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GTP cyclohydrolase I utilizes metal-free GTP as its substrate

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GTP cyclohydrolase I (GCH) is the rate-limiting enzyme for the synthesis of tetrahydrobiopterin and its activity is important in the regulation of monoamine neurotransmitters such as dopamine, norepinephrine and serotonin. We have studied the action of divalent cations on the enzyme activity of purified recombinant human GCH expressed in *Escherichia coli*. First, we showed that the enzyme activity is dependent on the concentration of Mg-free GTP. Inhibition of the enzyme activity by Mg^{2+}, as well as by Mn^{2+}, Co^{2+} or Zn^{2+}, was due to the reduction of the availability of metal-free GTP substrate for the enzyme, when a divalent cation was present at a relatively high concentration with respect to GTP. We next examined the requirement of Zn^{2+} for enzyme activity by the use of a protein refolding assay, because the recombinant enzyme contained approximately one zinc atom per subunit of the decameric protein. Only when Zn^{2+} was present was the activity of the denatured enzyme effectively recovered by incubation with a chaperone protein. These are the first data demonstrating that GCH recognizes Mg-free GTP and requires Zn^{2+} for its catalytic activity. We suggest that the cellular concentration of divalent cations can modulate GCH activity, and thus tetrahydrobiopterin biosynthesis as well.

Keywords: GTP cyclohydrolase I; magnesium; recombinant protein; tetrahydrobiopterin; zinc.

Metal ions are essential for many physiological functions of the brain. They may also induce or aggravate numerous neurodegenerative processes. Thus, it is important to understand the roles of metal ions in normal and pathological brain functions.

GTP cyclohydrolase I (GCH) is the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin (BH_4), and the cellular BH_4 content is regulated mainly by the activity of this enzyme. BH_4 is an essential cofactor for three aromatic amino-acid monooxygenases – phenylalanine, tyrosine, and tryptophan hydroxylases – and for nitric oxide synthase [1]. BH_4 deficiency caused not only a decrease in the activity of these enzymes but also a decrease in the protein levels of tyrosine hydroxylase and nitric oxide synthase [2,3]. Therefore, the availability of BH_4 affects the amounts of neurotransmitters such as catecholamines, serotonin, melatonin and nitric oxide. The role of BH_4 in the activity of nitric oxide synthase also makes BH_4 an important factor for the immune system and endothelial cell function.

Various hormones and cytokines are known to induce the expression of the GCH gene in neural, lymphocytic and endothelial cells, and in different cell lines, resulting in an increased BH_4 content [4–8]. At the post-transcriptional level, BH_4 was shown to inhibit, and phenylalanine to stimulate, GCH activity through interaction with GFRP, a GTP cyclohydrolase I feedback regulatory protein [9]. GCH, which is a homodecameric protein, shows positive cooperativity against the GTP substrate [10] and phenylalanine changes the substrate velocity curve from sigmoidal to hyperbolic [11].

Recent biophysical studies suggest a stimulatory effect of Zn^{2+} [12] and Ca^{2+} [13] on GCH activity. By crystallographic analysis using purified *Escherichia coli* enzyme [14], an N-terminally truncated form of the recombinant human enzyme [12], and a stimulatory complex of rat GCH and GFRP induced by phenylalanine [15], Zn^{2+} was shown to be bound to the active centre of the homodecameric GCH enzyme. As for Ca^{2+}, mutations of the recombinant rat enzyme in an EF-hand-like motif, which is absent in bacteria, inhibited both the binding of Ca^{2+} to the enzyme and enzyme activity [13]. In addition, inhibition of the enzyme activity by various divalent cations including Mg^{2+} and Zn^{2+} was reported, based on experiments using crude preparations from mammalian and bacterial tissues [16] and the enzyme purified from rat liver [10].

In the present study, we examined the effect of various divalent cations on purified recombinant human GCH expressed in *E. coli* to clarify the molecular mechanism of action of divalent cations on the GCH enzymatic activity. We showed that GCH activity was totally dependent on metal-free GTP and that Mg^{2+} inhibited the enzyme activity by reducing the concentration of metal-free GTP by complex formation. Mg–GTP complex and Mg^{2+} had little effect on the GCH activity at the concentrations tested here. Also, by performing a protein refolding assay for GCH, we demonstrated that a stoichiometric amount of Zn^{2+} was

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Abbreviations: BH_4, tetrahydrobiopterin; GCH, GTP cyclohydrolase I; GdnHCl, guanidine hydrochloride; NOS, nitric oxide synthase.

Enzyme: GTP cyclohydrolase I (EC 3.5.4.16).

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essential for the enzyme activity. Our data thus suggest that physiological and pathological changes in the levels of divalent cations including Mg^{2+} and Zn^{2+} may affect GCH activity and BH_4 levels *in vivo*.

Experimental procedures

Purification of recombinant human GCH

Recombinant human GCH was expressed in *E. coli* and purified as described previously [17]. We used this purified recombinant human enzyme for analysis of the action of divalent cations. Protein concentrations were determined by the method of Bradford [18], with bovine γ-globulin used as a standard.

Measurement of GCH activity

GCH activity was assayed as described previously [17]. The typical incubation mixture (total volume, 100 μL) contained 20 mM Tris/HCl (pH 7.5), 100 mM KCl, 1 $mg{\cdot}mL^{-1}$ BSA, and GTP as a substrate. The recombinant protein (10 μg) was incubated with various concentrations of GTP and divalent cations at 37 °C for 30 min.

Calculation of the concentrations of metal–GTP complex and metal-free GTP

Concentrations of metal-containing GTP, metal-free GTP, and GTP-free divalent cations in the reaction mixture for the measurement of the enzyme activity were determined by using the MAXCHELATOR program (WINMAXC ver.2.10 and SLIDERS ver.2.00, http://www.stanford.edu/~cpatton/maxc.html) [19]. Stability constants and enthalpy changes for metal–nucleotide complexes were obtained by referring to NIST Critically Selected Stability Constants of Metal Complexes: Version 6.0 (http://www.nist.gov/srd/nist46.htm). For calculation of concentrations of metal–GTP complex and metal-free GTP, we used stability constants and enthalpy changes of metal–ATP or proton–ATP complex as a substitute for those of the metal–GTP complex, because there were no data for stability constants and enthalpy changes of the Mg–, Zn–, Co– or Mn–GTP complexes in K^+ salt as a background electrolyte; however, stability constants of GTP with respect to Mg^{2+} in Na^+ salt as a background electrolyte and stability constants and enthalpy changes of GTP with respect to H^+ in K^+ salt as a background electrolyte were very similar to those of ATP in the database, and apparent stability constants of GTP with respect to Mg^{2+}, Mn^{2+} and Co^{2+} were almost the same as those of ATP given in a previous report [20]. Based on the condition of the incubation mixture for the enzyme activity described as above, parameters used in the calculation program were 37 °C, pH 7.5, and 0.110 ionic strength. Calculated values were considered to be accurate in a chelator-buffering range, which is within one order of magnitude of the K_d value for a metal–chelator complex.

Atomic absorption spectrophotometry

Zinc and calcium contents of the purified recombinant human GCH protein were determined by atomic absorption spectrophometry using a polarized Zeeman atomic absorption spectrometer, type Z-8100 (Hitachi, Tokyo, Japan).

Refolding assay

For the protein refolding assay in the presence of GroE, which is a chaperone protein, we referred to previous reports [21–24]. For denaturation, GCH was incubated on ice with 4 M guanidine hydrochloride (GdnHCl) for 30 min. The solution of denatured GCH was then diluted 100-fold with refolding buffer containing 50 mM Tris/HCl pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ATP, and a 2.5-fold molar excess of GroE. Equal molar amounts of GroES and GroEL (Takara Bio, Japan) were mixed for preparing the GroE complex. For refolding, the mixture was incubated at 25 °C for 60 min. Spontaneous refolding was performed in the absence of GroE.

For the experiment involving Zn^{2+} addition after refolding, the sample refolded in the presence of EGTA or Zn^{2+} was desalted by filtration through a spin-column (Micro Bio-spin 6, Bio-Rad). For elimination of Mg^{2+} and ATP, which are essential for the refolding reaction, as well as that of Zn^{2+} or EGTA, from the refolded samples, the spin-column was equilibrated with a solution containing 50 mM Tris/HCl pH 7.5, 50 mM KCl, and 1 mM dithiothreitol. After desalting, ions or chelators were added to aliquots of the filtered samples and preincubation was carried out at 25 °C for 5 min. Finally, aliquots of the samples (10 μL) were added to 90-μL volumes of the assay mixture for measurement of GCH activity, which was performed as described above.

Statistics

ANOVA followed by Bonferroni/Dunn's multiple comparison test was used for statistical evaluation of differences in the enzyme activity. $P < 0.05$ was accepted as statistically significant.

Results

Interaction of Mg^{2+} with the GTP substrate in solution is responsible for decrease in the GCH activity

GCH has enzyme activity in the absence of Mg^{2+}, whereas many other nucleotide hydrolyzing enzymes such as G proteins and kinases recognize Mg–GTP or Mg–ATP as the substrate. We first examined the effect of Mg^{2+} on the kinetics of enzyme activity of the purified recombinant human GCH. As shown in Fig. 1A, the dose–response curve for the GTP substrate was shifted to the right in the presence of 1 mM $MgCl_2$ and, to a much greater extent in the presence of 5 mM $MgCl_2$, whereas the enzyme activities at the high GTP concentrations remained unchanged. If Mg^{2+} acted directly on the enzyme we would expect the dose dependency of inhibition by $MgCl_2$ to be constant at various GTP concentrations. However, as shown in Fig. 1B, dose dependency for inhibition shifted to lower concentrations of $MgCl_2$ as the concentration of the GTP substrate was decreased. These results suggest that formation of the GTP–Mg^{2+} complex was responsible for the

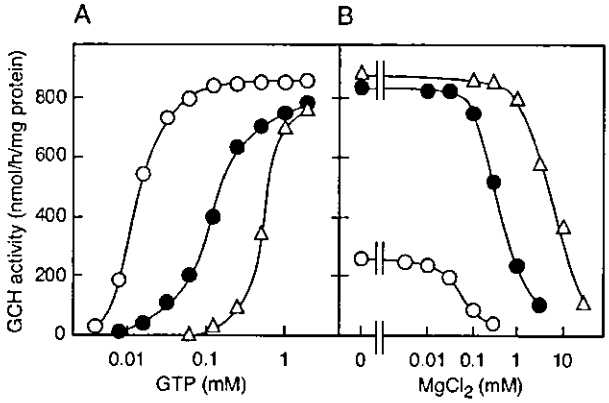

Fig. 1. Effect of Mg^{2+} on the enzyme activity of recombinant human GCH. (A) Purified recombinant enzyme was incubated in the absence (○) or presence of 1 mM (●) or 5 mM (△) $MgCl_2$ at the indicated concentrations of GTP. (B) Enzyme was incubated at 0.1 (○), 1 (●), or 10 (△) mM GTP in the presence of the indicated concentrations of $MgCl_2$. Each figure is representative of two independent experiments.

shift in the GTP dose–response curve at higher Mg^{2+} concentrations.

GCH recognizes Mg-free GTP

We next examined the dependency of the enzyme activity on Mg-free GTP. We assumed that the concentration of total GTP in the absence of $MgCl_2$ was equal to that of metal-free GTP, because 1 mM EDTA did not affect the dose–response curve for the GTP substrate (data not shown). We calculated the metal-free GTP concentrations in the presence of 200 μM $MgCl_2$. The concentrations of metal-free GTP at 15, 20, 30, 40, 50, 75, 100 and 125 μM total GTP were reduced in the presence of 200 μM total Mg^{2+} to 3.63, 4.92, 7.60, 10.4, 13.5, 21.8, 31.4 and 42.4 μM, respectively, in the presence of 200 μM $MgCl_2$ (Fig. 2A). We measured GCH activity under these conditions, and plotted it against total GTP (Fig. 2C) or Mg-free GTP (Fig. 2D). Although the enzyme activity was significantly decreased by the addition of $MgCl_2$ (Fig. 2C), the dependency of the enzyme activity on Mg-free GTP was similar in the presence and absence of $MgCl_2$ (Fig. 2D). The enzyme activity was, however, slightly decreased at > 15 μM Mg-free GTP in the presence of $MgCl_2$ compared with the values in the absence of $MgCl_2$ (Fig. 2D).

Next we measured enzyme activity at a constant concentration of Mg-free GTP and increasing concentrations of Mg–GTP complex and Mg^{2+}. When the concentration of Mg-free GTP was fixed at 10 μM, the concentrations of $MgCl_2$ in the reaction mixture were 35, 70, 105, 140, 175 and 210 μM at the total GTP concentrations of 15, 20, 25, 30, 35 and 40 μM, respectively (Fig. 3A and B). As shown in Fig. 3C, the GCH activity was almost unchanged when the concentrations of Mg-free GTP remained constant at 10 μM in the range 10–40 μM total GTP. These data suggest that the GCH activity was dependent on the concentration of Mg-free GTP and that neither Mg–GTP complex nor Mg^{2+} affected the enzyme activity under the conditions examined.

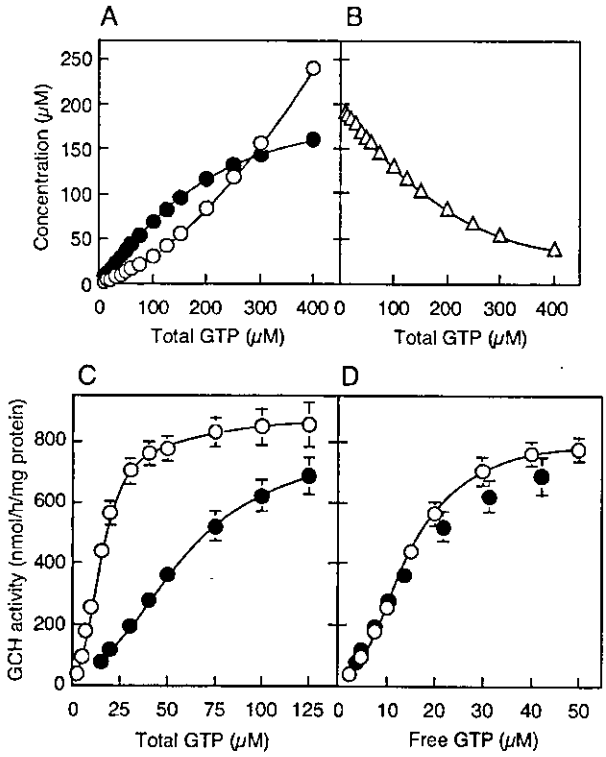

Fig. 2. GTP dose–response curves of the human GCH activity in the presence and absence of Mg^{2+}. (A and B) Concentrations of Mg-free GTP (○), Mg–GTP complex (●), and Mg^{2+} (△) at the indicated total GTP concentrations in the presence of 200 μM $MgCl_2$ were plotted. The concentrations were calculated as described in Experimental procedures. (C and D) Enzyme activity of the recombinant human enzyme was measured in the presence of 200 μM $MgCl_2$ over a range of total GTP of 15–125 μM (●) and in the absence of $MgCl_2$ over a range of total GTP of 2.5–125 μM (○). Concentrations of total and free GTP in the reaction mixture are plotted on the X-axis of (C) and (D), respectively. Results represent the mean ± SD of three independent experiments.

Various divalent cations at 0.5 mM inhibited enzyme activity when the GTP concentration was 0.1 mM (Fig. 4). Both $MgCl_2$ and $MgSO_4$ inhibited enzyme activity to a similar extent (Fig. 4), confirming that the inhibitory effect was caused by the Mg^{2+} ion. $MnCl_2$, $CoCl_2$, and $ZnSO_4$ inhibited the enzyme activity to a greater degree than $MgCl_2$ and $MgSO_4$ (Fig. 4). In contrast with the inhibition shown at 0.1 mM total GTP, we did not observe any inhibitory effect by any of the divalent cations examined at a higher concentration of the substrate, 1 mM total GTP (Fig. 4). The concentration of metal-free GTP in the presence of 0.5 mM Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} at 0.1 mM total GTP was estimated to be 12.7, 3.49, 4.89, and 1.46 μM, respectively. Nonetheless, the enzyme activities under these conditions showed good accordance with the metal-free GTP dose dependency (data not shown). At 1 mM GTP, metal-free GTP in the presence of 0.5 mM Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} was estimated to be 561, 561, 516, 519, and 510 μM, respectively. These data explain why there was no significant difference in the enzyme activity at 1 mM GTP in the presence

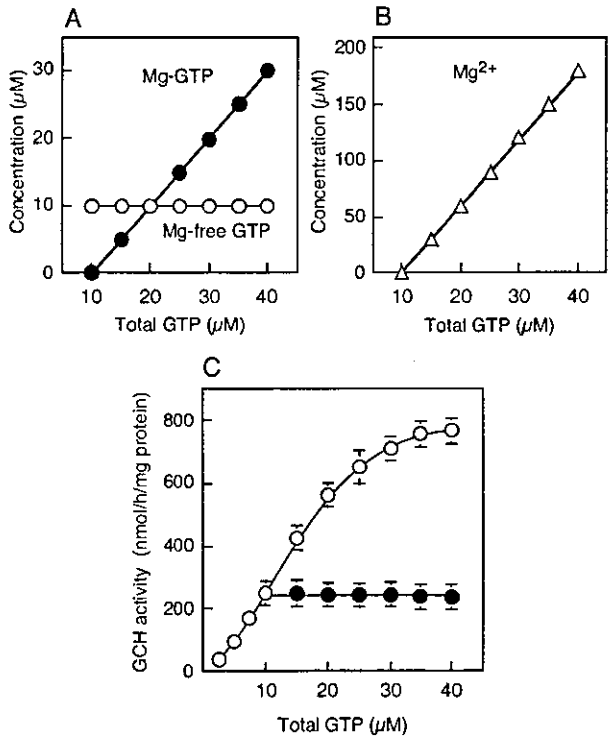

Fig. 3. Enzyme activity at a constant concentration of Mg-free GTP. To fix the concentration of Mg-free GTP at 10 μM constant, we adjusted the concentrations of $MgCl_2$ in the reaction mixture to 35, 70, 105, 140, 175, and 210 μM at the total GTP concentrations of 15, 20, 25, 30, 35, and 40 μM, respectively. Under these conditions at a constant 10 μM Mg-free GTP (A; ○), the concentrations of Mg-GTP complex (A; ●) and Mg^{2+} (B; △) were plotted. (C) GCH activity was measured at a constant 10 μM Mg-free GTP when the concentrations of Mg-GTP complex and Mg^{2+} were increased as described above (●). GCH activity without Mg^{2+} was also measured (○). Results represent the mean ± SD from three independent experiments.

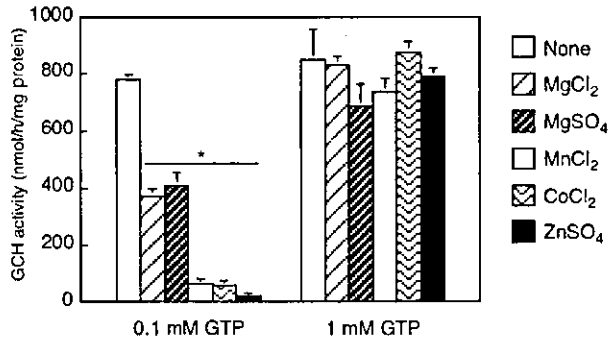

Fig. 4. Effect of various divalent cations on the enzyme activity of recombinant human GCH. Enzyme activity was measured at the GTP concentration of 0.1 or 1 mM in the presence of 0.5 mM $MgCl_2$, $MgSO_4$, $MnCl_2$, $CoCl_2$, or $ZnSO_4$. Results represent the mean ± SD of three independent experiments. *P* values were calculated based on the value for the vehicle only: $^*P < 0.001$.

of various cations: the substrate–velocity curve for metal-free GTP was in the plateau phase around 500 μM (Fig. 1).

Zn^{2+} bound to the purified recombinant human GCH

In contrast to the inhibitory effect of Zn^{2+} on enzyme activity when the ion was in molar excess over the GTP substrate, as was shown in Fig. 4, a recent crystallographic study showed that Zn^{2+} bound to the active centre of the bacterial and human GCH enzymes, with the conclusion that Zn^{2+} participated in the catalytic reaction [12]. Besides Zn^{2+}, Ca^{2+} at nanomolar concentrations was suggested to activate the enzyme [13]. We performed atomic absorption spectrophotometry using the purified recombinant human enzyme to examine whether GCH contained metal ions. By amino acid composition analysis, the concentration of the subunit of the GCH enzyme in the solution examined was calculated to be 14.4 ± 0.7 μM. The concentration of zinc in the solution was estimated to be 16.2 ± 1.4 μM by atomic absorption spectrophotometry, whereas that in the buffer control was not detectable (< 0.3 μM). The data indicate that the purified recombinant enzyme bound ≈ 1 zinc per subunit. On the other hand, calcium was not detectable in the enzyme solution (< 0.5 μM).

Requirement of Zn^{2+} for the GCH enzymatic activity

We next examined whether or not Zn^{2+} was essential for the enzyme activity of the human recombinant GCH. Zn^{2+} seemed to bind tightly to the enzyme protein, because preincubation of the recombinant enzyme with Zn^{2+} chelating agents such as EDTA, EGTA or N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) at 1 mM and 25 °C for 20 min had little effect on enzyme activity (data not shown). To examine the effect of Zn^{2+} on enzyme activity, we established a procedure for the refolding of GCH protein by using a chaperone protein, GroE. In this experiment, we measured enzyme activity at a concentration of GTP high enough, i.e. 1 mM, to give the V_{max} value, thus cancelling the inhibition by the complex formation of metal-free GTP substrate with Mg^{2+} at 0.5 mM and Zn^{2+} at < 30 μM, which were carried over from the refolding reaction. The recombinant enzyme incubated at 4 °C in 4 M GdnHCl was rapidly inactivated (within 5 min). The enzyme activity of the sample denatured for 30 min was ≈ 2% of that of the nondenatured one (Fig. 5A). The denatured sample diluted with the refolding buffer was incubated at 25 °C with or without Zn^{2+} or EGTA in the presence or absence of GroE, before measuring the GCH enzyme activity (Fig. 5B). In the presence of GroE, the enzyme activity recovered to a greater extent than in its absence. It was further elevated by the addition 10 μM $ZnSO_4$ to the refolding mixture, whereas it was inhibited by the addition of EGTA (Fig. 5B). The activity of the enzyme refolded in the presence of $ZnSO_4$ was 67.5 ± 12.3% of that of the nondenatured enzyme (Fig. 5B). Far less enzyme activity was recovered with 10 μM $ZnSO_4$ in the absence of GroE (Fig. 5B). When the refolding reaction was carried out at 4 °C instead of 25 °C, or without ATP or Mg^{2+} at 25 °C, the enzyme activity was not recovered (data not shown). In contrast with Zn^{2+}, Ca^{2+} at 10 μM in the refolding reaction did not have any effect on the enzyme activity (data not shown).

We next examined the amount of Zn^{2+} required for the stimulatory effect in the refolding assay. The presence of Zn^{2+} during the refolding procedure elicited a

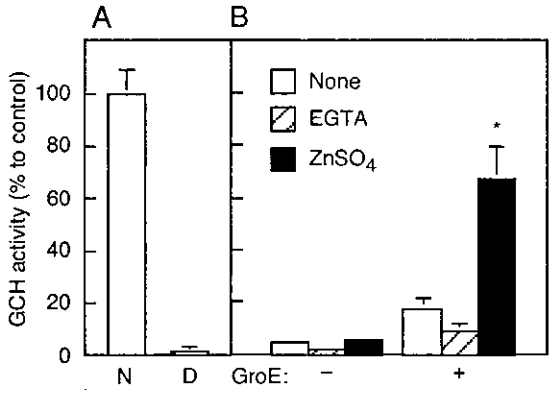

Fig. 5. GCH enzymatic activity was activated by addition of Zn^{2+} during protein refolding. (A) Enzyme activity of the denatured (D) or nondenatured (N) enzyme was measured. For the preparation of denatured samples, the enzyme was incubated on ice with 4 M GdnHCl for 30 min, and the mixture was then diluted with the refolding buffer (final concentration of GdnHCl, 40 mM). For preparation of the nondenatured control enzyme was diluted with refolding buffer containing 40 mM GdnHCl. (B) Enzyme activity of the refolded enzyme was measured. The denatured sample containing 10 μM $ZnCl_2$, 10 μM EGTA, or vehicle only was incubated at 25 °C for 60 min in the refolding buffer in the presence or absence of GroE. Results represent the mean ± SD of three independent experiments. *P* values were calculated based on the value for the vehicle only: $*P < 0.001$.

dose-dependent increase in the GCH enzyme activity, and the maximum effect was achieved at ≈ 10–30 μM (Fig. 6A). The apparent EC_{50} value of Zn^{2+} was estimated to be 235 nM at 70 nM GCH subunit (Fig. 6B).

Effect of Zn^{2+} addition on the GCH enzyme activity after refolding

To clarify whether the presence of Zn^{2+} during refolding was required for the recovery of enzyme activity, we

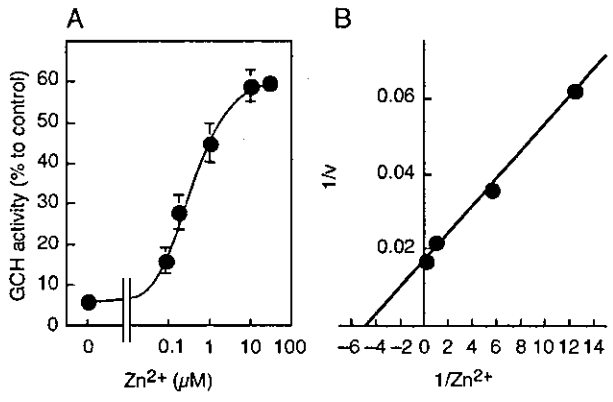

Fig. 6. Dose dependency of the stimulatory effect of Zn^{2+} on enzyme activity during refolding. We performed the refolding assay for the recombinant GCH in the presence of GroE at various Zn^{2+} concentrations. The Zn^{2+} concentration in the presence of 10 μM EGTA was presumed to be zero and that following the addition of the vehicle only was calculated to be 81 nM Zn^{2+} based on the data from the atomic absorption spectrometry. (A) The concentration of Zn^{2+} was plotted on a logarithmic scale. (B) Double reciprocal plot of (A). Results represent the mean ± SD of three independent experiments.

Table 1. Effect of the addition of Zn^{2+} ions after refolding on GCH activity. The denatured samples refolded in the presence of EGTA or Zn^{2+} following the desalting were prepared as described in Experimental procedures. The activities were determined in the presence of 1 mM GTP. The activity of the enzyme refolded in the presence of $ZnSO_4$ and then preincubated in vehicle only (none) was taken as 100%. Results represent the mean ± SD of three independent experiments. *P* values were calculated based on the value obtained by preincubation with vehicle only.

	GCH activity (% of control)	
Preincubation	Refolding with $ZnSO_4$	Refolding with EGTA
None	100.0 ± 0.5	21.3 ± 0.9
$ZnSO_4$	103.4 ± 1.8	64.5 ± 1.8*
EGTA	101.4 ± 0.6	17.8 ± 1.6

$*P < 0.001$.

examined the effect of Zn^{2+} addition on the GCH enzyme after refolding. The addition of 10 μM $ZnSO_4$ after refolding elevated the enzyme activity of the sample refolded with EGTA; however, it was less effective than the addition of Zn^{2+} during refolding (Table 1). EGTA had no significant effect on the enzyme activity of the samples after refolding (Table 1) or on the activity of the nondenatured enzyme (data not shown). The dose dependency of the effect of Zn^{2+} after refolding was similar to that during refolding (data not shown).

Discussion

In the present study, we demonstrated that GCH utilizes metal-free GTP as the substrate for the enzyme reaction. Inhibition of the GCH activity by divalent cations such as Mg^{2+} and Zn^{2+} was due to a reduction in the concentration of metal-free GTP substrate by complex formation. We also showed that Zn^{2+} at a micromolar level was required for the enzyme activity by discriminating it from the inhibitory action of Zn^{2+}. Our data are the first to show the requirement of Zn^{2+} for the enzyme activity of the wild-type enzyme.

Many nucleotide-hydrolyzing enzymes such as G proteins and kinases recognize Mg–GTP or Mg–ATP complex as their substrate. In contrast with these enzymes, GCH activity is dependent on the concentration of Mg-free GTP. Our data showed that the Mg–GTP complex and Mg^{2+} affected the enzyme activity very little at the concentrations tested here. The structure of the active centre for the *E. coli* GCH binding to dGTP also supports our findings, because Mg^{2+} assistance for binding to the GTP substrate was neither realized nor necessary [14].

However, there remains a possibility that the Mg–GTP complex at higher concentrations competitively antagonizes the Mg-free GTP substrate, because the enzyme activity was slightly decreased in the presence of 200 μM $MgCl_2$ at > 15 μM Mg-free GTP (Fig. 2D). When the concentration of total GTP was increased to 75, 100, 125 μM in the presence of 200 μM $MgCl_2$, the concentration of the Mg–GTP complex was calculated to be 53.1, 68.5, and 82.5 μM, respectively; and that of Mg-free GTP to be 21.8,

31.4 and 42.4 μM, respectively (Fig. 2D). As the Mg–GTP complex at < 30 μM had little affect on enzyme activity at 10 μM Mg-free GTP (Fig. 3), there is a possibility that > 50 μM Mg–GTP complex can be a competitive inhibitor or a low-affinity substrate for the GCH enzyme.

Other divalent cations such as Mn^{2+}, Co^{2+}, and Zn^{2+} also inhibited enzyme activity when present at a molar excess over the GTP substrate by a reduction in the concentration of metal-free GTP substrate by complex formation. Our data suggest that the inhibition by various divalent cations of bacterial and mammalian GCH enzymes shown previously [16] would also be due to a reduction in the availability of the metal-free GTP substrate by formation of complexes with the divalent cations.

Because Mg^{2+} is relatively abundant in cells it is considered to be the main cation affecting the concentration of metal-free GTP substrate for GCH *in vivo* under physiological conditions. A change in the intracellular concentration or distribution of Mg^{2+} may thus affect the enzyme activity of GCH. Interestingly, 6-pyruvoyltetrahydropterin synthase, which is the second enzyme in the pathway for BH_4 biosynthesis and which acts on the D-*erythro*-7, 8-dihydroneopterin triphosphate produced by GCH, requires Mg^{2+} for its activity [25,26] in spite of the inhibitory effect of Mg^{2+} on GCH. Our data suggest that there may be an optimal range of Mg^{2+} concentration for BH_4 biosynthesis *in vivo*.

Our data showed that Zn^{2+} is essential for the enzyme activity, in agreement with a suggestion from a previous crystallographic study [12]. Zn^{2+} was proposed to be involved in the catalytic reaction [12]; this proposition is supported by the results of mutation analysis of the *E. coli* enzyme [14] and most recently by the observation that mutational replacement of residues predicted to form the Zn^{2+} binding centre caused catalytic inactivation and reduced the capacity of the *E. coli* enzyme to bind zinc [27]. Although an apo-enzyme for Zn^{2+} was not generated by incubating with Zn^{2+} chelating agents such as EDTA and TPEN, we succeeded in showing the stimulatory effect of Zn^{2+} on the enzyme activity by using a protein refolding assay. Because the intracellular concentration of Zn^{2+} is relatively low, it is reasonable that Zn^{2+} dose-dependent elevation of the enzyme activity occurred in the range of ≈0.1–10 μM during the refolding procedure (Fig. 5) and during the preincubation after refolding with EGTA (data not shown). These data suggest that intracellular Zn^{2+} concentrations would be high enough to bind to the GCH enzyme and low enough to avoid the decrease in the enzyme activity by reduction of the intracellular metal-free GTP substrate, which was previously estimated to be ≈ 150 μM [28].

Because the zinc concentration in the diluted sample was calculated to be 81 ± 7 nM, the slight inhibition of enzyme activity detected after addition of 10 μM EGTA to the refolding solution (Fig. 5B) can be attributed to the chelating of Zn^{2+} (based on the Zn^{2+} dose–response curve). We also demonstrated that the addition of Zn^{2+} after refolding was effective for the sample refolded in the presence of EGTA. However, the apo-enzyme is probably unstable, because the addition of Zn^{2+} after refolding was less effective than that during refolding (Table 1). Auerbach *et al.* previously suggested that the absence of Zn^{2+} in the active centre of the GCH enzyme caused enzymatic inactivation by disulfide formation between the cysteine residues [14] which normally form the Zn^{2+} binding site.

Zn^{2+} would appear to have important roles in nitric oxide production *in vivo*, because Zn^{2+} also binds to nitric oxide synthase (NOS) [29], which utilizes BH_4 and is coinduced with GCH by cytokine-mediated stimulation. Zn^{2+} was reported to be required for the stability of NOS, but not for the catalytic reaction itself [30–32]. In contrast with NOS, our data demonstrate that Zn^{2+} is essential for the activity of the GCH enzyme. In addition to GCH, 6-pyruvoyltetrahydropterin synthase also binds Zn^{2+} [33]. Our present study emphasizes the importance of Zn^{2+} in nitric oxide production.

Ca^{2+} was not detectable in the enzyme solution containing 14.7 ± 0.7 μM GCH protein subunit. The presence of Ca^{2+} at micromolar concentrations in the refolding procedure as well as in the GCH enzyme reaction mixture had no effect on enzyme activity (data not shown). Therefore, Ca^{2+} at a micromolar concentration was demonstrated to be neither essential nor stimulatory for enzyme activity, and the inhibitory effect of EGTA during refolding on enzyme activity seemed to be independent of Ca^{2+} chelation. However, there is a possibility that Ca^{2+} might affect enzyme activity under certain conditions that were not examined in this study, because Ca^{2+} was previously shown to bind to rat GCH protein at very low concentrations, e.g. at the nanomolar level [13], which could have been present in any of the assay solutions used in this study. In order to clarify the effect of Ca^{2+} on enzyme activity, we need to conduct further experiments.

Under pathological and physiological conditions, changes in the concentrations of various divalent cations including Mg^{2+} and Zn^{2+} *in vivo* may affect GCH enzymatic activity, thus resulting in changes in the BH_4 level. There are many diseases known to involve alterations in metal metabolism, such as acrodermatitis enteropathica, in which there is a severe zinc deficiency. We suggest that part of the symptoms of these diseases may be caused by altered levels of GCH activity and BH_4 content. Further investigation into the relationship between divalent cations and GCH enzyme activity *in vivo* should be conducted in the future.

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ADENO-ASSOCIATED VIRAL VECTORS FOR PARKINSON'S DISEASE

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I. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder among the elderly, with an estimated 1% of the population over 60 years old suffering from PD. Progressive loss of nigrostriatal dopamine (DA) neurons leads to a severe decrease in the DA content of the striatum. Cardinal motor symptoms, including resting tremor, muscular rigidity, and bradykinesia, are produced by the reduction of striatal DA to about 20% of normal levels. Replacement of DA is therefore important to alleviate the motor symptoms of the disease. The introduction of the DA precursor, L-3,4-dihydroxyphenylalanine (L-DOPA), in the late 1960s represented a major therapeutic advance in the management of PD, providing clinical benefit to virtually all patients and reducing mortality. Since then, oral administration of L-DOPA has been the mainstay in pharmacotherapy for PD. However, as the disease progresses, L-DOPA is less efficiently converted to DA in the striatum and loses efficacy. Frequent systemic administration of high-dose L-DOPA causes oscillations in motor performance (Jenner, 2000; Langston *et al.*, 2000) and some deleterious complications such as hallucinations due to dopaminergic

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stimulation in the mesolimbic system (Carey *et al.*, 1995). Novel therapeutic interventions substituting for oral L-DOPA administration are therefore required.

PD is regarded as a suitable candidate for gene therapy. Unlike other neurological disorders that affect broad regions of brain, such as Alzheimer's disease, lipid storage disease, and multiple sclerosis, PD is primarily confined to the well-defined nigrostriatal dopaminergic system. Therapeutic genes do not need to be delivered to the entire brain, but can be targeted to portions of the basal ganglia. Stereotactic surgical techniques for approaching the basal ganglia are already established in clinical practice. With the development of microelectrode recording for neural activity, a number of surgical treatments such as pallidotomy, thalmotomy, and deep brain stimulation in the subthalamic nucleus have been employed in PD over the past century. In addition, well-characterized rodent and primate PD models are available for testing novel therapeutic interventions.

Apart from genetic mutations described in some familial cases, the causes of PD remain largely unknown. Purely genetic causes probably account for only a small number of PD patients, and multiple factors including environmental factors may contribute to the development of sporadic PD. For this reason, the current strategy of gene therapy for PD does not pursue the correction of specific genetic abnormalities, but rather aims at either restoring local production of DA by delivering genes for DA-synthesizing enzymes to the striatum (Kaplitt *et al.*, 1994; During *et al.*, 1998; Fan *et al.*, 1998; Mandel *et al.*, 1998; Leff *et al.*, 1999; Bankiewicz *et al.*, 2000; Shen *et al.*, 2000; Sanchez-Pernaute *et al.*, 2001; Kirik *et al.*, 2002; Muramatsu *et al.*, 2002), or providing neuroprotection to block or slow ongoing degenerative processes by providing genes for growth factors (Klein *et al.*, 1999; Mandel *et al.*, 1997, 1999; Kirik *et al.*, 2000; Wang *et al.*, 2002), antioxidant molecules, or antiapoptotic substances (Mochizuki *et al.*, 2001).

II. Recombinant Adeno-Associated Viral Vector

Among the various gene delivery vehicles, recombinant adeno-associated viral (rAAV) vector represents one of the most suitable means for direct introduction of therapeutic genes into the mammalian brain. The rAAV vector is the only viral vector system based on a nonpathogenic replication-defective virus. Efficient and long-term *in vivo* gene expression has been achieved without substantial toxicity or immune response (Kaplitt *et al.*, 1994; Du *et al.*, 1996; During *et al.*, 1998; Fan *et al.*, 1998; Leff *et al.*, 1999; Shen *et al.*, 2000).

AAVs are small, nonenveloped, single-stranded DNA viruses of the Parvoviridae family assigned to the genus *Dependovirus*, as productive infection requires coinfection with a helper virus such as adenovirus (Ad) or herpesvirus (Berns *et al.*, 1995). AAVs have been isolated from a variety of different species, including

primates, dogs, cows, horses, sheep, and chicken, and appear to be nonpathogenic in all cases. To date, eight primate AAVs (AAV-1 to -8) have been cloned and sequenced (Srivastava *et al.*, 1983; Muramatsu *et al.*, 1996; Rutledge *et al.*, 1998; Bantel-Schaal *et al.*, 1999; Chiorini *et al.*, 1997, 1999; Xiao *et al.*, 1999 Gao *et al.*, 2002). Among them, AAV-2 has been the most extensively studied and offers a major platform for the development of gene therapy vectors. The genomic organization of AAV-2 consists of two large open reading frames (ORFs) flanked by two inverted terminal repeats (ITRs). AAV-2 ITRs are 145-nt sequences that function in *cis* as the replication origin and are required for packaging of progeny AAV DNA into virus particles. The left ORF encodes four nonstructural Rep proteins, which are essential for the replication of the AAV genome and targeted integration into the host chromosome during latent infection. The right ORF encodes three viral capsid proteins, VP1, VP2, and VP3.

The conventional method of rAAV vector production involves double-plasmid transfection followed by helper Ad infection. The vector plasmid contains a transgene expression cassette flanked by AAV ITRs, whereas the packaging plasmid expresses Rep and VP proteins in *trans* to supply required AAV helper functions. More recently, a helper-free triple-plasmid transfection method has been developed (Matsushita *et al.*, 1998). By employing a helper plasmid containing the minimum Ad genomic sequences necessary for helper functions (E1A, E1B, E2, E4ORF6, VA RNA), undesirable contamination with wild-type Ad is avoided. Although high multiplicities of infection, estimated to be between 10^3 and 10^5 genome copies per cell, are required for efficient gene transfer via rAAV vector transduction, this system has the ability to produce pure, high-titer rAAV vectors that can be used for clinical applications. Vectors are purified by two sequential continuous CsCl gradient, or for clinical use discontinuous iodixanol gradient, ultracentrifugations. Vector titer is determined by quantitative DNA dot-blot hybridization of DNase I-treated vector stocks, and is routinely 10^{12} to 10^{13} genome copies per milliliter.

III. Restoration of DA Synthesis in the Striatum

A. Biosynthetic Pathway of DA

For efficient DA synthesis, three enzymes are necessary (Fig. 1). Tyrosine hydroxylase (TH) is the rate-limiting enzyme and first converts L-tyrosine, an essential amino acid present in all types of cells, to L-DOPA. Aromatic L-amino acid decarboxylase (AADC) then converts L-DOPA to DA. Guanosine triphosphate cyclohydrolase I (GCH) is the rate-limiting enzyme for synthesis of the essential TH cofactor tetrahydrobiopterine (BH4). Because low levels of endogenous BH4

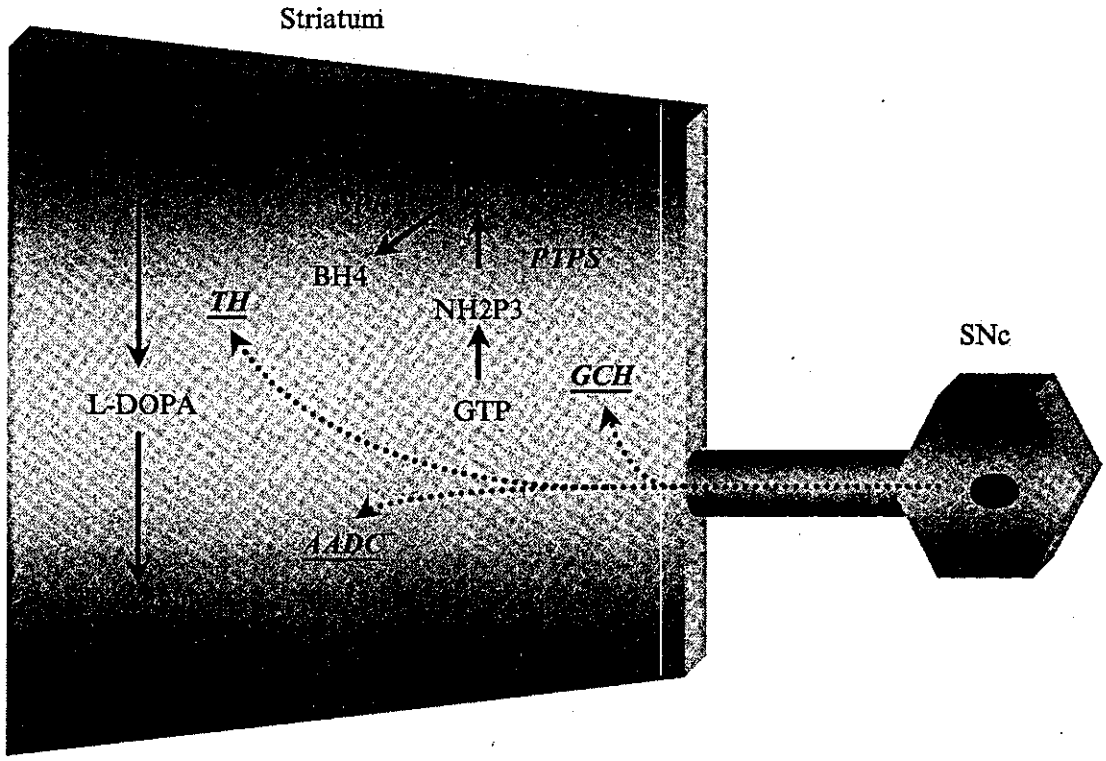

FIG. 1. Biosynthetic pathway of dopamine. Three enzymes (TH, AADC, and GCH) are necessary for efficient dopamine (DA) production. GCH is a rate-limiting enzyme for biosynthesis of BH4, an essential TH cofactor. These enzymes are transported from the substantia nigra to the striatum in an anterograde manner. TH, tyrosine hydroxylase; AADC, aromatic-L-amino-acid decarboxylase; BH4, tetrahydrobiopterin; GTP, guanosine triphosphate; GCH, GTP cyclohydrolase I; NH2P3, *d*-erythro-7,8-dihydroneopterin triphosphate; PPH4, 6-pyruvoyl tetrahydropterin; PTPS, 6-pyruvoyl tetrahydropterin synthase; SPR, sepiapterin reductase.

do not yield sufficient TH activity, GCH is considered to regulate TH activity via regulation of BH4 biosynthesis, thus indirectly controlling DA production in TH-containing DA neurons (Nagatsu *et al.*, 1997; Nagatsu and Ichinose, 1999). These enzymes are transported in an anterograde manner from the substantia nigra to the striatum. A severe loss of dopaminergic nerve terminals in advanced PD is associated with an 80–95% depletion of striatal TH and AADC activity (Zhong *et al.*, 1995; Kaddis *et al.*, 1997). AADC is present in DA-denervated striatum within nondopaminergic neurons and glial cells, but endogenous AADC activity in the striatum is considered insufficient (Nakamura *et al.*, 2000). Although L-DOPA may also function as a neurotransmitter, most L-DOPA produced in the striatum after gene transfer must be converted to DA *in situ* (Kaplitt *et al.*, 1994). GCH activity in the striatum is also decreased in PD and parallels decreases in levels of BH4, TH, and DA (Nagatsu *et al.*, 1987, 1997; Nagatsu and Ichinose, 1999). In a rat model, levels of BH4 and GCH activity in the striatum decrease to less than 27% and 32% of normal levels, respectively, after injection

of 6-hydroxydopamine (6-OHDA) into the substantia nigra, selectively destroying the DA neurons (Levine *et al.*, 1981). Although BH4 can cross the blood–brain barrier, BH4 in the brain is supplied predominantly by intracellular biosynthesis and uptake of exogenous BH4 from the blood was low (Hoshiga *et al.*, 1993). GCH gene transfer into striatal cells appears to offer a more efficient method of supplying BH4 than administration of exogenous BH4.

B. rAAV Vector Expressing DA-Synthesizing Enzyme

We produced rAAV vectors expressing TH (rAAV-TH), AADC (rAAV-AADC), GCH (rAAV-GCH), and LacZ (rAAV-LacZ) by helper-free method to investigate whether rAAV vector-mediated gene transfer of DA-synthesizing enzymes could restore local production of DA and produce behavioral recovery in PD model animals. The expression cassette of each rAAV vector contains cDNA encoding the exogenous gene with the human cytomegalovirus (CMV) immediate-early promoter, human growth hormone first intron, and simian virus 40 (SV40) polyadenylation signal sequence between two ITRs of the AAV-2 genome.

Expression of GCH via rAAV-GCH induced BH4 synthesis and enhanced DA production *in vitro*. When cells from the 293 human embryonic kidney cell line were transduced with various doses of rAAV-GCH (ranging from 0 to 5×10^5 genome copies per cell) together with fixed amounts of rAAV-TH and rAAV-AADC (5×10^5 genome copies per cell each), GCH activity and BH4 production gradually increased in concert with gradual increases in dose of rAAV-GCH. Intracelullar L-DOPA and DA levels increased significantly with increasing amounts of rAAV-GCH (Shen *et al.*, 2000).

C. Long-Term Behavioral Recovery in a Rat Model of PD

Hemiparkinsonian rats were made by the injection of 6-OHDA into the medial forebrain bundle. Mixtures of three rAAV vectors (rAAV-TH/-AADC/-GCH, 5×10^7 genome copies per site for each vector) were then injected into the lesioned striatum at three sites (Shen *et al.*, 2000). The rAAV vectors efficiently transduced striatal cells. A majority (>95%) of transduced cells were microtubule-associated protein (MAP2)-immunoreactive (IR) neurons and more than 90% of TH-IR cells were also positive for AADC and GCH, representing a high coexpression efficiency. This indicates that although the limited packaging capacity on the size of the DNA (<5 kb) renders the use of a single rAAV vector impossible for multiple gene transfer, cells could be simultaneously transduced using different rAAV vectors. TH-IR cells were still observed around injection sites in a wide area of the striatum, even 18 months after vector injection (Fig. 2). Rats

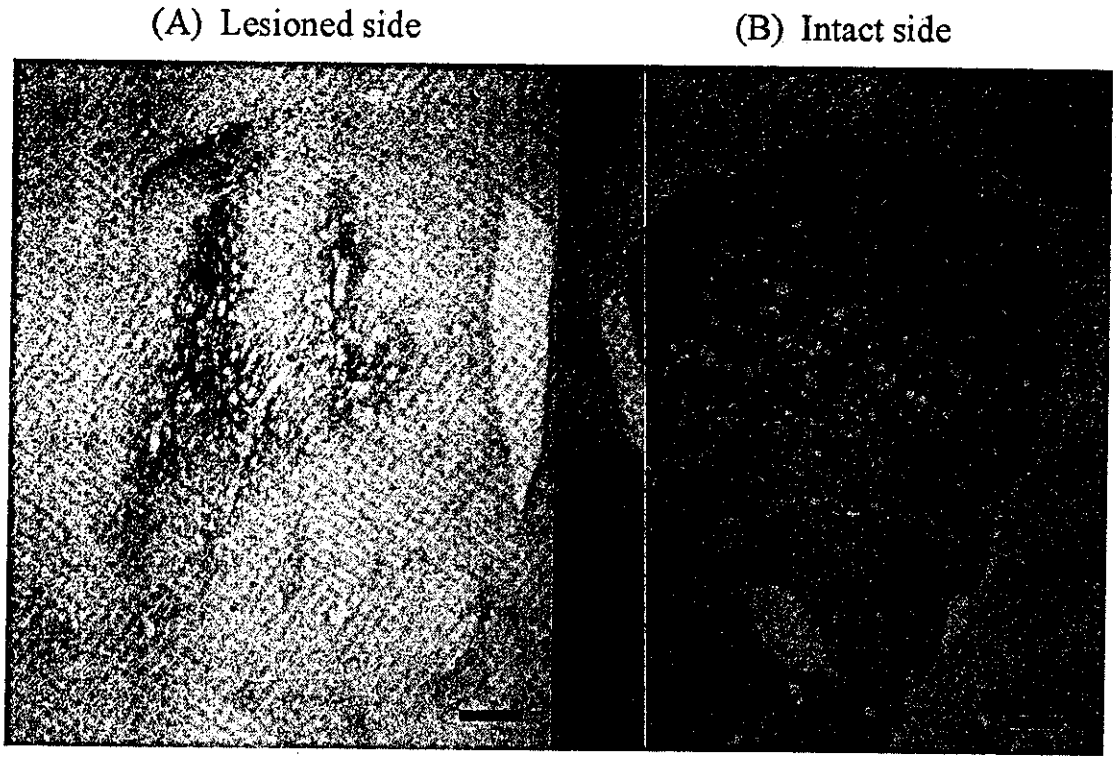

FIG. 2. TH immunostaining in the brain of 6-OHDA-lesioned rats 18 months after stereotaxic injection of rAAV-TH/-AADC/-GCH. (A) rAAV vectors were injected into the striatum of the lesioned side. Intense TH staining was observed around the two needle tracks. (B) Positive staining of the dopaminergic terminals was observed in the intact striatum. Scale bar = 2 mm.

receiving either rAAV-TH/-AADC or rAAV-TH/-AADC/-GCH demonstrated significant decreases in rates of apomorphine-induced rotation compared to pretreated levels, while the rotational behavior of rats injected with rAAV-LacZ was unaltered from baseline throughout the 18-month observation period following injection (Fig. 3). In addition, rats transduced with three rAAV vectors demonstrated a more remarkable decrease in rotation rate than those with two rAAV vectors. Levels of BH4 and DA in the lesioned striatum of rats injected with all three vectors were significantly greater than those in rats treated with rAAV-TH/-AADC or in uninjected control animals. Conversely, no significant difference in BH4 level was observed between animals injected with two vectors and control animals. Some researchers have reported that addition of AADC to systems expressing TH and GCH via genetically modified fibroblasts results in less L-DOPA production due to feedback inhibition of DA on TH (Wachtel *et al.*, 1997; Kang *et al.*, 2001). However, previous studies including our own have demonstrated that restoration of AADC and TH activity in the striatum is beneficial in 6-OHDA-lesioned rats (Fan *et al.*, 1998; Leff *et al.*, 1999), and the results presented here show that local production of striatal DA is actually enhanced by the additional expression of GCH compared with TH and AADC alone.

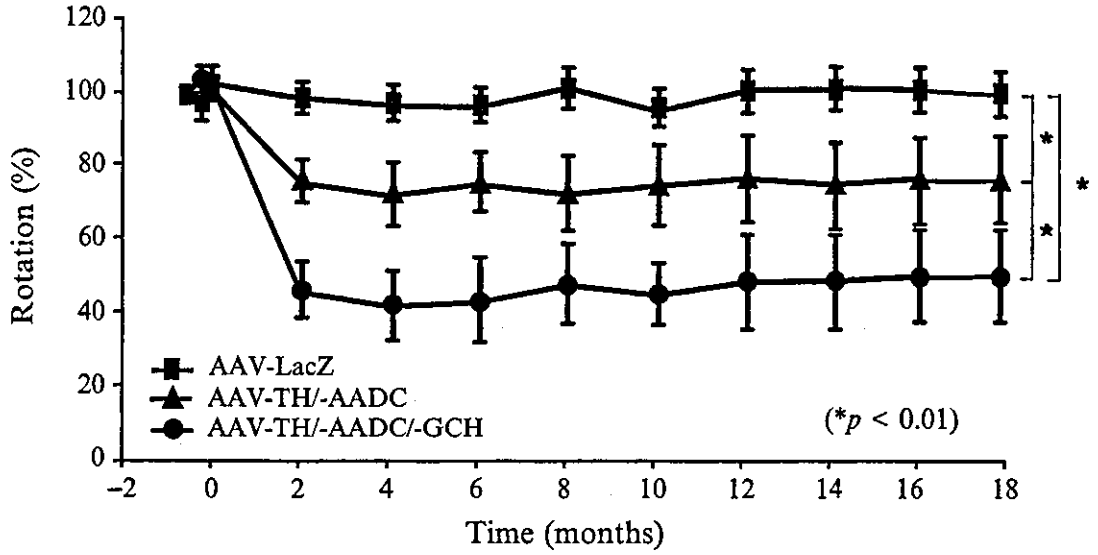

FIG. 3. Effects of intrastriatal injection of rAAV vectors on apomorphine-induced rotational behavior in 6-OHDA-lesioned rats. Rotation was evaluated every week before and after stereotaxic injection of rAAV-LacZ ($n = 5$), rAAV-TH/-AADC ($n = 8$), and rAAV-TH/-AADC/-GCH ($n = 8$).

In summary, cotransduction with three kinds of rAAV vectors results in more effective DA production and markedly increased behavioral recovery than transduction with rAAV-TH vector alone or combination of rAAV-TH and rAAV-AADC vectors. Behavioral recovery persisted at least 18 months after rAAV vector treatment.

D. BEHAVIORAL RECOVERY IN A PRIMATE MODEL OF PD

Encouraging results of rAAV vector-mediated gene transfer of DA-synthesizing enzymes in parkinsonian rats prompted us to extend preclinical explorations to a primate model of PD (Muramatsu *et al.*, 2002). Primate models are useful to evaluate motor functions more properly and to examine transduction efficiency in larger areas of striatum. Cynomolgus monkeys (*Macaca fascicularis*) had been trained to perform a fine motor task consisting of picking up four raisins or small pieces of apple with each of the two hands. Bilateral striatal lesions were made by intravenous injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a selective toxin of dopaminergic neurons, once a week until a stable parkinsonian syndrome was achieved. Monkeys were allowed to recover for 2 months after the last MPTP treatment to avoid the possibility that spontaneous recovery from acute toxicity of MPTP could mimic any behavioral effect of rAAV injection. Mixtures of three rAAV vectors (rAAV-TH/-AADC/-GCH) were then stereotaxically injected unilaterally into the putamen of parkinsonian monkeys. Each monkey received nine unilateral

injections of rAAV vectors in three tracks in the unilateral putamen. Each injection comprised 5 μl with a 1:1:1 mixture of rAAV-TH/-AADC/-GCH (1×10^{13} copies of each vector genome per ml). Thus, in total, 1.5×10^{11} genome copies of each vector were injected. As a control, 15 μl of AAV-LacZ (1×10^{13} vector genome copies/ml) or phosphate-buffered saline was injected into the contralateral putamen. Coexpression of enzymes in the unilateral putamen resulted in marked improvement in manual dexterity on the contralateral side to rAAV-TH/-AADC/-GCH vector injections. Monkeys picked up raisins better with the contralateral hand, in which tremor had disappeared. The ipsilateral limb remained disabled, suggesting that behavioral recovery of the contralateral limb cannot be explained by spontaneous recovery that may be expected to occur over time. Although 20% of thalamic projections from basal ganglia decussate in monkeys, unilateral transduction would primarily affect contralateral limb movement. Behavioral recovery persisted throughout the observation period (three monkeys were sacrificed for histological evaluation at 48 days, 65 days, and 50 days, respectively, and one monkey was kept alive more than 30 months after rAAV vectors injection). After rAAV vector injections, animals demonstrated apomorphine-induced dystonic postures, in which the body and face turned toward the ipsilateral side, and a circling tendency toward the ipsilateral side, indicating reduced DA receptor supersensitivity in the AAV-TH/-AADC/-GCH-injected putamen (Fig. 4). TH-IR, AADC-IR, and GCH-IR cells were present in a large region of the putamen (>90% of the putamen) (Fig. 5).

Although the convection-enhanced delivery method is reportedly efficient, a sufficient region can be transduced in primate brains by simple injection methods if high-titer rAAV vectors are available. The optimal TH:AADC:GCH ratio and the minimal volume of transduced region necessary for functional recovery are yet to be elucidated for human brains, in which the putamen is approximately 10 times larger than in monkey brains (Lange *et al.*, 1976; Lieberman *et al.*, 1995). However, a considerable therapeutic effect was obtained with a 1:1:1 ratio of rAAV vector doses and transducing a broad region of the human putamen by increasing the number of injection sites seems feasible. Except for slight infiltration of mononuclear cells and residual hemosiderin around the needle tract, hematoxylin and eosin staining revealed no signs of cytotoxicity in the rAAV vector-injected putamen. Microdialysis demonstrated that concentrations of DA in the rAAV-TH/-AADC/-GCH vector-injected putamen were increased compared with the control side. Monkeys did not show any complications related to rAAV vector injection, including dyskinesia.

In summary, rAAV vectors efficiently introduced genes for DA-synthesizing enzymes into the striatum of primates, resulting in restoration of motor function with robust transgene expression and elevated DA synthesis in the treated putamen.

IV. Neuroprotection by GDNF Gene Delivery

An alternative approach to the treatment of PD is the protection of nigrostriatal pathways from progressive degeneration (Dunnett and Bjorklund, 1999). Glial cell line-derived neurotrophic factor (GDNF) is a small glycoprotein that provides strong trophic support for DA neurons and is a candidate for this neuroprotective strategy (Bjorklund *et al.*, 2000; Bohn, 2000). However, the applications of GDNF protein are limited because of its short duration of activity and poor ability to cross the blood–brain barrier. An attempt to treat PD patients via direct injection of GDNF protein into the ventricles was unsuccessful (Kordower *et al.*, 1999). Moreover, because PD is a progressive disorder, GDNF should be delivered continuously to support diseased DA neurons. Repeated injections of GDNF proteins into the human brain are not practical and implanting infusion devices is likely to increase patient morbidity and risks of infection over the long term. Recombinant viral vectors are powerful tools for sustained GDNF production in the brain.

Previous studies have demonstrated that GDNF gene delivered via rAAV (Mandel *et al.*, 1997, 1999; Kirik *et al.*, 2000), adenoviral (Bilang-Bleuel *et al.*, 1997; Choi-Lundberg *et al.*, 1997, 1998; Kozlowski *et al.*, 2000; Connor *et al.*, 1999, 2001), herpes simplex viral (Natsume *et al.*, 2001), or lentiviral (Kordower *et al.*, 2000) vectors protects nigral DA neurons in rodent and primate models of PD. However, GDNF gene was administered prior to or shortly after injections of neurotoxin in these studies. Because substantial numbers of DA neurons are already lost before characteristic symptoms appear in PD, a clinically more important question is whether GDNF gene delivered in a delayed manner is capable of rescuing DA neurons and improving behavioral performance in animal models with extensive nigrostriatal DA denervation already present. In our study using a rat model, 4 weeks after creation of a unilateral striatal lesion using 6-OHDA, animals received injections of rAAV vectors expressing GDNF tagged with FLAG peptide (rAAV-GDNF*flag*) or β-galactosidase (rAAV-LacZ) into the lesioned striatum (Fig. 6). At that time point, the percentage of TH-IR neurons on the lesioned side had been reduced to 35% of the intact side. At 20 weeks after rAAV vector injection, extensive loss of TH-IR cells in the substantia nigra (SN) (20% of intact side) and TH-IR fibers in the striatum (14% of intact side) was observed in rats injected with rAAV-LacZ. In contrast, injection of rAAV-GDNF*flag* significantly increased numbers of TH-IR neurons in the SN and density of TH-IR fibers in the striatum, reaching nearly 57% and 49% of the contralateral side, respectively. Levels of DA and its metabolites in the striatum were markedly higher in the rAAV-GDNF*flag* group compared with the control group. Consistent with anatomical and biochemical changes, significant behavioral recovery was observed from 4–20 weeks following rAAV-GDNF*flag*

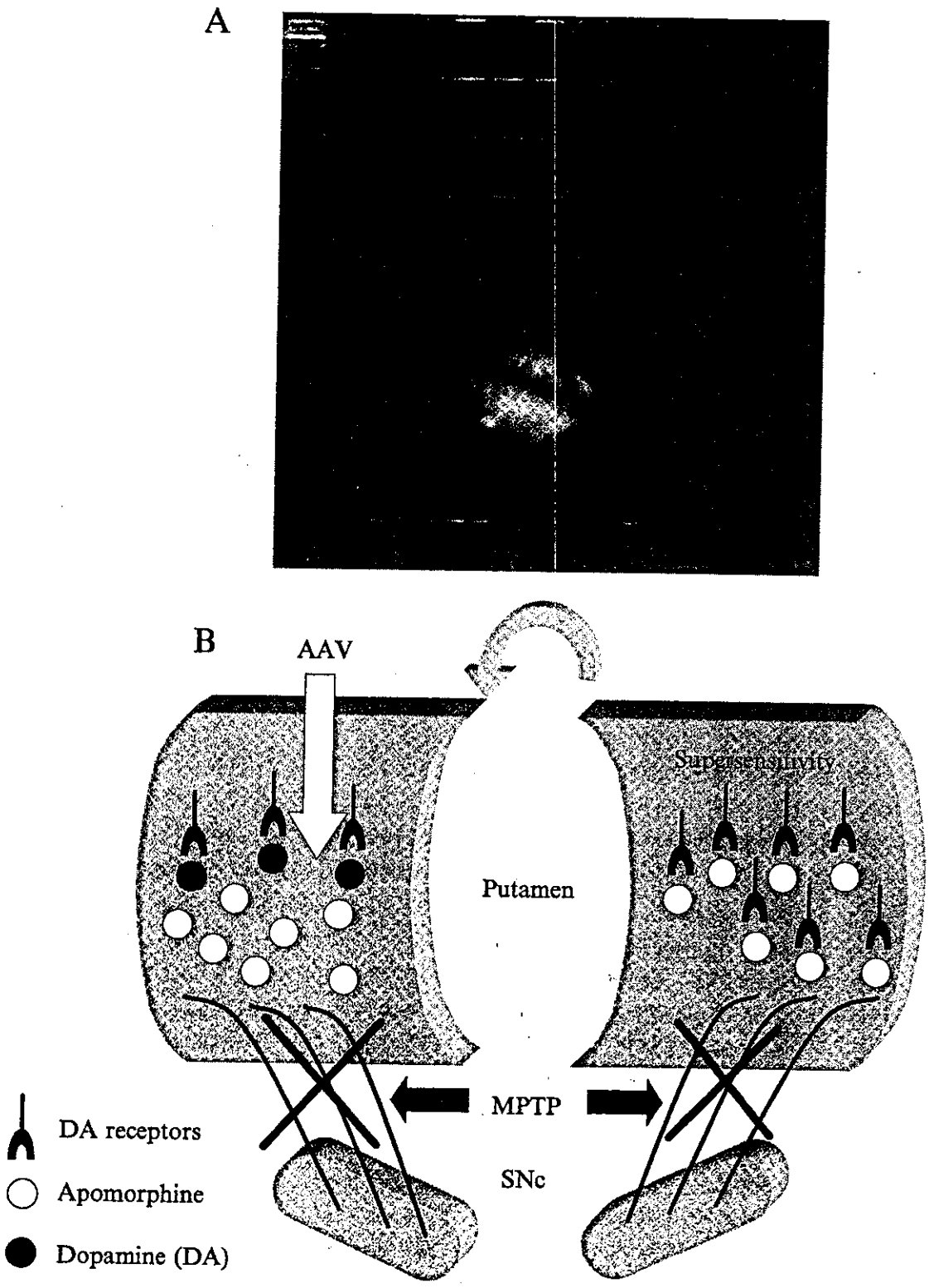

FIG. 4. Apomorphine-induced dystonic posture in an MPTP-treated monkey after gene delivery of DA-synthesizing enzymes into the unilateral putamen. (A) Administration of apomorphine, a dopamine receptor agonist, induced a dystonic posture, in which the body axis and face turned toward the left side after rAAV-TH/-AADC/-GCH injection into the left putamen. (B) Schematic representation of mechanisms of turning behavior. MPTP, a selective neurotoxin, induced

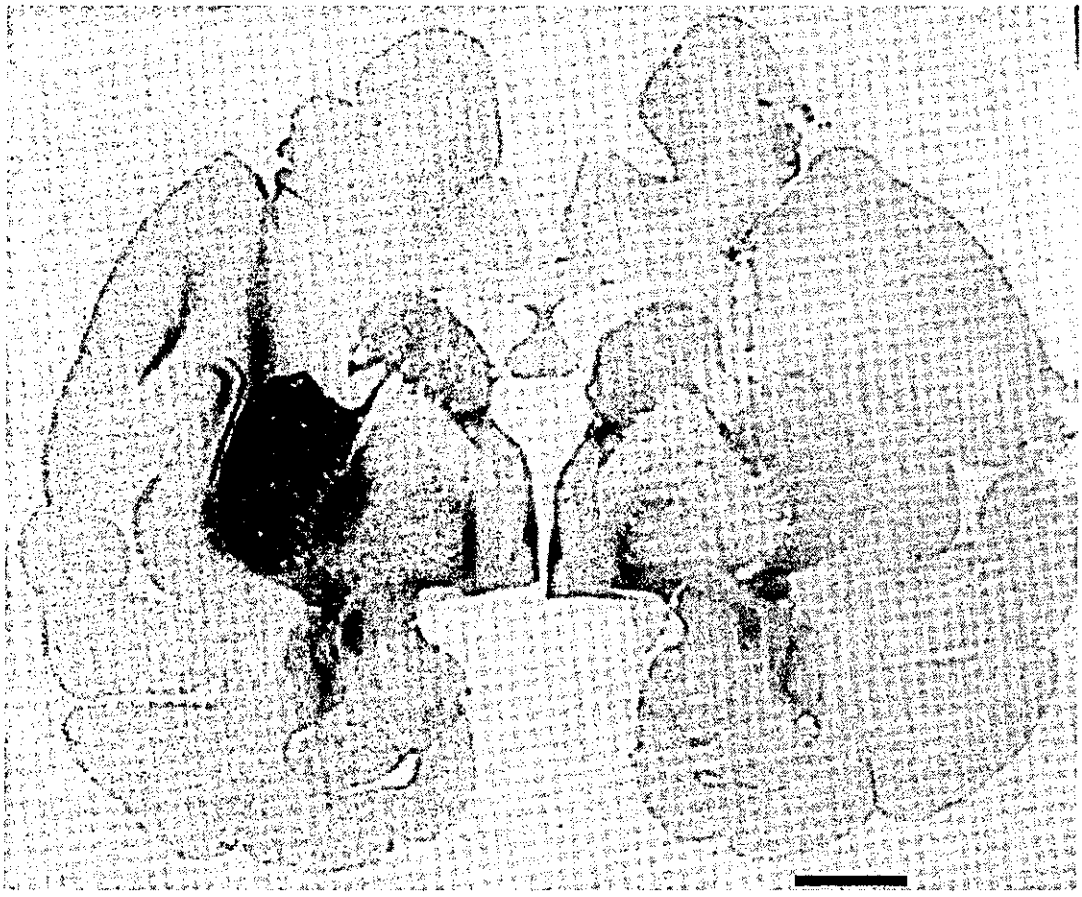

FIG. 5. Dense TH immunoreactivity in the unilateral putamen of the monkey, 65 days after rAAV-TH/-AADC/-GCH injection. Immunoreactivity in striatum on the control side was comprehensively lost.

injection. Dual-immunostaining for FLAG and TH demonstrated double positive cells in the SN. In contrast, no β-galactosidase signals could be detected in the SN of rAAV-LacZ-injected rats, although X-Gal-positive cells were detected in the striatum. Thus GDNF*flag* protein, rather than the rAAV vector per se, underwent retrograde transport to the SN, although the rAAV vector might demonstrate retrograde transport to some extent (Kaspar *et al.*, 2002). Striatal GDNF appears important for functional reinnervation. However, expression of GDNF in nigral DA neurons themselves may be detrimental, because intense local sprouting may prevent regeneration of lesioned axons toward the striatum (Grondin and Gash, 1998; Mandel *et al.*, 1999; Walton, 1999). Our results

supersensitivity of DA receptors in the striatum. Upregulation of DA receptors is shown here as simple elevation in receptor numbers, but may involve changes in downstream transduction pathways. This change was ameliorated by production of DA in the rAAV vector-treated side. Systemic administration of apomorphine caused an imbalance between the two striata, resulting in turning of the body axis toward the vector-treated side.

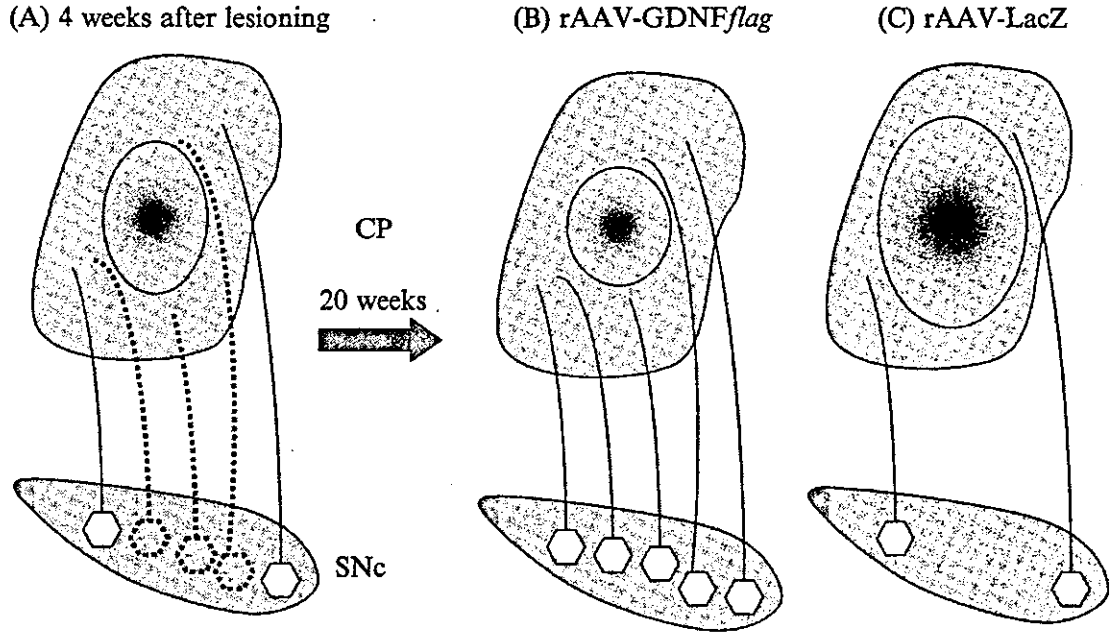

FIG. 6. Schematic drawing of the nigrostriatal pathway illustrating effects of rAAV vector-mediated delivery of GDNF gene in the striatum. At the time point of vector injection, i.e., 4 weeks after injection of 6-OHDA into the unilateral striatum, TH-IR neurons in the SN on the lesioned side had decreased to approximately 35% of the unlesioned side (A). Twenty weeks after rAAV vectors injection, TH-IR neurons in the SN and TH-IR nigrostriatal fibers in the striatum were restored to almost half the levels of the control side in rAAV-GDNF*flag* vector-injected rats (B), while further loss of TH-IR neurons and fibers was observed in rAAV-LacZ vector-injected rats (C). Some portions of the nigrostriatal connections remaining at 4 weeks postlesion might provide the substrate for sprouting and regrowth of TH-IR fibers in response to GDNF. CP, caudate-putamen; SNc, substantia nigra pars compacta.

indicate that delayed delivery of the GDNF gene using rAAV vectors halts the ongoing degeneration of nigrostriatal pathways, producing functional recovery even after substantial numbers of DA cells have been depleted.

V. Conclusions

Until recently, clinical trials of gene therapy for neurological disorders have been largely confined to rare metabolic diseases and brain tumors at advanced stages. However, advances in gene transfer methods, and particularly in the development of improved viral vectors, have expanded the potentials of gene therapy to include the treatment of a wide range of genetic and acquired diseases. At present, the rAAV vector represents one of the most powerful vehicles for delivering therapeutic genes into mammalian brains. In rodents and nonhuman primates, a substantial number of striatal neurons can be transduced with high

titer rAAV vectors through simple stereotaxic injection. Although chromosomal rearrangements in association with integration of rAAV have been indicated in a transformed cell line (Miller *et al.*, 2002), the safety and potential efficacy of rAAV vectors have been demonstrated both in animal models of several diseases and in clinical trials on patients with hemophilia (High, 2001).

Recent advances in the field of neural or embryonic stem cell research has raised the hope of cell replacement as an alternative therapy for PD (Bjorklund and Lindvall, 2000). In most protocols, dopaminergic cells are transplanted ectopically into the striatum instead of into the SN. If the primary mechanism underlying any expected functional recovery in these cell replacement therapies is restoration of dopaminergic neurotransmission, then direct gene delivery of DA-synthesizing enzymes into the striatum offers a much simpler and more straightforward approach. The significant functional recovery achieved with the triple transduction method in our primate model provides an optimistic outlook for gene therapy treatment of PD. If reconstruction of neural networks including nigrostriatal pathways is required to ameliorate more complex symptoms of PD such as dementia, the combination of gene therapy and cell transplantation appears to be the next strategy to overcome this considerable obstacle.

Although whether neurotoxin-induced PD models faithfully reflect the human disease requires further verification, neuroprotective gene therapy with neurotrophic factors holds further potential as a novel treatment of PD. Recently, neuroimaging techniques and genetic analyses for some familial cases have provided opportunities for detecting at-risk individuals before characteristic symptoms appear (Brooks, 2000; Hardy *et al.*, 2003). Earlier gene therapy with GDNF could obviously be applied to such at-risk individuals as easily as to PD patients. The elucidation of mechanisms whereby genetic mutations lead to the loss of DA neurons in familial forms of PD, in addition to the detection of factors increasing risk for PD, will supply fresh targets for gene therapy.

Developing vector constructs that allow regulation of gene expression should be necessary to avoid excess production of DA or GDNF. Studies on rAAV vectors with regulatable promoters are underway in several laboratories. For the DA replacement strategy, gene transfer of AADC alone in combination with oral administration of L-DOPA offers a shortcut toward reaching clinical trials (Bankiewicz *et al.*, 2000; Sanchez-Pernaute *et al.*, 2001). In this method, patients need to take L-DOPA for amelioration of symptoms, but DA production can be controlled by a dose of L-DOPA.

More recently, based on the observation that deep brain stimulation of the subthalamic nucleus (STN) provides antiparkinsonian effects, another palliative approach has been proposed that does not address DA replacement. This method involves rAAV vector-mediated gene transfer of glutamate decarboxylase, an enzyme for synthesis of inhibitory neurotransmitter GABA, to the STN