(--)-Stepholidine promotes proliferation and neuronal differentiation of rat embryonic striatal precursor cells in vitro

Hong Guo, Yi Yu, Lei Xing, Guo-Zhang Jin1 and Jiawei ZhouCA

Key Laboratory of Proteomics, Institute of Biochemistry and Cell Biology; 1Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, 200031, P.R. China

CA Corresponding Author: jzhou@sunm.shcnc.ac.cn

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The present study investigated the influence of (--)-stepholidine, an effective dopamine D1 receptor agonist and D2 receptor antagonist, on the development of neural precursor cells. Incubation of striatal neural precursor cells with stepholidine resulted in significant increase in the number of proliferating precursor cell spheres when in the presence of fibroblast growth factor-2. This action can be blocked by application of haloperidol. Treatment with stepholidine also increased the number of microtubule-associated protein-2-immunoreactive cells in the cultures and promoted marked increases in tyrosine hydroxylase expression. These findings suggest that stepholidine is involved in the regulation of proliferation of precursor cells. The effect appears to be mediated by dopamine receptors. Stepholidine also promotes the differentiation of precursor cells, however, this action may be independent of its effect on dopaminergic receptors. NeuroReport 13:2085–2089 © 2002 Lippincott Williams & Wilkins.

Key words: Differentiation; Neural precursor cells; Rat; Stepholidine; Tyrosine hydroxylase; Tissue culture

INTRODUCTION

In Parkinson’s disease (PD), neuronal loss in the substantia nigra and depletion of striatal dopamine (DA) levels produces characteristic symptoms. Several therapeutic strategies have been proposed, for example, the neuronal loss could be replaced by transplantation of neural cells. Epigenetic generation of tyrosine hydroxylase-expressing neurons derived from neural multipotent precursor cells is one of strategies to obtain the donor cells. Thus, identification of external signals involved in the regulation of neural stem cell proliferation and differentiation may allow for improved transplantation methods and augment the treatment of neurodegenerative disorders.

Embryonic striatum contains multipotent precursor cells, which can be expanded in fibroblast growth factor-2 (FGF-2)- and/or epithelial growth factor (EGF)-containing serum-free medium and induced to differentiate to neurons, astrocytes, and oligodendrocytes [1]. These findings support the view that extrinsic cues control the proliferation of neural precursors and progressively restrict their potential to generate various differentiated progenies [2]. There is evidence demonstrating that primary progenitor cells from the striatum could be induced to dopaminergic (DA) neurons by combined application of molecules, such as interleukin (IL)-1, IL-11, leukemia inhibitory factor [3] or bone morphogenetic proteins [4]. TH can also be induced in neuronal precursors derived from the embryonic striatum by exposure to FGF-2 and glial cell conditioned media [5]. Indeed FGF-2 regulates the development and differentiation of mesencephalic DA neurons [6,7].

Previous publications have shown that D1/D2 dopamine receptors are involved in tyrosine hydroxylase (TH) gene expression in either noradrenergic neurons of fetal locus coeruleus [8] or in DA neurons from the substantia nigra [9]. DA or apomorphine (APO), a non-selective DA receptor agonist, is able to elicit expression of TH, a marker for DA neurons, in fetal cerebrocortical cultures in the presence of brain-derived neurotrophic factor (BDNF) [10,11]. Interestingly, this TH-inducing effect of DA/BDNF is also observed in cultured striatal cells. FGF-2 or FGF-1, cooperating with other molecules including DA, elicits strong TH expression in these cells [12]. These findings suggest that DA receptors may play a role in the expression of the DA neurotransmitter phenotype in fetal neural cells.

(--)-Stepholidine (L-SPD), an active ingredient of the Chinese herb Stephania, possesses an agonistic action on the D1 receptor and an antagonistic action on D2 receptors. It augments activity of adenylyl cyclases (AC) and formation of cAMP [13–15]. It also exhibits D1 agonistic action on DARPP-32 phosphorylation [16]. These characteristics have prompted considerable interest in the development of L-SPD as a potential anti-schizophrenia drug [15]. In the present study, we sought to determine whether L-SPD influences differentiation of neural precursor cells of the...
striatum from rat embryos in vitro. It was found that L-SPD promoted proliferation as a co-factor and elicited expression of TH in the cultured striatal precursor cells.

MATERIALS AND METHODS

Cell culture: Sprague–Dawley rats were provided by the Animal Center of the Chinese Academy of Sciences. The rats were caged in groups with free access to food and water and kept in a temperature-controlled environment on a 12:12 h light-dark cycle. Rat fetuses were obtained at day 14 (E14, E0 = day of vaginal plug) of gestation. The striatum were dissected out and dissociated. All cells were incubated at 37°C in 95% air/5% CO2 and the medium changed twice a week. Cells were plated at a density of 1 x 105 cells/ml in 6 cm Petri dishes containing Dulbecco’s modified Eagle’s medium (DMEM/F12 (Life Technologies, MD, USA), 2% B27 and 5 ng/ml FGF-2. After 6 days in vitro (DIV), floating spheres were collected and incubated in 96-well plates for 10 DIV. The single cells in the upper phase were collected and incubated in 96-well plates, as described previously [17]. The cell suspension was left for 5 min to allow the undissociated spheres to settle. The single cells in the upper phase were collected and incubated in 96-well plates until the spheres became visible under the microscope at 3–4 DIV and cultured in the presence of both EGF and FGF-2. The spheres were dissociated mechanically and reseeded in fresh FGF-2-containing media at 50000 cells/ml, until secondary spheres were generated. The spheres were passaged at least twice prior to use in further studies. The passaged cells were seeded into 96-well plates (Nunc, Denmark) pre-coated with poly-L-lysine (10 μg/ml, Sigma, St. Louis, MO, USA) at 2 x 105 cells/cm² in DMEM/F12 supplemented with 10% fetal calf serum. The culture medium was switched to a defined medium containing DMEM/F12, 2% B27 and compound of interest at a density of 5000 cells/well, as described previously [17]. The culture medium was composed of DMEM/F12, 2% B27, 1% fetal bovine serum and compound of interest. Neither FGF-2 nor EGF was included.

To assess the proliferation of precursor cells, the cells were maintained in growth medium, composed of DMEM/F12, 2% B27, 10 ng/ml EGF, 10 ng/ml FGF-2, at a density of 5000 cells/well in 96-well plats, as described previously [17]. The number of spheres was counted after 7–10 DIV. Only spheres with a diameter > 75 μm were counted. To evaluate sphere’s renewal capacity, they were dissociated mechanically. The cell suspension was left for 5 min to allow the undissociated spheres to settle. The single cells in the upper phase were collected and incubated in 96-well plates for 10 DIV.

To study the differentiation of the neural precursor cells, the secondary spheres were transferred onto poly-L-lysine (10 μg/ml, Sigma) at 2 x 105 cells/cm² in DMEM/F12 supplemented with 10% fetal calf serum. The culture medium was switched to a defined medium containing DMEM/F12, 2% B27 and compound of interest 3 h after plating.

Immunohistochemistry and quantitation of immunoreactive cells: The cell populations of the DA neuron and neuron were characterized with antibodies against TH and microtubule-associated protein-2 (MAP-2), respectively. For immunohistochemical staining the cells were fixed with 4% paraformaldehyde. Mouse anti-MAP-2 antibody (1:1500, kind gift of Dr I. Fischer) and rabbit anti-TH (1:5000, Chemicon, Temecula, CA, USA) were used. The peroxidase was visualized by incubation with dianinobenzidine and intensified with nickel cobalt (Pierce, USA).

The number of MAP-2-positive or TH-positive cells in each culture was counted in two diametrical strips representing 31% of the total surface area of a well (96-well plate) or in the entire surface area of a culture well. They were expressed as the percentages of immunoreactive cells found in paired cultures treated with compound from control cultures (H2O, or vehicle), used as the reference value, i.e. 100%.

RNA isolation and RT-PCR: The spheres were harvested in denaturing solution. The total RNA was isolated using a Totally RNA isolation kit (Ambion, USA). The synthesis of single strand cDNA were performed. Specific oligonucleotide PCR primers were designed to amplify regions of coding sequence. D1 (nucleotides 269–464, Genbank accession number M38077): 5'-CTTTGTTGCTCTTGCTGAT-3' (forward); 5'-GGTCAATCTCTCTCATTG-3' (backward). D2 (nucleotides 953–1503, Genbank accession number X56065): 5'-GTTGTCCTAGAATCGTACTGCTG-3' (forward), 5'-GCTGCAATTCCAGATGAGCACA-3' (backward). Cycle condition consisted of 5 min at 95°C, 1 min at 95°C, 1 min at 54°C, 2 min at 72°C. The amplifications were carried through 35 cycles.

Statistical analysis: Results from the counting of TH- or MAP-2-positive cells were expressed as a percentage of the control values to stabilize the variance between individual experiments. The data were submitted to a one-way ANOVA followed by Dunnet’s post-hoc test. Difference were considered statistically significant at p < 0.05.

RESULTS

In the present study, the striata of the E14 rat embryo were cultured in the presence of both EGF and FGF-2. The spheres became visible under the microscope at 3–4 DIV and the number of spheres continued to increase over time. At 7 DIV, the average number of spheres per well was 74 ± 12.3 in the untreated cultures. However, application of L-SPD (0.5 μg/ml) increased the number of the spheres by up to 1.34-fold compared to the cultures without addition of L-SPD (p = 0.049; Fig. 1). Lower concentrations of L-SPD...
showed no effects. Addition of APO (0.05 μM), a non-selective DA receptor agonist, also increased the number of spheres by up to 1.57 ± 0.32-fold compared to the controls (Fig. 1). Conversely, haloperidol, a non-selective antagonist of DA receptors, was incapable of improving their proliferation (data not shown). Moreover, co-application of haloperidol with L-SPD or APO to the cultures abolished the proliferation-promoting effects of L-SPD and APO on the precursor cells (Fig. 1). These results suggest that activation of DA receptors may play a role in the proliferation of striatal precursor cells. However, neither L-SPD nor APO alone was able to affect the proliferation of these cells in the absence of FGF-2 and EGF, indicating that L-SPD or APO may act only as a co-factor for the mitogens FGF-2 and EGF in enhancing proliferation of striatal precursor cells.

Previous studies have shown that activation of D₁ receptors increases the level of cAMP, and application of L-SPD augments cAMP level in striatal neurons [18]. To determine whether the effect of L-SPD observed here was mediated by the up-regulation of cAMP levels, dibutyryl cAMP (dbcAMP, 1 mM) was thus applied to the precursor cell culture. The results shown in Fig. 1 demonstrated that it increased the number of spheres by up to 1.77-fold in the presence of FGF-2 and EGF compared to the controls (Fig. 1).

To examine the renewal capacity of the precursor cells, the spheres incubated in L-SPD- or APO-containing culture medium were dissociated individually and plated in the FGF-2/EGF-containing growth medium without L-SPD/APO. More than 80% of these spheres were able to generate secondary spheres. To determine whether the initial spheres were multipotent, single spheres were transferred on to poly-L-lysine-coated 96-well plates in medium containing 1% FBS (no FGF-2 or EGF). After 7 DIV, the cells were fixed and immunostained for MAP-2 and glial fibrillary acidic protein to reveal neurons and astrocytes. The results indicated that 99% of the spheres were well differentiated as evidenced by the presence of neurons and astrocytes (data not shown).

As shown in Fig. 1, DA receptor agonists (L-SPD, APO) were able to affect proliferation of progenitor cells. Therefore, we asked whether these cells express DA receptor mRNA. RT-PCR analysis revealed that two DNA fragments, 196 and 550 bp, corresponding to DA receptor D₁ and D₂ mRNA respectively, were specifically amplified (Fig. 2). This indicates that the striatal neural precursor cells expressed D₁ and D₂ receptors before they were committed to neuronal/glial cells.

To determine whether L-SPD is able to promote the neuronal differentiation, the spheres which had been previously passaged and expanded in FGF-2-containing medium were plated in 96-well plates. L-SPD (0.002–2 μg/ml) was then added to the culture. At this time point, no FGF-2 was included in the culture medium. Treatment with L-SPD increased the number of MAP-2-positive neurons by up to 2.25 ± 0.21-fold (to 55.91 ± 6.9% of the total cells) compared to untreated 6-day-old cultures (2.4 × 10⁴/cm², equivalent to 33.91 ± 8.5% of the total cells, Fig. 3, Fig. 4a,b). Addition of BDNF alone had minor effect on MAP-2 expression in the cultured precursor cells (1.37 ± 0.28-fold compared with control). The effects of L-SPD and BDNF seemed to be additive, since the number of MAP-2-positive cells increased up to 3.98 ± 0.34-fold in the presence of both BDNF and L-SPD (Fig. 3). In contrast, treatment of the culture with APO failed to alter expression of MAP-2 (data not shown). These results suggest that L-SPD promoted neuronal differentiation of the precursor cells in the absence of FGF-2 and EGF, demonstrating its distinct role from that in the proliferation of the precursor cells.

To examine whether L-SPD has influence on expression of TH, a marker for DA neurons, the spheres, were seeded in 96-well plates and addition of L-SPD was followed. After a 6-day incubation, the average number of TH-positive cells was 660 ± 56.54 cells/cm² in the control (0.30 ± 0.08% of the total cells; 2.7 ± 0.2% of total MAP-2-positive cells). Treatment with L-SPD (2 μg/ml) resulted in a 3.56-fold increase.
in the number of TH-positive cells over control (to 1.2 ± 0.08% of the total cells; 6 ± 0.2% of total MAP-2-positive neurons; Fig. 3, Fig. 4c,d). Haloperidol (1 μM), but not APO (0.05–5 μM), had a similar effect to L-SPD (to 1.02 ± 0.06% of the total cells; 8.1 ± 0.9% of total MAP-2-positive neurons). These results suggest that a D2 receptor antagonist may play a role in TH expression of striatal precursor cells. To verify this hypotheses, spiperone, a specific D2 receptor antagonist, was used to mimic the effect of L-SPD. The results demonstrated that spiperone had no influence on TH expression at any concentration (1.0–100.0 μM) examined. Furthermore, combined treatment of the cultures with spiperone and SKF38393 (a specific D1 agonist) also failed to reproduce the effect of L-SPD observed. These findings do not support the view that the inductive effect of L-SPD on TH expression is relevant to its dual actions on DA receptor. L-SPD may exert its action on TH expression through other mechanisms.

We have previously reported that DA receptor agonist and growth factors increased the TH expression in fetal rat and human cerebral cortical cells during specified sensitive developmental stages [10,11]. We thus examined whether the same treatment is able to alter TH expression in the striatal precursor cells. It was observed that BDNF alone increased the number of TH-positive neurons up to 1.45-fold compared to the untreated. However, combination of BDNF and L-SPD elevated the number of TH-positive neurons up to 4.61-fold in the precursor cell cultures (Fig. 3).

We also examined whether other substances have inducing effect on TH expression. It was observed that addition of either FGF-2 (0.1–10 ng/ml) or forskolin, an adenylyl cyclase activator alone, could not elicit the expression of TH in the precursor cell cultures. However, their combination increased the number of TH-positive cells up to 4.36 ± 0.59-fold compared to untreated. This action was potentiated by 8.32 ± 1.4-fold when L-SPD was co-administrated with FGF-2 and forskolin (Fig. 5). Moreover, in the presence of L-SPD, both FGF-2 and BDNF could increase the expression level by 4.21 ± 0.25 and 4.60 ± 0.09-fold, respectively. These results indicated that cAMP and growth factors are involved in TH expression in the cultured precursor cells.

**DISCUSSION**

The present study demonstrated that L-SPD promoted the proliferation of striatal-derived precursor cells and also influenced the expression of TH in these cells. This finding provides new evidence to enable the understanding of how L-SPD affects the development of neural cells. It also suggests that embryonic precursor cells may be manipulated in an alternative way, in regard to their proliferation and differentiation, toward the DA neurotransmitter phenotype.

EGF and FGF-2, two potent mitogens, have been widely used to propagate neural progenitors in vitro. It has been shown that the proliferative effect of EGF results from the activation of ERK and JNK pathway [19]. In contrast, FGF-2 influences cell propagation via Ras/Raf/MAPK and PLC pathway [20]. The transcription factor CREB (cAMP/Ca\(^{2+}\) response element binding protein) is the target of many molecules like MAPK and protein kinase A. The activation of CREB regulates cell proliferation, differentiation and survival responses in a variety of cell types in developing vertebrates (for review see [21]). Activation of the cAMP cascade also increases the proliferation of newborn cells in the adult mouse hippocampus, which is accompanied by activation of CREB phosphorylation [22]. These studies suggest that the cAMP-CREB pathway plays important roles in the proliferation of neural cells. There is evidence...
indicating that L-SPD is able to activate the D<sub>1</sub> receptor resulting in increased activity of the adenyl cyclases, enhanced generation of cAMP and activation of protein kinase A [16]. In the present study, L-SPD was shown to increase the number of spheres and its effects mimicked by dbcAMP. Thus, it is likely that L-SPD increases cAMP levels and activates the cAMP-CREB cascade, thereby promoting the proliferation of neural precursor cells.

The precursor cells in the embryonic striatum develop to GABAergic, glutamatergic, cholinergic neurons, and very few DA neurons in vivo and in vitro. However, the multipotentiality of these cells allows their differentiation toward DA phenotype following stimulation of exogenous factors present in cultures. Consistent with this notion, Daadi et al. showed that TH was induced in passaged neural precursors derived from the embryonic striatum by 24h exposure to FGF-2 and conditioned media of B49 glial cell line [23]. In their study, 19.6 ± 1.5% of the total number of neurons were TH-positive. Our data, shown in the present study, is consistent with their view that extrinsic signals may direct the choice of differentiation of cultured striatal precursor cells. We observed that 6 ± 0.2% of the neurons were induced to TH-expressing cells following combined treatment with L-SPD and FGF-2. These findings suggest that co-application of L-SPD and FGF-2 may be an alternative way to induce differentiation of striatal precursor cells toward DA neurons.

D<sub>1</sub>/D<sub>2</sub> dopamine receptors are believed to be responsible for changes such as augmentation of TH enzyme activity [24] and TH gene expression in catecholaminergic neurons [8,9]. However, the roles of DA receptors in the proliferation and subsequent specification of the DA phenotype of neural precursor cells have not been determined. In the present study, we observed that the cultured precursor cells from rat embryonic striatum expressed both D<sub>1</sub> and D<sub>2</sub> receptors, which may be involved in the promoted proliferation of the cultured precursor cells. Moreover, our data showed that administration of L-SPD or haloperidol, but not other DA receptor agonists/antagonists (e.g. APO), elicited TH expression in the precursor cells. Thus, the precise role of DA receptors in this process awaits further detailed investigation.

CONCLUSION
The present study suggests that L-SPD, a unique DA receptor modulator, may influence development of neural precursor cells by participating in the regulation of their proliferation and neuronal differentiation. The effect of the drug on the proliferation of the precursor cells appears to be mediated by DA receptors. This is consistent with the view that the DA receptors could play a role in stem cells proliferation. L-SPD also promotes the differentiation of the precursor cells, however, this action may be independent of its effect on DA receptors. The present study also provides an alternative way to obtain TH-expressing cells (presumably dopaminergic neurons) that may be used as cell sources for the treatment of Parkinson’s disease.

REFERENCES