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Adenovirus-Mediated Transduction with Human Glial Cell Line-Derived Neurotrophic Factor Gene Prevents 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Induced Dopamine Depletion in Striatum of Mouse Brain

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As a novel trial of neuroprotective therapy of neurodegenerative diseases, we have constructed a recombinant adenovirus vector (rAdv) bearing a neurotrophic factor gene to deliver the factor to rescue neurons *in vivo*. In the present study, human glial cell line-derived neurotrophic factor (hGDNF) was chosen to examine the applicability of our strategy to a mouse model of Parkinson's disease. During the construction of the rAdv, we found that the strong constitutive hGDNF expression unit somehow inhibited the appearance of the rAdv. Therefore we adopted a self-contained tetracycline-regulated expression system to acquire an rAdv expressing hGDNF. By analyzing the condition medium of SH-SY5Y cells infected with our constructed virus vector, we confirmed that biologically active GDNF was successfully expressed *in vitro*. For an animal study, we delivered this virus vector directly to the C57 black mouse brain and then exposed the animal to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to injure the nigrostriatal dopaminergic neurons. One week after the MPTP exposure, the neuroprotective effect of the virus vector was estimated by measurement of the dopamine content in the striatum of the mouse brain. The mice that had received our constructed virus had significantly higher dopamine levels in their striatum, demonstrating that our rAdv expressing hGDNF has therapeutic potential to protect the nigrostriatal dopaminergic neurons *in vivo*.

Neurotrophic factors have been shown to rescue neurons from various insults. Although the pathophysiological processes of neurodegenerative diseases are not fully understood, clinical trials using neurotrophic factors are currently in progress to prevent progression of the symptoms of the patients affected by neurodegenerative diseases, especially amyotrophic lateral sclerosis. To realize effective *in vivo* delivery of neurotrophic factors to the degenerating neurons, we have attempted to develop a recombinant adenovirus vector (rAdv) for the expression of a neurotrophic factor *in vivo* [1]. In this study, we further designed an *in vivo* experiment to investigate the possibility that administration of rAdv expressing neurotrophic factor(s) could provide a neuroprotective effect in a mouse model of Parkinson's disease. Since glial cell line-derived neurotrophic factor (GDNF) [2] has been shown to support the survival of midbrain dopaminergic neurons effectively *in vivo* [3-8], we examined the effect of adenovirus-mediated transduction of the human GDNF (hGDNF) gene into mouse brain on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced injury to the nigrostriatal neurons.

MATERIALS AND METHODS

Materials. MPTP was obtained from RBI, Natick, MA. The cosmid vector Ax1CAwt [9] was kindly donated by Dr. Izumu Saito (Institute of Medical Scinece, University of Tokyo); and another cosmid vector, Ax1cw [10], was obtained from RIKEN GENE BANK, Ibaraki, Japan. Plasmids required for the tetracycline-regulated expression system [11] were purchased from Gibco, Grand Island, NY, and Clontech, Palo Alto, CA. A human GDNF ELISA kit and recombinant hGDNF were obtained from Promega, Madison, WI.

Animals. Male C57Bl/6NJcl mice were purchased from Japan CLEA, Tokyo. They were maintained at the controlled temperature of 23°C with a 12/12 hr light/dark cycle and had free access to food and water throughout the experiments. They were subjected to the experiments at the age of 10-12 weeks.

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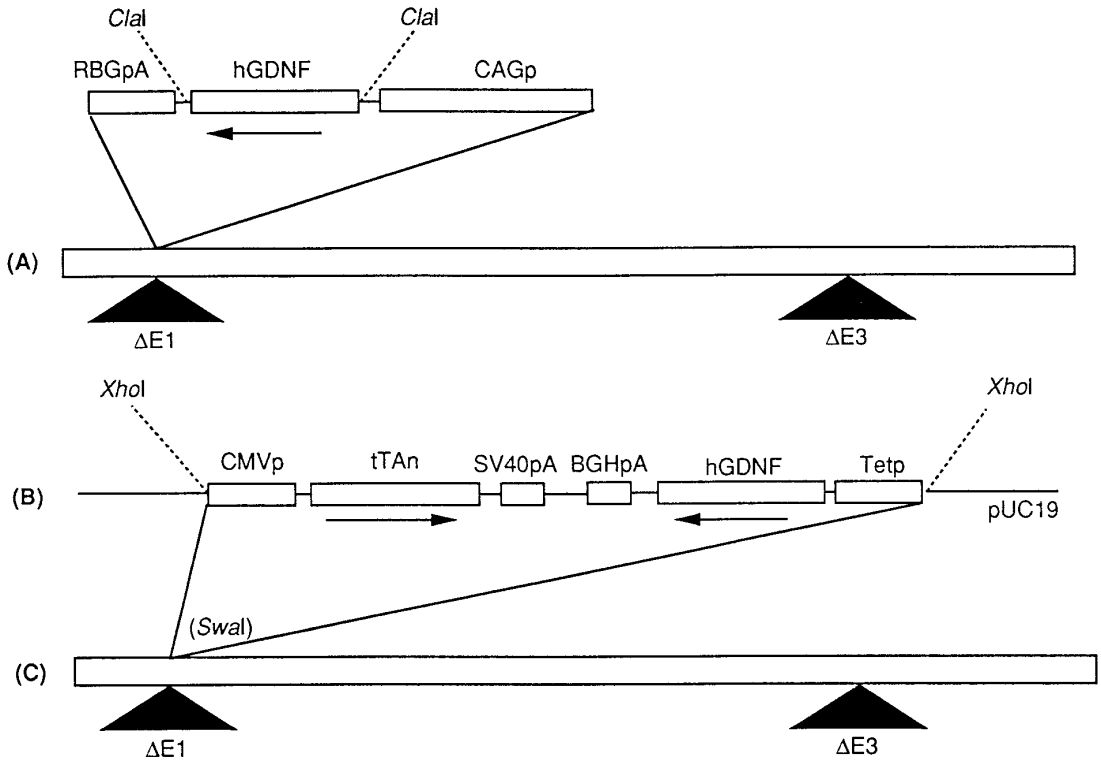

FIG. 1. Structure of AxCAG-hGDNF, pCTnT-hGDNF and AxCTnT-hGDNF. (A) AxCAG-hGDNF virus could not be obtained. CAGp: CAG promoter; hGDNF: human GDNF cDNA; RBGpA: rabbit β-globin gene-derived polyadenylation signal. (B) pCTnT-hGDNF is a pUC-based vector that contains both CMV promoter-driven tTAn gene and Tet promoter-driven hGDNF gene in the *Xho*I-*Xho*I region. CMVp: cytomegalovirus immediate early promoter and enhancer; tTAn: tetracycline transactivator gene with nuclear localization signal; SV40pA: SV40-derived polyadenylation signal; Tetp: tetracycline transactivator-dependent promoter; BGHpA: bovine growth hormone gene-derived polyadenylation signal. (C) Genomic structure of AxCTnT-hGDNF virus. AxCTnT-hGDNF is an E1, E3-deleted adenovirus vector that bears the *Xho*I-*Xho*I region of pCTnT-hGDNF in the deleted E1 region.

Preparation of virus vector. Recombinant adenovirus vectors bearing an hGDNF expression unit were constructed according to the same method as reported previously [1]. Briefly, pAxCA-hGDNF (Fig. 1A) was constructed by inserting human GDNF cDNA into the *Swa*I site of Ax1CAwt, which is a cosmid vector used for the construction of rAdv's [9]. The pUC19-based vector pCTnT-hGDNF was generated by combining the human cytomegalovirus immediate early promoter-driven tetracycline transactivator (tTA [11]) gene with tetracycline-responsive promoter (Tetp [11])-driven hGDNF cDNA as presented schematically in Fig. 1B. The tTA protein encoded by this plasmid was modified by adding a nuclear localization signal (MPKRPRPS [12]) to its N-terminus. pAxCTnT-hGDNF (Fig. 1C) was constructed by inserting the blunted *Xho*I-*Xho*I fragment of pCTnT-hGDNF into the *Swa*I site of Ax1cw, which is another cosmid vector employed for the construction of rAdv's [10]. Each cosmid vector DNA (8 μg) was co-transfected by the calcium phosphate precipitation method into 293 cells with 1 μg of *EcoT22*I-digested AxRSV-LacZ virus DNA tagging terminal proteins [1, 10]. After the genome structure of the viruses that were produced was confirmed by digestion with restriction enzymes, the viruses were propagated, concentrated, and stocked according to the same method as described previously [1].

Quantification of GDNF content in conditioned media of infected SH-SY5Y cell cultures. SH-SY5Y human neuroblastoma cells [13] in 25-cm^2 culture flasks were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The cells were then infected with AxCTnT-hGDNF or AxRSV-LacZ virus (1 multiplicity of infection). One day after the infection, the medium was completely replaced with 5 ml of fresh medium, and then the cultures were incubated further for 3 days. hGDNF concentration in the conditioned media was measured with a hGDNF ELISA assay kit according to the supplier's manual.

Biological activity of conditioned media toward primary rat nigral neurons. Biological activity of GDNF in the conditioned media was determined with the survival assay for rat embryonal midbrain dopaminergic neurons. Briefly, primary midbrain neurons from E18 rat embryos were maintained in 48-well culture wells [14]. Then the cells were incubated for one week in DF medium (1:1 mixture of DMEM and F12 medium) containing 5% horse serum, 1% FBS, 1% adult rat serum, and a 1% concentration of several dilutions of conditioned media. Thereafter the cells were immunostained with anti-rabbit tyrosine hydroxylase (TH) antibody [15], and the number of the TH-immunopositive neurons was counted in each well.

In vivo delivery of virus vectors and toxin exposure. For the study, mice were divided into four experimental groups (n=10): group S, sham-operated mice without MPTP exposure; group C, mice given control vehicle with MPTP exposure; group Z, mice given AxRSV-LacZ control virus with MPTP exposure; and group G, mice given AxCTnT-hGDNF virus with MPTP exposure. At day 0, 2 μl of Dulbecco's phosphate-buffered saline (DPBS) containing viruses [2×10^6 plaque-forming unit (p.f.u.)] or control vehicle (DPBS without virus) was injected slowly into the left striatum (AP: 0 mm, L: 2 mm, D: 3.6 mm to the bregma) through a 27-gauge needle of a Hamilton syringe. At days 2 and 3 (two consecutive days), MPTP (40 mg/kg) in sterile water was administered subcutaneously to the mice.

Dopamine concentration in striatum. At day 9, the mice in each group were killed and their brains removed. Then each side of the striatum was separately excised and collected on a cooled plate. The striatum was homogenized in 0.4 ml of 0.1 M perchloric acid containing 1 mM EDTA. The dopamine concentration of the supernatant was determined by reverse-phase HPLC with electrochemical detection according to the method reported [16]. Dopamine concentration in each side of the striatum was expressed as a percentage of the average concentration (ng/mg wet tissue) of the same side of the sham-operated mouse brain.

Statistical analysis. Among the mice exposed to MPTP, dopamine concentration in the striatum (% of sham-operated mice) was compared by ANOVA followed by Scheffe's post hoc test.

RESULTS

Instability of high-expression unit of hGDNF in rAdv in 293 cells. Initially we sought to obtain rAdv having the hGDNF gene under the control of the strong constitutive promoter CAG [17] by the same method as used for the construction of rAdv expressing brain-derived neurotrophic factor (AxCA-BDNF) [1]. However, we failed to obtain any recombinant virus bearing the complete hGDNF expression unit after four independent transfections. Our acquired recombinant viruses always lost either of the *Cla*I sites, suggesting that the CAG promoter-driven GDNF expression unit somehow inhibited the appearance of the recombinant virus bearing the complete expression unit. As an alternative approach, a semi-constitutive (semi-inducible) promoter system was designed in which a tetracycline-transactivator with a nuclear localization signal (tTAn) promotes the expression of the hGDNF gene that is placed downstream of the tTA-dependent promoter (Tetp) [11]. Using the cosmid vector harboring the above expression unit (self-contained tTAn-Tet expression unit), we could easily obtain the AxCTnT-hGDNF virus vector by a single transfection in the presence of tetracycline (10 μg/ml). AxCTnT-hGDNF could be propagated similarly as AxRSV-LacZ, and we obtained 1.2×10^{11} p.f.u./ml stock solution of the virus.

In vitro transduction of hGDNF gene into SH-SY5Y human neuroblastoma cells by rAdv. To confirm whether our constructed vector expressed hGDNF, we infected SH-SY5Y human neuroblastoma cells with the viruses; and the conditioned media of the infected cell cultures were then assayed for immunoreactivity toward hGDNF antibody. The conditioned media (5 ml) of the cells infected with AxCTnT-hGDNF possessed 973 ± 342 ng/ml of hGDNF, whereas the conditioned media of the cells infected with AxRSV-LacZ did not contain any detectable level of hGDNF. Further analysis was performed to examine whether the conditioned medium had the biological activity of supporting the survival of primary cultures of rat nigral neurons. We could confirm the presence of biologically active hGDNF protein in the conditioned media from the cell cultures infected with AxCTnT-hGDNF (Fig. 2).

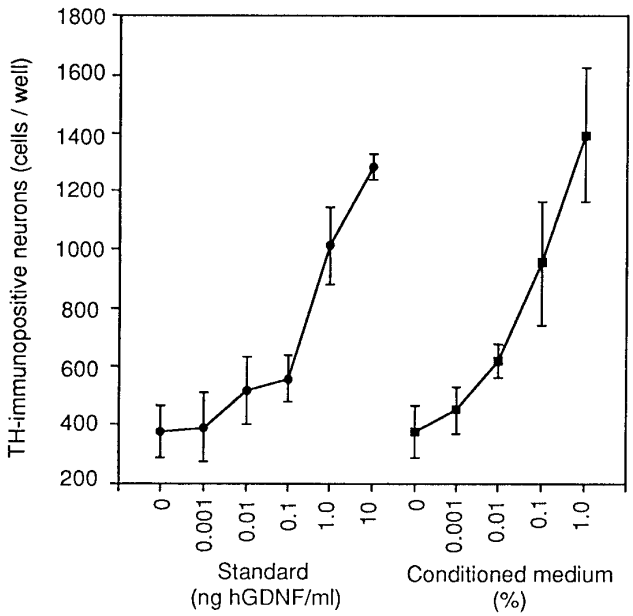

FIG. 2. Activity in conditioned medium for supporting survival of rat midbrain dopaminergic neurons in culture. The number of TH-immunopositive neurons (cells/well) in each group (n=4) is shown (mean ± S.D.). In the left half of the figure, the standard curve is presented. In the right half, the survival-supporting activity of the conditioned media of the cells infected with AxCTnT-hGDNF is shown.

Application of AxCTnT-hGDNF to a mouse model of Parkinson's disease. We planned the present animal experiment considering that the expression of transgenes in adenovirus vectors usually continues for 10-14 days in the central nervous system [18-20].

In the sham-operated mice, the insertion of the needle did not affect the content of dopamine in the striata (inserted side: 9930 ± 672 ng/g; contralateral side: 10084 ± 853 ng/g). Our adopted dose (40 mg/kg) of MPTP produced a marked reduction in dopamine levels to 7% of values for sham-operated mice in the DPBS-injected (control) mice (Fig. 3, group C). As shown in the figure, intrastriatal administration of AxCTnT-hGDNF virus significantly ($p<0.005$) lessened the MPTP-induced reduction in dopamine content in the injected side of the striatum (to 16%), whereas administration of AxRSV-LacZ control virus did not (to 9%).

To know whether administration of a higher dose of AxCTnT-hGDNF would provide a more beneficial effect, we also performed an experiment using 5×10^7 p.f.u. of the virus solution. However, in several repeats of this experiment, we found that the dopamine content in the virus-injected side was reduced (to 13%) and had become lower than that in the contralateral side of the striatum (to 18%). As for the injection of the higher dose of the control virus, the dopamine content of the both side became similarly low (to 6%), suggesting that the adenovirus vector inhibited the neuroprotective ef-

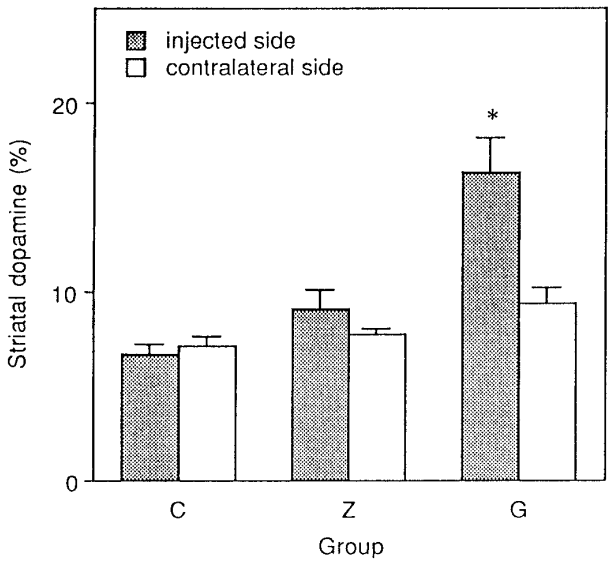

FIG. 3. Striatal dopamine levels of MPTP-treated mice. Striatal dopamine (ng/g wet tissue) in each experimental group is expressed as a percentage of the average of the value for the sham-operated group (mean ± S.E.). C: mice given control vehicle; Z: mice given AxRSV-LacZ virus; G: mice given AxCTnT-hGDNF virus. n=10, *$p<0.005$ (Scheffe's post hoc test).

fect of hGDNF when the high titer solution of the adenovirus vector was applied.

DISCUSSION

In this study, we explored the possibility that a humoral factor(s) delivered by rAdv could provide protective effects on deteriorated neurons *in vivo*. In a previous paper [1], we reported successful adenovirus-mediated transduction of one of the neurotrophic factor genes, that encoding BDNF, into the mouse brain *in vivo*. Here, using GDNF, a different neurotrophic factor, we transduced the gene of this factor into a mouse model of Parkinson's disease to confirm the applicability of our strategy. The presented data clearly demonstrate that intrastriatal administration of an rAdv expressing hGDNF can provide some protection for the mouse nigrostriatal neurons against MPTP-induced injury. GDNF has been reported to be one of the hopeful factors that provide beneficial effects to the nigrostriatal neurons in primate models of Parkinson's disease [21, 22]. Adenovirus-mediated transduction of the hGDNF gene should thus be applied also to these models to examine the therapeutic potential of our strategy.

Furthermore, a recent report showed that adenovirus-mediated transduction of the GDNF gene protected against 6-hydroxydopamine-induced damage to the nigrostriatal neurons of rats [23]. Administration of 6-hydroxydopamine to the brain produces reactive oxygen species in the neurons. Similarly, the neurotoxic activity of MPTP has been reported to be mediated by nitric oxide [24]. Reactive oxygen species and reactive nitrogen species are now considered to be deeply involved in neuronal cell death observed in some neurological diseases. These results suggest that GDNF has neuro-protective activity against various other insults caused by reactive oxygen species and reactive nitrogen species.

During the construction of the rAdv for the expression of hGDNF, we found that the high expression unit of hGDNF (CAG-hGDNF) inhibited the appearance of rAd virus having the complete expression unit. The 293 cells used as a host for the viruses originated from human embryonal kidney [25], and GDNF-knock out mice shows developmental anomaly of the kidney [26-28]. Together with our results, we suppose that GDNF can influence the phenotype of kidney-derived 293 cells to block adenovirus multiplication at some step(s).

For the construction of the rAdv for the expression of hGDNF, instead of using weaker constitutive promoters, we adopted a "self-contained tetracycline transactivator-dependent expression system". In this system, both tTAn gene and a gene of interest (hGDNF gene) are expressed from the same adenovirus genome. With this system, we could easily obtain rAdv expressing hGDNF, although we did not have full command of the expression of Tetp-driven transgenes in the genome of the rAdv (data not shown). However, according to a recent report [29], tTA with SV40 large T antigen-derived NLS (NtTA) is very effective for both activating and suppressing gene expression from rAdv by tetracycline with strict control. By replacing tTAn gene with NtTA gene, we may acquire rAdv as a self-contained and strictly controllable tetracycline-regulated expression system; and such vectors may be useful for strictly regulating the expression of transduced foreign genes *in vitro* and *in vivo*.

Besides the above difficulty in the construction of the rAdv expressing GDNF, we encountered another difficulty in *in vivo* experiments: when high titers of the virus solution was used, the dopamine content in the virus-injected side became lower than that in the contralateral side, indicating that our constructed virus might have some neurotoxic effects on the nigrostriatal neurons. We suppose that this cytotoxic activity may be partially attributed to the immunogenic or cytotoxic character of the first-generation adenovirus vectors. To overcome these difficulties, we are now developing safer vectors for delivery of neurotrophic factors.

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REFERENCES

1. Kojima, H., Inuzuka, S., Miwa, T., Furukawa, S., Hayashi, K., Kanegae, Y., Saito, I., Ohishi, N., Takamori, M., and Yagi, K. (1995) *Biochem. Biophys. Res. Commun.* **212,** 712–717.

2. Lin, L.-F., Doherty, D. H., Lile, J. D., Bektesh, S., and Collins, F. (1993) *Science* **260,** 1130–1132.
3. Hoffer, B. J., Hoffman, A., Bowenkamp, K., Huettl, P., Hudson, J., Martin, D., Lin, L.-F., and Gerhardt, G. A. (1994) *Neurosci. Lett.* **182,** 107–111.
4. Tomac, A., Lindqvist, E., Lin, L.-F., Ogren, S. O., Young, D., Hoffer, B. J., and Olson, L. (1995) *Nature* **373,** 335–339.
5. Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., Rosenthal, A., and Hefti, F. (1995) *Nature* **373,** 339–341.
6. Kearns, C. M., and Gash, D. M. (1995) *Brain Res.* **672,** 104–111.
7. Bowenkamp, K. E., Hoffman, A. F., Gerhardt, G. A., Henry, M. A., Biddle, P. T., Hoffer, B. J., and Granholm, A. C. (1995) *J. Comp. Neurol.* **355,** 479–489.
8. Sauer, H., Rosenblad, C., and Bjorklund, A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 8935–8939.
9. Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S., and Saito, I. (1995) *Nucleic Acids Res.* **23,** 3816–3821.
10. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1320–1324.
11. Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5547–5551.
12. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) *Science* **268,** 1766–1769.
13. Ross, R. A., Spengler, B. A., and Biedler, J. L. (1983) *J. Natl. Cancer Int.* **71,** 741–747.
14. Abiru, Y., Nishio, C., and Hatanaka, H. (1996) *Developmental Brain Res.* **91,** 260–267.
15. Hatanaka, H., and Arimatsu, Y. (1984) *Neurosci. Res.* **1,** 253–263.
16. Takeda, H., Matsumiya, T., and Shibuya, T. (1990) *J. Chromatogr.* **515,** 265–278.
17. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) *Gene* **108,** 193–200.
18. Davidson, B. L., Allen, E. D., Kozarsky, K. F., Wilson, J. M., and Roessler, B. J. (1993) *Nature Genet.* **3,** 219–223.
19. Akli, S., Caillaund, C., Vigne, E., Stratford-Perricaudet, L. D., Poenaru, L., Perricaudet, M., Kahn, A., and Peschanski, M. R. (1993) *Nature Genet.* **3,** 224–228.
20. Bajocchi, G., Feldman, S. H., Crystal, R. G., and Mastrangeli, A. (1993) *Nature Genet.* **3,** 229–234.
21. Gash, D. M., Zhang, Z., Cass, W. A., Ovadia, A., Simmerman, L., Martin, D., Russell, D., Collins, F., Hoffer, B. J., and Gerhardt, G. A. (1995) *J. Comp. Neurol.* **363,** 345–358.
22. Gash, D. M., Zhang, Z., Ovadia, A., Cass, W. A., Simmerman, L., Russell, D., Martin, D., Lapchak, P. A., Collins, F., Hoffer, B. J., and Gerhardt, G. A. (1996) *Nature* **380,** 252–255.
23. Choi-Lundberg, D. L., Lin, Q., Chang, Y. N., Hay, C. M., Mohajeri, H., Davidson, B. L., and Bohn, M. C. (1997) *Science* **275,** 838–841.
24. Schultz, J. B., Matthews, R. T., Muqit, M. M., Browne, S. E., and Beal, M. F. (1995) *J. Neurochem.* **64,** 936–939.
25. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) *J. Gen. Virol.* **36,** 59–72.
26. Sánchez, M. P., Silos-Santiago, I., Frisén, J., He, B., Lira, S. A., and Barbacid, M. (1996) *Nature* **382,** 70–73.
27. Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A.-C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H., and Westphal, H. (1996) *Nature* **382,** 73–76.
28. Moore, M. W., Klein, R. D., Fariñas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carvar-Moore, K., and Rosenthal, A. (1996) *Nature* **382,** 76–79.
29. Yoshida, Y., and Hamada, H. (1997) *Biochem. Biophys. Res. Commun.* **230,** 426–430.