microscopic margins and with an intact capsule was achieved by partial duodenectomy with preservation of the minor papilla. In order to consider less invasive methods, every effort should be made to accurately identify tumor origin and adjacent anatomic structures before and during surgery. Additional reports of these tumors occurring at uncommon sites may help to develop a standardized treatment for duodenal GIST.

References

1. Emory TS, Sobin LH, Lukes L, Lee DH, O'Leary TJ (1999) Prognosis of gastrointestinal smooth-muscle (stromal) tumors: dependence on anatomic site. Am J Surg Pathol 23:82–87
2. DeMatteo RP, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF (2000) Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. Ann Surg 231:51–58
3. Pidhorecky I, Cheney RT, Kraybill WG, Gibbs JF (2000) Gastrointestinal stromal tumors: current diagnosis, biologic behavior, and management. Ann Surg Oncol 7:705–712
4. Takahashi Y, Noguchi T, Takeno S, Uchida Y, Shimoda H, Yokoyama S (2001) Gastrointestinal stromal tumor of the duodenal ampulla: report of a case. Surg Today 31:722–726
5. Kim SH, Kim JH, Baik GH, Baek I, Hahn T, Oh SO, Kim JB, Park SH, Chang WK, Kim DJ, Park CK, Park HR (2004) Malignant gastrointestinal stromal tumor of the ampulla of Vater: a case report. Korean J Gastroenterol 43:66–70
6. Wellmann K, Gohla G, Wenk H (2004) Malignant gastrointestinal stromal tumor (GIST) of the papilla vateri. A rare tumor entity. Chirurg 75:196–199
7. Cavallini M, Cecera A, Ciardi A, Caterino S, Ziparo V (2005) Small periampullary duodenal gastrointestinal stromal tumor treated by local excision: report of a case. Tumori 91:264–266
8. Fletcher CD, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW (2002) Diagnosis of gastrointestinal stromal tumors: A consensus approach. Hum Pathol 33:459–465
9. Fletcher CD, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, LO'eary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW (2002) Diagnosis of gastrointestinal stromal tumors: a consensus approach. Int J Surg Pathol 10:81–89
10. Ueyama T, Guo KJ, Hashimoto H, Daimaru Y, Enjoji M (1992) A clinicopathologic and immunohistochemical study of gastrointestinal stromal tumors. Cancer 69:947–955
11. Newman PL, Wadden C, Fletcher CD (1991) Gastrointestinal stromal tumours: correlation of immunophenotype with clinicopathological features. J Pathol 164:107–117
12. Goh BK, Chow PK, Ong HS, Wong WK (2005) Gastrointestinal stromal tumor involving the second and third portion of the duodenum: treatment by partial duodenectomy and Roux-en-Y duodenojejunostomy. J Surg Oncol 91:273–275
13. Sakamoto Y, Yamamoto J, Takahashi H, Kokudo N, Yamaguchi T, Muto T, Makuuchi M (2003) Segmental resection of the third portion of the duodenum for a gastrointestinal stromal tumor: a case report. Jpn J Clin Oncol 33:364–366
14. Crosby JA, Catton CN, Davis A, Couture J, O'Sullivan B, Kandel R, Swallow CJ (2001) Malignant gastrointestinal stromal tumors of the small intestine: a review of 50 cases from a prospective database. Ann Surg Oncol 8:50–59
15. Singer S, Rubin BP, Lux ML, Chen CJ, Demetri GD, Fletcher CD, Fletcher JA (2002) Prognostic value of KIT mutation type, mitotic activity, and histologic subtype in gastrointestinal stromal tumors. J Clin Oncol 20:3898–3905
16. Liberati G, Lucchetta MC, Petraccia L, Nocchi S, Rosentzwig R, De Matteis A, Grassi M (2003) Meta-analytical study of gastrointestinal stromal tumors (GIST). Clin Ter 154:85–91
17. Fong Y, Coit DG, Woodruff JM, Brennan MF (1993) Lymph node metastasis from soft tissue sarcoma in adults. Analysis of data from a prospective database of 1772 sarcoma patients. Ann Surg 217:72–77

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Research Report

Intrapallidal metabotropic glutamate receptor activation in a rat model of Parkinson's disease: Behavioral and histological analyses

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ABSTRACT

Metabotropic glutamate receptors (mGluRs) have been recently implicated as robust therapeutic targets for Parkinson's disease (PD). Here, we explored how activation of mGluRs in globus pallidus (GP) affected the amphetamine-induced rotational behavior in the unilateral 6-hydroxydopamine (6-OHDA) lesion rat model of PD. The amphetamine-induced rotations were completely suppressed by the ipsilateral intrapallidal injection of the non-selective mGluR agonist, 1-aminocyclopentane-1S,3R-dicarboxylic acid (ACPD) and the selective group I mGluR agonist, (R,S)-3,5-dihydroxyphenylglycine (DHPG), but not the selective group III mGluR agonist, L-2-amino-4-phosphonobutyric acid (L-AP4). The suppressive effects were detected at 2, 4, 6, 8, and 12 h after ACPD injection, but returned to the control level at 24 h. A remarkable c-*fos* expression was found in the lesioned side of GP, subthalamic nucleus (STN), and substantia nigra pars reticulata (SNr) of rats that received the ACPD or DHPG injection, compared to rats treated with L-AP-4 or phosphate buffer-injection. The results indicate that the blockade of amphetamine-induced rotations might be at least partially mediated by group I mGluR activation. This study advances the use of selective group I mGluRs directed toward the GP for PD treatment.

1. Introduction

Metabotropic glutamate receptors (mGluRs) are one of the most essential excitatory neurotransmitters in the central nervous systems. mGluRs coupled with G protein exist in various sites of the brain, and are categorized into 3 subgroups (group I, II and III) and 8 subtypes (I: mGluR1 and 5, II: mGluR2 and 3, III: mGluR 4, 6, 7 and 8), which have been shown to participate in neuronal excitation and synaptic transmission (Ossowska et al., 2007). The expression of mGluRs in the basal ganglia implicates their close involvement in the nigrostriatal dopamine system (Feeley Kearney and Albin, 2003).

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Abbreviations: ACPD, 1-aminocyclopentane-1S,3R-dicarboxylic acid; 6-OHDA, 6-hydroxydopamine; DAPI, 4′, 6-diamidino-2-phenylindole, dilactate; EP, entopeduncular nucleus; EPSPs, excitatory postsynaptic potentials; GABA, gamma-aminobutyric acid; GP, globus pallidus; IPSCs, inhibitory postsynaptic currents; L-AP4, L(+)-2-amino-4-phosphonobutyric acid; mGluRs, metabotropic glutamate receptors; MAPK, mitogen-activated protein kinase; PD, Parkinson's disease; PB, phosphate buffer; PKA, protein kinase A; DHPG, (R,S)-3,5-dihydroxyphenylglycine; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulate; STN, subthalamic nucleus; TH, tyrosine hydroxylase

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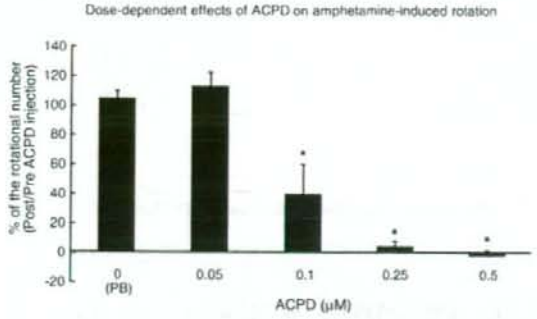

Fig. 1 – Dose-dependent effects of intrapallidal injection of mGluR agonists on amphetamine-induced rotational behavior. The injection of 0.25 and 0.5 μM of ACPD, the non-selective mGluR agonist, significantly suppressed the percentages of the amphetamine-induced rotational number, compared to that of 0.1 μM (p's<0.05), although there was no significant differences between 0.25 and 0.5 μM. Data are expressed as mean percentages (post/pre-ACPD-injection)±S.E. (n=3 each, ANOVA).

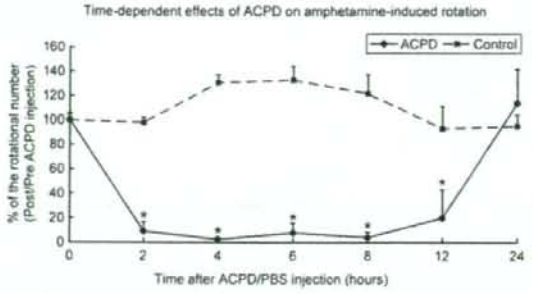

Fig. 2 – Time-dependent effects of intrapallidal injection of mGluR agonists on amphetamine-induced rotational behavior. Intrapallidal injection of 0.25 μM of ACPD significantly suppressed the percentages of the amphetamine-induced rotational number at 2, 4, 6, 8, and 12 h after ACPD injection, but not at 24 h, compared to that of the control group. Data are expressed as mean percentages (post/pre-ACPD-injection)± S.E. (n=at least 3 in each group, ANOVA).

Electrical activities of the subthalamic nucleus are abnormally exaggerated in patients with Parkinson's disease (PD) (Benabid et al., 2000). Glutamatergic outputs from the subthalamic nucleus to the projected nuclei usually control excitation via metabotropic or ionotropic receptors. Recent reports suggest that specific mGluR agonists or antagonists ameliorate the symptoms of PD (Bonsi et al., 2007; Feeley Kearney and Albin, 2003).

In PD, nigrostriatal degeneration causes the hyperactivity in the STN and globus pallidus (GP) via the suppression of the suppressive gamma-aminobutyric acid (GABA)ergic projections to the nuclei with subsequent motor symptoms. GP is a tonically active GABAergic nucleus that constitutes an integral part of the indirect pathway of the basal ganglia circuitry (Poisik et al., 2005). GP is also well known as a good target for PD using electrical stimulation or ablation (Rodriguez-Oroz et al., 2005), especially for the negative symptoms including akinesia and drug-induced dyskinesia. Thus, modulation of GP via essential mGluR agonists seems a logical and feasible therapeutic target for treating PD.

Previous electrophysiological investigations using rat brain slices revealed that activation of group III mGluRs reduces both glutamatergic and GABAergic transmission in the GP (Matsui and Kita, 2003). Based on these observations, we proceeded here to examine the behavioral and immunohistochemical effects of intrapallidal injection of the non-selective and selective mGluR agonists using a rat model of PD to reveal the mechanisms underlying the mGluR activation in the GP and its subsequent therapeutic benefits in the nigrostriatal dopamine system.

2. Results

2.1. *ACPD suppresses amphetamine-induced rotations in a dose-responsive manner*

A dose-response study was initially performed. Intrapallidal injection of 0.1, 0.25, and 0.5 μM of ACPD significantly suppressed the percentages of the amphetamine-induced rotational number (post/pre-ACPD injection; ANOVA, $F_{3,8}$=35.8, p<0.0001, p's<0.05, 0.1 μM: 40±16, 0.25 μM: 4.6±3.2, 0.5 μM: −2.0±3.6%, respectively) at 4 h after ACPD injection, compared to that of 0.05 μM (113±9.3%). The injection of 0.25 and 0.5 μM of ACPD significantly suppressed the percentages of the rotational number, compared to that of 0.1 μM (p's<0.05), although there were no significant differences of the percentages between 0.25 and 0.5 μM. Thus, dose-dependent effects of ACPD were demonstrated (Fig. 1).

2.2. *ACPD blocks amphetamine-induced rotations at 2-12 h after intrapallidal injection*

Subsequently, the effects of intrapallidal ACPD injection on amphetamine-induced rotation were explored over time. Repeated Measures of ANOVA revealed significant main effects ($F_{6,22}$=18.6, p<0.0001, Fig. 2) and ACPD injection significantly

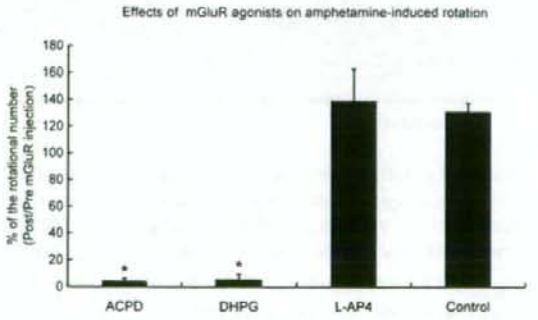

Fig. 3 – Suppressive effects of group I mGluR agonist on amphetamine-induced rotational behavior. DHPG, the selective group I mGluR agonist injection into the GP significantly suppressed the amphetamine-induced rotational behavior at 4 h after the mGluR injection, compared to those of the control group. Data are expressed as mean percentages (post/pre-ACPD-injection)± S.E. (n=3 each, ANOVA).

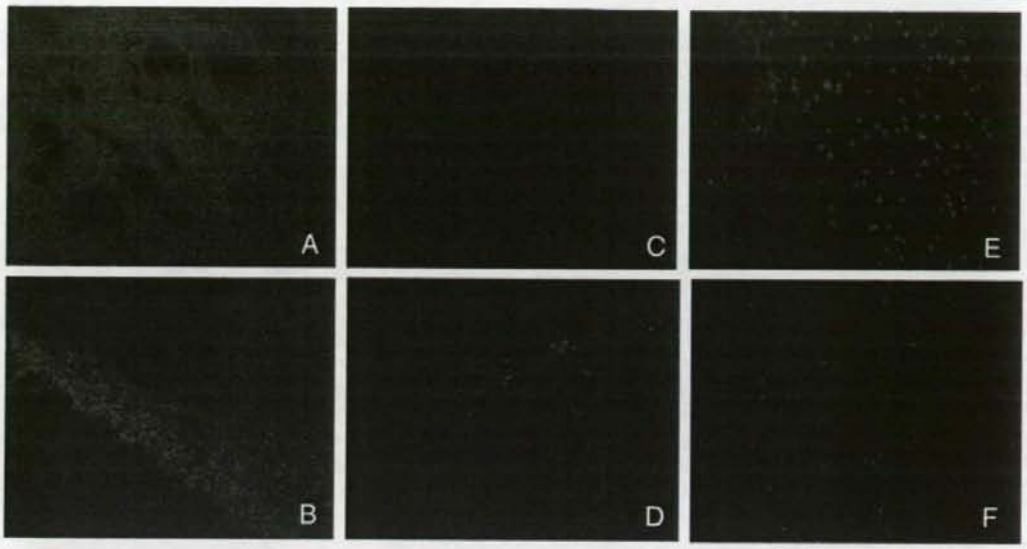

Fig. 4 – TH and c-*fos* staining in parkinsonian rats with intrapallidal mGluR agonist injection. TH staining of parkinsonian rats receiving ACPD reveals that TH-positive fibers in the lesioned striatum (C) and TH-positive neurons in the lesioned SNc (D) were apparently degenerated, compared to those in the intact striatum (A) and SNc (B). c-*fos* staining demonstrated the apparent enhancement in neuronal activity, compared to PB treatment, in ACPD-injected GP (E and F, respectively). Scale bar: 120 μm (A–D), 60 μm (E and F).

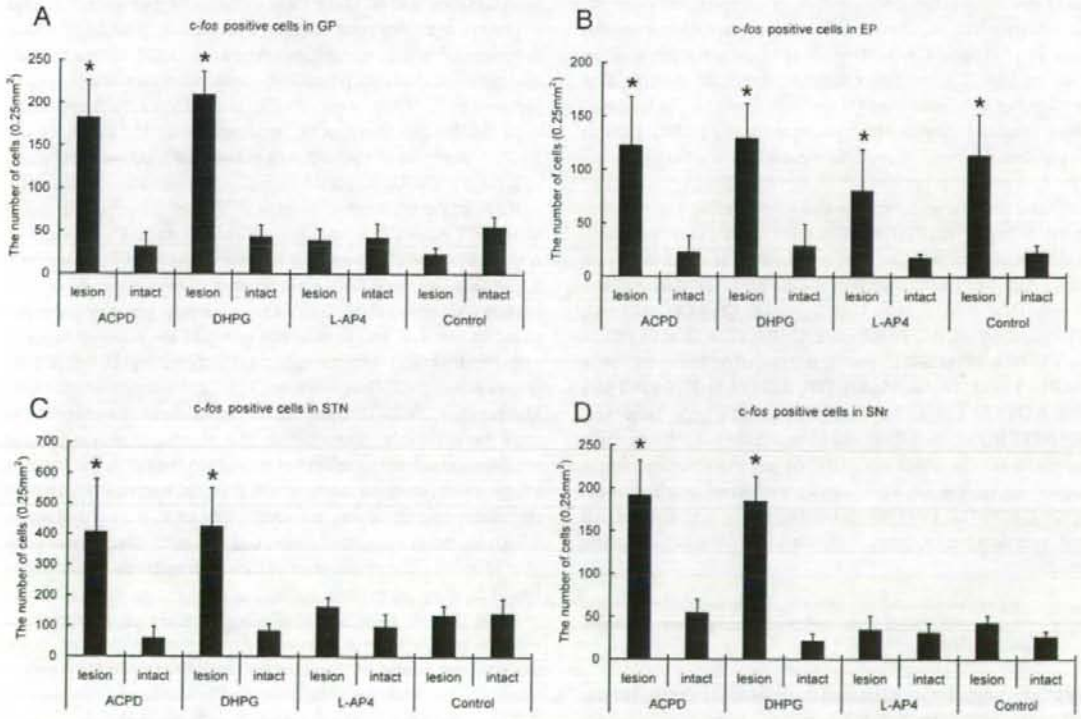

Fig. 5 – c-Fos positive cells in the GP, EP, STN and SNr. The number of c-*fos* positive cells in the GP, STN and SNr, but not EP in rats receiving ACPD or DHPG injection significantly increased in the lesion side, compared to the intact side, although L-AP4 or PB injection did not alter c-*fos* expression. Data are expressed as mean cell number ± S.E. (Mann–Whitney's U-test).

suppressed rotational behavior, compared to the control group (p's <0.01). Intrapallidal injection of 0.25 μM of ACPD significantly suppressed the percentages of the amphetamine-induced rotational number (post/pre-ACPD injection) at 2, 4, 6, 8, and 12 h after ACPD injection (9.2±7.6, 2.6±1.7, 8.0±8.0, 4.3±4.4, and 20.1±18. 3%, respectively), but not at 24 h (114±28%), compared to that of the control group (98±4.3, 131±6.3, 133±11.2, 122±15.5, 93±18.3, and 95±9. 3% at 2, 4, 6, 8, 12, and 24 h).

2.3. *ACPD and DHPG reduce amphetamine-induced rotations*

Next, the predominant selective mGluR agonist on amphetamine-induced rotations was explored. DHPG, the selective group I mGluR agonist injection into the GP significantly suppressed the percentages of the amphetamine-induced rotational number (post/pre mGluR injection, 5.7±1.6%, $F_{3,8}=212$, $p<0.0001$, Fig. 3) at 4 h after the mGluR injection, compared to those of the control group. In contrast, L-AP4, the selective group III mGluR agonist exerted no effects on the rotational behavior (138±10%, $p>0.05$ versus pre). There were no significant differences between the ACPD and DHPG group, indicating that the suppressive effects might be probably demonstrated via the selective group I mGluR agonist.

2.4. *TH and c-fos immunohistochemical investigations*

TH staining was performed to reveal the influence of the mGluR agonist injection on the degeneration of nigrostriatal dopaminergic systems (Fig. 4). There were no significant differences in the density of striatal TH-positive fibers in the striatum and the number of SNc TH-positive neurons across all groups. The percentages of the density of TH-positive fibers in the lesioned striatum relative to the intact striatum were 30±4.8% and those of TH-positive neurons in the SNc were 8±2.0%.

c-fos staining was performed to demonstrate the effects of intrapallidal mGluR injection on the surrounding brain structures, including GP, EP, STN, and SNr was counted and evaluated. The number of c-fos positive cells in the GP, STN and SNr in rats receiving ACPD or DHPG injection significantly increased in the lesion side (Mann–Whitney's U-test, $p<0.05$; GP, ACPD: 183±43, DHPG: 210±27; STN, ACPD: 408±172, DHPG: 130±32; SNc, ACPD: 192±40, DHPG: 185±28/0.25 mm^2), compared to the intact side (GP, ACPD: 32±16, DHPG: 44±13; STN, ACPD: 61±37, DHPG: 85±22; SNc, ACPD: 55±15, DHPG: 22±8/0.25 mm^2) (Fig. 5). However, L-AP4 and PB injection did not alter the number of c-fos positive cells ($p>0.05$ versus intact side). In contrast, the number of c-fos positive cells in the EP significantly increased in all groups, suggesting that the intrapallidal surgery, but not the mGluR agonist injection per se, most likely stimulated the cell signaling in the EP.

3. Discussion

In this study, suppressive effects of intrapallidal injection of the mGluR agonists on amphetamine-induced rotational behavior were explored using a rat model of PD. The suppressive effects were demonstrated at 2–12 h after single mGluR injection at the dose of 0.25 μM. The selective group I mGluR agonist, DHPG, but not group III agonist, L-AP4, was shown to mediate the suppressive effects. TH staining demonstrated that intrapallidal injection of mGluR agonists did not rescue against 6-OHDA-induced dopaminergic degeneration. On the other hand, c-fos staining revealed that the injection of mGluR agonists enhanced the cellular activity in the GP (equivalent to GPe in primates), STN, and SNr of the lesioned side, but not in the EP (equivalent to GPi in primates).

3.1. *Time- and dose-dependent effects of the mGluR agonist*

Our study revealed that intrapallidal injection of ACPD lasted for 12 h in a dose-responsive manner and but returned to near baseline levels at 24 h after ACPD injection. These results largely replicated previous observations demonstrating the effects of intrastriatal injection of ACPD were maintained over 24 h in a dose-responsive manner using rats (Sacaan et al., 1991). The differences between brain target regions for ACPD injection (GP versus striatum) between studies might have influenced the timing and dose effects of the drug.

3.2. *Function and distribution of mGluRs*

Accumulating evidence implicates mutiple roles of mGluRs in the CNS including modulation of transmission at glutamatergic synapses as presynaptic receptors, reducing GABA-release at inhibitory synapses and regulating neuronal excitability as postsynaptic receptors (Rouse et al., 2000). Eight mGluR subtypes were classified as group I for mGluR1 and 5, group II for mGluR 2 and 3, and group III for mGluR 4, 6, 7, and 8 (Ossowska et al., 2007). Recent studies also revealed that group I mGluRs were rich in the basal ganglia (Rouse et al., 2000). Accordingly, striatal mGluRs have been explored for the therapeutic applications in PD (Bonsi et al., 2007). Antagonist of mGluR5, but not mGluR1, both catergorized as group I mGluRs, inhibited contralateral rotations induced by L-DOPA administration (Dekundy et al., 2006) In another study using PET scans, the changes of striatal mGluR5 expression negatively correlated with DAT function in 6-OHDA-lesioned rats, suggesting compensatory mechanisms in dopaminergic degeneration (Pellegrino et al., 2007). Consequently, some antagonists of group I mGluR and agonists of group III are believed to have anti-Parkinsonian effects specifically targeting the striatum (Bonsi et al., 2007; Ossowska et al., 2007; Dekundy et al., 2006; Marino et al., 2003). To date, the functional properties of mGluRs have been mainly examined in the striatum, with very few studies focused on the effects of mGluRs in the GP. In the present study, we focused on intrapallidal mGluRs because the known regulatory role of GP on the excitability of STN and thalamus, which are brain structures closer to the output of the basal ganglia. Moreover, the emergence of GP as a feasible and efficacious target for deep brain stimulation has been recognized in clinical practice. Taken together, the present study on intrapallidal mGluRs provides a mechanistic support for this class of glutamate receptors and further clarifies the role of GP in PD therapy.

3.3. *The roles of group I and III mGluRs in the GP*

Previous reports revealed that group I and III mGluRs, but not group II, are expressed in the GP of rats (Bradley et al., 1999;

Testa et al., 1998). However, a recent study demonstrated that group II mGluRs are localized in glutamatergic pre-terminal axons and terminals in the GP suggesting their key participation in excitatory synaptic transmission (Poisik et al., 2005). Group I mGluRs are expressed at the postsynaptic membrane and once activated, the cell membranes of GP neurons become depolarized. Group III mGluRs (mGluR4) exist as heteroreceptors at the surface of synapse of the striatum and GP, and their activation leads to the suppression of the neuronal transmission through the reduced amount of GABA (Valenti et al., 2003). The activation of mGluRs induces diverse neuromodulatory effects in the GP. Upregulation of group I mGluRs in the GP increases the excitability of the postsynaptic membrane (Poisik et al., 2003) and decreases calcium currents (Stefani et al., 1998). Moreover, activation of group III mGluRs suppresses the amplitude of striatal stimulation-induced inhibitory postsynaptic currents (IPSCs), implying that STN inputs to the GP convey information through ionotropic receptor-mediated excitatory postsynaptic potentials (EPSPs) (Matsui and Kita, 2003). Accordingly, the control of GP activity level is achieved via postsynaptic group I mGluRs and the gain of both GABAergic and glutamatergic inputs through presynaptic group III mGluRs (Matsui and Kita, 2003).

3.4. *Functional differences of group I and III mGluR agonists in the striatum and GP*

In PD, striatopallidal neurons are overexcited (Ossowska et al., 2007). Group III mGluRs are expressed on the terminals of the neurons. Activation of group III mGluRs reduces GABAergic and glutamatergic transmission of the pallidal slice culture (Matsui and Kita, 2003). Intraventricular, intranigral, and intrapallidal injection of group III mGluR agonists reverse reserpine-induced akinesia (MacInnes et al., 2004). Furthermore, bilateral intrapallidal group III mGluR agonist injection abrogates the severe akinetic deficits induced by 6-OHDA lesion probably through modulating GABAergic neurotransmission (Lopez et al., 2007). A recent study also revealed intrapallidal group III mGluR agonist (ACPT-1) injection ameliorates haloperidol-induced catalepsy (Konieczny et al., 2007). The discrepancy of the results might lie in the differences of the experimental model (haloperidol-induced catalepsy vs. amphetamine-induced rotation of 6-OHDA-lesioned PD model of rats), of the drug (ACPT-1 vs. L-AP4 with different affinity to mGluR 4 and 7), and of the injected coordinates of GP. In our study, we focused on group I and III mGluR agonists in parallel with previous studies (Kaneda et al., 2005; Matsui and Kita, 2003). Based on these published reports, we initially hypothesized that intrapallidal injection of group III mGluR agonists would afford suppressive effects on amphetamine-induced rotations of parkinsonian rats. Surprisingly, however, group I mGluR agonists, but not group III, exerted strong suppressive effects, suggesting that the direct postsynaptic activation of pallidal neurons via group I mGluRs overrides the presynaptic suppression from the group III mGluRs. The discrepancy between our hypothesis and the results might be explained by highly distinct functions of mGluRs in the striatum and GP. The suppression of group I mGluRs in the striatum likely replicates the activation of this same class of glutamate receptors in the GP. As described later, there exists an analogy on c-*fos* expressions between striatal group I mGluR blockade and pallidal group I mGluR stimulation. Our results agree with the observation that systemic administration of the agonist mGluR8, one of group III mGluR subtypes, did not reverse amphetamine-induced hyperactivity (Robbins et al., 2007).

3.5. *Effects of group I mGluR injection into the GP of 6-OHDA-lesioned rats on amphetamine-induced rotations*

Amphetamine-induced hyperactivity was amplified in group I mGluR knockout mice through the impaired induction of preprodynorphin, compared to the wild-type mice, although there was no significant difference in the basal levels of spontaneous motor activity (Mao et al., 2001). These results indicate that group I mGluRs specifically modulate behavioral responses triggered by dopaminergic stimulation. Conversely, in the present study we show that group I mGluRs, abundant in the GP, were strongly stimulated, and suppressed amphetamine-induced rotations, thereby complementing the results seen in group I mGluR knockout mice.

3.6. *Upregulation of c-fos in the GP, STN and SNr in the mGluR agonist-injected side*

c-Fos is a transcription factor involved in the regulation of the downstream gene expression with *fos-jun* family genes and often used for the detection of changes of neuronal activity (Miwa et al., 1998). Phosphorylation of MAPK (mitogen-activated protein kinase) or PKA (protein kinase A) alters c-*fos* activity. mGluR agonists activate MAPKs through a calcium-insensitive pathway involving the transactivation of receptor tyrosine kinases (Wang et al., 2007), routinely used as an index of neuronal activity. Acute administration of amphetamine increases phosphorylated CREB, Elk-1 and ERK1/2 with subsequent elevation of c-*fos* expression. Intrastriatal blockade of group I, but not group III mGluRs significantly attenuate the immunoreactivity of c-*fos* (Choe et al., 2002), thus suggesting that group I mGluRs in the striatum, but not in the GP, suppress the neuronal activity. In contrast, our study revealed that group I mGluRs in the GP are a key participant in the enhanced neuronal activity of the GP, leading to the enhanced suppressive output directly affecting STN, SNr and EP. Similarly, group I mGluR agonists have been shown to induce phosphorylation of Akt, ERK1/2, and CREB in both CPu and NAc with subsequent c-*fos* upregulation (Pan et al., 2004). The differential changes in c-*fos* expression after intrapallidal mGluR agonist injection might have been affected by 6-OHDA-induced dopaminergic denervation and amphetamine injection in that after dopamine agonist treatment c-*fos* expression was increased in intact, but decreased in the lesioned GP (Wirtshafter and Asin, 1999). Because all animals included in our study received 6-OHDA lesion, the observed increments in c-*fos* expression can be ascribed to intrapallidal mGluR agonist injection.

4. Conclusions

Intrapallidal injection of the non-selective and selective group I mGluR agonists, but not group III, exerts strong suppressive effects on amphetamine-induced rotational behavior of 6-OHDA-lesioned rats in a dose- and time-responsive manner. The mGluR injection did not alter dopaminergic degeneration. On the other hand, c-*fos* expression was upregulated in the GP and the unilateral STN and SNc of non-selective and the selective group

I mGluR-injected parkinsonian animals, indicating enhanced neuronal activity. Our study clearly delineated the divergent functions of group I and III mGluR agonists, as well as provided insights on the vital roles of this class of glutamate receptors in the striatum and in the GP. The extensive expression of mGluRs in the basal ganglia strategically positions them as a major therapeutic target for PD.

5. Experimental procedure

5.1. *Animals and mGluR agonists*

Female Sprague–Dawley rats (n=96, Charles River, Japan) weighing 250-300 g were used. Animals were housed two per cage in a temperature- and humidity-controlled room with a 12 h light/dark cycle and they had free access to food and water. A non-selective mGluR agonist, 1-aminocyclopentane-1S,3R-dicarboxylic acid (ACPD, Sigma, MO, USA), a selective group I mGluR agonist, (R,S)-3,5-dihydroxyphenylglycine (DHPG, Tocris Bioscience, MI, USA) and a selective group III mGluR agonist, L(1)-2-amino-4-phosphonobutyric acid (L-AP4, Tocris Bioscience) were dissolved in 0.1 M phosphate buffer (PB) with pH adjusted using 4N NaOH.

5.2. *Unilateral 6-OHDA lesion*

Rats were pretreated with desipramine (25 mg/kg, i.p., Sigma) at 1 h before 6-hydroxydopamine (6-OHDA) injection (n=96). Animals were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and placed in a stereotaxic instrument (Narishige, Japan). Through a burr hole in the skull, a 10 μl Hamilton syringe was introduced into the right medial forebrain bundle (4.0 mm posterior from bregma, 1.3 mm lateral to the midline, and 8.4 mm ventral to the surface of the skull with the incisor bar at 2.8 mm) (Paxinos and Watson, 1998) and 6-OHDA (1.6 μl of 5 μg/μl dissolved in saline containing 0.2 mg/ml ascorbic acid; Sigma) was injected over 3 min The syringe was left in place for an additional 5 min prior to slow retraction (1 mm/ min). At 4 weeks after surgery, rats received an injection of apomorphine (0.03 mg/kg, s.c.) and rotational behaviors were measured. Animals exhibiting over 5 rotations per minute were used for the further study (n=75).

5.3. *Intrapallidal mGluR agonist injection*

Rats were placed in a sterotaxic instrument under general anesthesia with halothane (0.8–1.5%) in air and allowed to ventilate spontaneously. A 10 μl Hamilton syringe was introduced in the right GP through a burr hole in the skull and 0.5 μl of mGluR agonist at each dose (n=48) or 0.1 M PB (n=27) was injected for 4 min The syringe was left in place for 5 min and then slowly withdrawn. Stereotaxic coordinates for the injection were: 1.3 mm posterior from bregma, 3.4 mm lateral to the midline, and 7.2 mm ventral to the surface of the skull with the incisor bar at 2.8 mm (Paxinos and Watson, 1998).

5.4. *Behavioral evaluation*

First, amphetamine-induced rotational behaviors were assessed at 3 days before and again at 0, 2, 4, 6, 8, 12, and 24 h after intrapallidal ACPD or PB injection (0.25 μM/0.5 μl, n=3 respectively). The dose of ACPD was based on previous reports (Sacaan et al., 1991) and our initial dosing study (Fig. 1, n=3 for each dose). Animals received an injection of amphetamine (2.5 mg/kg, i.p., Dainippon-Seiyaku, Japan) and rotational behaviors were assessed for 90 min with a video camera. Full 360° turns in the direction ipsilateral to the lesion were counted. The percentages of the rotational number relative to that obtained at 3 days before ACPD injection were used for statistical analyses. In a parallel study, the selective group I or III mGluR agonist, DHPG or L-AP4 (0.25 μM/0.5 μl, respectively) was administered instead of ACPD at 4 h before amphetamine-induced rotational tests in order to reveal the predominant mGluR agonist in this study and analyzed similarly (n=6 in each group).

5.5. *Immunohistochemistry*

Immunohistochemical investigations were performed as described in our previous reports (Yasuhara et al., 2006, 2004). Immediately after measurement of rotational behavior at 4 h after the mGluR agonist or vehicle injection, rats were anesthetized deeply with pentobarbital and perfused transcardially with 200 ml of cold PBS, followed by 100 ml of 4% PFA in PBS. Brains were removed and post-fixed in the same fixative for 48 h followed by 30% sucrose in PB to be sunk. Six series of coronal sections were cut at a thickness of 40 μm in a freezing microtome and stored at −20 °C.

Sections at the level of the striatum or substantia nigra pars compacta (SNc) (striatum: 0 mm, SNc: −5 mm posterior to the bregma) were used for tyrosine hydroxylase (TH) staining and for c-*fos* staining, sections including GP, entopeduncular nucleus (EP), subthalamic nucleus (STN), substantia nigra pars reticulata (SNr) (−1.4, −2.8, −3.6 and −5.0 mm posterior to the bregma) were used in each rat. Free-floating sections for immunohistochemistry were incubated in an anti-TH antibody (rabbit polyclonal, 1:500, Chemicon, CA, USA) or anti-c-*fos* antibody (rabbit polyclonal, 1:500, Sigma) overnight at 4 °C with 10% normal goat serum (Vector). The sections were then washed in PBS 3 times with subsequent incubation in goat anti-rabbit IgG Alexa Fluor 594 (1:1000, Molecular Probes, OR, USA) and 4′, 6-diamidino-2-phenylindole, dilactate (DAPI, 1:1000, Molecular Probes) for 2 h, washed in 0.1 M PB and finally mounted onto clean glass slides. DAPI was used for counter staining. The slides were coverslipped using mounting medium (Prolong Antifade KitR, Molecular Probes). The number of nuclei with c-*fos* immunoreactivity in the bilateral GP, EP, STN, and SNr in 3 randomly selected squares (500×500 μm^2) in each section was counted using 2 consecutive sections for each structure and the averages were statistically analyzed. In addition, control studies included exclusion of primary antibody substituted with 10% normal goat serum in PBS and no immunoreactivity was observed in these controls.

5.6. Statistical analysis

Data are presented as means±standard errors. Data were evaluated statistically using either Mann–Whitney's U-test, Repeated measures with Two-Way ANOVA followed by a Scheffé *post hoc* test. Statistical significance was preset at $p<0.05$.

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REFERENCES

Benabid, A.L., Krack, P.P., Benazzouz, A., Limousin, P., Koudsie, A., Pollak, P., 2000. Deep brain stimulation of the subthalamic nucleus for Parkinson's disease: methodologic aspects and clinical criteria. Neurology 55, S40–S44.

Bonsi, P., Cuomo, D., Picconi, B., Sciamanna, G., Tscherter, A., Tolu, M., Bernardi, G., Calabresi, P., Pisani, A., 2007. Striatal metabotropic glutamate receptors as a target for pharmacotherapy in Parkinson's disease. Amino Acids 32, 189–195.

Bradley, S.R., Standaert, D.G., Rhodes, K.J., Rees, H.D., Testa, C.M., Levey, A.I., Conn, P.J., 1999. Immunohistochemical localization of subtype 4a metabotropic glutamate receptors in the rat and mouse basal ganglia. J. Comp. Neurol. 407, 33–46.

Choe, E.S., Chung, K.T., Mao, L., Wang, J.Q., 2002. Amphetamine increases phosphorylation of extracellular signal-regulated kinase and transcription factors in the rat striatum via group I metabotropic glutamate receptors. Neuropsychopharmacology 27, 565–575.

Dekundy, A., Pietraszek, M., Schaefer, D., Cenci, M.A., Danysz, W., 2006. Effects of group I metabotropic glutamate receptors blockade in experimental models of Parkinson's disease. Brain Res. Bull. 69, 318–326.

Feeley Kearney, J.A., Albin, R.L., 2003. mGluRs: a target for pharmacotherapy in Parkinson disease. Exp. Neurol. 184 (Suppl 1), S30–S36.

Kaneda, K., Tachibana, Y., Imanishi, M., Kita, H., Shigemoto, R., Nambu, A., Takeda, M., 2005. Down-regulation of metabotropic glutamate receptor 1alpha in globus pallidus and substantia nigra of parkinsonian monkeys. Eur. J. Neurosci. 22, 3241–3254.

Konieczny, J., Wardas, J., Kuter, K., Pilc, A., Ossowska, K., 2007. The influence of group III metabotropic glutamate receptor stimulation by (1S,3R,4S)-1-aminocyclo-pentane-1,3,4-tricarboxylic acid on the parkinsonian-like akinesia and striatal proenkephalin and prodynorphin mRNA expression in rats. Neuroscience 145, 611–620.

Lopez, S., Turle-Lorenzo, N., Acher, F., De Leonibus, E., Mele, A., Amalric, M., 2007. Targeting group III metabotropic glutamate receptors produces complex behavioral effects in rodent models of Parkinson's disease. J. Neurosci. 27, 6701–6711.

MacInnes, N., Messenger, M.J., Duty, S., 2004. Activation of group III metabotropic glutamate receptors in selected regions of the basal ganglia alleviates akinesia in the reserpine-treated rat. Br. J. Pharmacol. 141, 15–22.

Mao, L., Conquet, F., Wang, J.Q., 2001. Augmented motor activity and reduced striatal preprodynorphin mRNA induction in response to acute amphetamine administration in metabotropic glutamate receptor 1 knockout mice. Neuroscience 106, 303–312.

Marino, M.J., Williams Jr., D.L., O'Brien, J.A., Valenti, O., McDonald, T.P., Clements, M.K., Wang, R., DiLella, A.G., Hess, J.F., Kinney, G.G., Conn, P.J., 2003. Allosteric modulation of group III metabotropic glutamate receptor 4: a potential approach to Parkinson's disease treatment. Proc. Natl. Acad. Sci. U. S. A. 100, 13668–13673.

Matsui, T., Kita, H., 2003. Activation of group III metabotropic glutamate receptors presynaptically reduces both GABAergic and glutamatergic transmission in the rat globus pallidus. Neuroscience 122, 727–737.

Miwa, H., Fuwa, T., Nishi, K., Mizuo, Y., 1998. Effects of the globus pallidus lesion on the induction of c-Fos by dopaminergic drugs in the striatum possibly via pallidostriatal feedback loops. Neurosci. Lett. 240, 167–170.

Ossowska, K., Konieczny, J., Wardas, J., Pietraszek, M., Kuter, K., Wolfarth, S., Pilc, A., 2007. An influence of ligands of metabotropic glutamate receptor subtypes on parkinsonian-like symptoms and the striatopallidal pathway in rats. Amino Acids 32, 179–188.

Pan, Y., Berman, Y., Carr, K.D., 2004. Effects of the group I metabotropic glutamate receptor agonist, DHPG, and injection stress on striatal cell signaling in food-restricted and ad libitum fed rats. BMC Neurosci. 5, 50.

Paxinos, G., Watson, G., 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego. vol.

Pellegrino, D., Cicchetti, F., Wang, X., Zhu, A., Yu, M., Saint-Pierre, M., Brownell, A.L., 2007. Modulation of dopaminergic and glutamatergic brain function: PET studies on Parkinsonian rats. J. Nucl. Med. 48, 1147–1153.

Poisik, O.V., Mannaioni, G., Traynelis, S., Smith, Y., Conn, P.J., 2003. Distinct functional roles of the metabotropic glutamate receptors 1 and 5 in the rat globus pallidus. J. Neurosci. 23, 122–130.

Poisik, O., Raju, D.V., Verreault, M., Rodriguez, A., Abeniyi, O.A., Conn, P.J., Smith, Y., 2005. Metabotropic glutamate receptor 2 modulates excitatory synaptic transmission in the rat globus pallidus. Neuropharmacology 49 (Suppl 1), 57–69.

Robbins, M.J., Starr, K.R., Honey, A., Soffin, E.M., Rourke, C., Jones, G.A., Kelly, F.M., Strum, J., Melarange, R.A., Harris, A.J., Rocheville, M., Rupniak, T., Murdock, P.R., Jones, D.N., Kew, J.N., Maycox, P.R., 2007. Evaluation of the mGlu8 receptor as a putative therapeutic target in schizophrenia. Brain Res. 1152, 215–227.

Rodriguez-Oroz, M.C., Obeso, J.A., Lang, A.E., Houeto, J.L., Pollak, P., Rehncrona, S., Kulisevsky, J., Albanese, A., Volkmann, J., Hariz, M.I., Quinn, N.P., Speelman, J.D., Guridi, J., Zamarbide, I., Gironell, A., Molet, J., Pascual-Sedano, B., Pidoux, B., Bonnet, A.M., Agid, Y., Xie, J., Benabid, A.L., Lozano, A.M., Saint-Cyr, J., Romito, L., Contarino, M.F., Scerrati, M., Fraix, V., Van Blercom, N., 2005. Bilateral deep brain stimulation in Parkinson's disease: a multicentre study with 4 years follow-up. Brain 128, 2240–2249.

Rouse, S.T., Marino, M.J., Bradley, S.R., Awad, H., Wittmann, M., Conn, P.J., 2000. Distribution and roles of metabotropic glutamate receptors in the basal ganglia motor circuit: implications for treatment of Parkinson's disease and related disorders. Pharmacol. Ther. 88, 427–435.

Sacaan, A.I., Monn, J.A., Schoepp, D.D., 1991. Intrastriatal injection of a selective metabotropic excitatory amino acid receptor agonist induces contralateral turning in the rat. J. Pharmacol. Exp. Ther. 259, 1366–1370.

Stefani, A., Spadoni, F., Bernardi, G., 1998. Group I mGluRs modulate calcium currents in rat GP: functional implications. Synapse 30, 424–432.

Testa, C.M., Friberg, I.K., Weiss, S.W., Standaert, D.G., 1998. Immunohistochemical localization of metabotropic glutamate receptors mGluR1a and mGluR2/3 in the rat basal ganglia. J. Comp. Neurol. 390, 5–19.

Valenti, O., Marino, M.J., Wittmann, M., Lis, E., DiLella, A.G., Kinney, G.G., Conn, P.J., 2003. Group III metabotropic glutamate receptor-mediated modulation of the striatopallidal synapse. J. Neurosci. 23, 7218–7226.

Wang, J.Q., Fibuch, E.E., Mao, L., 2007. Regulation of mitogen-activated protein kinases by glutamate receptors. J. Neurochem. 100, 1–11.

Wirtshafter, D., Asin, K.E., 1999. Unilateral dopamine depletion paradoxically enhances amphetamine-induced Fos expression in basal ganglia output structures. Brain Res. 824, 81–88.
Yasuhara, T., Shingo, T., Kobayashi, K., Takeuchi, A., Yano, A., Muraoka, K., Matsui, T., Miyoshi, Y., Hamada, H., Date, I., 2004. Neuroprotective effects of vascular endothelial growth factor (VEGF) upon dopaminergic neurons in a rat model of Parkinson's disease. Eur. J. Neurosci. 19, 1494–1504.
Yasuhara, T., Matsukawa, N., Hara, K., Yu, G., Xu, L., Maki, M., Kim, S.U., Borlongan, C.V., 2006. Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. J. Neurosci. 26, 12497–12511.

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Comparison of the therapeutic potential of adult and embryonic neural precursor cells in a rat model of Parkinson disease

Laboratory investigation

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Object. The therapeutic effects of adult and embryonic neural precursor cells (NPCs) were evaluated and their therapeutic potential compared in a rat model of Parkinson disease.

Methods. Adult NPCs were obtained from the subventricular zone and embryonic NPCs were taken from the ganglionic eminence of 14-day-old embryos. Each NPC type was cultured with epidermal growth factor. The in vitro neuronal differentiation rate of adult NPCs was approximately equivalent to that of embryonic NPCs after two passages. Next, the NPCs were transfected with either green fluorescent protein or glial cell line–derived neurotrophic factor (GDNF) by adenoviral infection and transplanted into the striata in a rat model of Parkinson disease (PD) induced by unilateral intrastriatal injection of 6-hydroxydopamine. An amphetamine-induced rotation test was used to evaluate rat behavioral improvement, and immunohistochemical analysis was performed to compare grafted cell survival, differentiation, and host tissue changes.

Results. The rats with GDNF-transfected NPCs had significantly fewer amphetamine-induced rotations and less histological damage. Except for the proportion of surviving grafted cells, there were no significant differences between adult and embryonic NPCs.

Conclusions. Adult and embryonic NPCs have a comparable therapeutic potential in a rat model of PD.
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Key Words • **adult neural precursor cell** • **embryonic neural precursor cell** • **glial cell line–derived neurotrophic factor** • **Parkinson disease** • **rat** • **transplantation**

Neural stem cell transplantation is one of several methods currently under investigation as a therapy for CNS diseases.[16,29] Until recently only embryonic stem cells and fetal neural stem cells had been tested as sources of stem cells.[15,43] An in vitro culture system for adult NPCs was recently established, however, and there have been several studies related to CNS cell therapy with adult NPCs. Various techniques for performing cell transplantation more effectively have been examined, such as gene manipulation by transduction of neurotrophic factor genes[1,22,24] or induction of neuronal differentiation to obtain a specific cell phenotype.[30,34] Many issues remain unresolved, however. The application of embryonic stem cells induces a risk of tumor formation,[6,30] and there is a shortage of available donor tissue for fetal tissue transplantation studies.[5] Other problems include the host's immune reaction to the placement of foreign cells and the ethical debate surrounding the use of fetal tissues.[4,5]

Parkinson disease was the first CNS disorder for which cell transplantation was attempted in clinical trials. In an effort to replace degenerated dopamine-producing neurons, primary human fetal dopaminergic cells within mesencephalic cores have been transplanted into patients with PD.[16] There are two well-known fundamental problems that limit the effectiveness and use of fetal dopamine-producing cell transplants: difficulty recovering human fetal tissue and the poor survival of neurons. Many researchers working to overcome these problems have reported favorable results. Adult and embryonic NPCs cultured prior to transplantation promote both histological and behavioral recovery after intrastriatal transplantation in rats with 6-OHDA–in-

Abbreviations used in this paper: ANOVA = analysis of variance; CNS = central nervous system; E14 = 14-day-old embryos; EGF = epidermal growth factor; ELISA = enzyme-linked immunosorbent assay; GDNF = glial cell line–derived neurotrophic factor; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; IgG = immunoglobulin G; MHM = modified Hanks medium; NeuN = neuron-specific nuclear protein; NPC = neural precursor cell; PBS = phosphate-buffered saline; PD = Parkinson disease; SEM = standard error of the mean; SNC = substantia nigra pars compacta; TH = tyrosine hydroxylase; 6-OHDA = 6-hydroxydopamine.

duced loss of dopaminergic neurons.[20,28] Furthermore, compared with simple NPC transplantation, transplantation of *GDNF*-transduced NPCs promotes therapeutic efficacy in a rat model of PD.[1,22]

Methods for culturing adult human NPCs are now established,[18,21] and the use of autologous or allogeneic adult NPC transplantation has become a realistic option for overcoming the various issues related to the use of stem cells.[39] However, few reports exist demonstrating whether different donor sources have different therapeutic potentials in cell therapy. We therefore compared the applicability of adult and embryonic NPCs as sources for cell therapy in a rat model of PD and examined gene transfection efficiency. Either the *GDNF* (reported to have therapeutic effects in patients with PD[42]) or the *GFP* gene was transfected into donor cells via adenovirus infection. The transfected donor cells were then implanted into rat striata,[1] and the adult and embryonic cell sources were compared with regard to therapeutic effects.

Materials and Methods

Animal Preparation

We used 55 adult female Sprague–Dawley rats (weighing 200–250 g at the beginning of the experiment; Charles River Laboratories) as donors of adult NPCs and as recipients in a rat model of PD. Embryonic NPCs were obtained from six E14 Sprague–Dawley rats. The animals were housed 2 per cage in a temperature- and humidity-controlled room maintained on a 12-hour light/dark cycle. The animals had free access to food and water. All procedures were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Okayama University. All efforts were made to minimize the number of animals used and their suffering.

Neural Precursor Cell Culture and Analysis

The procedures used to generate neurospheres from embryonic and adult forebrain tissues were adopted as described previously, with minor modifications.[27,32,38]

Embryonic NPC Cultures. Briefly, the ganglionic eminences were dissected from the E14 embryos in PBS containing 0.6% glucose, penicillin (50 U/ml), and streptomycin (50 U/ml; Life Technologies) and then transferred to MHM composed of Dulbecco modified Eagle medium-F12 (1:1), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (5 mM), insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM; all from Sigma/Aldrich Chemical Co., except glutamine, which was from Life Technologies). The tissues were mechanically triturated into single cells with a fire-polished pipette. Cells were cultured for 7 days in vitro at a density of 100 cells/μl in 20 ng/ml EGF-containing media (human recombinant; PeproTech).

Adult NPC Cultures. Adult rats were killed by decapitation after anesthesia had been induced with intraperitoneal injection of sodium pentobarbitol (30 mg/kg). The brains were dissected, placed in sterile PBS containing 1% penicillin/streptomycin solution on ice, and transferred to a sterile tissue culture hood. The subventricular zone was then dissected grossly. Each whole brain was placed in a brain matrix, and two coronal cuts were made, the first posterior to the rhinal fissure and the second anterior to the hippocampus. The resulting tissue blocks were checked grossly to verify that no hippocampal tissue was included. Two parasagittal cuts were made just lateral to the ventricles; one horizontal cut was made at the level of the corpus callosum, and one horizontal cut was made below the level of the ventricles. The tissue was then minced with 2 sterile scalpels, transferred into MHM containing 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid (Sigma) and incubated for 15 minutes at 37°C. After complete trituration with a micropipette, the suspension was transferred into the same volume of media containing 0.7 mg/ml trypsin inhibitor (Roche Diagnostics). This suspension was centrifuged at 600 rpm for 5 minutes, resuspended, and then plated at a density of 20,000 cells/ml in a 6-well plate in EGF-containing media. The generated neurospheres (primary spheres) were passaged by mechanical dissociation and reseeded as single cells at a density of 10,000 cells/ml for embryos and 20,000 cells/ml for adults in EGF-containing media. Passaged cells were processed for differentiation experiments as described below and illustrated in Fig. 1.

Counting Neurons In Vitro

Primary spheres were dissociated and either differentiated to assess neuron production or replated in EGF-containing media to generate secondary spheres. To observe neuronal differentiation in vitro, dissociated primary spheres were plated onto poly-L-ornithine-coated coverslips in MHM containing 1% fetal bovine serum at a density of 100,000 cells/ml/well in 24-well plates. After 72 hours, cells were fixed in 4% paraformaldehyde solution and washed in PBS. The cells were incubated with an antibody directed against β-tubulin (mouse monoclonal IgG, 1:500 dilution; Sigma) with 10% normal horse serum (Vector Laboratories). After several rinses in PBS, the cells were incubated in sheep anti–mouse IgG Cy3 conjugate (1:1000 dilution; Sigma) and diaminophenylindol (1:200 dilution; Sigma). The cells were then washed in PBS, mounted on albumin-coated slides, and sealed to the slides. Immunoreactive neurons were counted for each randomly selected high-power field (10,000 μm^2), and the immunostaining was photographically documented. Using the same procedure, secondary and tertiary spheres were dissociated and neuron production assessed. In control studies, 10% normal horse serum in PBS was substituted for primary antibody. No immunoreactivity was observed in the controls.

Evaluation of Transfected GFP *and* GDNF *Gene Expression by ELISA*

Adenovirus-mediated gene transfection was performed. Adult and embryonic NPCs were exposed to the infectious viral particles in 40-ml MHM at 37°C for 12 hours; cells were infected with recombinant adenovirus vectors carrying either *GFP* or *GDNF* at a multiplicity of infection of 10^3. The media were then removed, and the cells were washed once with MHM and replated in MHM without EGF. Forty-eight hours after the gene-transfected NPCs were reseeded in vitro, culture supernatants were collected for analysis. Commercial GDNF ELISA kits (Promega) were used to quantify the concentration of GDNF in each of the samples. All samples were run in triplicate.

Cell Transplantation and 6-OHDA Injection

All rats were deeply anesthetized with intraperitoneal injections of sodium pentobarbital (30 mg/kg) and placed in a stereotactic instrument (Narishige). A midline skin incision was made in each animal's skull and a bur hole was drilled. Using a 5-μl Hamilton syringe (87900; Hamilton), 1 μl of the cell suspension (10^4 cells/μl) was injected over a 10-minute period into four sites in the right striatum at the following coordinates measured from the bregma: 0.7 mm anteroposterior, 2.0 mm mediolateral, −2.5 and −5.5 mm dorsoventral, −0.3 mm anteroposterior, 3.0 mm mediolateral, and −2.5 mm and −5.5 mm dorsoventral.[26] After implantation, the cannula was left in place for 5 minutes and slowly withdrawn (1 mm/minute). Seven days after implantation, following pretreatment with desipramine (25 mg/kg, intraperitoneally administered; Sigma–Aldrich) and 20 μg of a 6-OHDA solution (4 μl of 5 μg/μl 6-OHDA dissolved in 0.9% saline containing 0.2 mg/ml ascorbic acid; Sigma–Aldrich) was injected into the right striatum with a 28-gauge Hamilton syringe at the following coordinates from the bregma: 0.0 mm anteroposterior, 2.7 mm mediolateral, and −6.0 mm dorsoventral. The injection rate was 1 μl/minute, and the syringe was left in place for 5 minutes before it was slowly withdrawn (1 mm/minute).

The rats were divided into 4 groups of 6 animals according to the cells injected: 1 group received adult *GDNF*-transfected cells, 1 group received adult *GFP*-transfected cells, 1 group received embryonic *GDNF*-transfected cells, and 1 group received embryonic *GFP*-transfected cells.

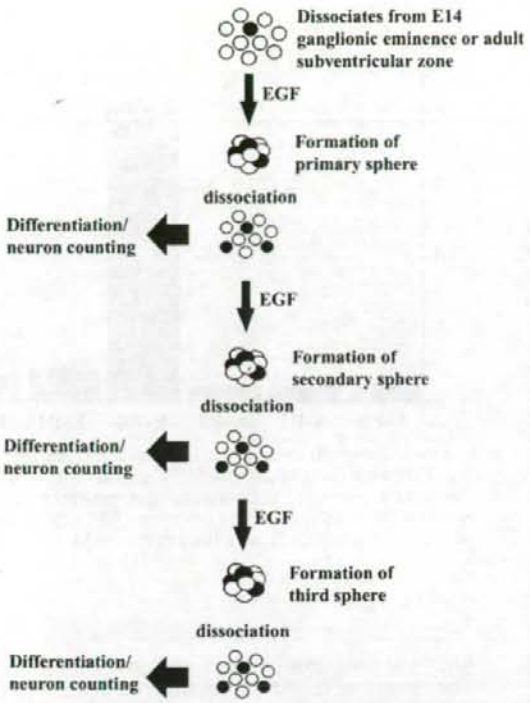

FIG. 1. Schematic of the experimental protocol for the in vitro assessment of neuronal differentiation in this study. A neural stem cell is expanded by the formation of a clonally derived cell cluster, called a sphere, in EGF-containing growth medium. The primary spheres generated are dissociated and plated in populations (5×10^4 cells/ml) for neuronal differentiation assessment or reseeded for secondary sphere generation in EGF medium. The neuronal differentiation rates of both secondary and tertiary spheres were assessed in the same manner.

Measurement of GDNF Production In Vivo by ELISA

Seven days after cell transplantation, 4 rats from each group were killed by decapitation. The brains were rapidly removed and sliced into 2-mm sections, and the transplanted side of the striatum was punched out (punch diameter: 3 mm), snap-frozen in liquid nitrogen, and stored at −80°C for subsequent extraction and quantification of GDNF by ELISA. For optimal protein extraction, the tissue was homogenized on ice in PBS containing protease inhibitors (Complete Mini; Roche) at pH 7.4. Homogenates were centrifuged at 12000 × gravity for 10 minutes at 4°C, and the supernatants were immediately removed and assayed in duplicate, along with calibrated cytokine standards.

Amphetamine-Induced Rotation

Rats were tested for amphetamine-induced rotation (2.5 mg/kg; Dainippon-Seiyaku) at 1, 2, 4, and 6 weeks after 6-OHDA injection. Rotation was assessed for 90 minutes using a video camera, and full 360° turns in the direction ipsilateral to the lesion were counted.

Fixation and Sectioning

At 6 weeks after 6-OHDA injection, deep anesthesia was induced in all rats, and they were perfused through the ascending aorta with 200 ml of cold PBS, followed by 100 ml of 4% paraformaldehyde in PBS. Brains were removed and postfixed in the same fixative for 3 days, followed by fixation in 30% sucrose in PBS for 1 week. Six series of coronal sections were cut at a 40-μm thickness with a freezing microtome and stored at −20°C.

Immunohistochemical Analysis

The sections processed for fluorescent staining were incubated with an anti-GFP antibody (either rabbit polyclonal, 1:500 dilution, MBL International; or mouse monoclonal, 1:200 dilution, Molecular Probes), an anti-NeuN antibody (mouse monoclonal, 1:100 dilution; Chemicon), or an anti-GFAP antibody (rabbit polyclonal, 1:500 dilution; Sigma) with 10% normal goat serum (Vector Laboratories). After several rinses in PBS, sections were incubated in donkey anti–rabbit IgG fluorescein isothiocyanate conjugate (1:500 dilution), biotinylated donkey anti–mouse (1:1000 dilution), or donkey anti–rabbit IgG (1:500 dilution). After several rinses in PBS, the sections were incubated in Cy3 conjugate streptavidin (1:2000 dilution; all from Jackson ImmunoResearch Laboratories). All nuclei were stained with diaminophenylindol (1:1000 dilution; Molecular Probes) in PBS for 5 minutes and then washed again with PBS. The sections were processed for TH, GFP, or GDNF; pretreated with 0.3% H_2O_2 in methanol for 30 minutes; and incubated with 10% normal goat serum in PBS for 1 hour at room temperature to block nonspecific binding. The sections were incubated with anti-TH antibody (rabbit polyclonal, 1:1000 dilution; Chemicon), anti-GFP antibody (mouse monoclonal, 1:200 dilution; Molecular Probes), or anti-GDNF antibody (rabbit polyclonal, 1:200 dilution; Molecular Probes) in PBS. The sections were then incubated with biotinylated anti–mouse IgG (1:200 dilution) or anti–goat IgG (1:100 dilution), followed by incubation in an avidin–biotin complex solution (Vector Laboratories). Sections were visualized using 3,3′-diaminobenzidine (D5905; Sigma), dehydrated with a graded ethanol series, and then passed through pure xylene. In the control studies, the primary antibody was replaced with 10% normal goat serum in PBS. There was no immunoreactivity in the controls.

Morphological Analysis

The density of TH-positive fibers in the striata of animals with transplanted cells was determined and analyzed using a computerized analysis system (Olympus SP-1000), as described previously.[31] The level of the bregma +0.5 mm in the atlas was selected for quantitative analysis. Two areas of the grafted side and the equivalent areas on the contralateral side were analyzed. The TH-positive neurons of the lesioned and intact SNC were counted under bright-field illumination using an objective lens (magnification × 40). The TH-positive cell counts in the lesioned SNC were expressed as a percentage of the intact side.

Quantification of Surviving Cells

Few transplanted cells were identified in sections that were 500 μm or more away from the transplantation site. Therefore, surviving cells located within 480 μm of the site were counted. A 1-in-6 series of coronal sections (40-μm-thickness) from 480 μm rostral to 480 μm caudal to the transplant coordinates (anteroposterior: +0.7 and −0.3 mm, 8 sections total) was collected. All GFP-positive transplanted cells were located in this range. Dead cells and red blood cells produce autofluorescence under a fluorescence microscope. Therefore, cells were counted before phenotype-specific immunostaining to distinguish green (GFP)-single-positive surviving cells from autofluorescent dead cells. Cells that were positive for green fluorescence and negative for red fluorescence were considered to be surviving cells. In contrast, green/red double-positive cells were excluded as dead cells or red blood cells. The numbers of GFP-positive (surviving), NeuN/GFP-positive (mature neuronal differentiated), and GFAP/GFP-positive (glial differentiated) cells were counted.

Statistical Analysis

Values are presented as the means ± SEMs. Comparisons of in vitro neuronal differentiation after repetitive passages were made between the adult and embryonic NPCs using an unpaired t-test. Statistical analysis of experiments involving multiple groups was

performed using ANOVA, followed by the Tukey honest significant difference post-hoc analysis to compare all groups with each other or a repeated measures ANOVA to compare the changes in amphetamine-induced rotational behavior.

Results

Neuronal Differentiation Rate In Vitro

Before transplantation, cells obtained from the brain must be cultured in vitro by repeated passaging until there are sufficient cells. Therefore, we first examined whether the neuronal differentiation rate of adult and embryonic NPCs changed after repeated passage. The neuronal differentiation rates of adult NPCs were 1.2 ± 0.5% for the primary sphere, 1.2 ± 0.2% for the secondary sphere, and 1.0 ± 0.1% for the tertiary sphere (Fig. 2). The neuronal differentiation rate of adult NPCs did not change, even after several passages; there was no significant difference between each passage stage. In contrast, the neuronal differentiation rates of embryonic NPCs were 20.5 ± 2.6% (primary sphere), 2.2 ± 0.3% (secondary sphere), and 1.9 ± 0.3% (tertiary sphere). Repeated passaging remarkably reduced the neuronal differentiation rates of embryonic NPCs. There was no difference between adult and embryonic NPCs in the tertiary sphere in terms of the neuronal differentiation potential in vitro (adult compared with embryonic tertiary spheres from 3 rats; $p = 0.996$). These results demonstrated that when adult and embryonic NPCs are passaged twice, the 2 donor sources have equivalent neuronal differentiation potential in vitro.

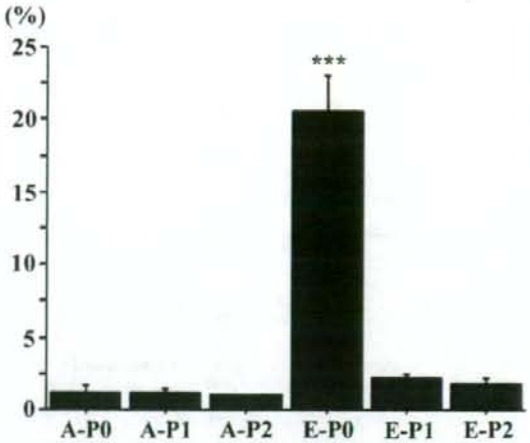

FIG. 2. Bar graph showing the differentiation rates after passaging (P) of adult (A-) and embryonic (E-) primary (0), secondary (1), and tertiary spheres (2). Repeated passages remarkably reduced the neuronal differentiation rates of embryonic NPCs (E-P0 compared with E-P1, $p < 0.0001$, 3 rats). Error bar: + SEM. ***$p < 0.0001$.

Transfected GDNF Expression In Vitro and In Vivo

Various gene manipulation methods have been used in cell transplantation studies. In this experiment, *GFP* or *GDNF* was transfected into donor cells by adenovirus infection, and the expression efficiency of each transfected gene was compared between adult and embryonic NPCs. The amount of GDNF produced by each cell in vitro was 67.8 ± 14.1 pg/unit from adult GFP, 79.7 ± 23.6 pg/unit from embryonic GFP, 2403.3 ± 125.3 pg/unit from adult GDNF, and 2448.3 ± 108.6 pg/unit from embryonic GDNF (4 rats; unit = 24 hour/10^6 cells, Fig. 3 *left*). The adult GFP and embryonic GFP groups produced very small amounts of GDNF. These amounts were considered to be due to spontaneous secretion from the NPCs. On the other hand, the adult and embryonic GDNF groups produced significantly higher

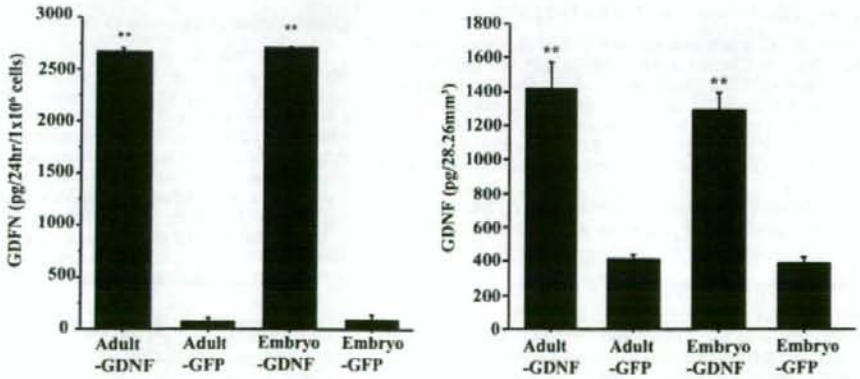

FIG. 3. *Left:* Amount of GDNF produced in vitro. The adult and embryonic *GDNF*-transfected cells produced significantly higher amounts of GDNF than both *GFP*-transfected groups. There was no statistical difference between the *GDNF*- or *GFP*-transfected groups. **$p < 0.0001$; error bar + SEMs. *Right:* Amount of GDNF produced in vivo. In vivo evaluation also revealed a significantly higher amount of GDNF in the groups that received adult and embryonic *GDNF*-transfected cells than in both groups receiving *GFP*-transfected cells. There was no statistical difference, however, between the *GDNF*- or *GFP*-transfected groups. **$p < 0.0001$; error bar + SEMs.

amounts of GDNF compared with the adult and embryonic GFP groups, respectively (adult GDNF compared with adult GFP, $p < 0.001$; embryonic GDNF compared with embryonic GFP, $p < 0.001$). There was no statistically significant difference between the adult and embryonic GDNF groups. These results confirmed that adult and embryonic NPCs have approximately the same potential to express a gene that has been transfected in vitro using adenovirus infection.

We then compared the amount of GDNF produced by transfected cells 1 week after intrastriatal transplantation. The amount of GDNF produced in vivo was 411.8 ± 32.2 pg/unit in the adult GFP group, 396.9 ± 37.5 pg/unit in the embryonic GFP group, 1414.3 ± 163.0 pg/unit in the adult GDNF group, and 1292.4 ± 106.3 pg/unit in the embryonic GDNF group (unit; 28.26 mm^3, 6 rats/group, Fig. 3 *right*). The amount of GDNF detected in the adult and embryonic GFP groups was thought to be supplied mainly from the host brain tissue because, based on the in vitro data, only a small portion of the total amount detected was produced by the grafted cells. On the other hand, the increased amounts of GDNF detected in the *GDNF*- compared with the *GFP*-transfected groups was due to the increased quantities supplied by *GDNF*-transfected cells. Although the adult GDNF group tended to produce a larger amount of GDNF than the embryonic GDNF group, the difference between the two groups in vivo and in vitro was not significant. These results confirmed that transfected-gene expression levels in the host brain are approximately the same in adult and embryonic NPCs.

Amphetamine-Induced Rotation

Amphetamine-induced rotation tests were performed to assess changes in rat behavior. There was no obvious change in the spontaneous behavior among the four groups (data not shown). There was no significant difference between the adult and embryonic GFP groups in either the difference or change in number of rotations. Also, there was no significant difference between the adult and embryonic GDNF groups in number of rotations or in the change in the number of rotations after treatment. On the other hand, the number of rotations in both *GDNF*-transfected groups was reduced compared with the *GFP*-transfected groups (adult GDNF compared with adult GFP, $p < 0.01$; adult GDNF compared with embryonic GFP, $p < 0.001$; embryonic GDNF compared with adult GFP, $p < 0.05$; embryonic GDNF compared with embryonic GFP, $p < 0.01$; repeated measure ANOVA, Fig. 4). These results indicate that the donor cell sources in both the *GFP*-transfected and *GDNF*-transfected groups had the same potential therapeutic efficiency.

Tyrosine Hydroxylase Immunohistochemistry in the Striatum and SNC

Six weeks after lesioning with 6-OHDA, TH immunohistochemistry was performed to analyze the dopaminergic fibers in the striatum. The density of TH-positive fibers in the striatum lesioned with 6-OHDA was compared with the contralateral side using a computerized image analysis system.[7,31] The preservation of TH-positive fibers in animals transplanted with the adult GDNF or embryonic GDNF cells was significantly greater than in animals transplanted with adult GFP or embryonic GFP cells (adult GDNF compared with adult GFP, $p < 0.0001$; embryonic GDNF compared with embryonic GFP, $p < 0.0001$; Fig. 5A). There were no significant differences between adult and embryonic GFP groups or GDNF groups (adult GDNF compared with embryonic GDNF, $p = 0.45$; adult GFP compared with embryonic GFP, $p = 0.38$; Fig. 5A).

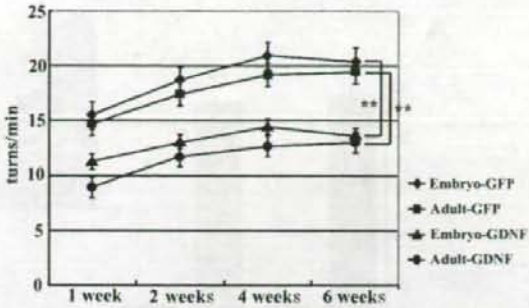

FIG. 4. Graph of the time course of the amphetamine-induced rotational behavior in rats with 6-OHDA–induced loss of dopaminergic neurons. There was a statistically significant reduction in amphetamine-induced rotations in animals implanted with *GDNF*-transfected cells (adult and embryonic) compared with animals that received *GFP*-transfected cells. Data are shown as means ± SEMs expressed as rotation numbers; 6 animals/group. ** $p < 0.01$.

The *GDNF*-transfected grafts induced greater protection in the SNC, as indicated by TH immunohistochemical analysis. The number of TH-positive neurons on the ipsilateral side of grafted rats was analyzed as a percentage of the neurons on the contralateral side. There was significant sparing of TH-positive neurons in the SNC of the lesioned side in the rats transplanted with the *GDNF*-transfected graft (adult GDNF 58.2 ± 6.9%, embryonic GDNF 57.2 ± 7.9% versus the contralateral side in 6 rats; Fig. 6A) as compared with rats transplanted with the *GFP*-transfected graft (adult GFP 15.5 ± 2.8% and embryonic GFP 15.8 ± 2.3%, versus the contralateral side in 6 rats; Fig. 6A). There was no significant difference between the adult and embryonic NPC groups (Fig. 6A). Together with the results of behavioral assessment, these results indicate that adult and embryonic NPCs have the same potential for therapeutic efficiency.

Graft Survival and Morphological Analysis of Grafted Cells 6 Weeks Posttransplantation

Transplanted cells continued to express the transfected *GFP*- or *GDNF*-gene 6 weeks after intrastriatal transplantation. Therefore, survival and morphological changes could be observed. Grafted cells migrated in the dorsoventral direction along the needle track and also dispersed laterally, adjusting to the structure of the host striatum (Fig. 7A–D). There were significantly more GFP-positive cells in the adult GFP group (682.7 ± 19.1 cells) than in the embryonic GFP group (620.8 ± 19.3 cells, $p < 0.05$). Considering that the sections were collected as a 1-in-6 series and that a GFP labeling rate was 25%,[19] the estimated survival rates of the grafted cells were 4.41 ± 0.22% for adult NPCs and 3.58 ± 0.26% for embryonic NPCs. Trans-

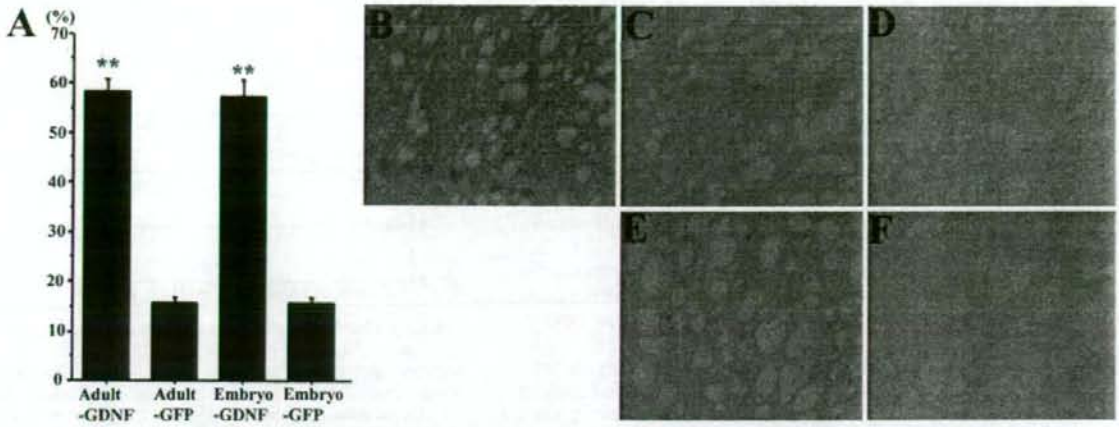

FIG. 5. A: Bar graph showing the preservation of TH-positive fibers in the host striatum in the 4 groups of rats. No statistical difference was detected between the adult and embryonic GDNF groups or between the adult and embryonic GFP groups. Data are shown as means ± SEMs expressed as percentages of the contralateral side. ** $p < 0.0001$. B–F: Photomicrographs. Staining for TH in the striatum on tissue obtained from the nonlesioned side (B), adult GDNF (C), adult GFP (D), embryonic GDNF (E), and embryonic GFP (F) are shown. In the GDNF groups there were more TH fibers than in the GFP groups, but there were no morphological differences between adult and embryonic NPCs in any of the groups.

planted cells extended processes into the host tissue. Furthermore, in both the adult and embryonic GDNF groups, the grafted cells extended longer and smoother processes than did the *GFP*-transfected groups (Fig. 7E–H).

Neuronal and Astrocytic Differentiation

Green fluorescent protein/NeuN double-positive neuronal differentiated cells and GFP/GFAP double-positive astrocytic differentiated cells were located mainly at the periphery of the cell cluster (Fig. 8A–C, G–I). Only a small number of GFP-positive surviving cells that were located inside the cell cluster expressed GFAP (Fig. 8G–I). Almost none of the cells inside the cluster showed NeuN or GFAP staining. Rates of differentiation into GFP/NeuN double-positive neurons were 0.89 ± 0.22% in the adult GFP group, and 0.91 ± 0.19% in the embryonic GFP group. The GFP/NeuN double-positive cell bodies extended long processes, indicating morphological integration into the host

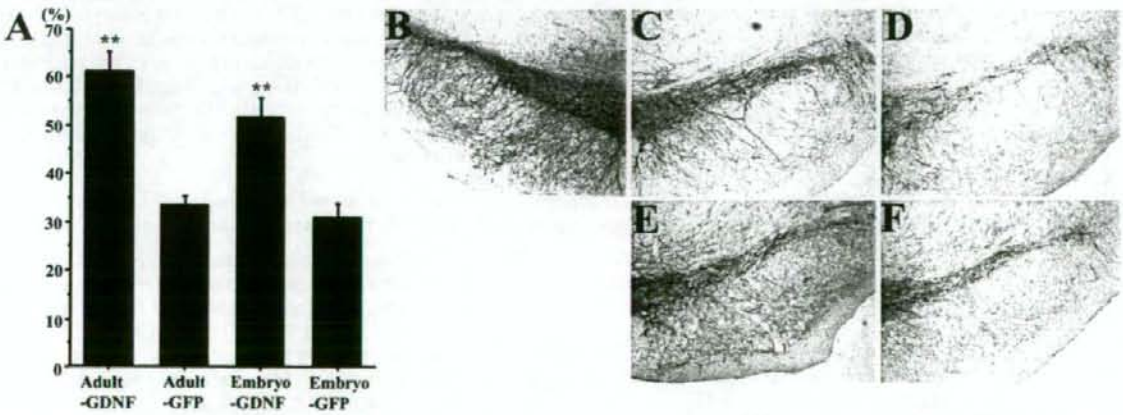

FIG. 6. A: Bar graph showing the preservation of TH-positive neurons in the host SNC. Adult and embryonic GDNF transplantation induced a greater preservation of these neurons and had more neuroprotective effects in vivo than adult and embryonic GFP cell transplantation. On the other hand, there was no statistical difference between adult and embryonic GDNF groups or between adult and embryonic GFP groups. Data are shown as means ± SEMs expressed as percentages of the contralateral side. **$p < 0.0001$. B–F: Photomicrographs. Staining for TH in the SNC on the nonlesioned side (B), adult GDNF (C), adult GFP (D), embryonic GDNF (E), and embryonic GFP (F) are shown.

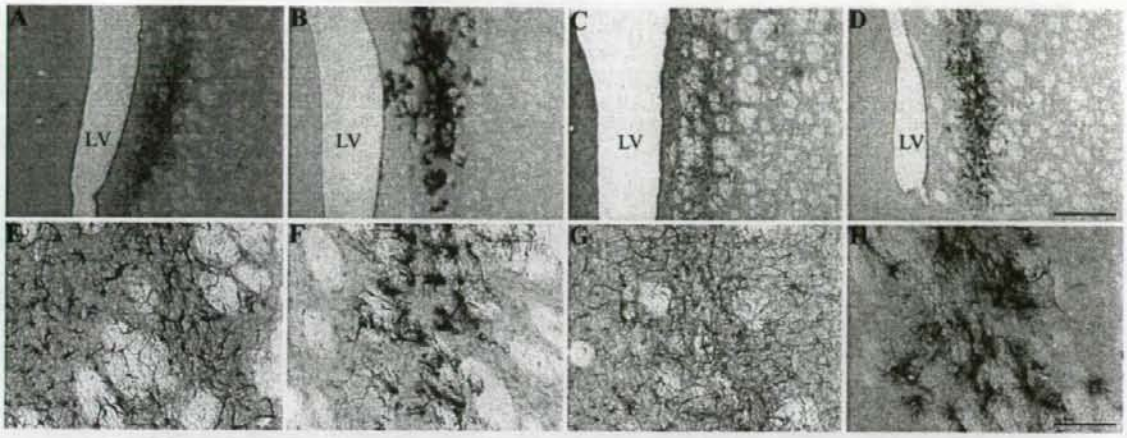

FIG. 7. Photomicrographs of the appearance of intrastriatal grafts 6 weeks after implantation. Adult GDNF (A, E), adult GFP (B, F), embryonic GDNF (C, G), and embryonic GFP (D, H). Upper images at low magnification show that grafted cells migrated in a dorsoventral direction along the needle track and also dispersed laterally, settling within the host striatum. Lower images at higher magnification show that in both *GDNF*-transfected grafts, the grafted cells extended longer and smoother processes than the *GFP*-transfected grafts. A–D: bar = 1.0 mm; E–H: bar = 100 μm. LV = lateral ventricle.

tissue (Fig. 8D–F). The differentiation rate into GFP/GFAP double-positive cells was 8.7 ± 1.3% in the adult GFP group and 9.1 ± 1.4% in the embryonic GFP group. The GFP/GFAP double-positive cells extended multiple short processes, typical of astrocytes (Fig. 8J–L). There was no significant difference in the differentiation rate between adult GFP and embryonic GFP groups (NeuN, $p = 0.964$; GFAP, $p = 0.846$). Immunofluorescent staining in the *GDNF*-transfected groups revealed no obvious GDNF-expressing implanted cells; therefore, we could not evaluate their phenotype.

Discussion

In the present study, adult and embryonic NPCs were compared for potential use as donor sources for CNS cell therapy. The results demonstrated that the potential of adult NPCs was comparable to that of embryonic NPCs as a donor source with respect to the neuronal differentiation rate, graft growth kinetics after implantation in the host striatum, and the efficiency of adenoviral gene transfection.

Comparison of Differentiation Potential Between Donor Sources

The use of primary adult or embryonic NPCs in cell therapy is not realistic because a large quantity of donor tissue or many donors are required. Therefore, for practical reasons donor tissues must be grown in vitro by subculturing prior to implantation.[16] We cultured adult and embryonic NPCs and compared the changes in the neuronal differentiation rate in vitro for each generation. The results indicated that the neuronal differentiation potential of the 2 sources was equivalent after 2 passages. Although the neuronal differentiation rate of the adult NPCs was almost unchanged after passaging, the differentiation rate of the embryonic NPCs was remarkably reduced after the first passage compared with the high differentiation rate of primary cells. One reason for this might be that the mitogen EGF was used in the cultures. Adult NPCs and embryonic NPCs are both very heterogeneous cell populations that are differentially affected by various growth factors and cytokines.[8,13,14,36] In such heterogeneous populations, it can be assumed that cells that divide after EGF stimulation—so-called EGF-responsive precursors—proliferated and finally formed a homogeneous cell population, although the differentiation stage varied among cells.[25,40] In other words, the addition of EGF created a selective culture system. We believe that the EGF-culture system influenced the results by producing a comparatively uniform cell population in vitro.

Transplanted NPCs produce neurotrophic factors and various cytokines, and such signals are expected to have both protective and restorative effects within host tissue.[17,35] After implantation, grafted cells differentiate into neurons or glia. Such cells might promote restoration of the host tissue.[9,11] Therefore, at first we performed adult or embryonic NPC transplantation in a rat model of Parkinson disease. The results of *GFP*-transfected NPC transplantation demonstrated that the behavior and histological characteristics of the host animals that received adult or embryonic *GFP*-transfected NPCs were not significantly different. These findings suggest that adult and embryonic NPCs have an equivalent potential for therapeutic transplantation. The percentages of NeuN-positive neurons and GFAP-positive astrocytes were not significantly different between the two sources. These results suggest that adult and embryonic NPCs have equal potential to differentiate into a specific phenotype, which is induced by the adult striatal microenvironment.

The reason that the neuronal differentiation rate of grafted cells was lower than that in vitro is thought to be the adult striatal microenvironment, which does not induce

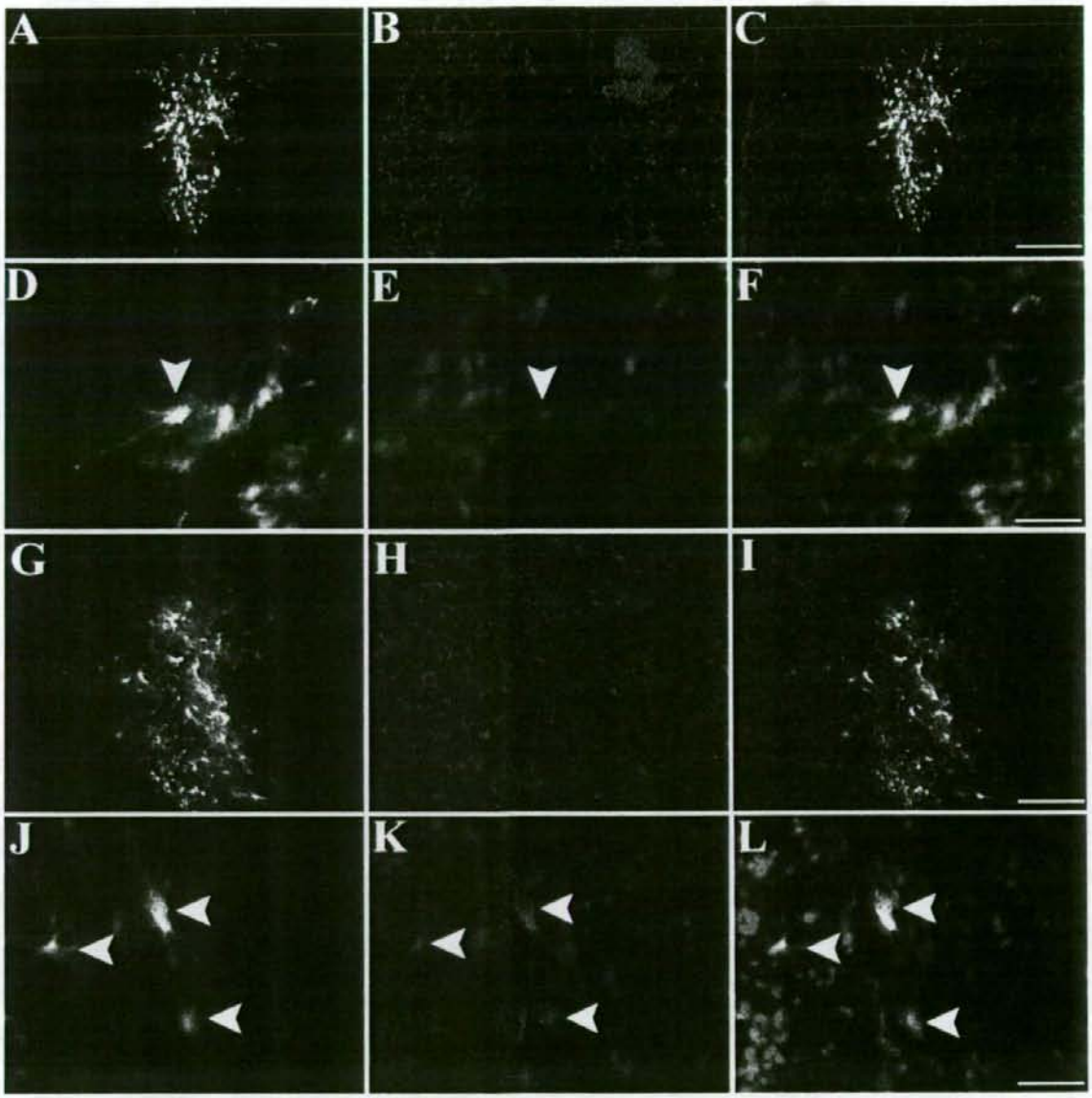

FIG. 8. Photomicrographs showing differentiation of adult GFP grafts. The GFP-positive cell clusters disperse and arrange within the host striatum (A and G). No positivity for NeuN was observed within the cluster (B). The GFP/NeuN double-positive cells are located mainly in the periphery and outside of the cluster (C is a merged view of A and B). D–F: High magnification images showing a GFP/NeuN double-positive cell extending a long, thick process *(arrowhead)*. D: GFP. E: NeuN. F: Merged view of D and E, indicating grafted cells that differentiated into mature neurons and integrated into the host tissue. G–I: Although astrocytic differentiation of grafted cells is observed inside the cluster, they are located mainly in the cluster periphery (G: GFP; H: GFAP; I is a merged view of G and H). J–L: High magnification images showing GFP/GFAP double-positive cells extending multiple short processes that are typical of astrocytes (*arrowheads*, J: GFP; K: GFAP; L is a merged view of J and K). A–C and G–I: bar = 200 mm; D–F, J–L: bar = 30 mm.

neuronal differentiation very well compared with the stem cell medium containing 1% fetal bovine serum. In addition, because the ability of the inside of the grafted cell cluster to induce differentiation was decreased by the host inflammatory reaction resulting from implant manipulation, almost all grafted cells that remained inside the cluster were negative for NeuN and GFAP expression.[19] The host tissue reaction is thought to be one cause of the reduced neuronal dif-

ferentiation rate of grafted cells compared with the rate in vitro.

Compatibility With the Host Tissue

The E14 ganglionic eminence from which embryonic donor cells were taken is the striatal primordium. The adult subventricular zone, from which the adult donor cells were taken, maintains adult NPCs and supplies the adult striatum with new neurons. Basically, transplantation of both donor sources into the striatum is an orthotopic and homologous transplantation.[25,33] The proportion of surviving cells, however, was higher for adult NPCs than embryonic NPCs. The reason for the difference in survival between the 2 sources might be the compatibility of donor cells with the host tissue. In other words, this finding implies that adult NPCs are a better match with the host environment—an adult CNS. Although statistical analysis of individual assessments (both the behavioral and histological assessments) did not reveal a significant difference between adult and embryonic NPC transplantation, adult NPC transplantation resulted in a trend toward greater improvement in every assessment compared with embryonic NPC transplantation, implying that the tissue compatibility of the donor cells affected the results.

A frequent concern is that long-term propagation of stem cells in vitro might result in tumor formation. For example, extensive culturing of neural stem cells leads to genetic changes that alter cell growth and differentiation patterns.[23] Tumor formation was not observed, however, in any culture system of either fetal or adult NPCs, or after transplantation into any groups in this study. Thus, the risk of tumorigenesis should be quite low and comparable for both cell sources.

Transfected Gene Expression

Adenoviral *GDNF* transfection was performed to examine possible differences in the properties of the 2 donor sources in ex vivo gene therapy. There are various ex vivo gene manipulation methods, using viruses (adenovirus, retrovirus, and lentivirus) or plasmids,[10] and each method has a specific transfection pathway and induces a host cell reaction.[2] Transfected *GDNF* expression was equivalent between adult and embryonic NPCs both in vitro and in vivo. These results demonstrated that there was no difference between adult and embryonic NPCs in terms of adenovirus infection-induced gene transfection. This finding suggests that adult and embryonic NPCs have similar cell surface antigens that are necessary for adenovirus infection and the two cell types have a similar ability to recognize the exogenous antigen.

In adult NPC and embryonic NPC transplantation experiments, the expression level of the transfected *GDNF* gene, measured by the quantification of GDNF produced in vivo after transplantation, was not significantly different, and both grafts had approximately equivalent protective effects based on the behavioral and histological assessment. These results demonstrated that the two sources have an equivalent potential as a cell-based drug delivery source. Furthermore, the transfected gene was stably expressed during this observation period because the *GDNF*-transfected cells were detected by diaminobenzidine staining as were *GFP*-transfected cells 7 weeks after transplantation. In both adult and embryonic transplantation groups, *GDNF*-transfected cells extended long and smooth processes compared with *GFP*-transfected cells. Grafted cells did not migrate in a specific direction, but dispersed using the host striatal structure as a scaffold because there were no specific factors produced to induce cell migration, as in cases of ischemia and trauma. In both adult and embryonic transplantation, however, *GDNF*-transfected cells migrated a longer distance than did *GFP*-transfected cells. These results suggest that GDNF, which is produced by the grafted cells, promotes the morphological integration and migration of the grafted cells in an autocrine or paracrine manner, in addition to protective and restorative effects on TH-positive cells and host tissue fibers.[3,12]

Promotion of Therapeutic Effect in PD

In the present study we demonstrated that embryonic and adult cell sources have similar characteristics in transplantation therapy in a model of PD. As previously reported by other groups, we also demonstrated that *GDNF*-transduced grafts have a greater therapeutic effect than *GFP*-transduced grafts in both adult and embryonic NPC transplantations. Although many other groups have performed similar experiments, each group has used different culture systems and gene transfection methods. Therefore, it has been difficult to perform simple quantitative comparisons between different cell sources. Because we applied the same methods in different cell sources, our results are reliable and useful for comparing the properties between the 2 cell sources.

The original goal of treatment for PD, however, was to replace damaged dopamine-secreting neurons. Until now, neuronal differentiation of adult NPCs expanded from the subventricular zone has not been observed when transplanted into nonneurogenic intact striatum, and the differentiation rate into neurons is quite low ($< 1\%$) in a rat model of PD.[28] These results are consistent with the data we present here. On the other hand, priming human fetal NPCs in vitro 6–7 days prior to transplantation greatly increases the neuronal differentiation of these cells after transplantation into the striatum.[41] This finding suggests that inducing NPCs to differentiate prior to transplantation might initiate an intrinsic mechanism for continuing neuronal differentiation in vivo. In fact, cultured embryonic NPCs differentiate into dopaminergic neurons to a greater extent in dopamine-depleted striatum than in intact striatum.[20] These data suggest that environmental factors in damaged brain regions must also be considered in developing restorative transplantation techniques for cultured NPCs.

To increase the differentiation rate of TH-positive dopaminergic neurons and promote the therapeutic effects of cell transplantation, it will be important to assess the different responses between the 2 cell sources against effective factors using various transplantation schedules, pretreatment drugs, and posttransplantation drug infusions. Furthermore, comparisons of electrophysiological integration and TH-positive differentiation of both sources in vivo will be useful for demonstrating therapeutic effect in the treatment of PD.

Richardson and associates[28] reported that the secretion of neurotrophins by NPCs restores dopamine transporter expression in nigrostriatal axon terminals damaged by 6-OHDA lesioning. These data suggest that NPC transplan-

tation has various effects besides integration into the host neuronal circuitry. In addition, the glial differentiation rate is important because the various important functions of glial cells in the CNS have been unraveled.[37] In the future, various benefits and mechanisms of therapeutic effect of NPC transplantation therapy will require further elucidation.

Conclusions

Our findings in the present study demonstrate that adult and embryonic NPCs have comparable potential for use as donor cells in cell therapy for PD. Authors of recent studies have begun to unravel the properties of human NPCs, and the development of culturing techniques for adult NPCs is progressing.[18,21] Adult human NPC transplantation is a viable possibility for CNS cell therapy.

Acknowledgments

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Reference

1. Akerud P, Canals JM, Snyder EY, Arenas E: Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. **J Neurosci 21:**8108–8118, 2001
2. Asahara T, Kalka C, Isner JM: Stem cell therapy and gene transfer for regeneration. **Gene Ther 7:**451–457, 2000
3. Bakshi A, Shimizu S, Keck CA, Cho S, LeBold DG, Morales D, et al: Neural progenitor cells engineered to secrete GDNF show enhanced survival, neuronal differentiation and improve cognitive function following traumatic brain injury. **Eur J Neurosci 23:** 2119–2134, 2006
4. Barker RA, Widner H: Immune problems in central nervous system cell therapy. **NeuroRx 1:**472–481, 2004
5. Bjorklund A, Lindvall O: Cell replacement therapies for central nervous system disorders. **Nat Neurosci 3:**537–544, 2000
6. Brederlau A, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, et al: Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. **Stem Cells 24:**1433–1440, 2006
7. Date I, Felten SY, Felten DL: The nigrostriatal dopaminergic system in MPTP-treated mice shows more prominent recovery by syngeneic adrenal medullary graft than by allogeneic or xenogeneic graft. **Brain Res 545:**191–198, 1991
8. Doetsch F, Petreanu L, Caille I, Garcia-Verdugo JM, Alvarez-Buylla A: EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. **Neuron 36:**1021–1034, 2002
9. Eriksson C, Bjorklund A, Wictorin K: Neuronal differentiation following transplantation of expanded mouse neurosphere cultures derived from different embryonic forebrain regions. **Exp Neurol 184:**615–635, 2003
10. Falk A, Holmstrom N, Carlen M, Cassidy R, Lundberg C, Frisen J: Gene delivery to adult neural stem cells. **Exp Cell Res 279:** 34–39, 2002
11. Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Björklund A: Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. **J Neurosci 19:**5990–6005, 1999
12. Korochkin LI, Revishchin AV, Okhotin VE: Neural stem cells and their role in recovery processes in the nervous system. **Neurosci Behav Physiol 36:**499–512, 2006
13. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH: Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. **J Neurosci 17:** 5820–5829, 1997
14. Kukekov VG, Laywell ED, Suslov O, Davies K, Scheffler B, Thomas LB, et al: Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. **Exp Neurol 156:**333–344, 1999
15. Lindvall O: Parkinson disease. Stem cell transplantation. **Lancet 358 Suppl:**S48, 2001
16. Lindvall O, Kokaia Z, Martinez-Serrano A: Stem cell therapy for human neurodegenerative disorders—how to make it work. **Nat Med 10 Suppl:**S42–S50, 2004
17. Lu P, Jones LL, Snyder EY, Tuszynski MH: Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. **Exp Neurol 181:** 115–129, 2003
18. Moe MC, Varghese M, Danilov AI, Westerlund U, Ramm-Pettersen J, Brundin L, et al: Multipotent progenitor cells from the adult human brain: neurophysiological differentiation to mature neurons. **Brain 128:**2189–2199, 2005
19. Muraoka K, Shingo T, Yasuhara T, Kameda M, Yuan W, Hayase H, et al: The high integration and differentiation potential of autologous neural stem cell transplantation compared with allogeneic transplantation in adult rat hippocampus. **Exp Neurol 199:**311–327, 2006
20. Nishino H, Hida H, Takei N, Kumazaki M, Nakajima K, Baba H: Mesencephalic neural stem (progenitor) cells develop to dopaminergic neurons more strongly in dopamine-depleted striatum than in intact striatum. **Exp Neurol 164:**209–214, 2000
21. Nunes MC, Roy NS, Keyoung HM, Goodman RR, McKhann G II, Jiang L, et al: Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. **Nat Med 9:**439–447, 2003
22. Ostenfeld T, Tai YT, Martin P, Deglon N, Aebischer P, Svendsen CN: Neurospheres modified to produce glial cell line-derived neurotrophic factor increase the survival of transplanted dopamine neurons. **J Neurosci Res 69:**955–965, 2002
23. Palmer TD, Takahashi J, Gage FH: The adult rat hippocampus contains primordial neural stem cells. **Mol Cell Neurosci 8:**389–404, 1997
24. Park KI, Himes BT, Stieg PE, Tessler A, Fischer I, Snyder EY: Neural stem cells may be uniquely suited for combined gene therapy and cell replacement: evidence from engraftment of neurotrophin-3-expressing stem cells in hypoxic-ischemic brain injury. **Exp Neurol 199:**179–190, 2006
25. Parmar M, Sjoberg A, Bjorklund A, Kokaia Z: Phenotypic and molecular identity of cells in the adult subventricular zone. in vivo and after expansion in vitro. **Mol Cell Neurosci 24:**741–752, 2003
26. Paxinos G, Watson C: **The Rat Brain in Stereotaxic Coordinates.** New York: Academic Press, 1998
27. Reynolds BA, Tetzlaff W, Weiss S: A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. **J Neurosci 12:**4565–4574, 1992
28. Richardson RM, Broaddus WC, Holloway KL, Fillmore HL: Grafts of adult subependymal zone neuronal progenitor cells rescue hemiparkinsonian behavioral decline. **Brain Res 1032:**11–22, 2005
29. Rossi F, Cattaneo E: Opinion: neural stem cell therapy for neurological diseases: dreams and reality. **Nat Rev Neurosci 3:**401–409, 2002
30. Roy NS, Cleren C, Singh SK, Yang L, Beal MF, Goldman SA: Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. **Nat Med 12:**1259–1268, 2006
31. Shingo T, Date I, Yoshida H, Ohmoto T: Neuroprotective and restorative effects of intrastriatal grafting of encapsulated GDNF-producing cells in a rat model of Parkinson's disease. **J Neurosci Res 69:**946–954, 2002

32. Shingo T, Sorokan ST, Shimazaki T, Weiss S: Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. **J Neurosci 21:**9733–9743, 2001
33. Stenman J, Toresson H, Campbell K: Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. **J Neurosci 23:**167–174, 2003
34. Studer L, Tabar V, McKay RD: Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. **Nat Neurosci 1:**290–295, 1998
35. Toda H, Tsuji M, Nakano I, Kobuke K, Hayashi T, Kasahara H, et al: Stem cell-derived neural stem/progenitor cell supporting factor is an autocrine/paracrine survival factor for adult neural stem/progenitor cells. **J Biol Chem 278:**35491–35500, 2003
36. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, et al: Direct isolation of human central nervous system stem cells. **Proc Natl Acad Sci U S A 97:**14720–14725, 2000
37. Verkhratsky A, Toescu EC: Neuronal-glial networks as substrate for CNS integration. **J Cell Mol Med 10:**826–836, 2006
38. Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, et al: Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. **J Neurosci 16:** 7599–7609, 1996
39. Westerlund U, Svensson M, Moe MC, Varghese M, Gustavsson B, Wallstedt L, et al: Endoscopically harvested stem cells: a putative method in future autotransplantation. **Neurosurgery 57:**779–784, 2005
40. Winkler C, Fricker RA, Gates MA, Olsson M, Hammang JP, Carpenter, MK, et al: Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain. **Mol Cell Neurosci 11:**99–116, 1998
41. Wu P, Tarasenko YI, Gu Y, Huang LY, Coggeshall RE, Yu Y: Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. **Nat Neurosci 5:**1271–1278, 2002
42. Yasuhara T, Shingo T, Muraoka K, Kobayashi K, Takeuchi A, Yano A, et al: Early transplantation of an encapsulated glial cell line-derived neurotrophic factor-producing cell demonstrating strong neuroprotective effects in a rat model of Parkinson disease. **J Neurosurg 102:**80–89, 2005
43. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA: In vitro differentiation of transplantable neural precursors from human embryonic stem cells. **Nat Biotechnol 19:**1129–1133, 2001

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