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Infusion of epidermal growth factor and basic fibroblast growth factor into the striatum of parkinsonian rats leads to in vitro proliferation and differentiation of adult neural progenitor cells

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Abstract

This study investigated the proliferation and differentiation of adult neural progenitor cells (aNPCs) derived from the striatum and substantia nigra (SN) of parkinsonian rats. We found that aNPCs isolated from the two areas of parkinsonian rats readily formed nestin-enriched neurospheres in vitro and exhibited an ability to differentiate into either neurons or astrocytes. Injection of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) into the striatum of parkinsonian rats prior to the harvesting striatal aNPCs significantly increased the neurosphere formation rate and multiple differentiation capacity of these aNPCs when cultured in vitro. These data suggest that striatal and nigral adult NPCs in parkinsonian rats retain the abilities of proliferation and differentiation in vitro. In addition, exogenously applied growth factors could up-regulate the developmental potential of aNPCs. We conclude that our data supports the notion that endogenous cell replacement therapies may be useful for the future treatment of Parkinson's disease (PD).

Keywords: Adult neural progenitor cells; Neurospheres; Parkinson's disease; Growth factors; Striatum; Substantia nigra

The discovery of neurogenesis in adult mammalian brain has provoked much interest because of the possibility of using neural stem cells in replacement therapies for Parkinson's disease (PD) [3]. Increasing evidence has shown that adult neural progenitor cells (aNPCs) exist in many areas of mammalian adult brain and exhibit the capacity to generate mature neural cells de novo [19]. In addition, some studies suggest that aNPCs have considerable plasticity. For example, they can be stimulated to proliferate in response to injury signals to replace cells lost in certain experimental models [12], or to differentiate into certain types of neurons in a favorable niche.

However, aNPCs appear to lack the capacity to reconstruct the damaged tissue in neurodegenerative disorders spontaneously. One possibility is that the local toxic environment may suppress the biological functions of aNPCs and their regenerative capacity. Alternatively, extracellular signals needed for initiating or facilitating proliferation and differentiation of aNPCs might be insufficient, thus limiting the ability of aNPCs to compensate for lost cells at the lesion site. One particular study had shown that aNPCs derived from substantia nigra (SN) differentiated into neurons following transplantation into the rat hippocampus, whereas when transplanted into SN, they generated only new mature glial cells [11]. This indicated that local environmental signals play a critical role to promote the differentiation of aNPCs into neuronal cells. In order to manipulate the endogenous aNPCs for the therapeutic purposes, we believe it to be crucial to determine the nature of the extracellular signals that stimulate cell division and regulate the fate of aNPCs. Indeed, several studies have shown that growth and neurotrophic factors can induce proliferation and differentiation of aNPCs. Moreover, aNPCs in the subventricular zone (SVZ) express EGF and bFGF receptors [7] and can respond to EGF and bFGF signals with increased division and neuronal differentiation in vitro [15,17,20]. These studies suggest that EGF and bFGF can serve as the positive extracellular signals that enhance the functional capacities

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of aNPCs. We hypothesize that intrastriatal administration of EGF and bFGF may potentially mobilize the aNPCs in the parkinsonian rats when local environmental signals generated by injury are not sufficient to induce the activation of aNPCs.

The hallmark of PD is a progressive loss of dopaminergic neurons [1]. Cell replacement, especially inducing aNPCs towards neuronal differentiation in situ as a therapeutic strategy, has shown great promise to restore the dopamingeric system in PD. In order to activate endogenoeous aNPCs, two issues need to be addressed. First, it is essential to determine whether aNPCs are affected by pathological conditions in neurodegenerative disorders. Second, it is important to determine whether aNPCs can proliferate and/or differentiate in response to environmental stimuli. To address these issues, we investigated the in vitro proliferation and differentiation of aNPCs derived from the striatum or the SN of normal and 6-hydroxydopamine (OHDA)-lesioned, i.e., parkinsonian rats to test the injury effect on them. We also studied the effects of EGF and bFGF administrated into the striatum before harvesting aNPCs by comparing the neurosphere formation rate and differentiation potential in vitro.

Adult female Sprague–Dawley rats, weighing 200–250 g, were obtained from the Laboratory Animal Center, Peking University, and were housed under a 12 h light/dark cycle with access to food and water ad libitum. Procedures involving animal survival surgery were approved by the Committee on Animal Care and Usage of Peking University Health Science Center.

To establish parkinsonian models, rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the tooth bar set at −2.3 mm. A total amount of 12 μg of 6-OHDA (Sigma, St. Louis, MO), dissolved in 4 μl of 0.1 mg/ml L-ascorbate saline, was injected stereotaxically into the right medial forebrain bundle (MFB) [10] at the following coordinates: AP = −4.3 mm, ML = −1.5 mm, and DV = −7.5 mm. The injection rate was 1 μl/min and the syringe needle was left in position for an additional 5 min before slow retraction. The skull surface was then covered with fibrosponge and the skin was sutured.

Two weeks later, intrastriatal administration of EGF and bFGF was performed to observe the effect of EGF and bFGF on aNPCs. An 8-mm long stainless-steel tube with an external diameter of 0.25 mm was stereotaxically implanted in the striatum of successful parkinsonian rats at the lesion side. The coordinates were set as AP = +1.0 mm, ML = −3.0 mm, DV = −5.0 mm. The tube was fixed with dental cement and was sealed with a smaller injection cannula. Rats received a 4 μl injection of 100 ng/μl EGF and 100 ng/μl bFGF at the rate of 1 μl/min through the injection cannula each day for 5 consecutive days.

After 5 days intrastriatal injection of EGF and bFGF, aNPCs of striatum and SN were isolated as previously described [16]. Briefly, striatum and ventral mesencephalic tissues were dissected using a dissection microscope, dissociated into cell suspensions by trypsinization and triturating, then plated on culture dishes with Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (FBS) (Hyclone, Logan, UT) overnight. On the second day, floating cells were collected, washed, and subsequently cultured in serum-free medium DMEM/F12 (1:1) containing a supplement of B27 (1:50; Gibco, Life Technologies, Rockville, MD), 20 ng/ml of EGF (Gibco) and 20 ng/ml of bFGF (Gibco) for 2 weeks. Cells capable of forming neurospheres were allowed to expand for a month and cultures were fed every 3 days. Neurospheres with a diameter greater than 30 μm were counted in five fields of view per well (center and at the relative coordinates of 3, 6, 9, and 12 o'clock) under a light microscope every week for 4 weeks. Neurospheres were collected from culture by harvesting with trypsin–EDTA solution and suspended to a final density of 10^5 cells/ml in serum-free medium with BrdU addition (5 μM). To assess the differentiation ability, neurospheres generated in the BrdU-supplemented media were plated onto poly-L-lysine-coated glass coverslips and exposed to differentiation conditions (DMEM/F12 culture media supplemented with 5% FBS and 0.5 μM all-*trans* retinoic acid) for 7 days.

Cultured cells or neurospheres were fixed in 4% paraformaldehyde, and processed for immunofluorescent detection. Immunofluorescent labeling against BrdU and the cell type specific markers was performed according to the protocol previously described [9]. Briefly, samples were pretreated with 50% formamide in 2× SSC for 2 h at 65 °C, followed by rinses in 2× SSC for 15 min, and then incubated with 2N HCl for 30 min at 37 °C and 0.1 M borate buffer for 10 min. After blocked with 10% normal goat serum and incubated with a primary antibody solution, the corresponding secondary antibodies conjugated with fluorescence (1:200, Pharmingen) were added. The primary antibodies used were mouse anti-BrdU (1:500, Sigma) to recognize proliferating cells, rabbit anti-glial cell line-derived fibrilliary acidic protein (GFAP, 1:200, Zymed Laboratories Inc.) to identify astrocytes, mouse anti-βIII-tubulin (1:200, Chemicon, Temecula, CA) to identify neurons, mouse anti-nestin (1:200, Chemicon) to detect progenitor cells, or mouse anti-O4 (1:200, Sigma) to recognize oligodendrocytes. Images were examined under confocal laser scanning microscopy (CLSM, Leica, Heideberg, Germany). BrdU-immunoreactive (IR) cells and double-stained positive cells (BrdU and GFAP, BrdU and βIII-tubulin) were counted.

Data were expressed as means ± S.E.M. Statistical significance was assessed by one-way ANOVA followed by Newman–Keuls post hoc test. Statistical difference between the two groups was tested by *t* test. An alpha value of $P < 0.05$ was considered statistically significant.

The aNPCs isolated from the striatum and SN of normal or parkinsonian rats divided into two cells after 2 days, and gradually increased their numbers to form neurospheres after 2 weeks in vitro (Fig. 1A). To verify whether the

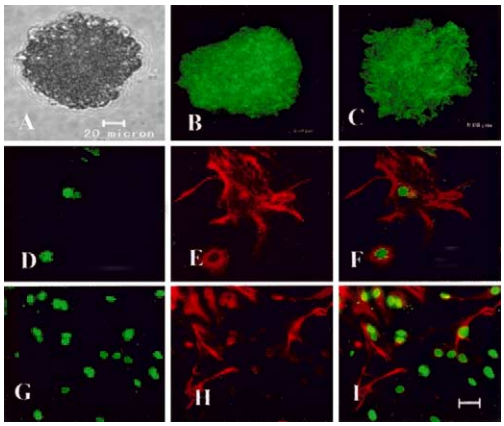

Fig. 1. Proliferation and differentiation of rat striatal aNPCs. Striatum-derived rat aNPCs proliferated into neurospheres when cultured in a serum-free medium (A). Most cells in the neurosphere were BrdU-IR (B) and expressed the neural progenitor cell marker nestin (C). When differentiation for 7 days, BrdU-IR cells (D and G) derived from neurospheres co-expressed the phenotypic markers: GFAP for astrocytes (E and F) or βIII-tubulin for neurons (H and I). Scale bar = 30 μm.

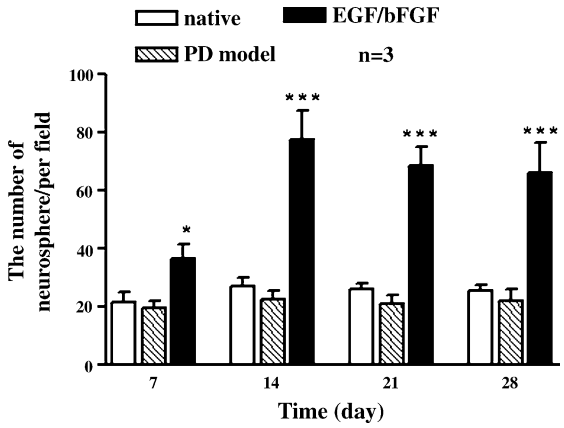

Fig. 2. Neurosphere formation of rat striatal aNPCs. A significant increase in the number of neurospheres was found in striatum-derived cultures after intrastriatal supplement with EGF/bFGF. $^{*}P < 0.05$ and $^{***}P < 0.001$ as compared to naïve rats.

neurospheres resulted from proliferation of aNPCs in culture, the mitotic marker BrdU was added to the culture media (5 μM) for 7 days. Neurospheres and dispersed cells derived from neurospheres were then stained for BrdU immunoreactivity. Nearly all the cells were BrdU positive, suggesting that these cells underwent DNA synthesis during their proliferation to form neurospheres (Fig. 1B). The immaturity of striatal or nigral neurospheres from normal or dopamine-lesioned rats, i.e., parkinsonian rats was demonstrated by the strong expression of an immature neuroepithelium marker nestin in neurospheres (Fig. 1C).

To compare the neurosphere formation rates between normal and parkinsonian rats, the number of neurospheres in culture was counted. The results showed no statistically significant difference in the number of neurospheres derived from the SN between the two groups of rats (data not shown). Similarly, no significant difference was found in the formation rate of neurospheres by the striatum-derived NPCs between the two groups of rats (Fig. 2). These date indicated that an injury signal per se did not induce a significant increase in proliferation of aNPCs in parkinsonian rats.

We then conducted double immunofluorescent labeling to determine the fate of divided cells. Under differentiation condition, BrdU-labeled cells were found to be co-immunoreactive to either an astrocyte-specific marker GFAP or a neuron-specific marker βIII-tubulin, indicating the multiple differentiations of newly generated cells into either GFAP-IR astrocytes or βIII-tubulin-IR neurons, respectively (Fig. 1D–I). However, co-expression of oligodendrocyte-specific marker O4 was not found in any BrdU-IR cells (data not shown). The number of double stained positive cells in every 100 randomly chosen BrdU-IR cells was counted. There was a significant increase in the number of GFAP-IR astrocytes differentiated from aNPCs of parkinsonian rats (41.67 ± 2.85) as compared with those from normal rats (31.00 ± 3.46). However, there was no significant difference in the number of βIII-tubulin-IR neurons between these two groups (Fig. 3). These results suggest that an injury signal may serve as a pro-glial signal in the procedure of aNPCs multiple differentiation.

Next, we examined whether intrastriatal administration of EGF and bFGF could increase the proliferation and differentiation of striatal or nigral aNPCs from parkinsonian rats in vitro. A combination of EGF and bFGF injected into the striatum (400 ng/day, 5 days) significantly increased the neurosphere formation rate of striatal aNPCs (Fig. 2), whereas the neurosphere formation of aNPCs derived from the SN did not show any significant change (data not shown). To investigate the effect of EGF and bFGF on the striatal aNPCs differentiation potential, double-labeled cells for the presence of BrdU and a lineage-specific marker were counted. The

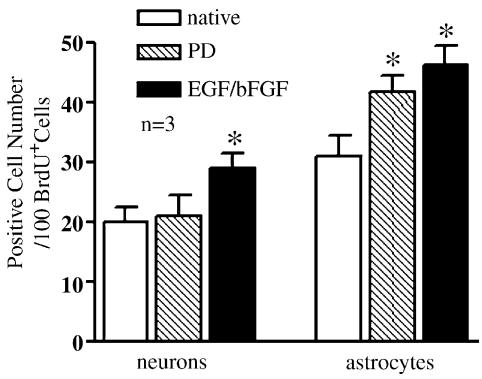

Fig. 3. Multiple differentiation of rat striatal aNPCs. The number of BrdU and βIII-tubulin double positive cells in the EGF/bFGF-injected group increased significantly relative to both naïve and parkinsonian rats. The number of BrdU and GFAP double positive cells also increased in the EGF/bFGF-injected group as compared with naïve rats, although there was no difference as compared with parkinsonian rats. $^{*}P < 0.05$ relative to naïve rats.

percentage of BrdU-IR cells that co-expressed a neuronal or astroglial marker was 29 or 46% in EGF/bFGF-treated rats, respectively, which was significantly higher than 19 or 31% as was observed in naive rats ($P < 0.05$), suggesting that EGF and bFGF may up-regulate the multiple differentiation capacity of aNPCs.

Recent studies have shown that aNPCs can self-renew and, after mitogen withdrawal, differentiate into neurons, astrocytes and oligodendrocytes in vitro [8,14]. In addition, aNPCs can proliferate and differentiate in response to environmental and pharmacological manipulations, and consequently replace cells lost in some experimental lesion paradigms [5,6]. However, it is unclear whether aNPCs are present in the brain of neurodegenerative disorders such as PD and, if so, whether they can proliferate and/or differentiate in response to environmental stimuli as do their normal counterparts.

It assumed that in neurodegenerative disorders, aNPCs might be at a deficit because they are also a target of the pathological process or incapable of responding appropriately to the putative signals given by the degenerating neurons [2]. In the present study, we demonstrated that despite of a dramatic decrease in the numbers of doparminergic cells in parkinsonian rats (data not shown), no significant difference was found in striatal- or nigral-derived neurosphere formation between parkinsonian and normal rats. We also showed that the aNPCs cultured from the striatum and SN of parkinsonian rats maintained the properties of stem cells such as proliferation and differentiation, as assessed by the expression of specific neural markers of mature lineages (GFAP, βIII-tubulin) and immaturity. These data suggest that aNPCs within the nigrostriatal system are not affected in this model.

The evidence supporting the presence of aNPCs in the parkinsonian brain is quite important, since it provides a potential opportunity to induce these cells to proliferate and differentiate in situ to replace the lost cells and hopefully restore the lost function. However, under normal physiologic conditions, aNPCs remain quiescent and are relatively few in number in nonneurogenic areas of the brain. One crucial issue for realizing this therapeutic strategy is to increase the efficiency of inducing these progenitor cells to proliferate and differentiate into neurons. Several studies in vivo have demonstrated that injury itself may play an important role in these processes [18] and that the local environmental milieu may contain pro-neuronal or pro-glial signals directing the differentiation of endogenous progenitor cells. For example, there was gliogenesis in the nigrostriatal doparminergic system after neurotoxic injuries induced by 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [13] and neurogenesis in the cortex undergoes targeted apoptotic degeneration [12]. However, our data suggest that an injury signal may be insufficient to induce aNPCs to proliferate and differentiate into neurons.

It has been reported that exogenously administered growth and neurotrophic factors can influence the proliferation and differentiation of progenitor cells either under normal conditions or in disease states. Infusion of EGF and bFGF into the adult ventricular system could stimulate the mitotic gliogenesis and neurogenesis, respectively [4]. Likewise, there was a rapid proliferation and migration of forebrain stem cells towards the transfer growth factor (TGF)-α infusion site in the striatum resulting in an increased number of differentiated neurons [5]. Our data demonstrated that EGF and bFGF were capable of inducing the proliferation of aNPCs in the striatum of parkinsonian rats. The neurosphere formation was increased only in those rats that had received EGF and bFGF injection, suggesting that these two growth factors were needed to mobilize the proliferation of endogenous neural progenitors. In addition, we observed that there was a significant increase in the number of newly generated neurons and astrocytes. This observation suggested that EGF and bFGF exhibited an up-regulating effect on aNPCs to undergo neuronal differentiation. Therefore, our data is concordant with the observations of others where it was shown that the local microenvironment plays a crucial role in directing endogenous aNPCs to proliferate and differentiate [11]. Thus, in order to modify the microenvironment of specific regions of the brain in those patients with neurodegenerative diseases, an intriguing repair strategy will be required. We envision this operating in a strategy whereby the patients' own neural stem cells may be stimulated to proliferate and differentiate into desired cell types so that the lost cells can be replaced and the lost or diminished function restored.

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