Neuromol Med
DOI 10.1007/s12017-007-8003-0

ORIGINAL PAPER

Reversible Unilateral Nigrostriatal Pathway Inhibition Induced Through Expression of Adenovirus-mediated Clostridial Light Chain Gene in the Substantia Nigra

Jun Yang · Qingshan Teng · Mary E. Garrity-Moses · Shearwood McClelland III · Thais Federici · Erin Carlton · Jonathon Riley · Nicholas M. Boulis

Received: 10 August 2006 / Accepted: 16 April 2007

Abstract Clostridial light chain (LC) inhibits synaptic transmission by digesting a vesicle-docking protein, synaptobrevin, without killing neurons. We here report the feasibility of creating a rat hemiparkinsonism model through LC gene expression in the substantia nigra (SN), inhibiting nigrostriatal transmission. 40 adult Sprague Dawley rats were divided into four groups for SN injections of PBS, 6-hydroxydopamine (6-OHDA), or adenoviral vectors for the expression of LC (AdLC), or GFP (AdGFP). Amphetamine and apomorphine induced rotations were assessed before and after SN injection, revealing significant rotational alterations at 8 or 10 days after injection in both AdLC and 6-OHDA but not PBS and AdGFP groups. Induced rotation recovered by one month in AdLC rats but persisted in 6-OHDA rats. Histological analysis of the SN revealed LC and GFP expression with corresponding synaptobrevin depletion in the LC, but not the GFP groups. Tyrosine hydroxylase (TH) and dopamine transporter (DAT) immunohistochemistry (IHC) showed markedly decreased staining in ipsilateral SN and striatum in 6-OHDA but not AdLC or AdGFP rats. Similarly, compared with contralateral, ipsilateral striatal dopamine level only decreased in 6-OHDA but not AdLC, AdGFP, or PBS treated rats. Thus, LC expression induces nigral synaptobrevin depletion with resulting inhibition of nigrostriatal synaptic transmission. Unlike 6-OHDA, LC expression inhibits synaptic activity without killing neurons. This approach, therefore, represents a potentially reversible means of nigrostriatal pathway inhibition as a model for Parkinson's disease. Such a model might facilitate transient and controlled nigral inhibition for studying striatal recovery, dopaminergic re-innervation, and normalization of striatal receptors following the recovery of nigrostriatal transmission.

Keywords Clostridial toxin · Hemiparkinsonism · Synaptic transmission · Targeted gene expression

J. Yang · Q. Teng · M. E. Garrity-Moses · S. McClelland III · T. Federici · E. Carlton · J. Riley · N. M. Boulis
Departments of Neuroscience and Neurosurgery, Cleveland Clinic, Lerner Research Institute, 9500 Euclid Avenue, Cleveland, OH 44195, USA

N. M. Boulis (✉)
Department of Neuroscience and Center for Neurological Restoration, Cleveland Clinic, Lerner Research Institute, NB 2-126, 9500 Euclid Avenue, Cleveland, OH 44195, USA
e-mail: Boulisn@ccf.org

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is primarily characterized by the degeneration of dopaminergic neurons in the nigrostriatal pathway. However, what triggers the degeneration of these neurons and the pathogenesis of PD are not completely understood. Therefore, the current treatments for PD are aimed primarily at dopamine replacement for symptomatic relief. Animal models form the basis for much of our understanding of the pathophysiology of PD and facilitate the development of novel therapies. Several animal models are available for studying the pathogenesis of PD (Beal 2001; Shimohama et al. 2003; Sindhu et al. 2005). In the rat, unilateral depletion of striatal dopamine (DA) by intranigral injection of 6-OHDA has been used extensively, as a model of PD. However, the rapid destruction of dopaminergic neurons following 6-OHDA injection prevents the study of recovery in nigrostriatal dopaminergic innervation, as might occur following growth factor or cell

replacement strategies. Some moderate PD models have been proposed for early therapeutic intervention studies, but remain suboptimal and with varied reliability in different species (Ghorayeb et al. 2001; Shimohama et al. 2003; Bove et al. 2005). Nigral synaptic inhibition, rather than dopamine cell death, could provide a reversible model of dopamine depletion, allowing the study of recovery and analysis of the intricate process of striatal re-innervation. In addition, such a model could be applied in both primates and rodents and provide a means for the study of downstream changes that occur in the basal ganglia following dopamine depletion and regeneration.

Striatal dopamine depletion results in a variety of changes in the striatum including well-documented dopamine receptor supersensitivity, increased c-fos expression (Meloni and Davis 2000), alterations in cholinergic function (Muma et al. 2001), and alterations in glutamate synapses (Meshul et al. 1999). The successful pharmacological therapies for Parkinson's disease have focused on replacing lost dopaminergic control of basal ganglia circuits. However, the progressive loss of functional nigrostriatal synapses limits the durability of these therapies and our understanding of how striatal neurons respond to the replacement of dopaminergic input. Fetal mesencephalic grafts survive in the Parkinsonian striatum replacing lost dopaminergic synapses (Freed et al. 2001). However, contrary to expectations, these grafts have provided limited functional improvement in human clinical trials (Olanow et al. 2003). This apparent failure may derive from the inability of current grafts to adequately reconstruct the nigrostriatal control system. Therefore, the information that can be derived from these experiments is limited. In contrast, transient and controlled nigral inhibition creates an alternative model for studying striatal recovery in the context of dopaminergic re-innervation. A reversible model of PD, based on nigrostriatal synaptic inhibition, would permit the study of how the striatum recovering from dopamine depletion as well as the critical windows that may govern effective recovery.

We have previously reported focal synaptic inhibition through adenovirus-mediated gene transfer of LC in the central nervous system (Teng et al. 2005; Zhao et al. 2006). LC inhibits synaptic transmission by digesting synaptobrevin (VAMP), a vesicle-docking protein responsible for neurotransmitter release, without killing neurons (Sudhof 1995; Lin and Scheller 2000). An adenoviral vector driving expression of LC (AdLC) induces time- and concentration-dependent digestion of mouse brain synaptobrevin. Lumbar spinal cord AdLC injection induces a transient paralysis secondary to neuromuscular synaptic inhibition. LC expression within one of the critical midbrain nuclei of the acoustic startle reflex selectively inhibits the reflex by depleting synaptobrevin. Despite the existence of a variety of critical brainstem structures in close proximity, no alterations in either general health, arousal level, or general motor function are observed. Additional experiments have demonstrated the ability of LC expression to inhibit vesicle mediated growth hormone release in a model of acromegaly, and suppress epileptic activity following cortical penicillin injection (Federici et al. 2006; Yang et al. 2007). Both in vitro and in vivo experiments demonstrated neuronal survival after LC gene expression as well as a reversible synaptic inhibition (Teng et al. 2005; Zhao et al. 2006). Therefore, vector mediated LC gene delivery to the nigrostriatal pathway may represent a novel tool to create a reversible rat hemiparkinsonism model.

The present study tests the hypothesis that vector mediated nigral LC expression could create a reversible hemiparkinsonism model through inhibiting nigrostriatal transmission. We have demonstrated that nigral LC expression resulted in significant alterations in rotation induced by both amphetamine and apomorphine. In contrast with 6-OHDA, induced rotational behaviors resolved by one month after injection in AdLC but not 6-OHDA rats. LC expression in SN failed to reduce TH and DAT staining in both SN and striatum, suggesting that LC expression did not kill neurons in nigrostriatal dopaminergic system.

Materials and Methods

Vector Design

A 1496 bp synthetic gene encoding the LC fragment of tetanus toxin protein was cloned into an adenovirus vector pACCMVpLpA(-)loxP.SSP. For easier identification of gene expression, the GFP sequence was cloned into this vector downstream of the LC sequence under the control of IRES (internal ribosomal entry sequence). The construction and production of AdLC is described elsewhere in detail (Teng et al. 2005). AdGFP, used as a control vector, contains the GFP gene under the control of CMV promoter. Adenoviral vectors were produced through Cre mediated in vitro recombination (Hardy et al. 1997) and purified through a standard cesium chloride centrifugation procedure.

Animal Surgery

Animal experiments were carried out in accordance with the Animal Research Committee of the Cleveland Clinic Foundation rules for the care and use of laboratory animals and rules of biosafety. A total of 40 adult male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 321–355 g were used in this study. They were housed in a temperature-controlled environment maintained under a 12 h light-dark cycle with food and water available ad lib. All efforts were

made to minimize the number of animals used and minimize suffering.

Injection of 6-OHDA into unilateral SNpc and medial forebrain bundle (MFB) is traditionally used to generate PD rat models (Deumens et al. 2002; Shimohama et al. 2003; St-Hilaire et al. 2005). We therefore used 6-OHDA rat model as a positive control in our experiment. Animals in 6-OHDA group were pre-treated with desipramine hydrochloride (20 mg/kg i.p.) 30 min before 6-OHDA, in order to protect noradrenergic neurons. 6-OHDA (2 μg/μl) was dissolved in saline (0.9% NaCl) containing ascorbic acid (0.2%). 40 rats were divided randomly into four groups for injections of AdLC, AdGFP (vector control), 6-OHDA, and PBS (negative control). Rats were anesthetized with isoflurane (3% in O_2) and placed in the Kopf stereotactic frame (David Kopf Instruments, Tujunga, CA). Four μl of AdLC (7.5×10^9 PFU/ml), AdGFP (7.5×10^9 PFU/ml), PBS and 6-OHDA (8 μg) were injected into right SNpc (AP: -5.3; ML: 2.2; and DV: –7.5) and MFB (AP: –4.4; ML: 1.2; and DV: –7.5) (Paxinos 1982; Yurek and Fletcher-Turner 2004; St-Hilaire et al. 2005). Injections were performed with an oocyte microinjector (Nanoject; Drummond, Broomall, PA) mounted with glass micropipettes. A glass micropipette puller (PP–83; Narishige, Tokyo, Japan) was used to create tapered tips on micropipettes, which were then beveled to 100 μm under microscopic visualization. Micropipettes were advanced using a micromanipulator (N-152; Narishige, Tokyo, Japan). Injections were performed gradually over a 15 min-period to allow diffusion of the viral solution and minimize surrounding tissue trauma. A dissecting microscope (Stereozoom 6; Leica, Buffalo, NY) was used for all surgical procedures.

Behavioral Assays

Rotational behaviors were assessed by inducing with amphetamine or apomorphine on alternate days before and after SN injection. Drug-induced rotations were measured using an automatic rotometer (Rotometer photobeam activity system, San Diego Instruments, San Diego, CA) which consists of rotation bowls and tethers attached to the torso of the rats. Rats were placed in an independent hemispheric bowl and left to adapt for 20 min. At that time, the rats were given either amphetamine (5 mg/kg/i.p., before, 8 and 34 days post-injection) or apomorphine (1 mg/kg/s.c., before, 10 and 36 days post-injection). Circling behavior was then monitored for 30 min. Rotational asymmetry scores were calculated as net ipsilateral rotations (turns contralateral to the lesion subtracted from those towards the lesion side). Tests performed at selected time points are based on our previous experiments that adenoviral gene expression in the spinal cord peaks in the first to second weeks, AdLC induced motor deficit recovers by one month after injection (Teng et al. 2005). Relatively high doses of drugs were chosen to avoid possibly dose-related inconsistent effects of amphetamine or apomorphine induced rotation, due to the partial striatal degeneration (Ghorayeb et al. 2001; Deumens et al. 2002; Sindhu et al. 2005).

Histological and Immunohistochemical Analysis

Six rats were sacrificed 12 days post injection for histological and dopamine analysis; four rats were kept to test long-time behavioral effects in each group. The rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and underwent trans-cardiac perfusion with 0.9% saline followed by 2% paraformaldehyde. After they were post-fixed and cryoprotected in 20% sucrose solution overnight, brains were blocked and frozen in optimal cutting temperature gel (Sakura Finetek Inc., Torrance, CA). Brains were later serially sectioned in the coronal plane at 20 μm using a cryostat (Leica Microsystems, Nussloch, Germany). The sections were then thaw-mounted onto pre-cleaned superfrost plus micro slides. GFP expression was used to monitor the distribution and intensity of gene expression. The striatum, mesencephalon tissues, and sections identified as having GFP expression were immunohistochemically stained for tyrosine hydroxylase (TH), dopamine transporter (DAT), D1 dopamine receptor (D1DR), and synaptobrevin/VAMP-1 (vesicle-associated membrane protein) with Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions. After rinsing with PBS, sections were incubated in 0.002% proteinase K solution for 10 min at 37°C, followed by incubation in 1% H_2O_2 in methanol for 10 min. Tissue sections were then blocked with blocking serum for 20 min. Primary antibodies were diluted in blocking solution, including rabbit anti-synaptobrevin/VAMP-1 (diluted in 1:200), rabbit anti-DAT (diluted in 1:100), mouse anti-D1DR (diluted in 1:50, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-TH (diluted in 1:200, Pel-Freez Biologicals, Rogers, AR). Negative controls were performed by applying blocking solution alone to different sections in the same slide.

Sections were washed with PBS before diluted biotinylated secondary goat anti-rabbit or mouse antibodies were applied for 30 min at room temperature. For TH, DAT, and D1DR staining, sections were incubated with avidin-HRP (ABC mix of VECTASTAIN Elite ABC Reagent) for 30 min at room temperature. Immunohistochemical staining was visualized with 3′3-diaminobenzidine (DAB) mixed solution (DAB Substrate Kit, Vector Laboratories, Burlingame, CA) for 5–10 min. Finally, slides were counterstained with Vector Methyl Green Solution (Vector Laboratories, Burlingame, CA) and coverslipped with Permount (Fisher Scientific, Pittsburgh,

PA). For synaptobrevin/VAMP-1 staining, sections were incubated with diluted Texas Red Avidin D (Vector Laboratories, Burlingame, CA) for 20–30 min at room temperature. After washing with PBS, slides were coverslipped with Gel/Mount (Biomeda, Foster City, CA) and observed under fluorescence microscope.

Enzyme Immunoassay (EIA) for Dopamine

Rats were perfused intracardially with cold 0.9% saline, and the brains were removed and snap frozen in cold 2-Methylbutane inside dry ice. Striata were dissected at –20°C, according to a stereotactic atlas (Paxinos 1982). Dopamine levels were evaluated using an EIA kit according to the manufacturer's instruction (LDN, Nordhorn, Germany) (Cha et al. 2005; Rowell et al. 2005). Tissues were homogenized in RIPA buffer with protease inhibitors. The lysates were adjusted to the same dilution with RIPA buffer based on the BCA protein assay. About 10 µl of each sample was used per evaluation, and all samples were analyzed in 96-well microtiter plates.

Statistical Analyses

All data were presented as mean ± sd and analyzed by ANOVA (analysis of variance) and Student's t-test. $P < 0.05$ was considered as the level of significance.

Results

Amphetamine Induced Rotation

Amphetamine induced rotational asymmetry was assessed before, 8 and 34 days after injection (Fig. 1A). All data were presented as mean ± sd of net number of ipsilateral rotations/min. After challenging with amphetamine, significant ipsilateral rotations were induced in the 6-OHDA group at both time points (–0.94 ± 0.76, before; 12.59 ± 2.12, day 8 after; 11.78 ± 3.29, day 34 after, $P < 0.01$). In contrast, significant contralateral rotations were observed at 8 days post injection in the AdLC group (–0.21 ± 0.49, before; –2.73 ± 1.72, day 8 after, $P = 0.04$). Similar with our previous motor function experiment (Teng et al. 2005), induced rotational behavior resolved at 34 days after AdLC injection compared with before injection (0.71 ± 0.86, $P = 0.23$). There were no significant differences of rotation in PBS group (–0.19 ± 0.69, before; –0.10 ± 0.49, day 8 after, $P = 0.88$; 0.03 ± 0.13, day 34 after, $P = 0.76$) and AdGFP group (–1.14 ± 2.15, before; –0.89 ± 1.43, day 8 after, $P = 0.77$; –1.38 ± 1.67, day 24 after, $P = 0.8$).

Amphetamine increases cytoplasmic levels and the release or efflux of DA, and inhibits reuptake of DA (Miller et al. 1999). Consistent with numerous previous studies, after challenging with amphetamine, ipsilateral rotations were induced in 6-OHDA rats due to dopaminergic neuron destruction. In contrast, contralateral rotations were observed after challenging with amphetamine in AdLC treated rats, which is similar with MPTP treated animals. Contralateral turns indicated a significant remaining dopamine innervation after MPTP administration (Iancu et al. 2005). Moreover, after AdLC injection, contralateral turns might also be due to blocking DA release and resulted accumulation of DA in SN (Fig. 3E).

Apomorphine Induced Rotation

Apomorphine induced rotational asymmetry was assessed before, 10 and 36 days after injection (Fig. 1B). All data were presented as mean ± sd of net number of ipsilateral

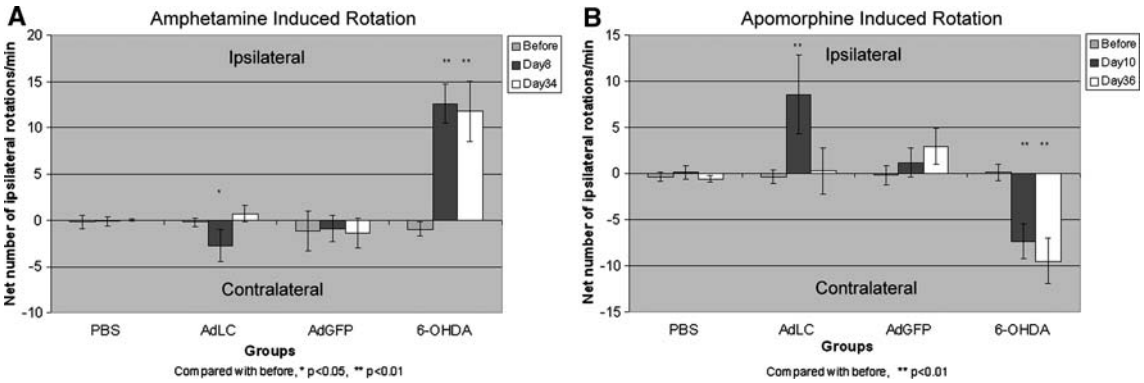

Fig. 1 Drug-induced rotation bias. (A) After AdLC injection, amphetamine induced contralateral rotations at 8 days, and the induced rotational behavior resolved by 34 days post injection. In contrast, after 6-OHDA injection, amphetamine induced ipsilateral rotations persisted at 8 and 34 days post injection. (B) Similarly, amphetamine induced ipsilateral rotations at 10 days, and the induced rotational behavior resolved by 36 days after AdLC injection. Whereas, after 6-OHDA injection, amphetamine induced contralateral rotations persisted at 10 and 36 days post injection. All data were presented as mean ± sd of net number of ipsilateral rotations/min (turns contralateral to the lesion subtracted from those towards the lesion side)

rotations/min. After challenging with apomorphine, significant contralateral rotations were induced at both time points in the 6-OHDA group (0.09 ± 0.92, before; –7.35 ± 1.89, day 10 after; –9.47 ± 2.45, day 36 after, $P < 0.01$). In contrast, significant ipsilateral rotations were observed at 10 days in AdLC injected rats (–0.40 ± 0.75, before; 8.54 ± 4.29, day 10 after, $P < 0.01$). Similar with amphetamine induced rotational behavior, apomorphine induced rotational asymmetry recovered at 36 days after AdLC injection compared with before injection (0.23 ± 2.54, $P = 0.7$). Again, there were no significant differences of rotation in the PBS (–0.42 ± 0.48, before; 0.09 ± 0.68, day 10 after, $P = 0.3$; –0.60 ± 0.33, day 36 after, $P = 0.7$) and AdGFP groups (–0.22 ± 0.99, before; 1.17 ± 1.59, day 10 after, $P = 0.3$; 2.97 ± 1.95, day 36 after, $P = 0.06$).

Following injection of 6-OHDA, marked DA depletion (over 90%) in the striatum induces supersensitivity in DA receptors, and hence apomorphine can induce contralateral rotation (Creese et al. 1977; Kirik et al. 1998). By contrast, MPTP injection might result in the downregulation of striatal DA receptors and apomorphine induced ipsilateral rotation (Shimohama et al. 2003). Our 6-OHDA treated rats showed contralateral rotations after challenging with apomorphine, consistent with previous reports. Similarly, AdLC treated rats showed ipsilateral rotations after challenging with apomorphine, identical with MPTP, 3-NP, or quinolinic acid models (Ghorayeb et al. 2001; Waldner et al. 2001).

LC Gene Expression Decreased Synaptobrevin Level at the Injection Site

Rats were sacrificed after behavioral data collection to detect transgene expression and synaptobrevin/VAMP level. Robust GFP expression was detected in the injected site of the mesencephalon in the AdGFP and AdLC treated rats (Fig. 2A, D). In order to confirm the enzymatic activity of LC gene product, anti-synaptobrevin/VAMP immunohistochemistry was performed. Consisted with our previous studies (Teng et al. 2005; Zhao et al. 2006; Yang et al. 2007), LC expression significantly decreased synaptobrevin level in the area of GFP expression compared with neighboring areas without GFP expression (Fig. 2D–F) in AdLC treated rats. In contrast, there were no appreciable synaptobrevin level changes in the areas with and without GFP expression in the brain of AdGFP animal (Fig. 2A–C). Thus, LC expression depleted synaptobrevin levels in the injected area.

TH, DAT, and D1DR Immunohistochemical Staining in the Mesencephalon and Striatum

IHC with the anti-TH antibody showed symmetric staining in bilateral SNpcs (Fig. 3A) in AdGFP rats, but markedly decreased staining in ipsilateral SNpc (Fig. 3 C) in 6-OHDA rats. In AdLC rats, IHC with TH antibody showed increased staining in the ipsilateral SNpc (Fig. 3E) compared with contralateral side. Similarly, TH-IHC showed symmetric staining in bilateral striata in AdGFP (Fig. 3B) and AdLC (Fig. 3F) rats, but markedly decreased staining in ipsilateral striatum in 6-OHDA (Fig. 3D) rats. Therefore, consistent with previous observations, LC expression depleted synaptobrevin in the injected area but spared neurons.

In order to further clarify the effect of AdLC nigral injection on striatal dopaminergic system, more specific pre-synaptic dopaminergic marker, DAT, and post-synaptic marker, D1DR, were selected for IHC (Miller et al. 1999). The results showed symmetric staining of DAT in bilateral striata in AdGFP (Fig. 4A) and AdLC (Fig. 4E) rats, but

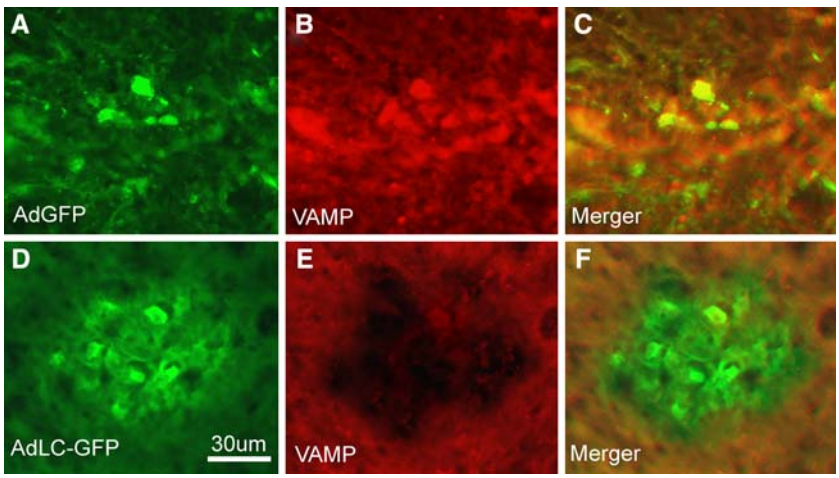

Fig. 2 Transgene expression and the digestion of synaptobrevin. AdGFP (A) and AdLC (D) mediated GFP expression in the injected areas of the mesencephalon. (B) robust synaptobrevin/VAMP expression in the areas with and without GFP expression in the brain of AdGFP animal. (C) merging of A and B. (D-F) LC expression significantly decreased synaptobrevin/VAMP level in the area of GFP expression compared with neighboring areas without GFP expression in brain of AdLC treated rat.

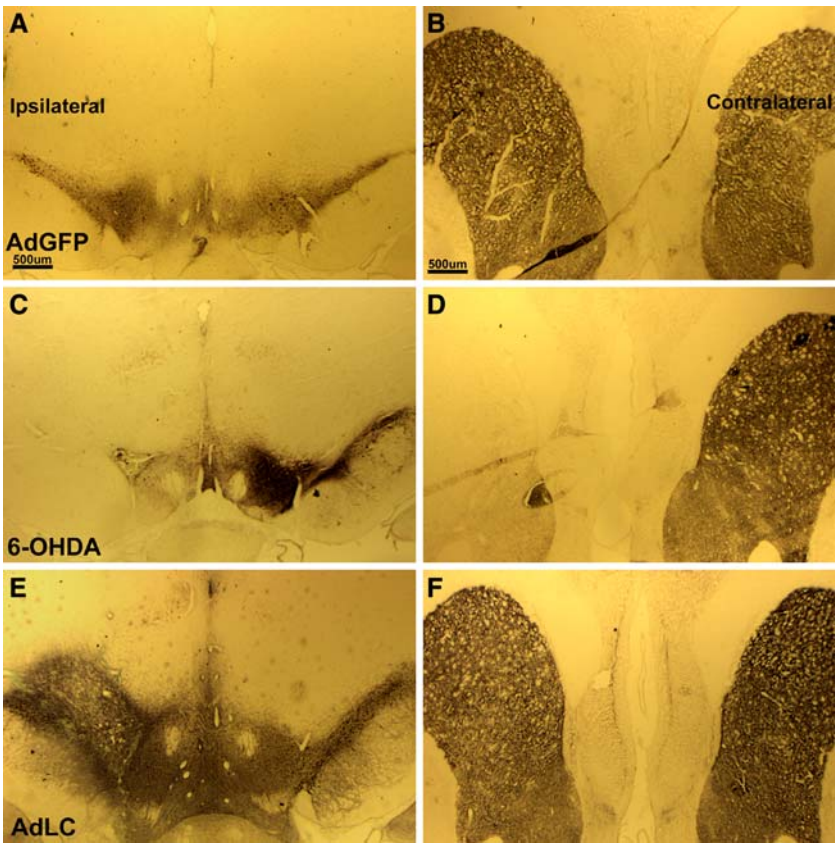

Fig. 3 SN AdLC injection did not kill neurons as showed by TH IHC staining in the SNs and striata. IHC with antibody of anti-TH showed symmetric staining in bilateral SNpcs (A) and striata (B) in AdGFP rats, but markedly decreased staining in ipsilateral SNpc (C) and striatum (D) in 6-OHDA rats. IHC with TH antibody showed rather increased than decreased staining in ipsilateral SNpc (E) and striatum (F) compared with contralateral side in AdLC rats. Therefore, unlike 6-OHDA, SN AdLC injection did not kill neurons in SN or striata

markedly decreased staining in ipsilateral striatum in 6-OHDA (Fig. 4C) rats. D1DR-IHC showed symmetric staining in bilateral striata in AdGFP (Fig. 4B), 6-OHDA (Fig. 4D), and AdLC (Fig. 4F) rats. Thus, LC nigral expression failed to kill either pre-synaptic or post-synaptic neurons in nigrostriatal dopaminergic system.

Striatal Dopamine Level

Bilateral striatal dopamine levels in homeostasis (two days after drug challenge) were evaluated and the contralateral striatal dopamine content were used as internal control (Fig. 5). All data were presented as percentage of ipsilateral side to contralateral side. The results showed no significant differences of striatal dopamine level in both sides in AdLC, AdGFP, and PBS rats, but significant decrease in ipsilateral striatum in 6-OHDA rats. Therefore, LC expression altered synaptic transmission of dopaminergic neurons, but did not insult neuronal health or dopamine storage in striata.

Discussion

The present study demonstrates that vector mediated nigral LC expression can inhibit nigrostriatal dopamine transmission without killing dopamine neurons. Stereotactic vector administration achieved gene expression in an anatomically discrete distribution. Nigral LC expression resulted in significant alterations in rotation induced by both amphetamine and apomorphine. Consistent with our previous spinal cord function experiment (Teng et al. 2005), induced rotational behavior in AdLC rats resolved by one month after injection. Immunohistochemistry revealed a focal reduction in synaptobrevin/VAMP levels in regions of LC expression, consistent with the previous demonstration of in vitro and in vivo rat brain synaptobrevin/VAMP digestion (Teng et al. 2005; Zhao et al. 2006; Yang et al. 2007). Synaptobrevin depletion which occurred in AdLC but not AdGFP injected animals confirmed that depletion results from LC expression rather than nonspecific adenoviral transgene expression. In contrast with 6-OHDA, LC expression in SN failed to reduce TH and DAT staining, pre-synaptic markers, in both SN and striatum, suggesting that LC expression did not kill neurons in nigrostriatal dopaminergic system. In addition, compared with contralateral, ipsilateral striatum dopamine level only decreased in 6-OHDA but not in AdLC and AdGFP treated rats, confirming that neither LC expression nor adenoviral gene delivery had toxic effects on DA neurons. Taken together, these findings confirm that LC mediated synaptobrevin digestion can achieve targeted neural inhibition

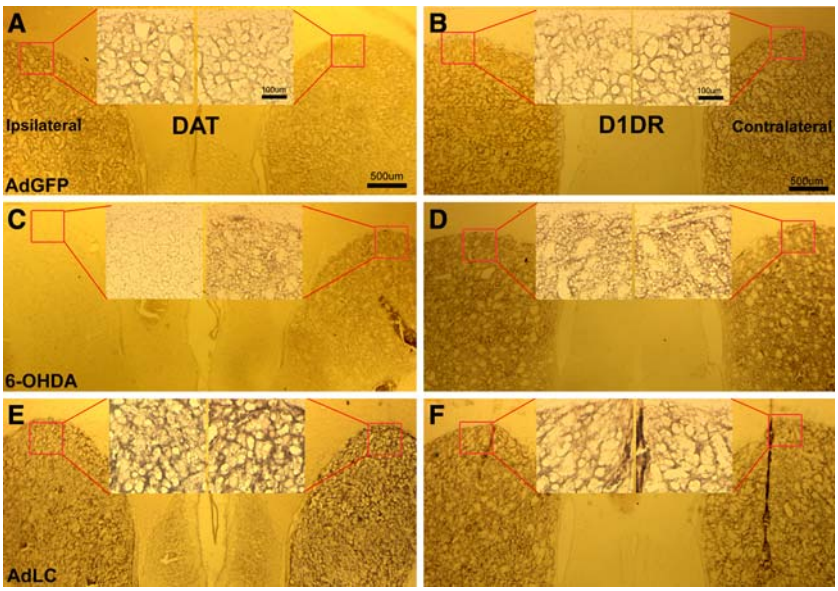

Fig. 4 SN AdLC injection did not kill either pre- or post-synaptic neurons in striatal dopaminergic system as showed by DAT and D1DR IHC staining in the striata. IHC with specific antibody of DAT for pre-synaptic dopaminergic neuron showed symmetric staining in bilateral striata in AdGFP (A) and AdLC (E) rats, but markedly decreased staining in ipsilateral striatum compared with contralateral side in 6-OHDA (C) rat. D1DR IHC showed symmetric staining in bilateral striata in AdGFP (B), 6-OHDA (D), and AdLC (F) rats, demonstrating structural intactness of post-synaptic dopaminergic neurons after injections

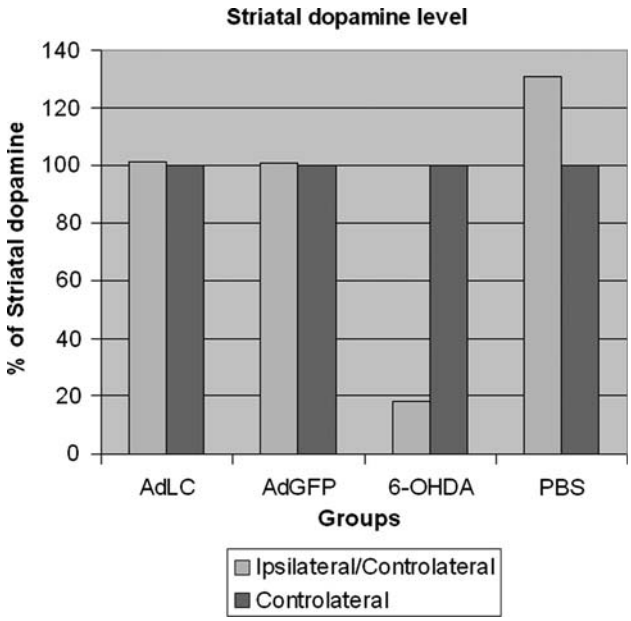

Fig. 5 Striatal Dopamine Level. Homeostatic dopamine levels in bilateral striata were evaluated two days after the drug challenge (12 days post injections). The contralateral striatal dopamine content was served as inherent control, and all data were presented as percentage of ipsilateral side to contralateral side. The results showed no significant differences of striatal dopamine levels in both sides in AdLC, AdGFP, and PBS rats, but significant decrease in ipsilateral striatum in 6-OHDA rats, indicating that nigral LC expression altered synaptic transmission of dopaminergic neurons, but did not insult neuronal health or dopamine storage in striata

without killing neurons as previously documented. Since the inhibition of striatonigral synaptic transmission can be achieved through transgene dependent synaptic inhibition without compromising neuronal health, advanced generation vectors with inducible transgene expression would be expected to provide a means of reversible and inducible suppression of striatal dopamine release.

The clostridial neurotoxins include botulinum neurotoxins (BoNT) and tetanus neurotoxin (TeNT). TeNT consists of light chain (LC, ~ 50 kDa) and heavy chain (HC, ~ 100 kDa) molecules endowed with different functions (Montecucco and Schiavo 1993; Schiavo et al. 1994). HC is responsible for neuronal membrane binding and membrane translocation, while LC acts as a zinc-dependent endopeptidase. TeNT-LC cleaves the single Gln-76-Phe-77 bond of synaptobrevin, a membrane protein of small synaptic vesicles involving neurotransmitter release. Cleavage of synaptobrevin disrupts neurotransmitter release through disruption of the vesicle docking and priming complex, thus inhibiting neurotransmission (Sudhof 1995; Humeau et al. 2000; Lin and Scheller 2000; Rossetto et al. 2001). However, inadvertent release of LC is not capable of inhibiting surrounding neurons since this fragment is not capable of neuronal binding and uptake without HC. The tropism of the TeNT protein is dependent on the HC, while the tropism of LC vectors is dependent on the vector. Thus, vector mediated LC dependent synaptobrevin digestion occurs in an anatomical area restricted to the region of gene expression. Our results showed that stereotactic AdLC administration in SN achieved gene expression in an anatomically discrete distribution and corresponding synaptobrevin digestion, accordingly resulting in inhibition of nigrostriatal transmission. Improved specificity of gene-based nigrostriatal inhibition could be achieved through vectors designed for targeted delivery specifically to DA neurons. Our laboratory has designed adeno-associated viruses for selective motor neuron tropism by inserting peptides with defined tropism for motor neurons into the

capsid (Davis et al. 2005). These same techniques could potentially be applied to the development of vectors with selective dopaminergic tropism. Similarly, cell type specific expression can be achieved through selective promoters. Zhuang et al. (2005) have recently described selective gene expression in dopaminergic cells with the dopamine transporter promoter using knock-in mice. This same approach could be applied to selective vector mediated expression in DA neurons.

Our previous in vitro and in vivo experiments documented both neuronal survival and functional recovery following LC expression, suggesting that the observed functional inhibition results from the disruption of synaptic vesicle docking rather than neuronal death (Teng et al. 2005; Zhao et al.2006). Spinal cord LC expression resulted in hindlimb sensorimotor dysfunction localized by electrophysiology to the neuromuscular junction. AdLC induced motor dysfunction recovers by one month after injection. Further, in both previous studies of AdLC in spinal cord and the midbrain, functional effects paralleled the duration of transient adenoviral gene expression. The fact that the functional effects were transient proved that the AdLC effect was not cytolytic in character. The lack of neurotoxicity in the AdLC vector is further supported by the study of neurons in culture and in the spinal cord. LC expression caused no increase in the either TUNEL staining or Caspase 3 activation, and no reduction in neuronal density. In the current study, AdLC nigral injection inhibited the nigrostriatal pathway. Amphetamine or apomorphine induced rotational behavior in AdLC rats resolved by one month after injection. TH, DAT or D1DR staining was not reduced in the SN or striata, suggesting that pre- or post-synaptic neurons in nigrostriatal dopamine system were alive and metabolically active at the time of observed nigrostriatal inhibition. Therefore, vector mediated nigral LC expression can inhibit nigrostriatal dopamine transmission without killing dopamine neurons.

Currently, the most popular animal models of PD depend on neurotoxins (Deumens et al. 2002; Shimohama et al. 2003). In the rat, unilateral depletion of striatal dopamine by intranigral injection of 6-OHDA has been used extensively. Although it has been very useful in pharmacological, neuroprotection, and transplant studies, the irreversible nature of the neurotoxin model prevents the study of recovery in nigrostriatal dopaminergic innervation. In order for LC nigral expression to constitute a feasible strategy for creating a reversible animal model of PD, it must function through synaptic disruption, rather than neuronal death. Consistent with the Yamamoto's results (Yamamoto et al. 2003), our previous and current works documented both neuronal survival and functional recovery following LC expression, suggesting that the observed functional inhibition resulted from the disruption of synaptic vesicle docking rather than neuronal death. In order for LC mediated nigrostriatal synaptic inhibition to provide an advance over the existing neurotoxic models of DA depletion, gene expression must be sustained and controllable. Advanced generation vectors and inducible/repressible promoter systems may provide these features. Transgene expression by both lentiviral (Blomer et al. 1997) and adeno-associated viral (Peel and Klein 2000) vectors in the brain occurs over years. In these vectors, new inducible/repressible promoters can provide the ability to switch gene expression on and off. These systems include the Tet-on and Tet-off systems (Baron and Bujard 2000). More recently, the Rheoswich system has provided a system with multiple potential activator ligands that are permeable to the blood brain barrier (Potter et al. 2006). These evolving controllable promoter systems, together with advanced generation vectors, could provide a means for a controlling nigrostriatal DA production over a long period of time, varying the intervals of DA depletion. Such a system could be employed in both rodents and primates, providing a more durable and humane model of Parkinson's Disease than irreversible toxin mediated dopamine depletion. This model will also provide advantages in the study of downstream striatal changes that result from DA deprivation and regeneration.

Acknowledgment This research was supported by the grant from NINDS KO8 NS43305.

References

Baron, U., & Bujard, H. (2000). Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances. *Methods in Enzymology, 327*, 401–421.

Beal, M. F. (2001). Experimental models of Parkinson's disease. *Nature Reviews. Neuroscience, 2*, 325–334.

Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M., & Gage, F. H. (1997). Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *Journal of Virology, 71*, 6641–6649.

Bove, J., Prou, D., Perier, C., & Przedborski, S. (2005). Toxin-induced models of Parkinson's disease. *NeuroRx, 2*, 484–494.

Cha, G. H., Kim, S., Park, J., Lee, E., Kim, M., Lee, S. B., Kim, J. M., Chung, J., & Cho, K. S. (2005). Parkin negatively regulates JNK pathway in the dopaminergic neurons of Drosophila. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 10345–10350.

Creese, I., Burt, D. R., & Snyder, S. H. (1977). Dopamine receptor binding enhancement accompanies lesion-induced behavioral supersensitivity. *Science, 197*, 596–598.

Davis, A., Stachler, M., Shi, W., Liu, J., Boulis, N., & Bartlett, J. (2005). Manipulating AAV2 tropism for enhanced delivery of AAV vectors to the spinal cord. *Molecular Therapy, 11*, S46.

Deumens, R., Blokland, A., & Prickaerts, J. (2002). Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Experimental Neurology, 175*, 303–317.

Federici, T., Teng, Q., Garrity-Moses, M., Krudy, C., Yang, J., & Boulis, N. M. (2006). Adenovirus-Light Chain Inhibition of Growth Hormone Release from Pituitary Adenoma Cells. *Molecular Therapy,13* Supplement 1, S54.

Freed C., Greene P., Breeze R., Tsai W., DuMouchel W., Kao R., Dillon S., Winfield H., Culver S., Trojanowski J., Eidelberg D., & Fahn S. (2001). Transplantation of Embryonic Dopamine Neurons for Severe Parkinson's Disease. *The New England Journal of Medicine, 344*, 710–719.

Ghorayeb, I., Puschban, Z., Fernagut, P. O., Scherfler, C., Rouland, R., Wenning, G. K., & Tison, F. (2001). Simultaneous intrastriatal 6-hydroxydopamine and quinolinic acid injection: a model of early-stage striatonigral degeneration. *Experimental Neurology, 167*, 133–147.

Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., & Phipps, M. L. (1997). Construction of adenovirus vectors through Cre-lox recombination. *Journal of Virology, 71*, 1842–1849.

Humeau, Y., Doussau, F., Grant, N. J., & Poulain, B. (2000). How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie, 82*, 427–446.

Iancu, R., Mohapel, P., Brundin, P., & Paul, G. (2005). Behavioral characterization of a unilateral 6-OHDA-lesion model of Parkinson's disease in mice. *Behavioural Brain Research, 162*, 1–10.

Kirik, D., Rosenblad, C., & Bjorklund, A. (1998). Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Experimental Neurology, 152*, 259–277.

Lin, R. C., & Scheller, R. H. (2000). Mechanisms of synaptic vesicle exocytosis. *Annual Review of Cell and Developmental Biology, 16*, 19–49.

Meloni, E., & Davis, M. (2000). Enhancement of acoustic startle response by dopamine agonists after 6-hydroxydopamine lesions of the substantia nigra pars compacta: corresponding changes in c-Fos expression in the caudate-putamen. *Brain Research, 879*, 93–104.

Meshul, C., Emre, N., Nakamura, C., Allen, C., Donohue, M., & Buckman, J. (1999). Time-dependent changes in striatal glutamate synapses following a 6-hydroxydopamine lesion. *Neuorscience, 88*, 1–16.

Miller, G. W., Gainetdinov, R. R., Levey, A. I., & Caron, M. G. (1999). Dopamine transporters and neuronal injury. *Trends in Pharmacological Sciences, 20*, 424–429.

Montecucco, C., & Schiavo, G. (1993). Tetanus and botulism neurotoxins: a new group of zinc proteases. *Trends in Biochemical Sciences, 18*, 324–327.

Muma, N., Lee, J., Gorman, L., Heidenreich, B., Mitrovic, I., & Napier, T. (2001). 6-hydroxydopamine-induced lesions of dopaminergic neurons alter the function of postsynaptic cholinergic neurons without changing cytoskeletal proteins. *Experimental Neurology, 168*, 135–143.

Olanow, C., Goetz, C., Kordower, J., Stoessl, A., Sossi, V., Brin, M., Shannon, K., Nauert, G., Perl, D., Godbold, J., & Freeman, T. (2003). A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Annals of Neurology, 54*, 403–414.

Paxinos, G. (1982). *The rat brain in stereotaxic coordinates*. San Diego: Academic Press.

Peel, A. L., & Klein, R. L. (2000). Adeno-associated virus vectors: activity and applications in the CNS. *Journal of Neuroscience Methods, 98*, 95–104.

Potter, D. W., DiPietro, M. A., Seitzer, J. L., Katakam, A., & Kumar, P. (2006). Adenoviral Delivery of the RheoSwitch Therapeutic System to Mouse Brain and Liver. *Molecular Therapy, 327*, 401–421.

Rossetto, O., Seveso, M., Caccin, P., Schiavo, G., & Montecucco, C. (2001). Tetanus and botulinum neurotoxins: turning bad guys into good by research. *Toxicon, 39*, 27–41.

Rowell, C., Carpenter, D. M., & Lamartiniere, C. A. (2005). Chemoprevention of breast cancer, proteomic discovery of genistein action in the rat mammary gland. *The Journal of Nutrition, 135*, 2953S–2959S.

Schiavo, G., Rossetto, O., & Montecucco, C. (1994). Clostridial neurotoxins as tools to investigate the molecular events of neurotransmitter release. *Seminars in Cell Biology, 5*, 221–229.

Shimohama, S., Sawada, H., Kitamura, Y., & Taniguchi, T. (2003). Disease model: Parkinson's disease. *Trends in Molecular Medicine, 9*, 360–365.

Sindhu, K. M., Saravanan, K. S., & Mohanakumar, K. P. (2005). Behavioral differences in a rotenone-induced hemiparkinsonian rat model developed following intranigral or median forebrain bundle infusion. *Brain Research, 1051*, 25–34.

St-Hilaire, M., Landry, E., Levesque, D., & Rouillard, C. (2005). Denervation and repeated L-DOPA induce complex regulatory changes in neurochemical phenotypes of striatal neurons: implication of a dopamine D1-dependent mechanism. *Neurobiology of Disease, 20*, 450–460.

Sudhof, T. C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature, 375*, 645–653.

Teng, Q., Tanase, D. K., Liu, J. K., Garrity-Moses, M. E., Baker, K. B., & Boulis, N. M. (2005). Adenoviral clostridial light chain gene-based synaptic inhibition through neuronal synaptobrevin elimination. *Gene Therapy, 12*, 108–119.

Waldner, R., Puschban, Z., Scherfler, C., Seppi, K., Jellinger, K., Poewe, W., & Wenning, G. K. (2001). No functional effects of embryonic neuronal grafts on motor deficits in a 3-nitropropionic acid rat model of advanced striatonigral degeneration (multiple system atrophy). *Neuroscience, 102*, 581–592.

Yamamoto, M., Wada, N., Kitabatake, Y., Watanabe, D., Anzai, M., Yokoyama, M., Teranishi, Y., & Nakanishi, S. (2003). Reversible suppression of glutamatergic neurotransmission of cerebellar granule cells in vivo by genetically manipulated expression of tetanus neurotoxin light chain. *The Journal of Neuroscience, 23*, 6759–6767.

Yang, J., Teng, Q., Federici, T., Najm, I., Chabardes, S., Moffitt, M., Alexopoulos, A., Riley, J., & Boulis, N. M. (2007). Viral Clostridial Light Chain Gene-based Control of Penicillin-induced Neocortical Seizures. *Molecular Therapy, 15*, 542–551.

Yurek, D. M., & Fletcher-Turner, A. (2004). Comparison of embryonic stem cell-derived dopamine neuron grafts and fetal ventral mesencephalic tissue grafts: morphology and function. *Cell Transplantion, 13*, 295–306.

Zhao, Z., Krishnaney, A., Teng, Q., Yang, J., Garrity-Moses, M., Liu, J. K., Venkiteswaran, K., Subramanian, T., Davis, M., & Boulis, N. M. (2006). Anatomically discrete functional effects of adenoviral clostridial light chain gene-based synaptic inhibition in the midbrain. *Gene Therapy, 13*, 942–952.

Zhuang, X., Masson, J., Gingrich, J., Rayport, S., & Hen, R. (2005). Targeted gene expression in dopamine and serotonin neurons of the mouse brain. *Journal of Neuroscience Methods, 143*, 27–32.