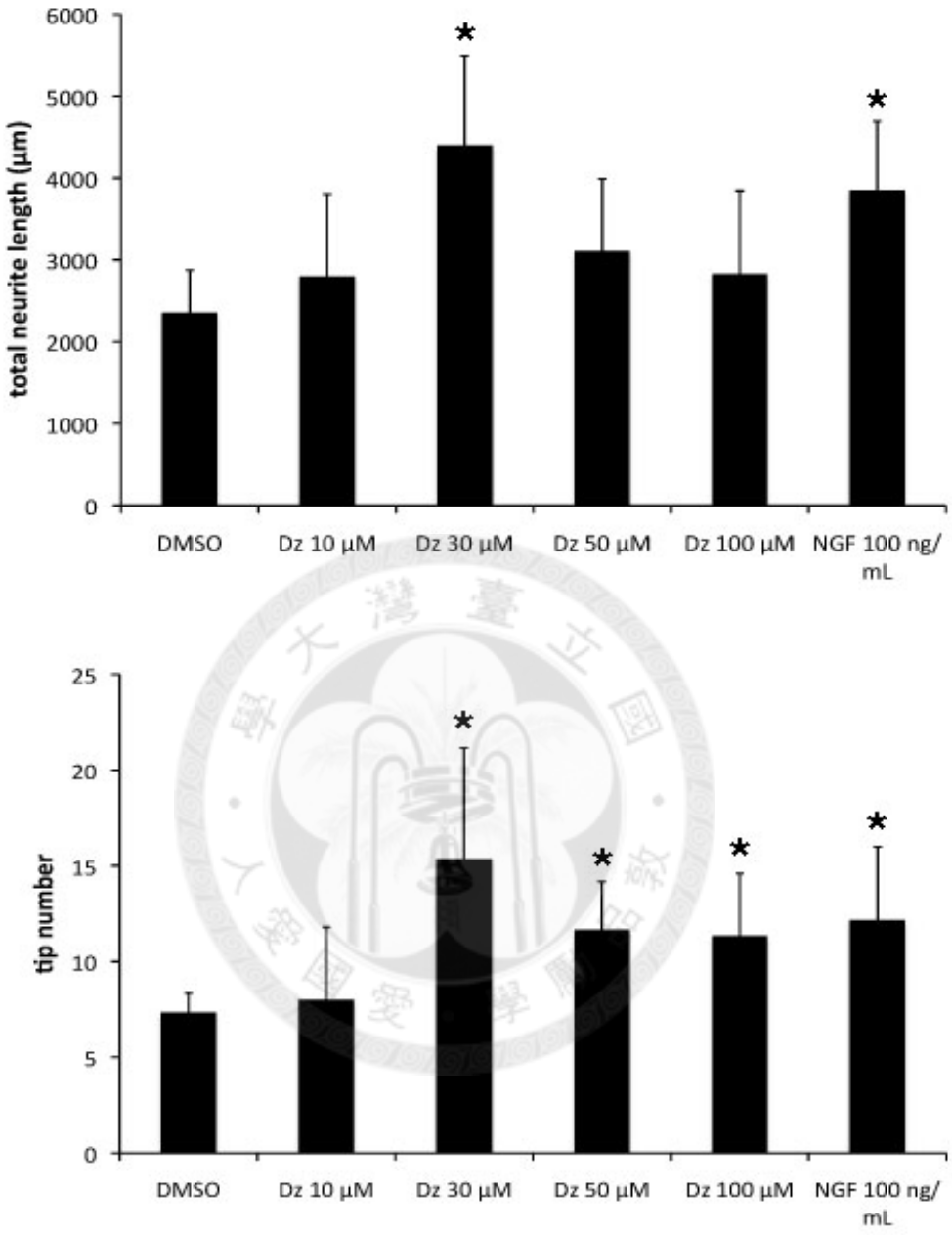

Fig 3. Quantitative analysis of total neurite length and tip number of neurite branches per neuron following various treatments by DMSO, daidzein, and NGF. Three independent experiments were performed. Six neurons were chosen from each group in one representative experiment for analysis. *, $p < 0.05$.

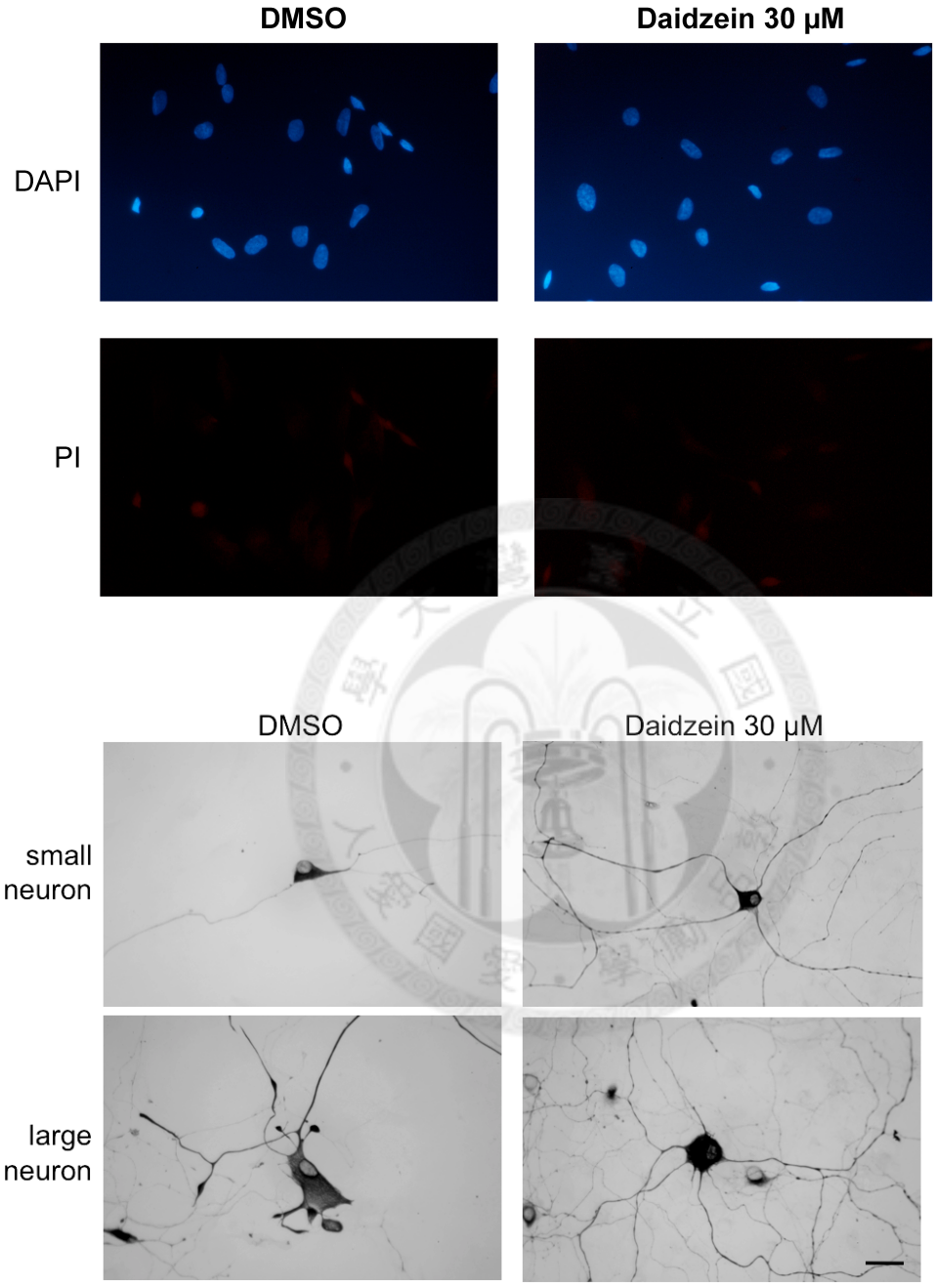

Fig 4. Upper panel: effect of drug treatment on neuronal cell survival. Cultured DRG neurons treated with DMSO or daidzein 30 μM for 6 hours were stained with DAPI and PI stain. No obvious apoptosis or necrosis of neuronal cells were present in either condition. Lower panel: response of large and small DRG neurons to daidzein 30 μM treatment. Both types of neurons showed enhanced neurite outgrowth.

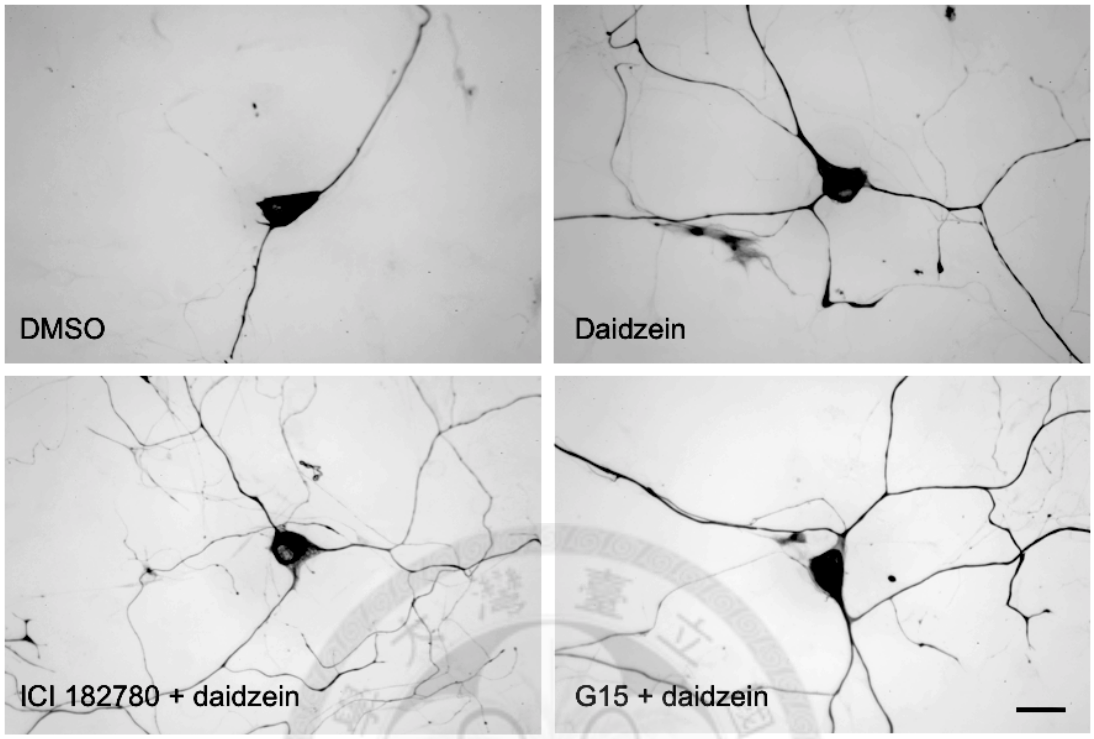

Fig 5. Induction of DRG neuronal neurite outgrowth by daidzein, and effect of the ER antagoists ICI 182780 and G15 on daidzein-induced neurite outgrowth. DRG neuronal cultures were treated for 24 h with DMSO, 30 μM of daidzein, 1 μM of ICI 182780 for 30 min before 30 μM of Daidzein treatment (Daidzein + ICI 182780), or 100 nM of G15 for 30 min before 30 μM of Daidzein treatment (Daidzein + G15), then immunostained for NF-L. Scale Bar = 30 μm. Both ER inhibitors did not block the neuritogenic effect of daidzein.

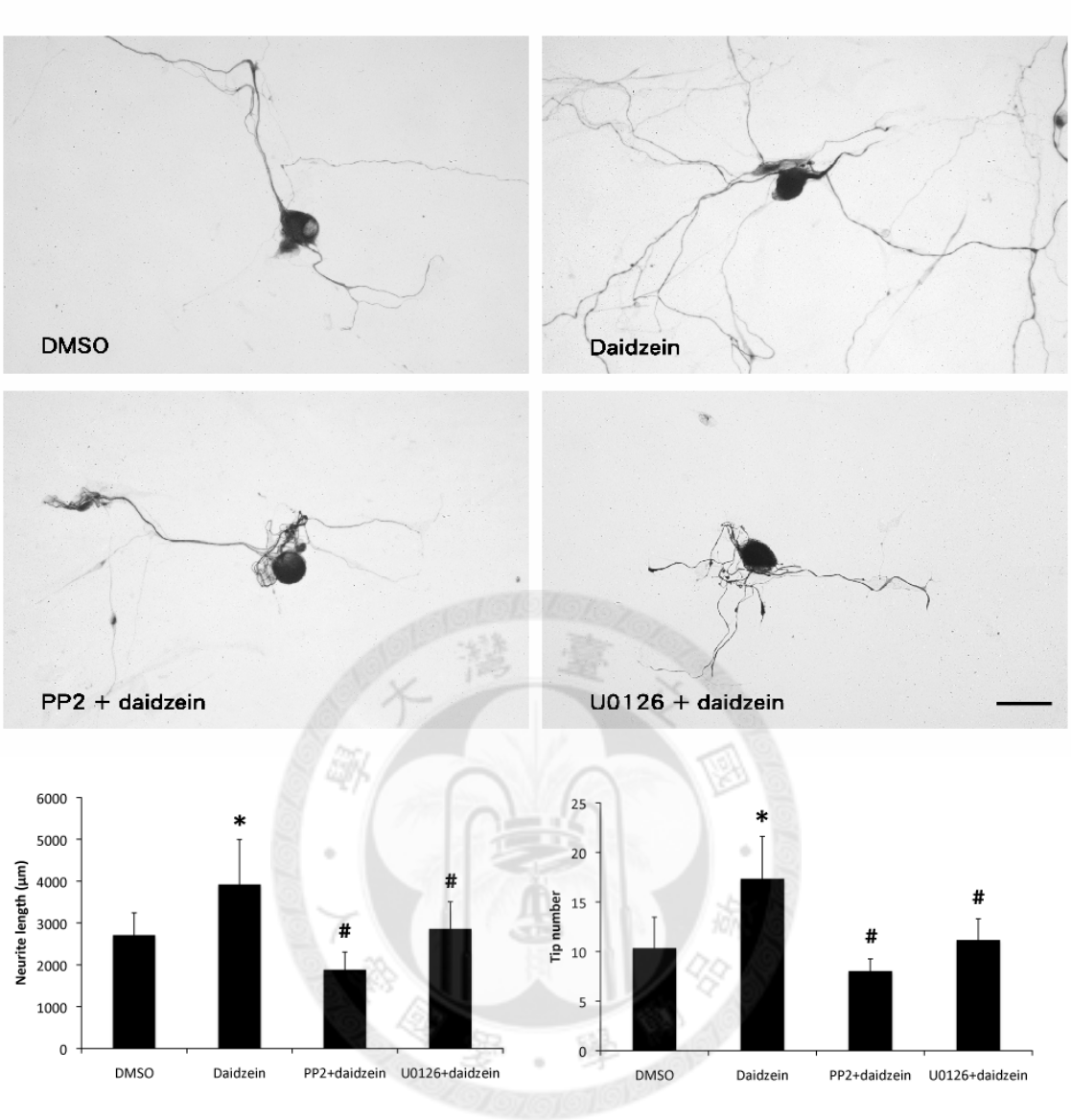

Fig 6. Blockage of neurite outgrowth of DRG neurons by the Src inhibitor PP2 and MEK inhibitor U0126 on daidzein-induced neurite outgrowth. DRG neuronal cultures were treated for 24 h with DMSO, 30 μM of daidzein, 10 μM of PP2 for 30 min before 30 μM of daidzein treatment (daidzein + PP2), or 5 μM of U0126 for 30 min before 30 μM of daidzein treatment (daidzein + U0126), then immunostained for NF-L. Scale Bar = 30 μm. Quantitative analysis of total neurite length and tip number of neurite branches per DRG neuron showed PP2 and U0126 were able to block the neuritogenic effect of daidzein. Three independent experiments were performed. Six neurons were chosen from each group in one representative experiment for analysis. *, $p < 0.05$ vs DMSO; #, $p < 0.05$ vs daidzein.

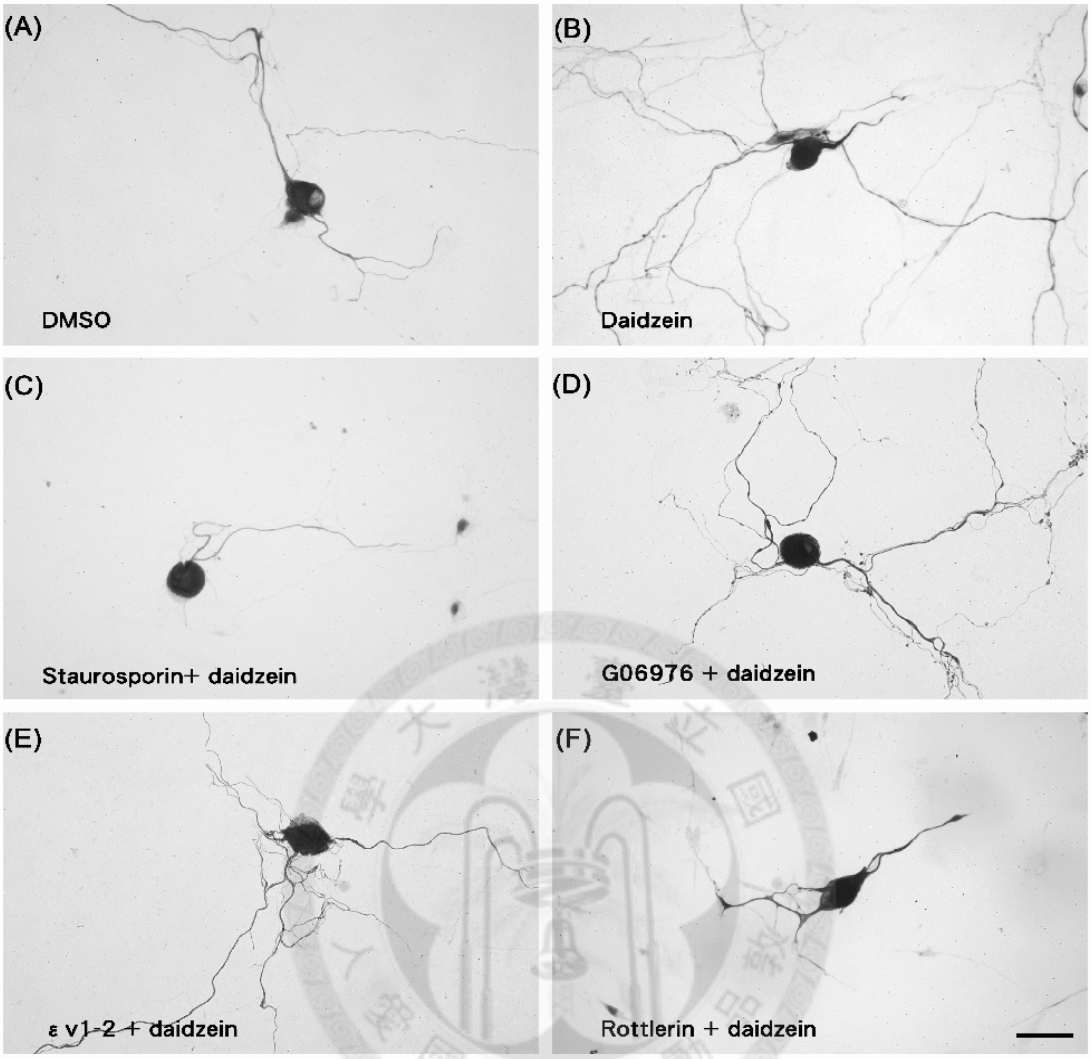

Fig 7. Blockage of neurite outgrowth of DRG neurons by various PKC inhibitors on daidzein-induced neurite outgrowth. DRG neuronal cultures were treated for 24 h with DMSO, 30 μM of daidzein, 0.1μM of pan-PKC inhibitor staurosporin for 30 min before 30 μM of daidzein treatment (Staurosporin + daidzein), 1μM of PKC α/β inhibitor Go6976 for 30 min before 30 μM of daidzein treatment (Go6976 + daidzein), 2 μM of PKCε inhibitor εVM1-2 for 30 min before 30 μM of daidzein treatment (ε v1-2 + daidzein), or 2 μM of PKCδ inhibitor rottlerin for 30 min before 30 μM of daidzein treatment (Rotterlin + daidzein), then immunostained for NF-L. Scale Bar = 30 μm. Only pretreatment of staurosporin and rottlerin effectively inhibited the neuritogenic effect of daidzein.

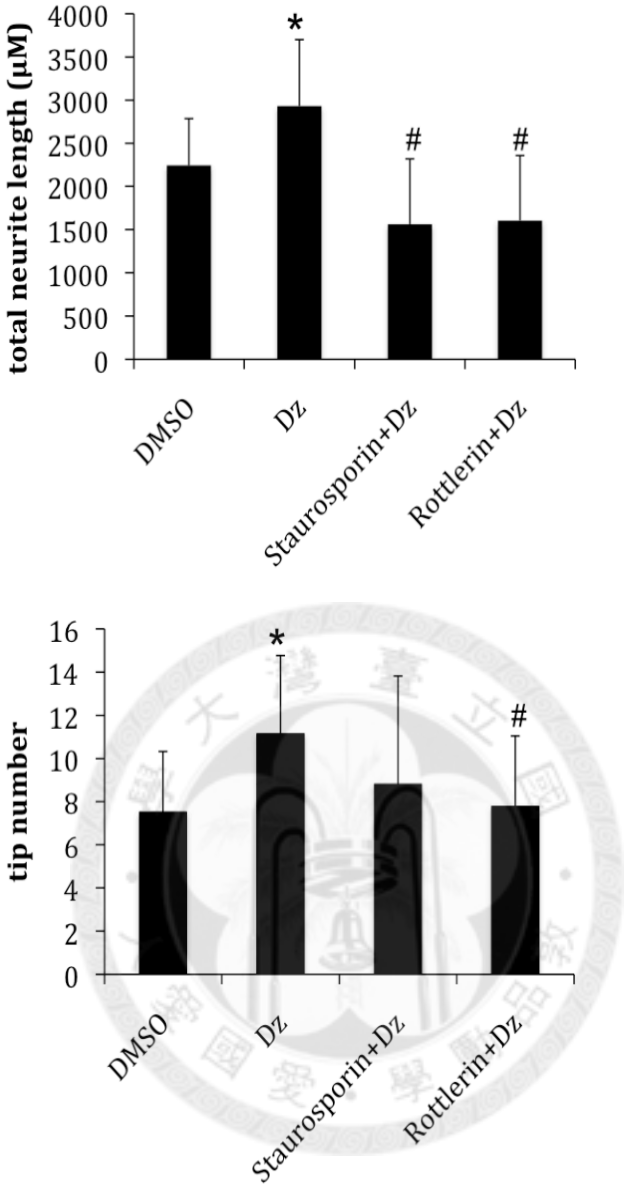

Fig 8. Quantitative analysis of total neurite length and tip number of neurite branches per neuron following various treatments by DMSO, daidzein (Dz), staurosporin pretreatment then daidzein (Staurosporin + Dz), and rottlerin pretreatment then daidzein (Rottlerin + Dz). Three independent experiments were performed. Six neurons were chosen from each group in one representative experiment for analysis. *, $p < 0.05$ vs DMSO; #, $p < 0.05$ vs daidzein.

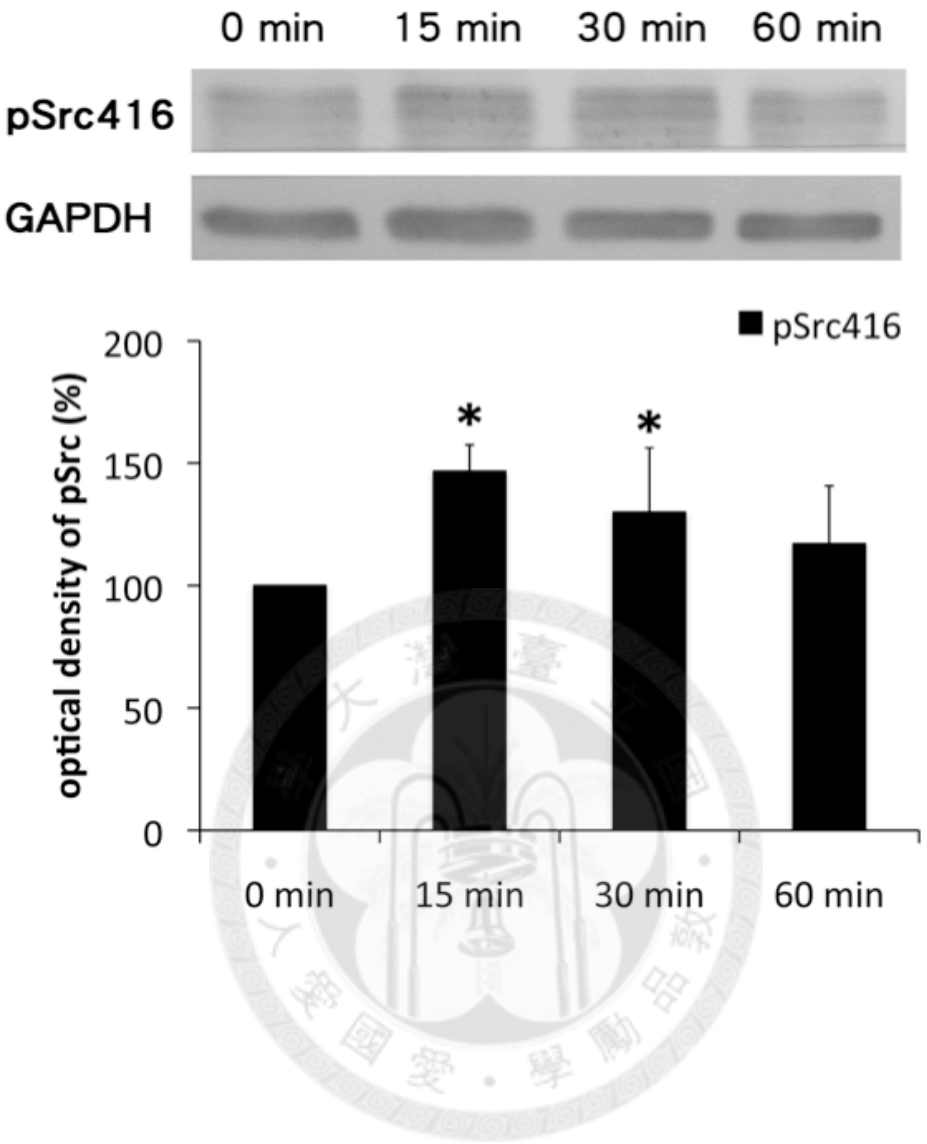

Fig 9. Phosphorylation of Src induced by daidzein analyzed by Western blot analysis. DRG neuronal cultures were treated with 30 μM of daidzein for 0, 15, 30, or 60 min, then the cell homogenate was analyzed for phosphorylated Src (pSrc416). GAPDH, loading control. Upper panel showed a representative blot from one experiment. Lower panel showed optical densities of the densitometric scans of the pSrc416 bands normalized to that of the GAPDH band. *, $p < 0.05$. $n = 4$.

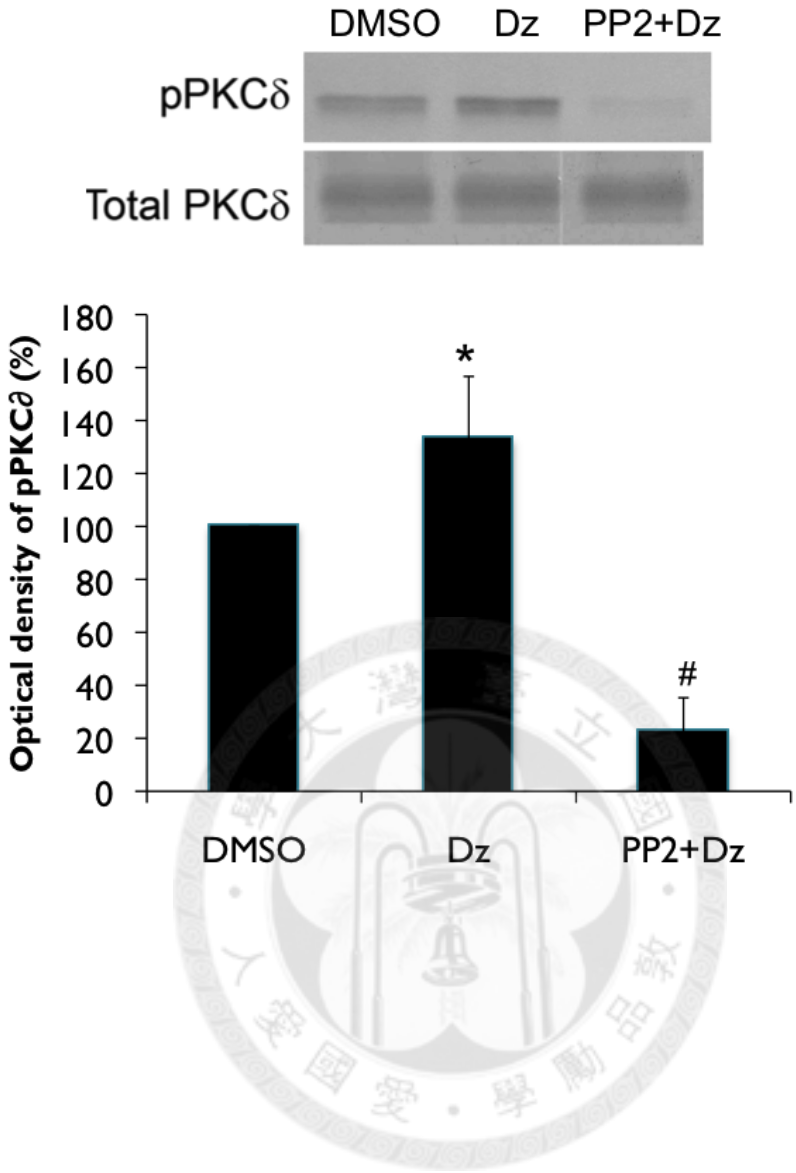

Fig 10. Effects of Src kinase inhibitor PP2 on daidzein-induced phosphorylation of PKCδ. DRG neuronal cultures were treated for 60 min with DMSO (DMSO), 60 min with daidzein (Dz), or 30 min with 10 μM of PP2 then 60 min with daidzein (PP2 + Dz) analyzed by Western blot analysis. The cell homogenate was analyzed for phosphorylated and total PKCδ. Upper panel showed a representative blot from one experiment. Lower panel showed optical densities of the densitometric scans of the PKCδ bands. *, $p < 0.05$ vs DMSO; #, $p < 0.05$ vs daidzein. $n = 4$.

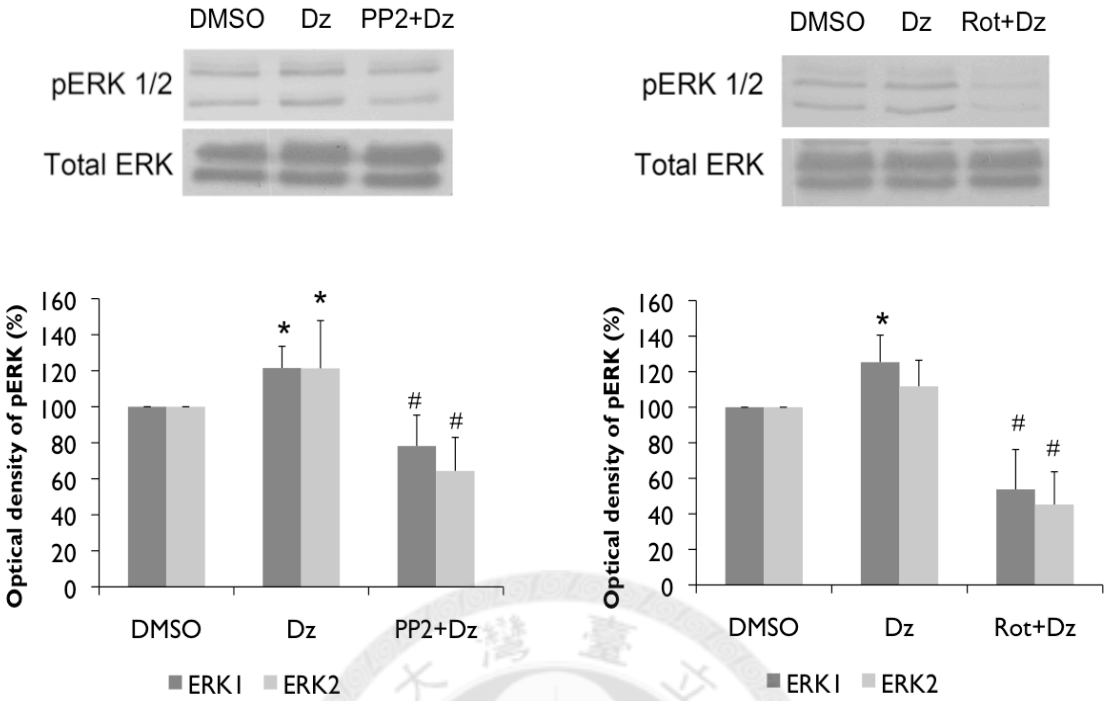

Fig 11. Effects of Src kinase inhibitor PP2 and PKCδ on daidzein-induced phosphorylation of ERK. DRG neuronal cultures were treated for 60 min with DMSO (DMSO), 60 min with daidzein (Dz), 30 min with 10 μM of PP2 then 60 min with daidzein (PP2 + Dz), or 30 min with 2 μM of rottlerin then 60 min with daidzein (Roz + Dz) analyzed by Western blot analysis. The cell homogenate was analyzed for phosphorylated and total ERK1 and ERK2. Upper panel showed a representative blot from one experiment. Lower panel showed optical densities of the densitometric scans of the ERK bands. *, $p < 0.05$ vs DMSO; #, $p < 0.05$ vs daidzein. $n = 3$.

Chapter 5. Conclusion and future perspectives

The result of our study indicated that both schisandrin and daidzein could facilitate neurite outgrowth in cultured rat neurons. In both cultured dorsal root ganglion and hippocampal neurons, schisandrin stimulated neuritogenesis. While different types of neurons from DRG and hippocampus responded differently in response to the same molecule schisandrin, the same DRG neuron also reacted differentially in response to different neurotrophic agent (schisandrin and daidzein). In contrast to previous reports, we discovered that daidzein's neuritogenic effect was not mediated through estrogen receptors. These findings illustrate the complexity of biological systems that a simple change of one variable would result in a different response, so all details need to be considered thoroughly in order to conduct a sound scientific research.

Although the signaling mechanisms of schisandrin and daidzein are elucidated, how each molecule activates its corresponding kinase cascade requires further research. For schisandrin, it activates the PI3K-Akt-CREB pathway in DRG neurons and CaMKII-PKCε-MEK-CREB pathway in hippocampal neurons, and also causes calcium inflow into both types of cells. Theoretically, calcium is required for activation of CaMK proteins, and has also been reported to activate PI3K (Danciu et al., 2003). Though the calcium inflow occurred before the beginning of kinase activation, the cause-effect relationship warrants further study by growing neurons in calcium free culture medium, or using extracellular calcium ion chelator (BAPTA) or intracellular calcium ion chelator (BAPTA/AM). For daidzein, how it turns on the function of Src kinase remained unsettled. Besides estrogen receptor, other candidate includes receptor tyrosine kinase, e.g. epidermal growth factor (EGF) receptor, hepatocyte growth factor

(HGF) receptor, and receptor tyrosine phosphatase (Goldshmit et al., 2004, Maejima et al., 2003, Yang et al., 2005). Creation of a daidzein-BSA (bovine serum albumin) construct may be the first step to study the involvement of any membrane receptor in daidzein signaling.

The positive neuritogenic effect of both schisandrin and daidzein shown in neuronal cell culture systems also warrants further study using animal models to explore their effects in neural regeneration. As the schisandrin containing herbal complex Shengmai-san facilitated regeneration of corticospinal tract in rats with spinal cord injury (Seo et al., 2009) and daidzein enhanced regrowth of retinal ganglion neuron in rats with optic nerve injury (Ma et al., 2010), it is likely that both molecules are able to have impact on neural regeneration following common diseases like stroke and spinal cord injury. However, our preliminary result didn't demonstrate behavioral or histological recovery from spinal cord injury with schisandrin treatment. Modification of the delivery route, drug dosage, and treatment duration of schisandrin will be tried to fully delineate its capacity of regeneration. Since herbs and foods containing schisandrin and daidzein have long been used in human beings and the pharmacokinectics of daidzein is well studied, these agents will be ready for clinical trials once they are proved to improve outcome after stroke or spinal injury in animal models.

Schisandrin also increase the expression of post-synaptic density and uptake of fluorescent marker into pre-synaptic terminal in hippocampal neurons. The signaling mechanism of synaptogenesis by schisandrin also requires further investigation in the neuronal culture system. The increase of synapse formation also implies a role of schisandrin in enhancing memory and learning function in normal individual as swell in

patients suffering from Alzheimer's disease and other types of dementia. Previous study showed that memory impairment due to scopolamine could be reversed by schisandrin treatment (Egashira et al., 2008). The mechanism is related to inhibition of acetylcholinesterase by schisandrin. Gamma-schisandrin was reported to reduce production of amyloid beta-protein by inhibition of gamma-secretase in M146L cells (Liu et al., 2006b). It would be interesting see if schisandrin could ameliorate memory impairment in animal model of Alzheimer's disease and how it works to achieve this effect.

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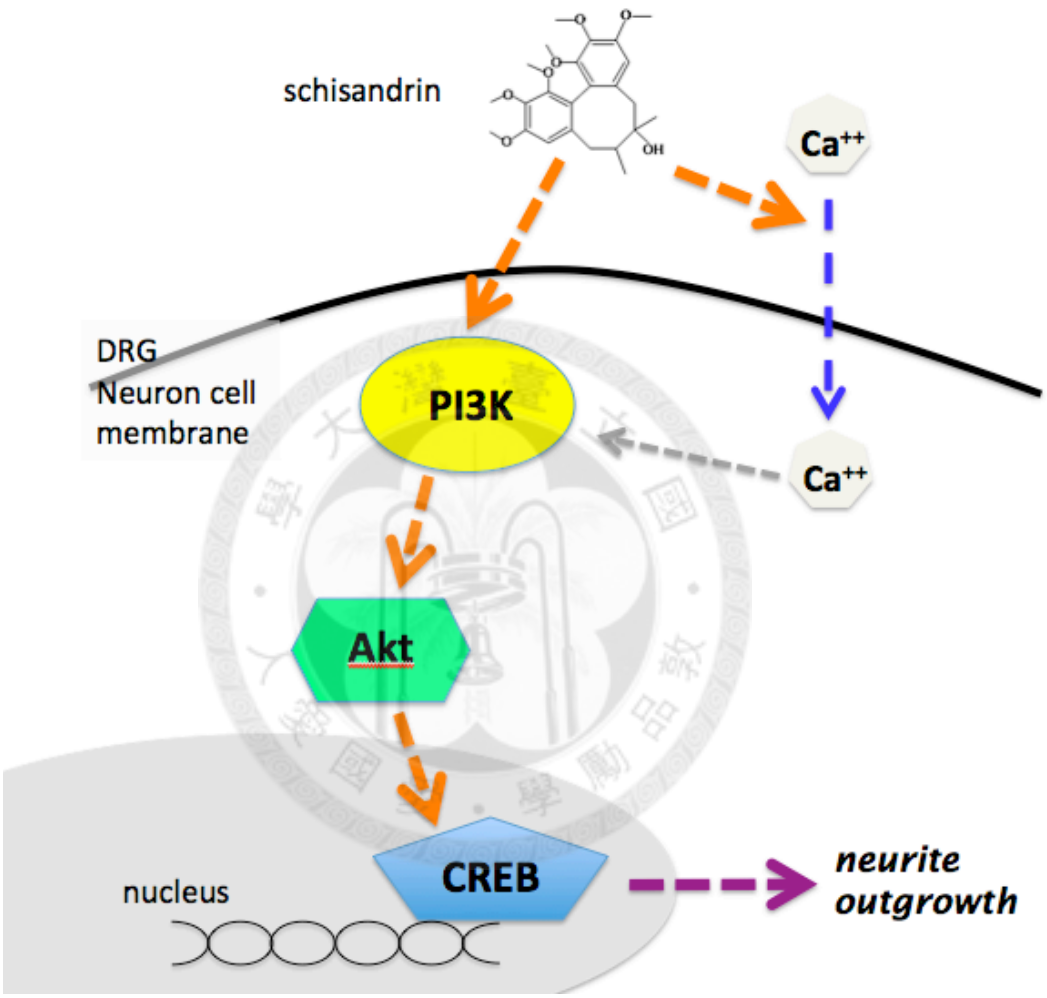
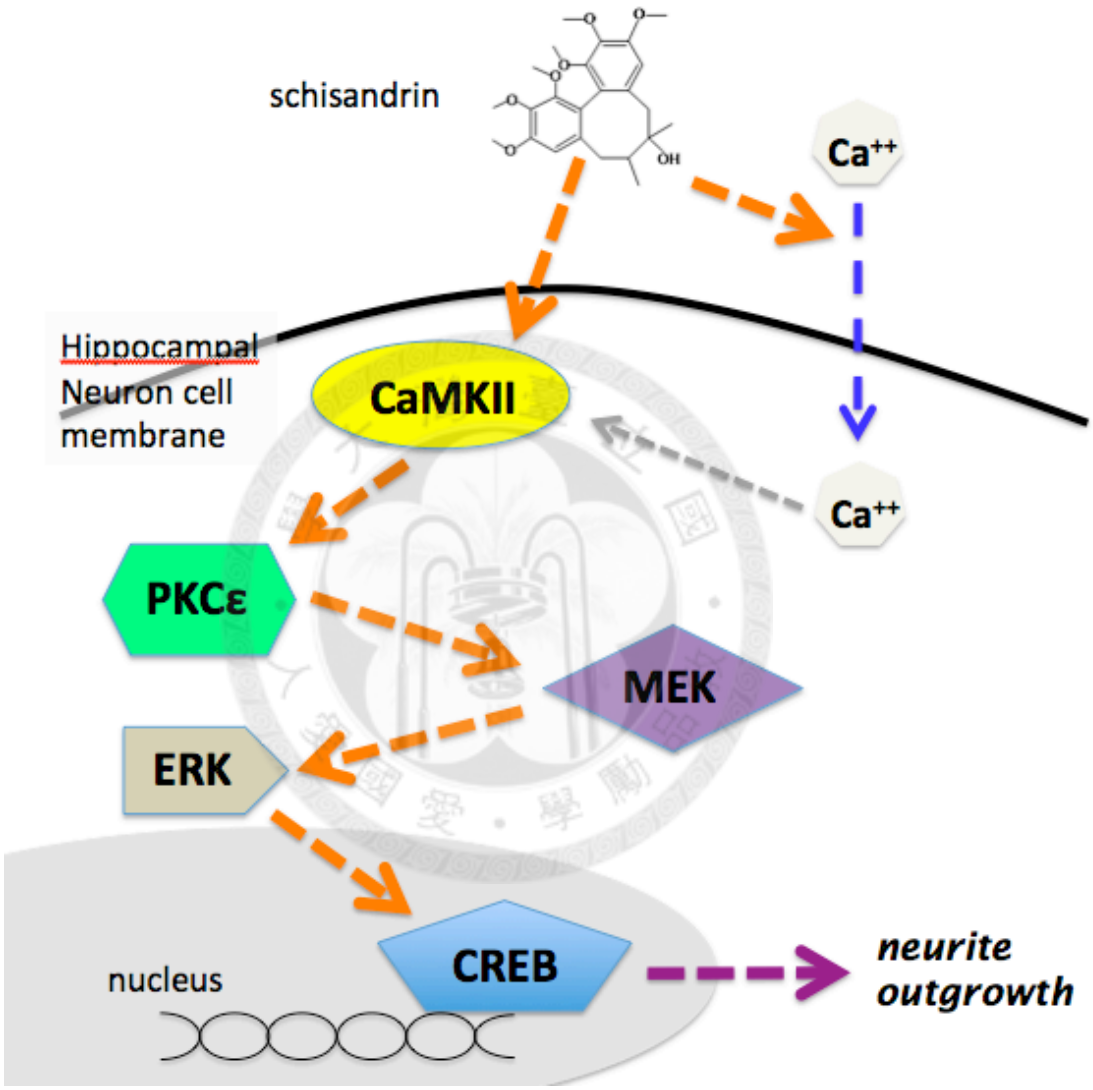
附錄

I. Signaling pathway of schisandrin-induced neurite outgrowth in cultured DRG neurons



II. Signaling pathway of schisandrin-induced neurite outgrowth in cultured Hippocampal neurons



III. Signaling pathway of daidzein-induced neurite outgrowth in cultured DRG neurons