# Subthalamic GAD Gene Therapy in a Parkinson's Disease Rat Model

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**The motor abnormalities of Parkinson's disease (PD) are caused by alterations in basal ganglia network activity, including disinhibition of the subthalamic nucleus (STN), and excessive activity of the major output nuclei. Using adeno-associated viral vector–mediated somatic cell gene transfer, we expressed glutamic acid decarboxylase (GAD), the enzyme that catalyzes synthesis of the neurotransmitter GABA, in excitatory glutamatergic neurons of the STN in rats. The transduced neurons, when driven by electrical stimulation, produced mixed inhibitory responses associated with GABA release. This phenotypic shift resulted in strong neuroprotection of nigral dopamine neurons and rescue of the parkinsonian behavioral phenotype. This strategy suggests that there is plasticity between excitatory and inhibitory neurotransmission in the mammalian brain that could be exploited for therapeutic benefit.**

Degeneration of specific groups of cells characterizes many neurological disorders. In PD, neurons of the substantia nigra pars compacta (SNc) are particularly vulnerable, leading to marked depletion of dopamine in the primary projection region, the striatum. As a result, the major inhibitory-output nuclei of the basal ganglia, the substantia nigra pars reticulata (SNr) and internal segment of the globus pallidus (GPi), are driven by a disinhibited and thereby overactive subthalamic nucleus (*1*–*4*) whose projection axons end in asymmetric, excitatory synapses on target neurons in the SNr (*5*). Marked improvement of the motor symptoms of PD occurs following either STN ablation (*6*, *7*), electrical inhibition with high-frequency stimulation (*8*, *9*), or pharmacological silencing by local lidocaine or muscimol infusion (*10*). Here, we describe a genetic approach to test the hypothesis that the glutamatergic neurons of the STN can be induced to express GAD, and thereby change from an excitatory nucleus to a predominantly inhibitory system that releases GABA at its terminal region in the substantia nigra (SN), leading to suppression of firing activity of these SN neurons. Moreover, we show that such an intervention also results in neuroprotection with resistance to 6-hydroxydopamine (6-OHDA)–induced degeneration of dopaminergic neurons.

We used recombinant adeno-associated virus (rAAV) to transduce neurons in the STN (*11*). This vector not only provides for highly efficient and stable gene transfer (*12*, *13*), but also results in minimal inflammatory and immunological responses (*14*). GABA, the brain's major inhibitory transmitter, can be generated by two isoforms of GAD, GAD65 and GAD67 (supporting text online). We generated rAAV vectors (*11*) containing both GAD65 and GAD67 cDNAs using the cytomegalovirus enhancer/chicken β-actin (CBA) promoter (*15*) and a woodchuck hepatitis virus postregulatory element (WPRE) (*16*) to further enhance expression (fig. S1A).

Mouse neural progenitor C17.2 cells were infected with both the GAD65 and GAD67 vectors with functional expression of the transgene confirmed by immunocytochemistry with antibodies specific to each GAD isoform and GABA (*11*) (fig. S1, B to J). GABA release was quantified by high-performance liquid chromatography (*11*, *17*) (fig. S1K).

Adult male rats were stereotactically injected into the left STN with GAD65, GAD67, or control GFP (green fluorescent protein) vectors. Four to 5 months after surgery, expression of the transgenes was determined by immunofluorescence (*11*). Robust expression confined to the STN was obtained for all transgenes (Fig. 1, A to I) with a nuclear halo in the GAD65-transduced neurons consistent with membrane-bound enzyme (Fig. 1F), whereas both GAD67 (Fig. 1I) and GFP (Fig. 1C) filled the cell soma completely. The STN neurons send a major projection to the SNr, with efferents also to

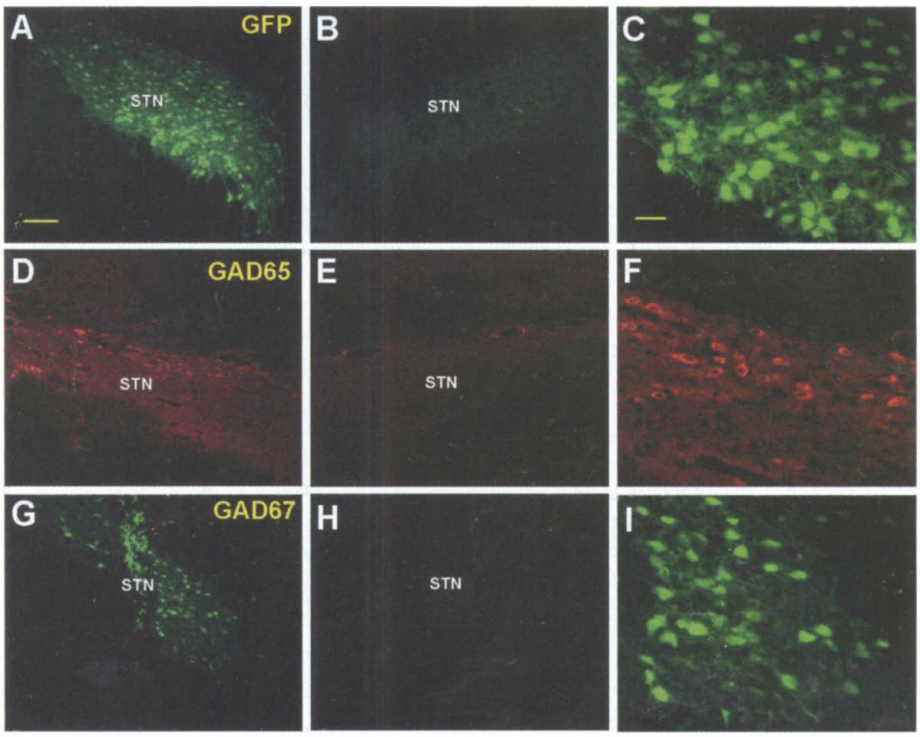


**Fig. 1.** rAAV-mediated transgene expression in the STN. AAV vectors were injected into the left STN. (**A**, **D**, **G**) Low-power ipsilateral (left) STN (bar, 100 μm). (**B**, **E**, **H**) Contralateral (**C**, **F**, **I**) High-power ipsilateral (bar, 30 μm). (A to C) GFP fluorescence. (D to F) GAD65 immunoreactivity. (G to I) GAD67 immunoreactivity.

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the SNc, as shown by the fiber staining in the rats expressing GFP, which acts as an anterograde tracer (Fig. 2, H to J). Sections were also analyzed with antibodies to CD4, CD8, major histocompatibility complex class I, and ED-1, a macrophage and microglial marker (fig. S2). In addition, sera were tested for the presence of antibodies to the AAV capsid or transgene (*18*). There was no significant immunoreactivity, humoral responses, or cellular infiltration in any animal, consistent with our previous report on lack of immunogenicity following rAAV injection into the mammalian brain (*14*).

To test the hypothesis that the expression of GAD in the STN induced by rAAV-mediated gene transfer induces a phenotypic shift, we assessed the responses of unlesioned control and 6-OHDA–lesioned parkinsonian rats that had received GAD65, GAD67, GFP, or saline 4 to 5 months earlier, using a combination of microdialysis and electrophysiology (*11*). Microdialysis probes were introduced into the SNr (Fig. 2, H and J), and a stimulating electrode implanted into the ipsilateral STN (Fig. 2, H and I). Samples were analyzed for glutamate and GABA (*11*). Representative data from individual unlesioned control rats (Fig. 2A), or from parkinsonian rats treated with either saline (Fig. 2B), GFP (Fig. 2C), GAD65 (Fig. 2D), or GAD67 (Fig. 2E), are shown, as well as the pooled data with means ± SEM of GABA (Fig. 2F) and glutamate concentrations (Fig. 2G). In the unlesioned control rats, as well as in saline- and GFP-treated parkinsonian rats, there was no significant increase in either GABA or glutamate release with STN stimulation. In contrast, following GAD65 gene transfer there was a (4.0 ± 1.5)-fold increase in GABA release associated with the STN stimulation ($P < 0.05$) (Fig. 2F).

A subgroup of rats had recording electrodes placed in the SNr, in addition to the STN stimulating electrode (*11*) (supporting text online). Single-unit recording from SNr cells during STN stimulation led to excitatory responses in 74% of SNr cells recorded in naïve unlesioned rats (Fig. 3A). This is consistent with the lack of GAD expression in the STN and the presence of glutamate immunoreactivity and asymmetrical synapses in the axon terminals (*5*, *19*). In parkinsonian rats, the responses of the SNr cells to STN stimulation were similar to that of unlesioned rats, with 83% showing excitatory responses ($P = 0.065$, Chi-square analysis); however, there was a more robust bursting response to each stimulation of the STN compared with the single-spike responses in naïve rats (Fig. 3B). GFP rats exhibited SNr responses that were indistinguishable from those of the saline parkinsonian rats, with 83% and 6% showing excitatory and inhibitory responses, respectively. The ratio of excitatory to inhibitory responses was markedly altered following GAD65 gene transfer (Fig. 3A). The excitatory single-unit recordings were reduced to 17% of responses in the GAD65 rats ($P < 0.0001$). In contrast, inhibitory responses observed in only 5, 10, and 6% of cells recorded in unlesioned, saline, and GFP parkinsonian rats, respectively, were increased to 78% in the GAD65 rats ($P <$ 0.001). In addition to the marked increase of inhibitory responses, the GAD65 rats also showed prolonged inhibition following STN stimulation, lasting 100 to 200 ms. Recordings from the GAD67-treated rats revealed a predominant excitatory response (62%) (Fig. 3). Inhibitory responses in GAD67 rats were in-

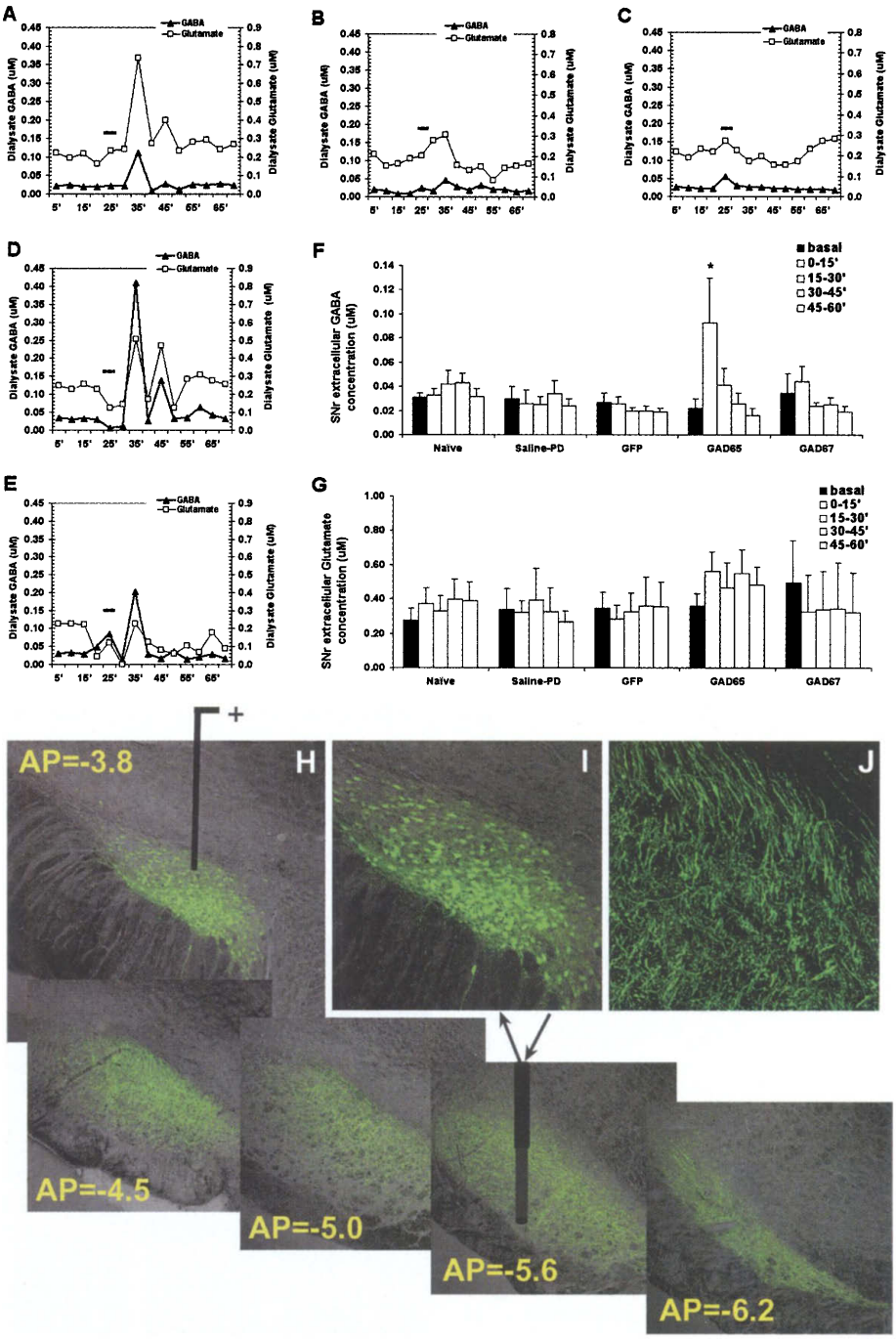


**Fig. 2.** Representation of electrode and dialysis probe implantation and SN microdialysis. (A to D) Sequential 5-min dialysate GABA and glutamate concentrations of a representative single animal in (**A**) unlesioned control rats, (**B**) saline, (**C**) GFP, (**D**) GAD65, and (**E**) GAD67 parkinsonian rats. The horizontal bar indicates the 5-min stimulation period. (**F** and **G**) Mean ± SEM of 15-min pooled data ($n = 4$ for each of the five groups) for GABA and glutamate, respectively. (**H**) Sequential sections through the midbrain of a GFP rat demonstrating the transduced STN (high power, **I**) and placement of stimulating electrode, and microdialysis probe lying in the SNr with high power (**J**) showing the intensity of fiber staining in the SN. Asterisk, $P < 0.05$, repeated measures ANOVA.

A

| Group | Excitatory | Inhibitory | No Response |
|---|---|---|---|
| Control (n=5) | 14 (74%) | 1 ( 5%) | 4 (21%) |
| Saline (n=5) | 25 (83%) | 3 (10%) | 2 (7%) |
| GFP (n=3) | 15 (83%) | 1 ( 6%) | 2 (11%) |
| GAD67 (n=4) | 13 (62%) | 7 (33%) | 1 (5%) |
| GAD65 (n=4) | 3 (17%) | 14 (78%) | 1 (6%) |

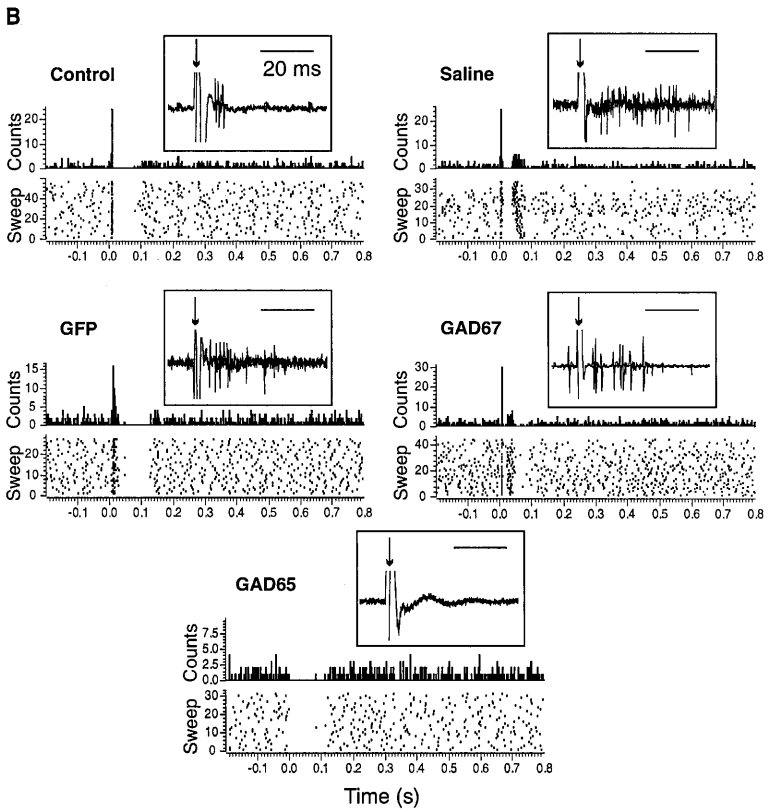


**Fig. 3.** (**A**) SNr electrophysiology in unlesioned control rats and saline-, GFP-, GAD65-, and GAD67-treated parkinsonian rats. Summary of the response of SNr neurons to electrical stimulation of the STN. The number of neurons that were excited, inhibited, or did not respond to STN stimulation are listed. The overall percentage of neurons in each category is in parentheses. (**B**) Histograms (2-ms bin width) and raster plots of SNr impulse activity in unlesioned control rats and saline-, GFP-, GAD65-, and GAD67-treated parkinsonian rats. In each case, the data shown are summarized from at least 30 presentations of the stimulus. All mean excitatory spike latencies were <7 ms from the onset of the stimulus, which is consistent with a monosynaptic connection of the STN to the SNr. (**Insets**) Electrophysiology traces from SNr neurons during STN stimulation are shown for each experimental group. Three overlay sweeps are shown. The arrowmark represents the stimulus artifact. The stimulus artifact is not included in the rasters and histograms because of the waveform matching program used selected only spikes for analysis (Spike 2, version 4; CED Inc., Cambridge, UK).

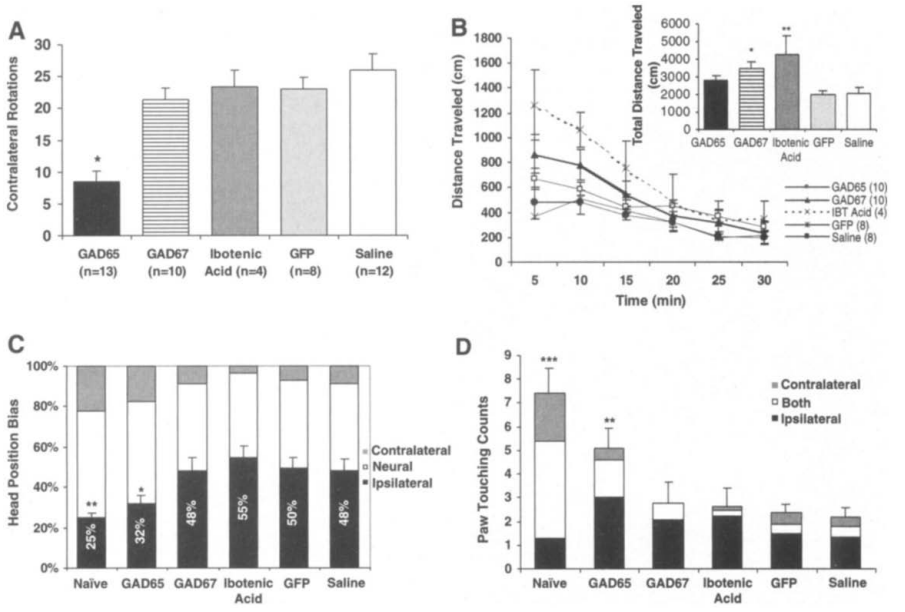


**Fig. 4.** GAD65 transduction of the STN inhibits 6-OHDA–induced parkinsonian asymmetry. (**A**) Apomorphine-induced contralateral rotations. (**B**) Locomotor activity. (**C**) Head position bias. (**D**) Paw touching behavior. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ANOVA with post hoc Fisher's PLSD test.

creased to 33% of all recordings ($P < 0.02$), consistent with an intermediate phenotype between the saline and GAD65 rats.

To further characterize the effects of the shift toward a mixed phenotype of the STN projection to the SNr, we examined whether the increased inhibitory tone might influence the ability of midbrain dopaminergic neurons to withstand a neurotoxic insult. Young adult male rats received intraSTN GAD65 ($n$ = 13), GAD67 ($n$ = 10), GFP ($n$ = 8), saline ($n$ = 12), or ibotenic acid ($n$ = 4) (to further control for nonspecific lesion effects associated with the surgery and gene transfer). Three weeks after surgery, the ipsilateral (left) medial forebrain bundle (MFB) was lesioned with 6-OHDA (*11*). Outcome measures of the lesion severity and potential neuroprotection included analysis of behaviors dependent on an intact and symmetrical midbrain dopaminergic pathway. Both spontaneous and drug-induced behaviors were assessed at 8 to 16 weeks after 6-OHDA lesioning (*11*).

MFB lesions in naïve, GFP-, or saline-treated control animals leads to impaired general locomotor activity, specific deficits in contralateral limb use, and apomorphine-induced rotations contralateral to the denervated side. These rotations provide a highly reproducible and quantitative surrogate marker of the dopaminergic deficit (*20*). In the GAD65 rats, rotation rates were decreased by ~65% compared with both the saline and GFP control rats ($P < 0.05$) (Fig. 4A). Rotation rates in the ibotenic acid and GAD67 rats were unchanged from those of controls. Total locomotor activity was increased in ibotenic acid–lesioned ($P < 0.005$) and GAD67 ($P < 0.05$) rats, with a trend toward an increase in the GAD65 rats ($P$ = 0.16) compared with controls (Fig. 4B). The head position bias test (*21*) showed marked asymmetry in the controls with an ipsilateral bias, which was almost completely normalized in the GAD65 rats ($P < 0.05$) but not altered significantly in the other groups (Fig. 4C). Similarly, forelimb use assessed by quantitative paw touch counting was improved only in the GAD65 rats ($P < 0.01$) (Fig. 4D). Examination of the midbrain in these rats with unbiased stereology for cell counting (*22*) revealed pro-

found nigral dopaminergic cell loss in the saline- and GFP-treated rats, with >99% loss of tyrosine hydroxylase (TH) immunoreactivity of the SNc for both groups and 93 ± 4% and 94 ± 4% loss in the ventral tegmental area (VTA) of saline- and GFP-treated rats, respectively (Fig. 5, A and B), compared with the contralateral side (Fig. 5C). Fluorogold (FG) was injected into the striatum 2 weeks before analysis, but after the 6-OHDA lesion (Fig. 5, middle panels) to provide definitive proof of neuronal degeneration in this model and not simply loss of dopaminergic phenotype as defined by TH immunoreactivity. Moreover, when administered after lesioning, intrastriatal FG injection with subsequent labeling of cells in the SN provides confirmation of intact surviving neurons retaining projections to the striatum. In the GAD65 rats, 35 ± 14% [$P < 0.01$, analysis of variance (ANOVA) with Fisher's post hoc test versus GFP-treated rats] and 80 ± 11% ($P < 0.0001$) of dopaminergic neurons survived in SNc and VTA, respectively (Fig. 5, D and E). For the GAD67 rats, there was no significant protection of SNc neurons, with less than 1% survival, but there was a 42 ± 3% ($P < 0.02$) survival of VTA neurons (Fig. 5F). The ibotenic acid–treated rats had no protection of the SNc, but showed a trend toward protection of the VTA, with 19 ± 9% surviving neurons compared with the 6 ± 4% and 7 ± 4% surviving VTA neurons in the GFP- and saline-treated groups, respectively. Although at least one study has suggested dopaminergic neuroprotective efficacy of STN lesions (*23*), these investigators used intraSTN kainic acid and a less severe, partial intrastriatal 6-OHDA lesion model. In contrast, others have shown either no neuroprotection conferred by a quinolinic acid STN lesion (*24*), or a weak 23% neuroprotective effect of intraSTN ibotenic acid in the partial intrastriatal 6-OHDA lesion model (*25*). Thus, the marginal efficacy of a STN lesion (confined to the A10 cells in the VTA and not reaching statistical significance) in a much more severe MFB 6-OHDA lesion model suggests that the robust nigral neuroprotection we observed with GAD65 is not just due to a drop in the excitatory drive from the STN, but that the inhibitory neurotransmission in this pathway induced by GAD65 gene transfer is critical.

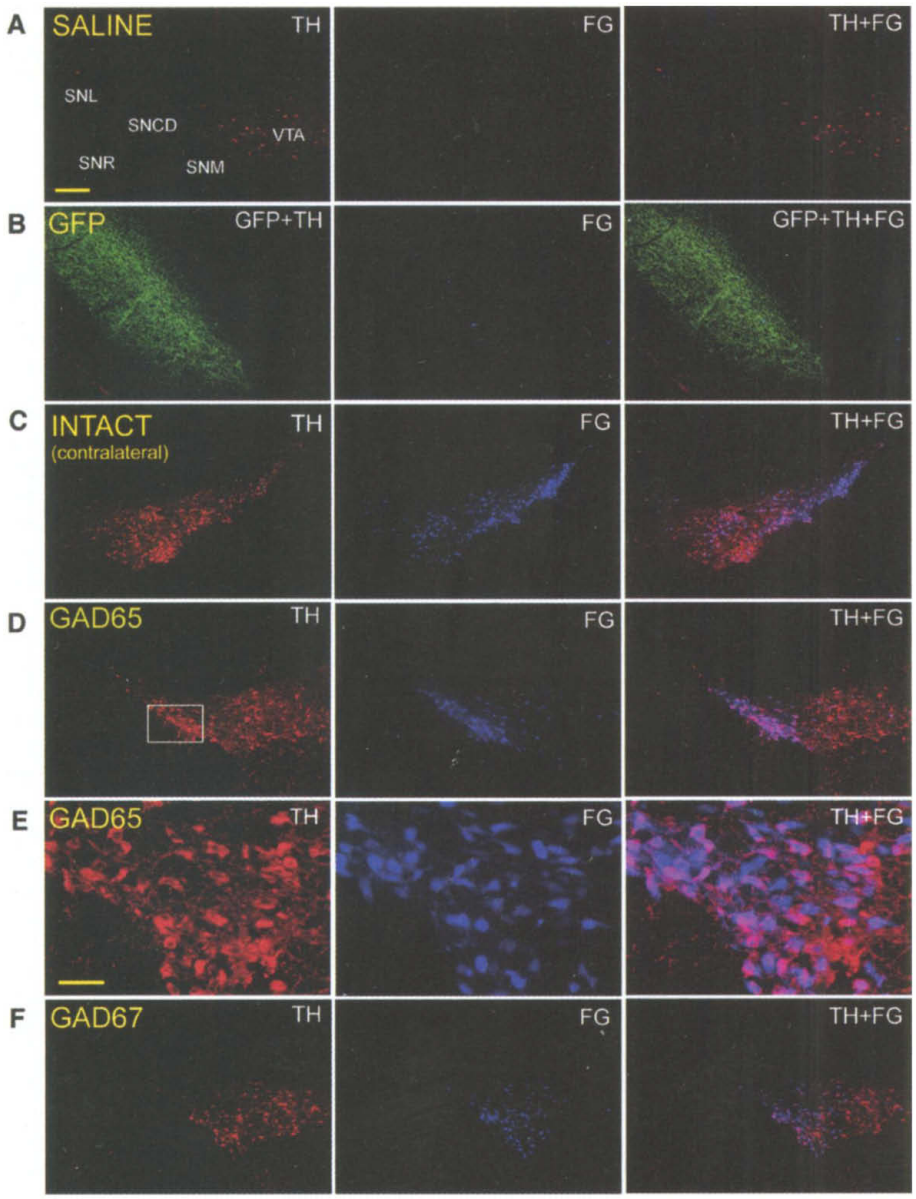


**Fig. 5.** GAD65 mediates increased survival of SN and VTA tyrosine hydroxylase (TH)–positive neurons. TH immunofluorescence and fluorogold (FG) double-labeled images show survival of TH neurons. TH alone (left), FG (middle), and combined TH and FG (right). (**A**) Lesioned hemisphere of a representative saline-injected parkinsonian rat. (**B**) GFP. (**C**) Representative intact contralateral hemisphere. (**D**) Low-power and (**E**) high-power GAD65. (**F**) GAD67. SN, substantia nigra; SNM, medial SN; SNCD, dorsal pars compacta SN; SNL, lateral SN; SNR, pars reticulata SN. Bar, 200 μm [(A) to (D) and (F)], 50 μm (E).

Although in our study we used gene transfer to elicit the phenotypic shift, it has been previously shown that certain groups of neurons generally considered excitatory and glutamatergic can also express GAD transcripts. Specifically, hippocampal dentate granule and CA1 cells express very low levels of GAD65 and GAD67 mRNA (*26*). Moreover, these cells can also express low levels of the protein, but increase expression with electrical stimulation (*27*) or following seizures (*28*, *29*). Hence, there is plasticity with the potential for heterotransmission of an inhibitory transmitter in well-characterized excitatory pathways in pathophysiological states.

The success of STN deep-brain stimulation and subthalamotomy for patients with advanced PD, together with symptomatic relief mimicked by infusion of the $GABA_A$ agonist muscimol into the STN (*10*) or directly into the SNr in Parkinsonian monkeys (*30*), suggests that a gene-transfer strategy that enhances GABA transmission in the STN and its terminal regions may be similarly effective. The limited efficacy of intraSTN ibotenic acid to protect against a subsequent MFB 6-OHDA lesion suggests that GAD65 gene transfer may be more effective than simple ablation or local electrical silencing. At present, there are no treatments for PD shown to definitively attenuate disease progression. Although our data suggest some promise, both rodent and nonhuman primate studies are insufficient to predict neuroprotection in the clinic. Such an answer will require large blinded clinical trials and will be the ultimate goal of such a gene-transfer approach.

Our data, including behavior, immunohistochemistry, in vivo neurochemistry, and single unit–recording electrophysiology, are strongly supportive of the concept of hetero-

transmission and inherent plasticity of the mammalian nervous system, with transfer of a single gene in a specific population of cells leading to a marked phenotypic change from largely excitatory to predominantly inhibitory transmission. Whether this shift induces additional phenotypic changes including ultrastructure typical of inhibitory neurons will be a focus of future studies. GAD gene transfer into glutamatergic excitatory neurons leading to an inhibitory bias with altered network activity and a neuroprotective phenotype holds potential for treatment of PD and other neurological conditions associated with excessive excitation.

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**Supporting Online Material**

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Materials and Methods
SOM Text
Figs. S1 and S2
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