Panaxadiol Glycosides that Induce Neuronal Differentiation in Neurosphere Stem Cells

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Received March 29, 2007

Bioassay-guided fractionation, combined with screening based on EGF-responsive neural stem cells (NSCs) differentiation assay, has been used to search for active molecules from *Panax notoginseng*. Ginsenosides Rg3 (**1**), Rk1 (**2**), and Rg5 (**3**) were identified as potential neurogenic molecules. The degrees of their neurogenic effects were found to be **3** > **2** > **1**. The neurogenic effect of **3** represents a biphasic dose- and time-dependent regulation. Transient exposure of NSCs to 8 μM **3** for 24 h followed by 1 μM and 72 h incubation was the optimal procedure for the induction of neurons in NSCs, and compound **3** resulted in an approximately 3-fold increase in neurogenesis at the expense of astrogliogenesis. The neurogenic effect of **3** was completely eliminated by the Ca^{2+} channel antagonist nifedipine. These findings imply that **3** may be utilized as a pharmacological agent in studying the molecular regulation of neurogenesis of brain stem cells and, subsequently, for treatment of neurodegenerative diseases.

Neural stem cells (NSCs) are primary progenitors that are capable of self-renewal and of differentiation into neurons, astrocytes, and oligodendrocytes in the embryonic, neonatal, and adult brain.[1,2] They may offer new prospects as therapies for disorders in the central nervous system (CNS).[3,4] Endogenous NSCs might be partially activated to proliferate, differentiate, and migrate in response to different injuries such as traumatic, ischemic, or inflammatory events.[5,6] However, spontaneous neurogenesis and gliogenesis of NSCs are not robust enough to self-repair damaged neural tissue in the adult CNS.[7,8] One of the promising strategies for the treatment of neuronal loss could be to use neural stem cell transplantation or, even better, to induce or to enhance the neurogenic potentials of NSCs *in situ*.[3,8,9] It is of great interest to identify bioactive molecules that can direct differentiation of NSCs into neuronal phenotypes. NSCs can proliferate *in vitro* in response to growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), and retain their multilineage potency to differentiate into neurons, astrocytes, and oligodendrocytes.[10,11] It has been well documented that FGF-responsive neurosphere stem cells generate neurons at high frequency, whereas EGF-responsive neurosphere stem cells (erNSCs) spontaneously differentiate to astroglial cells and have very low frequency of neuronal differentiation.[12,13] This property of erNSCs makes them a good *in vitro* pharmacological screening model to search for neuronal differentiation-promoting agents. Small molecules of natural origin have recently received attention as potential cell fate modifiers of NSCs.

The Chinese medicinal herb "Sanchi", derived from the dried roots of *Panax notoginseng* (Burk.) F. H. Chen, has been used for the treatment of trauma and bleeding due to various injuries. The major bioactive constituents of *P. Sanchi-ginseng* are dammarane-type triterpene saponins,[14] generally referred to as ginsenosides. The ginsenosides have been reported to have various pharmacological effects on the CNS including memory improvement,[15,16] neuritogenesis,[17,18] and neurotrophic and neuroprotective action.[19−21] The ginsenosides were also shown to possess antioxidant,[22] anti-inflammatory,[23] and estrogen-like bioactivities.[24] These findings suggested that the ginsenosides might have a beneficial effect on the damaged brain. In addition, recent studies have demonstrated that ginsenoside Rg1 promotes the proliferation of rat hippocampal progenitor cells both *in vitro* and *in vivo*.[25−27] Orally administered ginseng has been reported to enhance neurogensis of the dentate gyrus in rats,[28] although active compounds have not yet been identified. Therefore, ginsenosides, small molecules isolated from *P. Sanchi-ginseng* or *P. ginseng*, might have high potential for regulating endogenous NSCs or progenitor cells. Moreover, our previous studies showed that the total saponins from cultured *P. Sanchi-ginseng* were found to exhibit significant astrogliogenesis-promoting effects in NSCs cultures; among them, ginsenoside Rd, one of the principal components in *P. Sanchi-ginseng*, which was further characterized as an active component, promotes astrocyte differentiation of NSCs.[29] In a continuing study, we have found that a hot MeOH extract of the roots of wild *P. Sanchi-ginseng* produced a significant astrogliogenesis effect and a slight increase in the number of neurons in NSCs cultures.

The objective of the present study is to search for active components with a bioassay-guided fractionation, combined with screening based on an erNSCs differentiation assay from wild *P. Sanchi-ginseng* root, and to explore neuronal differentiation potentials of these molecules in erNSCs cultures.

Results and Discussion

The dried, powdered roots of wild *P. Sanchi-ginseng* were Soxhlet extracted with petroleum ether (PE), CH_2Cl_2, and MeOH. These extracts were tested for biological activity using the erNSCs differentiation assay, according to the scheme described in Figure 1. The PE extract was insoluble in culture medium. The CH_2Cl_2 extract (10 μg/mL) exhibited significant cytotoxic activity for erNSCs. Both were not further investigated. Meanwhile, exposure of erNSCs to the MeOH extract (many dammarane-type triterpene saponins have been characterized from the MeOH extract, so-called *Sanchi-ginseng* saponin, named SGS in the present study) at 10 μg/mL was found to result in a significant increase of $GFAP^+$ astrocytes (49.8 ± 5.4% vs 39.4 ± 1.9%, $p < 0.01$) and a slight increase of $TUJ/MAP2^+$ neuronal cells (14.2 ± 2.0% vs 10.8 ± 1.0%) (Table 1). On the other hand, immunostaining revealed that SGS-induced $TUJ/MAP2^+$ neurons displayed more complex morphological features: numerous longer neurites and more dendritic branches, compared with the controls (Supporting Information Figure S1d). These observations suggested that SGS may contain active molecules possessing the neuronal differentiation-promoting activity.

To characterize active molecules having neurogenesis-inducing activity, we performed a bioassay-guided isolation of SGS in order not to miss any active compounds. The SGS extract was subjected to normal-phase Si gel column chromatography and gave 11 fractions. Of the 11 fractions, fraction SGS-3 was found to exhibit considerable neurogenesis-promoting effects (Table 1) and was then purified successively by preparative TLC and semipreparative reversed-phase HPLC. Three main active subfractions (3B-2, 3B-3, and 3B-4) were obtained. Among them, the fraction SGS-3B-2 was a mixture, according to LC-MS analysis (unpublished data). It was not further investigated owing to the limited quantity available. Subfraction SGS-3B-3 was identified as 20(*S*)-ginsenoside

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10.1021/np070135j CCC: $37.00
Published on Web 07/31/2007

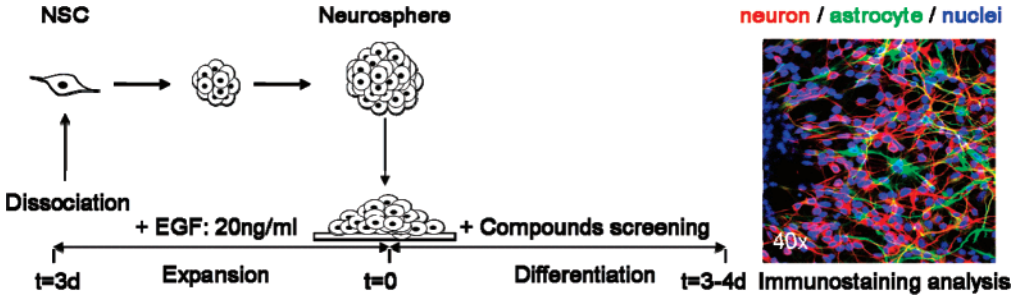


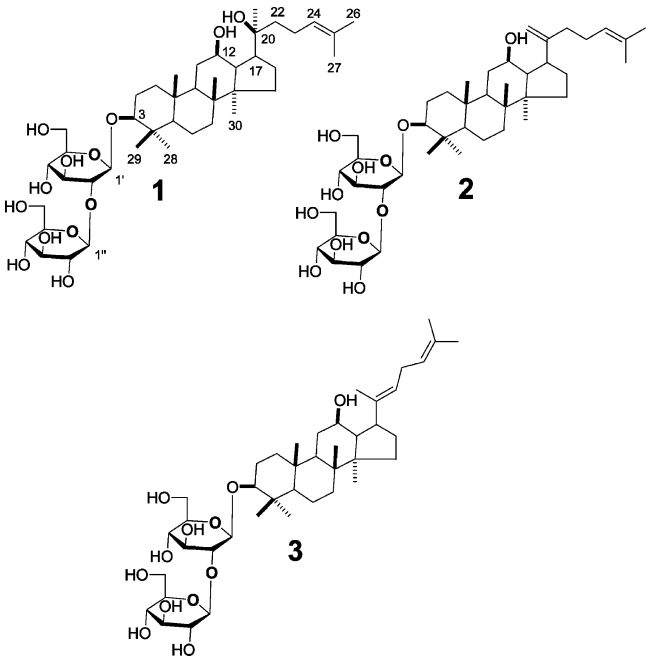
Figure 1. Schematics of a cell-based bioassay and neurosphere stem cell culture schedules. Neurospheres were generated from mouse E14.5 telencephalonic vesicles, expanded for 3 DIV in response to EGF (20 ng/mL). For differentiation assays, 50−100 neurospheres were plated onto poly-L-ornithine-coated coverslips, and differentiated for 3−4 DIV at time of tests, then fixed and analyzed with triple immunostaining for neurons (anti-TUJ/MAP2 coupled to Alexa 555, in red), astrocytes (anti-GFAP coupled to Alexa 488, in green), and nuclei (TOPRO-3, in blue).

Table 1. Neurogenesis and Astrogliogenesis Effects of Fractions and Ginsenosides in erNSCs Cultures

sample	TUJ+MAP2$^+$/ total cells	GFAP$^+$/ total cells	TOPRO$^+$ (total cells)
control	10.8 ± 1.0%	39.4 ± 1.2%	156 ± 10
RA[a]	16.5 ± 1.4%*[d]	36.3 ± 2.2%	148 ± 7
SGS[b]	14.2 ± 2.0%	49.8 ± 5.4%*	189 ± 16
SGS-3[b]	16.8 ± 1.7%*	37.5 ± 1.7%	145 ± 14
SGS-3B[b]	22.7 ± 1.7%**	31.4 ± 1.5%	147 ± 9
SGS-3B-2[b]	24.7 ± 2.8%**	26.9 ± 1.9%*	139 ± 16
Rg3 (**1**)[c]	16.1 ± 1.8%*	35.9 ± 2.7%	140 ± 12
Rk1 (**2**)[c]	28.4 ± 2.4%**	28.8 ± 2.6%*	107 ± 6*
Rg5 (**3**)[c]	30.5 ± 2.8%**	25.2 ± 2.2%*	132 ± 10

[a] RA, retinoic acid (1 μM) as internal standard. [b]SGS, *P. Sanchi-ginseng* saponins, 10 μg/mL incubation for 3 days. [c]Treatment with 8 μM for 24 h followed by 1 μM incubation for an additional 72 h. Each value represents mean ± SEM of the percentage of the marker-positive cells or total number of cells counted in 8−12 random neurospheres of each experiment; at least three independent experiments were performed. [d]Significant difference from treatment vs control, *$p < 0.01$, **$p < 0.001$, one-way ANOVA with Bonferroni−Dunn test.

Rg3 (**1**, 0.027% from dried root powder).[30,31] The most active subfraction, SGS-3B-4, was rechromatographed on RP-HPLC to afford two known compounds, ginsenoside Rk1 (**2**, 0.0042%)[32] and Rg5 (**3**, 0.0027%).[33] Their structures were identified by 1- and 2-D NMR, MS, IR, and comparisons with published data and/or authentic samples.



The neuronal differentiation potentials of compounds **1**−**3** were again evaluated by erNSCs differentiation assays. Compounds **2** and **3** exhibited significantly greater neurogenic effects than retinoic acid, an internal standard[34] (Figures 2A, 2B and Table 1). The order of neurogenic activity is **3** > **2** > **1**. As shown in Figure 2A, immunocytochemical experiments indicated that treatment with **3** resulted in a significant numerical increase of TUJ/MAP2$^+$ neurons (30.5 ± 2.8% vs 10.8 ± 1.0%, compared with the controls, $p < 0.001$) with a considerable concomitant decrease in the number of GFAP$^+$ astrocytes (25.2 ± 2.2% vs 39.4 ± 1.2%, compared with the controls, $p < 0.01$) in NSCs cultures. Exposure of NSCs to **3** at 8 μM for 24 h showed a potent neurogenic effect, without cytotoxicity, as indicated by similar total cell numbers (Table 1); however, compound **2** under the same conditions (8 μM for 24 h) showed signs of cell toxicity, as indicated by a drop in the total number of cells (107 ± 6 vs 156 ± 10, $p < 0.01$), although **2** exhibited a considerable increase in percentage of TUJ/MAP2$^+$ neurons (28.4 ± 2.4% vs 10.8 ± 1.0%, compared to the controls, $p < 0.001$, Table 1). Compound **1**, with a C-20 hydroxy group, showed less activity on the neuronal differentiation of erNSCs, compared to the dehydrated compound **2** or **3** (Table 1). This suggested that the presence of a double bond at C-20(22) may be beneficial to neuronal differentiation activity in erNSCs cultures. It is noted that ginsenoside Rd, a 20-*O*-glycoside, promoted astrocyte maturation and astrogliogenesis in erNSCs cultures,[29] in contrast to the neurogenesis-inducing activity of **3**. Thus, changes in ginsenoside structure, however small, significantly affect bioactive expression.

The neurogenic effect of **3** exhibits biphasic dose- and time-dependent response (Figure 3). First, exposure of erNSCs to different concentrations (0.1−15 μM) of **3** for 72 h revealed a dose-dependent biphasic regulation in the induction of TUJ/MAP2$^+$ neurons. At concentrations of 1 to 8 μM, compound **3** significantly promoted neuronal differentiation of erNSCs and reached its maximum level at 8 μM, whereas the effects of lower concentrations (<0.1 μM) were not statistically different from those produced by the controls (Figure 3A). At doses higher than 10 μM, there was a reversal effect. The number of TUJ/MAP2$^+$ neurons was decreased with an increase in the concentration of **3** from 10 to 15 μM (Figure 3A), which even exhibited cytotoxicity to erNSCs (Supporting Information Figure S2B). Second, a biphasic time-dependent regulation of neurogenic effects was also observed at 8 μM of **3** (Figure 3B). A 3 h pulse exposure of NSCs to 8 μM of **3**, which continued to differentiate in medium up to 72 h, did not produce a considerable increase in the number of neurons. Upon transient exposure of erNSCs to 8 μM of **3** for 12, 24, or 48 h, TUJ/MAP2$^+$ neurons were significantly induced. However, continuous exposure of erNSCs to 8 μM of **3** for 72 h, or more, induced a decrease of neurons (Figure 3B) and total number of cells (Supporting Information Figure S3B) and inhibited the development of neurite and dendritic branches (Supporting Information Figure S3C). An optimal effect was achieved when erNSCs were first transiently treated with 8 μM of **3** for 24 h, followed by 72 h of differentiation in the presence of 1 μM of **3**. Under these conditions, compound **3** resulted in enhanced neurogenesis at the expense of astrogliogenesis, as evidenced by a 3-fold increase in the number of neurons and an

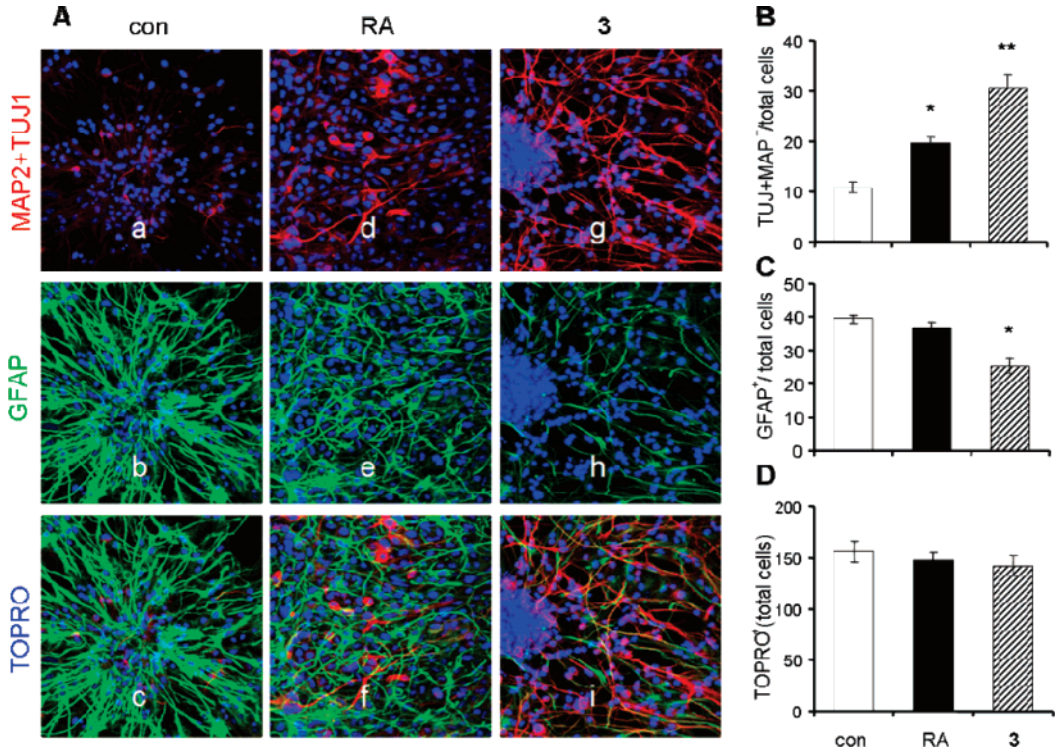


Figure 2. Compound **3** promotes neuronal differentiation and inhibits astrocyte differentiation in neurosphere stem cells. (A) Triple immunostaining of neurospheres for neurons (anti-TUJ/MAP2, a, d, and g, in red), astrocytes (anti-GFAP, b, e, and h, in green), and nuclei (TOPRO-3, in blue). Cells were treated with 0.1% EtOH (control, a−c) and retinoic acid (RA, 1 μM, d−f) for 4 days and with 8 μM of **3** for 24 h followed by 72 h incubation with 1 μM (g−i). (B−D) Quantitative analysis showed that **3** significantly induced an increase in the number of TUJ/MAP2$^+$ neurons (B) and a decrease of GFAP$^+$ astrocytes (C) without changing the total number of cells (D). Each bar represents mean ± SEM of the percentage of marker-positive cells and total cells counted in 8−12 random neurospheres of each experiment; at least three independent experiments were performed. Significant difference from **3** and retinoic acid (RA) vs control (con), *$p < 0.01$ and **$p < 0.001$. One-way ANOVA with Bonferroni−Dunn test.

approximately 2-fold decrease in astrocyte cells (Figure 2B). These data suggested that the neuronal differentiation program induced by compound **3** displayed a strong dose- and time-dependent process in the *in vitro* neurosphere differentiation bioassay.

Interestingly, after exposure of erNSCs to 5−8 μM compound **3** for 12−24 h, cells displayed the "neuron-like" morphological features under optical microscopy (Figure 4A), and this was verified by antibodies against TUJ and MAP2 [Figures 2A (g) and 4D]. TUJ/MAP2 immunstaining showed that **3** greatly enhanced the development of neurons. Numerous long neurite and dendritic branches could be observed in neuronal cells induced by compound **3** [(Figures 2A (g), 4D, and 4E], compared with those in controls (Figure 4C) and in the continuous treatment with 8 μM of **3** for 72 h (Supporting Information Figure S3C).

Recent evidence shows that expression of voltage-gated Ca^{2+} channels and Ca^{2+} influx through calcium channels is correlated with NSCs differentiation toward the neuronal lineage. Ca^{2+} influx can activate downstream signal to the nucleus, regulate gene expression, and subsequently affect cell fate.[35,36] Moreover, Ca^{2+} dynamic excitation acts to inhibit expression of the glia fate genes *HES1* and *Id2* and to up-regulate the neuronal differentiation gene *NeuroD*.[35] This led us to perform a preliminary study on the mechanism of action of **3**.

In order to examine whether neurogenesis of NSCs induced by **3** would be involved in Ca^{2+} influx, neurospheres were preincubated with the voltage-gated Ca^{2+} channel inhibitor nifedipine (5 μM) for 15 min prior to addition of **3** (8 μM). After differentiation for 4 days *in vitro*, nifedipine was surprisingly found to completely eliminate the neurogenic effect induced by **3** (29.6 ± 2.3 vs 4.3 ± 0.4, compared to **3**+nif group, $p < 0.001$) (Figures 5A and 5C). Similarly, nifedipine alone induced a drop in the percentage of TUJ/MAP2$^+$ neuron to 4.0 ± 1.2% (Figure 5C). These data indicate that Ca^{2+} influx via voltage-gated Ca^{2+} channel induced by **3** is required for its neurogenesis of NSCs. Next, to verify whether application of **3** could trigger an elevation of $[Ca^{2+}]_i$ in NSCs, we measured intracellular $[Ca^{2+}]_i$ by using the fura-2 calcium imaging technique.[37] Cells loaded with fura-2 were superfused with 5 μM of **3** for 15 min at room temperature. As shown in Figures 6A and 6B, an average elevation of $[Ca^{2+}]_i$ was observed within 10 min following perfusion of compound **3** (Figure 6A), and exposure of erNSCs to **3** produced a significant time-dependent rise of $[Ca^{2+}]_i$, expressed as the fura-2 ratio *F*350/*F*380 from 1.0 to 3.4 within 20 min. However, the intracellular dye-Ca^{2+} signal decreased after withdrawal of **3** (Figures 6A and 6B). $[Ca^{2+}]_i$ response induced by **3** was blocked by addition of nifedipine (10 μM) (Figure 6A). These data strongly indicated that application of **3** triggers calcium influx via voltage-gated calcium channels, subsequently contributing to the induction of neuronal differentiation in erNSCs. In accordance with our results, D'Ascenzo et al. have very recently demonstrated that Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels plays a key role in promoting neuronal differentiation of NSCs.[36] Moreover, it has been reported that the increased number of new neurons was observed in the dentate gyrus 1 month after a 7-day course of Ca^{2+} channel agonist treatment.[35] Again, these findings are also consistent with observations that GABA-induced excitation can trigger Ca^{2+} influx, increase the expression of *NeuroD*, and subsequently lead to neuronal phenotype commitment in progeny of the proliferating progenitors in the hippocampus.[38] Our experimental data support the notion of an excitation−neurogenesis coupling in NSCs.[39]

Ginsenoside Rg5 (**3**) is a minor component of *P. Sanchi-ginseng* and raw *ginseng* (*P. ginseng* C. A. Meyer). Several recent studies have suggested that ginsenoside Rg5 protected neurons,[40] decreased beta-amyloid peptide production,[41] and exhibited anti-inflammation activity.[42] The neurogenic activity of **3** has not been reported previously. Compound **3** selectively directed differentiation of erNSCs into neurons and inhibited astrogliogenesis in a dose- and time-dependent manner (Figures 2 and 3). Application of **3** can

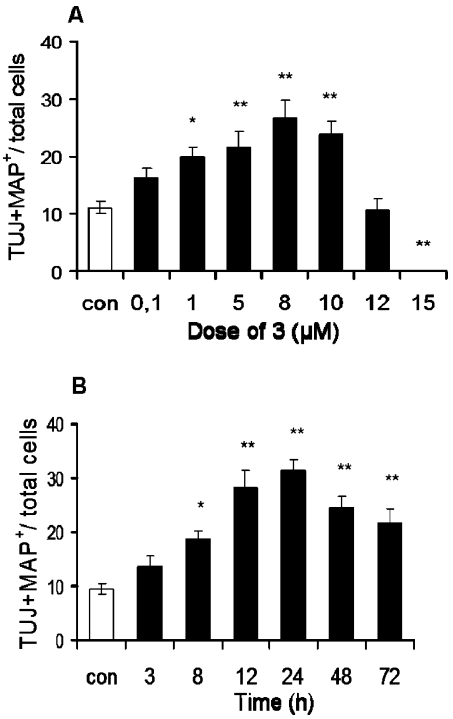


Figure 3. Biphasic dose- and time-response analysis of **3**-induced neuronal differentiation in erNSCs. (A) The addition of **3** (0.1−8 μM) for 72 h produced a dose-dependent increase in TUJ/MAP2$^+$ neurons and reaches a maximum at 8 μM. Doses higher than 10 μM of **3** for 72 h resulted in a reversal effect. (B) Treatment with **3** at 8 μM for 3, 8, 12, 24, 48, and 72 h produced a significant time-dependent increase in neurons and reached a maximum at 24 h. The quantitative data represent means ± SEM of the percentage of TUJ/MAP2$^+$ neurons in 8−12 random neurospheres of each experiment. Significant difference from **3** vs control (con), *p < 0.01, and **p < 0.001. One-way ANOVA with Bonferroni−Dunn test.

activate voltage-gated Ca^{2+} channels and give rise to elevations of intracellular $[Ca^{2+}]_i$ of NSCs (Figure 6). These finding imply that **3** may be utilized as a potential pharmacological agent and be applicable in studying signaling pathways of neurogenesis in cellular systems and animal models. Moreover, compound **3** may offer good prospects for the treatment of neurodegenerative disorders, which are characterized by neuron loss, such as in traumatic injuries and in Alzheimer's disease.

In summary, we have isolated and identified ginsenoside Rg5 (**3**) as a neurogenic molecule, which can induce neuronal differentiation in EGF-responsive neurosphere stem cells cultures. We are currently attempting to further identify the molecular basis for its activity and the biochemical mechanisms of neurogenesis induced by compound **3**.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. Melting points were determined on a micro-melting point apparatus and are uncorrected. IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer as KBr disks. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR, DEPT, COSY, NOESY, HMBC, and HSQC experiments were performed on a Bruker Avance-500 spectrometer instrument. Mass spectra were recorded on a MALDI-TOF instrument. Si gel TLC was performed on Si gel 60 F_{254} with visualization by a solution of 1% vanillin (in H_2SO_4−EtOH) and heating. Column chromatography was on Merck Si gel 60. Semipreparative HPLC was performed on a Varian Polaris HPLC system (Varian) equipped with a Polaris C8-A column (250 × 10.0 mm i.d. stainless steel, 5 μm particles; Varian). The gradient mobile phase was MeCN−H_2O (0.01% HCOOH) from 25%:75% to 20%:80% (v/v), the flow rate was 2 mL/min, and the elution was monitored with a Varian 9050 variable-wavelength UV−visible detector at 203 nm. Eluted fractions were collected and concentrated by lyophillization.

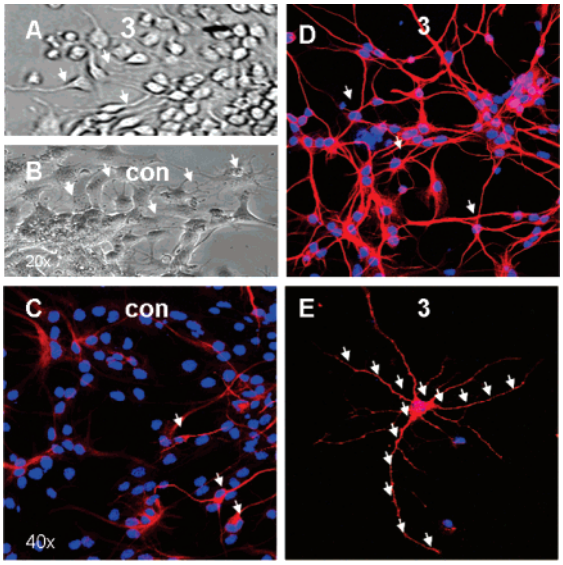


Figure 4. Neurite growth and dendritic branches of TUJ/MAP2$^+$ neurons were dramatically increased in neurosphere stem cells treated with **3**. (A, B) Phase-contrast micrographs of differentiating NSCs. After 24 h of treatment with **3**, cells displayed a neuron-like morphology (A), while a large, flat or stellate morphology was observed in the controls (con) (B). (C) TUJ/MAP2$^+$ neurons poorly developed neurite and dendritic trees in the controls. (D) Numerous long neurites could be observed in neuronal cells induced by **3**. (E) Individual TUJ/MAP2$^+$ neurons displayed very complex dendritic branches with multiple secondary and tertiary trees, relative to that seen in controls.

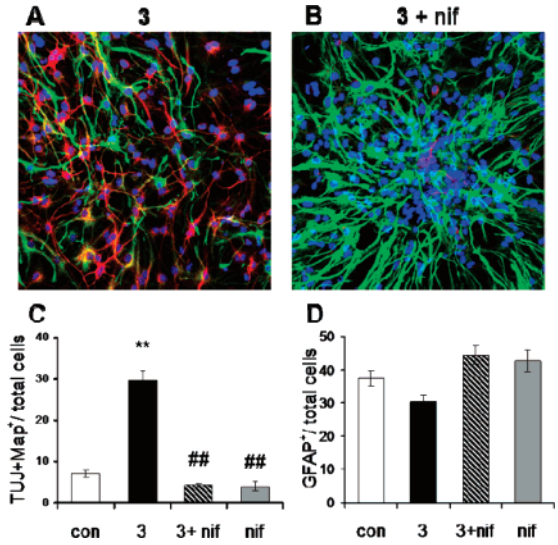


Figure 5. Neurogenesis effect of **3** was completely eliminated by nifedipine (nif) in erNSCs. Nifedipine (5 μM) was added to medium 15 min before **3** (8 μM). After 24 h treatment, the concentration of **3** was then reduced to 1 μM, and nifedipine concentration was sustained at 5 μM for additional 72 h incubation. (A, B) Representative photomicrographs of triple immonostaining in erNSCs with **3** (A) or **3**+nif treatment (B). (C, D) Quantitative analysis of TUJ/MAP2$^+$ neurons and GFAP$^+$ astrocytes over total number of cells. Each bar represents mean ± SEM of the percentage of the marker-positive cells counted in 8−12 random neurospheres of each experiments. *p < 0.01, **p < 0.001 from **3** vs control (con); ##p < 0.01 from **3** vs **3**+nif and **3** vs nif. One-way ANOVA with Bonferroni−Dunn test.

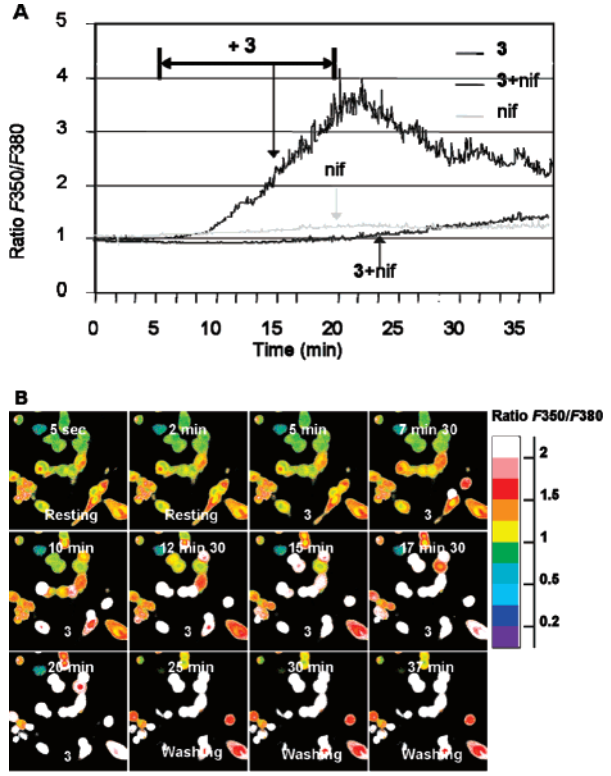


Figure 6. Compound **3** application induced an elevation of $[Ca^{2+}]_i$ in erNSCs. (A) Mean values of $[Ca^{2+}]_i$ response from a population of cells stimulated with **3** (5 μM) (**3**, $n = 24$ cells). **3**-induced $[Ca^{2+}]_i$ response is blocked by the L-type calcium channel antagonist nifedipine (nif, 10 μM) (**3**+nif, $n = 21$ cells). Graph shows fura-2 fluorescence intensity expressed as the ratio $F350/F380$; an increase in $F350/F380$ indicates a rise in $[Ca^{2+}]_i$. (B) Calcium images of the same **3**-treated cells are shown, with time running from left to right and top to bottom. The experiments were repeated three times; a representative example is shown.

Plant Material. Wild *P. Sanchi-ginseng* [*P. notoginseng* (Burk.) F. H. Chen] roots were collected in Wenshang, Yunnan Province, China, in September 2005. Voucher specimens are preserved at our institute. The samples were dried and pulverized before use.

Extraction and Bioassay-Guided Isolation. The powder of the root of *P. Sanchi-ginseng* (255.0 g) was Soxhlet extracted with petroleum ether (PE), CH_2Cl_2, and MeOH. The extracts were evaporated *in vacuo* to yield PE (0.73 g), CH_2Cl_2 (0.48 g), and MeOH extracts (31.00 g, SGS), respectively. These extracts were tested using the neurosphere stem cell differentiation assay. SGS extract was found to exhibit activity and then separated by chromatography on a normal-phase Si gel column and eluted with solvents of a CH_2Cl_2−MeOH gradient. A total of 225 fractions (50 mL/fraction) were collected; fractions with the same or similar R_f values on TLC were pooled, resulting in 11 fractions, which were also further tested for their neurogenesis activity. Fraction SGS-3 (2.0 g) was the most active, with 16.8 ± 1.7% of TUJ/MAP2$^+$ neurons at 10 μg/mL. It was further rechromatographed on preparative TLC (20 × 20, 0.5 mm) with $CHCl_3$−MeOH−H_2O (13:7:2, v/v/v) as the eluant and gave fractions SGS-3A (71.5 mg), -3B (519.2 mg), -3C (714.9 mg), and -3D (449.8 mg). Fraction SGS-3B ($R_f = 0.45$, $CHCl_3$−MeOH−H_2O = 13:7:2, v/v/v) was found to be the most active and was further purified by reversed-phase HPLC in a semipreparative system using a gradient of MeCN−H_2O (0.01% HCOOH) (from 25%: 75% to 20%:80%, v/v), affording nine subfractions, The three subfractions (3B-2, 3B-3, 3B-4) possessed significant activity; LC-MS analysis showed that fraction SGS-3B-2 was a mixture of several minor components and was not further analyzed. Subfraction SGS-3B-3 (t_R = 38.9 min; 68.0 mg, 0.027% from dried root powder) was identified as 20(*S*)-ginsenoside Rg3 (**1**).[30,31] The most active subfraction, SGS-3B-4, was further purified by RP-HPLC and afforded fraction SGS-3B-4a (t_R = 43.8 min; 10.6 mg, 0.0042%) and fraction SGS-3B-4b (t_R = 44.3 min; 7.0 mg, 0.0027%). The major component of fraction SGS-3B-4a (>90% HPLC) was identified as 3β,12β-dihydroxydammar-20-(21),24-diene-3-*O*-β-D-glucopyranosyl(1→2)-β-D-glucopyranoside (ginsenoside Rk1, **2**)[32] by comparison with an authentic sample of ginsenoside Rk1 (gift from Professor J. H. Park) and spectroscopic data. Fraction SGS-3B-4b (>98% HPLC) was identified as 3β,12β-dihydroxydammar-20(22),24-diene-3-*O*-β-D-glucopyranosyl(1→2)-β-D-glucopyranoside (ginsenoside Rg5, **3**)[33] on the basis of spectroscopic analysis by 1-D and 2-D NMR, IR, and MS.

NSCs Culture. Mouse embryonic neural stem cells were prepared as described by Reynolds and Weiss.[43,44] Briefly, embryonic telencephalonic vesicles taken from pregnant mice at gestational age 14.5 days were dissected and mechanically dissociated. Cells were then plated in 25 mL flasks (Falcon) at a cell density of 10^6/10 mL in the defined medium DMEM:F12 (1:1, v/v; Invitrogen), supplemented with 25 μg/mL insulin (Sigma), 100 μg/mL holotransferin (Sigma), 20 nM progesterone (Sigma), 60 μM putrescine (Sigma), 30 nM selenium (Sigma), 100 units/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma) with 20 ng/mL EGF (Sigma) and were cultured in a humidified (5% CO_2, 37 °C) incubator. After 7 days culturing *in vitro* (DIV), the free-floating undifferentiated cell clusters (referred to as primary neurospheres) were collected from flasks, centrifuged, resuspended, and dissociated mechanically and chemically with a cell dissociation solution (Sigma) into a single-cell suspension. The cells stimulated by EGF further formed secondary neurospheres after 3−4 DIV culture in 75 mL flasks (Falcon) precoated with poly(2-hydroxyethyl methacrylate) (1.6 mg/cm^2; Sigma), and EGF-responsive neurosphere stem cells were passaged routinely every 3−4 days, up to 30.

NSCs Differentiation Assay and Treatment. The neuronal differentiation-promoting effects of extracts and column subfractions were monitored using an *in vitro* EGF-responsive neurosphere differentiation assay. Briefly, 50−100 neurospheres per well were deposited on poly-L-ornithine-coated coverslips in 24-well plates and differentiated in the defined medium with 0.5% fetal bovine serum (FBS) and 2 ng/mL EGF for 3−4 days. Tested fractions and compounds were prepared in 100% EtOH and diluted in defined medium immediately before each experiment (final EtOH concentration was <0.1%, v/v) at different concentrations (10−20 μg/mL for extracts and less pure fractions, 5−10 μg/mL for purer fractions, 0.1−15 μM for pure compounds) with a final volume of 2 mL in each well. For dose−response experiments, cells were treated for 72 h with the following concentrations of compound **3**: 0.1, 1, 5, 8, 10, 12, and 15 μM. Time course experiments were carried out after cells were treated with 8 μM of **3** for 3, 8, 12, 24, 48, and 72 h; cells then differentiated in defined medium up to 72 h. For optimal induction procedure, cultures were treated with 8 μM of **3** for 24 h and then additionally incubated with 1 μM of **3** for 72 h. The antagonist nifedipine (5 μM) was added to neurospheres 15 min before treatment with **3** at 8 μM. Retinoic acid (1 μM) was used as a internal standard,[34] and EtOH at a concentration of 0.1% as the control.

Immunocytochemistry and Confocal Microscopy. Cells grown on poly-L-ornithine-coated coverslips were fixed for 20 min with 4% paraformaldehyde in PBS at room temperature and were subsequently permeabilized for 5 min with 0.5% Triton X-100 in PBS and blocked by applying 3% BSA for 30 min. Cells were incubated for 2 h at room temperature in PBS containing 1% BSA and the appropriate mixture of primary antibodies as follow: mouse monoclonal anti-βIII-tubulin (TUJ1, neuronal marker, 1:400; Covance); mouse monoclonal anti-microtubule-associated protein 2a+2b (MAP2, neuronal marker, 1:600; Sigma); and rabbit polyclonal anti-glia fibrillary acidic protein (GFAP, astrocyte marker, 1:1000; Dako). After cells were rinsed with PBS five times, secondary antibodies conjugated with anti-mouse Alexa 555 or anti-rabbit Alexa 488 (1:1000; Molecular Probes) in PBS containing 1% BSA were added to tested cells for 1 h in the dark. Then cells were subsequently washed in PBS three times and counterstained for 20 min with TOPRO-3 (1:10000; Molecular Probes) to identify nuclei. Finally cells were washed twice in PBS and the coverslips were mounted on the slides with Aquapolymount (Polyscience). The cell samples were viewed for triple immunofluorescence, and images were acquired under a Zeiss LSM 510 confocal microscope (Oberkochen, Germany) with a 40× oil objective lens.

Calcium Imaging in Living NSCs. $[Ca^{2+}]_i$ in erNSCs was determined by fluorescence ratio imaging of the calcium indicator dye fura-2-AM as described by de Barry.[37] After dissociation of neurospheres, cells were replated at a density of 50 000 cells/cm^2 onto poly-L-ornithine-coated coverslips and incubated for 24 h, then 2 μM fura-2-

AM was loaded for 30 min at 37 °C in Krebs-HEPES buffer (106 mM NaCl, 4.5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 11 mM d-glucose, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, pH 7.4, equilibrated with 5% CO_2). Cells were washed and incubated with Krebs-HEPES buffer for 20 min at 37 °C to remove remaining fura-2 ester. After three washes, the loaded cells were mounted in a perfusion chamber on an inverse microscope (Axiovert 35M; Zeiss). Cells were superfused with Kerbs-HEPES buffer at a flow rate of 1 mL/min. Fura-2 fluorescence was excited by a light source at 350 ± 5 and 380 ± 5 nm. The fluorescence emission was observed using a dichroic mirror at 500 nm and a long pass filter at 510 nm. Fluorescence images of $[Ca^{2+}]_i$ were acquired in real time at 5 s intervals with an EM-CCD camera (EMimage, Hamamatsu, Photonics) and analyzed with the Fluostar software (Molecular Devices). A rise in the ratio of *F*350/380 indicates a rise in $[Ca^{2+}]_i$. Compound **3** (5 μM) and nifedipine (10 μM) were applied by superfusion Krebs buffer, and cells were pretreated with nifedipine (10 μM) for 15 min prior to the perfusion with **3** (5 μM)+nifedipine (10 μM).

Cell Count and Statistical Analysis. For quantitative analysis, 8−12 randomly selected neurospheres were captured from each experiment, and at least three independent experiments were performed. Quantification of cells was based on counting the number of TOPRO-3 stained nuclei and the specific immunostained cells by using ZEISS LSM software. All experimental data were expressed as mean ± SEM. One-way ANOVA followed by the Bonferroni−Dunn test was used to assess significance of the differences between groups; differences with $p < 0.01$ were regarded as statistically significant. Analysis was conducted using StatView 5.0 software (Abacus Concepts, Inc.).

Acknowledgment. The authors are grateful to Prof. J. H. Park (Seoul National University, Korea) for a gift of ginsenoside Rk1. We wish to thank Prof. E. Marchioni (Université Louis Pasteur, Strasbourg) for providing the RP-HPLC instrument. Also we thank J. Bouissac and T. K. Tabopda for technical assistance, and X.-R. Chen, E. Mohier, L. Grandbarbe, and D. Coowar for helpful discussions. We acknowledge the laser confocal microscopy facility service of the IBMP (Strasbourg, France). We thank Prof. M. Rohmer (Université Louis Pasteur, Strasbourg) for critical reading of the manuscript.

Supporting Information Available: Supplemental data include compound characterization and three figures. These materials are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Gage, F. H. *Science* **2000**, *287*, 1433−1438.
(2) Merkle, F. T.; Alvarez-Buylla, A. *Curr. Opin. Cell Biol.* **2006**, *18*, 704−709.
(3) Weiss, S. *Nat. Biotechnol.* **1999**, *17*, 850−851.
(4) Brustle, O.; McKay, R. D. *Curr. Opin. Neurobiol.* **1996**, *6*, 688−695.
(5) Leker, R. R.; McKay, R. D. *Curr. Neurovasc. Res.* **2004**, *1*, 421−427.
(6) Arvidsson, A.; Collin, T.; Kirik, D.; Kokaia, Z.; Lindvall, O. *Nat. Med.* **2002**, *8*, 963−970.
(7) Armstrong, R. J.; Barker, R. A. *Lancet* **2001**, *358*, 1174−1176.
(8) Jagasia, R.; Song, H.; Gage, F. H.; Lie, D. C. *Trends Mol. Med.* **2006**, *12*, 400−405.
(9) Arlotta, P.; Magavi, S. S.; Macklis, J. D. *Ann. N. Y. Acad. Sci.* **2003**, *991*, 229−236.
(10) Reynolds, B. A.; Tetzlaff, W.; Weiss, S. *J. Neurosci.* **1992**, *12*, 4565−4574.
(11) Reynolds, B. A.; Weiss, S. *Science* **1992**, *255*, 1707−1710.
(12) Rao, M. S. *Anat. Rec.* **1999**, *257*, 137−148.
(13) Kuhn, H. G.; Winkler, J.; Kempermann, G.; Thal, L. J.; Gage, F. H. *J. Neurosci.* **1997**, *17*, 5820−5809.
(14) Ng, T. B. *J. Pharm. Pharmacol.* **2006**, *58*, 1007−1019.
(15) Tohda, C.; Matsumoto, N.; Zou, K.; Meselhy, M. R.; Komatsu, K. *Neuropsychopharmacology* **2004**, *29*, 860−868.
(16) Ying, Y.; Zhang, J. T.; Shi, C. Z.; Qu, Z. W.; Liu, Y. *Yao Xue Xue Bao* **1994**, *29*, 241−245.
(17) Zou, K.; Zhu, S.; Meselhy, M. R.; Tohda, C.; Cai, S.; Komatsu, K. *J. Nat. Prod.* **2002**, *65*, 1288−1292.
(18) Tohda, C.; Hashimoto, I.; Kuboyama, T.; Komatsu, K. *Neuropsychopharmacology* **2006**, *31*, 1158−1164.
(19) Rudakewich, M.; Ba, F.; Benishin, C. G. *Planta Med.* **2001**, *67*, 533−537.
(20) Salim, K. N.; McEwen, B. S.; Chao, H. M. *Brain Res. Mol. Brain Res.* **1997**, *47*, 177−182.
(21) Tian, J.; Fu, F.; Geng, M.; Jiang, Y.; Yang, J.; Jiang, W.; Wang, C.; Liu, K. *Neurosci. Lett.* **2005**, *374*, 92−97.
(22) Chen, X. C.; Zhou, Y. C.; Chen, Y.; Zhu, Y. G.; Fang, F.; Chen, L. M. *Acta. Pharmacol. Sin.* **2005**, *26*, 56−62.
(23) Rhule, A.; Navarro, S.; Smith, J. R.; Shepherd, D. M. *J. Ethnopharmacol.* **2006**, *106*, 121−108.
(24) Chan, R. Y.; Chen, W. F.; Dong, A.; Guo, D.; Wong, M. S. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 3691−3695.
(25) Shen, L. H.; Zhang, J. T. *Yao Xue Xue Bao* **2003**, *38*, 735−738.
(26) Shen, L. H.; Zhang, J. T. *Neurol. Res.* **2004**, *26*, 422−428.
(27) Cheng, Y.; Shen, L. H.; Zhang, J. T. *Acta. Pharmacol. Sin.* **2005**, *26*, 143−149.
(28) Qiao, C.; Den, R.; Kudo, K.; Yamada, K.; Takemoto, K.; Wati, H.; Kanba, S. *Neurosci. Res.* **2005**, *51*, 31−38.
(29) Shi, Q.; Hao, Q.; Bouissac, J.; Lu, Y.; Tian, S.; Luu, B. *Life Sci.* **2005**, *76*, 983−995.
(30) Kang, D. I.; Lee, J. Y.; Yang, J. Y.; Jeong, S. M.; Lee, J. H.; Nah, S. Y.; Kim, Y. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 1194−1201.
(31) Anufriev, V. P.; Malinovskaya, G. V.; Denisenko, V. A.; Uvarova, N. I.; Elyakov, G. B.; Kim, S. I.; Baek, N. I. *Carbohydr. Res.* **1997**, *304*, 179−182.
(32) Park, I. H.; Kim, N. Y.; Han, S. B.; Kim, J. M.; Kwon, S. W.; Kim, H. J.; Park, M. K.; Park, J. H. *Arch. Pharm. Res.* **2002**, *25*, 428−432.
(33) Kim, S. I.; Jeong, H. P.; Ryu, J. H.; Park, J. D.; Lee, Y. H.; Park, J. H.; Kim, T. H.; Kim, J. M.; Baek, N. I. *Arch. Pharm. Res.* **1996**, *19*, 551−553.
(34) Takahashi, J.; Palmer, T. D.; Gage, F. H. *J. Neurobiol.* **1999**, *38*, 65−81.
(35) Deisseroth, K.; Singla, S.; Toda, H.; Monje, M.; Palmer, T. D.; Malenka, R. C. *Neuron* **2004**, *42*, 535−552.
(36) D'Ascenzo, M.; Piacentini, R.; Casalbore, P.; Budoni, M.; Pallini, R.; Azzena, G. B.; Grassi, C. *Eur. J. Neurosci.* **2006**, *23*, 935−944.
(37) de Barry, J.; Janoshazi, A.; Dupont, J. L.; Procksch, O.; Chasserot-Golaz, S.; Jeromin, A.; Vitale, N. *J. Biol. Chem.* **2006**, *281*, 18098−18111.
(38) Tozuka, Y.; Fukuda, S.; Namba, T.; Seki, T.; Hisatsune, T. *Neuron* **2005**, *47*, 803−815.
(39) Deisseroth, K.; Malenka, R. C. *Neuron* **2005**, *47*, 775−777.
(40) Kang, J. S.; Yeo, S. J.; Jeong, Y. H.; Kang, J. Y.; Jeong, S. J.; Lee, K. A.; Zu, H. M.; Bao, H. Y.; Hwang, G. S.; Han, Y. N.; Lee, S. K.; Oh, K. W.; Park, J. H.; Myung, C. S.; Cho, J. S. K.R. Patent 063, 041, 2006.
(41) Landry, D.W.; Kim, T. W.; Deng, S. U.S. Patent 0,014,729, 2006.
(42) Shin, Y. W.; Bae, E. A.; Kim, D. H. *Arch. Pharm. Res.* **2006**, *29*, 685−690.
(43) Reynolds, B. A.; Weiss, S. *Dev. Biol.* **1996**, *175*, 1−13.
(44) Louis, S. A.; Reynolds, B. A. *Methods Mol. Biol.* **2005**, *290*, 265−280.

NP070135J