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OVER-EXPRESSION OF THE POTASSIUM CHANNEL Kir2.3 USING THE DOPAMINE-1 RECEPTOR PROMOTER SELECTIVELY INHIBITS STRIATAL NEURONS

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Abstract—Dysfunction of basal ganglia circuits underlies a variety of movement disorders and neuropsychiatric conditions. Selective control of the electrical activity of striatal outflow pathways by manipulation of ion channel function presents a novel therapeutic approach. Toward this end, we have constructed and studied *in vitro* an adenoviral gene transfer vector that employs the promoter region of the dopamine-1 receptor to drive expression of the inward rectifier K^+ channel Kir2.3. The use of this neuronal promoter confers cell-type specificity and a physiological level of trans-gene expression in rat primary striatal cultures. The electrophysiological properties were confirmed in transfected human embryonic kidney cells, in which an inwardly-rectifying, Cs^+-sensitive current was measured by voltage clamp. Current clamp studies of transduced striatal neurons demonstrated an increase in the firing threshold, latency to first action potential and decrease in neuronal excitability. Neurotoxin-induced activation of c-Fos, a marker of neuronal activity, was blocked in transduced neurons indicating that the decrease in electrical excitability was physiologically significant. When used *in vivo*, this strategy may have the potential to positively impact movement disorders by selectively changing activity of neurons belonging to the direct striatal pathway, characterized by the expression of dopamine-1 receptors.

Key words: adenovirus, gene therapy, inwardly rectifying potassium channel, basal ganglia, cell culture, c-Fos.

A variety of neurological and neuropsychiatric disturbances are linked to pathological electrical activity in specific basal ganglia circuits. One circuit termed the "direct" or "GO" pathway (Frank, 2005; Obeso et al., 1997) is overactive in hyperkinetic movement disorders such as tardive dyskinesia, chorea, Tourette's syndrome (Mink, 2001), and levodopa-induced dyskinesia (Obeso et al., 2000b). A large body of evidence documents that changes of excitability in the basal ganglia outflow pathways are the hallmark of Parkinson's disease (PD) and the immediate consequence of dopaminergic denervation (Obeso et al., 1997). In the akinetic PD state the GO pathway is hypoexcitable (Mallet et al., 2006). The GO pathway is generally conceptualized as a circuit connecting the striatum, pallidum, thalamus, and motor-associated cortex. Striatal circuits that contain these direct pathway neurons are segregated anatomically along a rostral–caudal dimension, in a pattern correlating with the function that they subserve. Striatal output neurons that belong to the direct pathway are characterized by a relatively high expression of the dopamine-1 (D_1) receptor and are activated by dopamine released from the nigro-striatal pathway. Clinical entities featuring over-activity in this pathway can be medically treated using dopamine receptor antagonists, but this approach has major shortcomings due to the lack of specificity of dopamine receptor agonists and antagonists which act indiscriminately across all basal ganglia circuits (Holmes et al., 2004; Romanelli et al., 2005). Recent surgical advances (e.g. deep brain stimulation) have clearly demonstrated that modifying electrical activity in anatomically discrete basal ganglia regions can have dramatic clinical results in PD (Perlmutter and Mink, 2006). Pallidal electrical stimulation can reduce symptoms of levodopa-induced dyskinesia (Metman and O'Leary, 2005), tardive dyskinesia (Eltahawy et al., 2004), Huntington's disease (Moro et al., 2004), and Tourette's syndrome (Houeto et al., 2005), although the precise mechanism of benefit is not understood (Garcia et al., 2005). We hypothesized that a gene therapy approach that selectively modifies basal ganglia circuit activity could be achieved by using promoter-specific control of expression of activity-modifying ion channels. *In vivo*, specificity to target cell types in specific basal ganglia pathways would also be enhanced by anatomical selection of the injection sites. In the treatment of movement disorders for example, stereotactic injection of a gene vector can be anatomically confined to motor circuits while avoiding effects on limbic and cognitive circuits (Romanelli et al., 2005). This approach offers the promise of clinical utility for controlled intervention, but requires the identification of appropriate and effective promoters and the choice of therapeutic gene products that have particular relevance to the neural circuitry involved in the disease.

In order to demonstrate feasibility, assess effectiveness of promoter–ion channel constructs selected for relevance to basal ganglia, and move the gene therapy agent to a form useful for future trials *in vivo*, we focused on

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Abbreviations: AAA, triple alanine; CMV, cytomegalovirus; D_1, dopamine-1; GYG, glycine–tyrosine–glycine; IRES, internal ribosomal entry site; PD, Parkinson's disease; ROI, region of interest.

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creation, characterization and optimization of adenoviral gene transfer vectors designed to modify the electrical activity of the "direct" striatal pathway. This vector uses the D_1 receptor promoter to confer neuron specific over-expression of an inward rectifier K^+ channel (Kir) predicted to suppress excitability, and a dominant negative construct predicted to increase neuronal excitability. We have characterized the functional effects of these vectors using the NS20Y neuroblastoma cell line and dispersed primary cultures of rodent striatal neurons as model systems. We (Falk et al., 2006) and others (Joyce and Rayport, 2000; Geldwert et al., 2006) have characterized fundamental properties of primary cultures of striatal neurons *in vitro* previously showing that the cells maintain molecular markers and electrophysiology firing patterns comparable to those described for striatal neurons *in vivo* (Falk et al., 2006). This model system facilitates characterization of the introduced promoter and ion channel gene construct at a defined cellular level, and enables optimization of the molecular agents needed before the subsequent testing *in vivo* planned for future studies.

EXPERIMENTAL PROCEDURES

Generation of pAdD_1-Kir and AdD_1-Kir

The D_1 receptor promoter (accession number: *S46131*) was amplified from rat genomic DNA using oligonucleotide primers corresponding to −787 to −10 bp. The 777 base pairs (bp) D_1 PCR product was then cloned by single base overhang into a pGEM-T easy vector (Promega, Madison, WI, USA) and the whole insert was sequenced to check for errors. The D_1 promoter was then subcloned into a promoterless adenoviral shuttle vector pacAd5mcs-IRESeGFPpA (provided by Gene Transfer Vector Core, University of Iowa, Iowa City, IA, USA) to verify expression. Kir2.3 had previously been cloned (Falk et al., 1995). A Strep-tag (IBA, St. Louis, MO, USA) was added on to the N-terminus of the channel to distinguish it from endogenous Kir2.3 channels. The whole insert was sequenced to check for errors. Strep-tag-Kir2.3 was then subcloned into pacAd5D_1-IRESeGFPpA and into pacAd5CMV-IRESeGFPpA (provided by Gene Transfer Vector Core). The dominant negative mutation of the K^+ channel pore residues glycine–tyrosine–glycine (GYG; McLerie and Lopatin, 2003; Kuzhikandathil and Oxford, 2000; Xue et al., 2002) to triple alanine (AAA) was carried out by site-specific mutagenesis as described elsewhere (Preisig-Muller et al., 2002). The Ad5 adenovirus preparations (AdD_1-eGFP, AdD_1-Kir) were then custom made by the Gene Transfer Vector Core at the University of Iowa (Anderson et al., 2000). In their hands, an infectious titer of 1×10^{10} pfu/ml (infectious units/ml) as determined by HEK293 cell plaque assay for their standard procedure is equivalent to 1×10^{12} particles/ml.

Propagation and transfection of cell lines and cultured primary neurons

HEK 293 and NS20Y cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were grown in Dulbecco's Minimal Essential Media (DMEM) with 10% (v/v) fetal bovine serum (FBS) and split every 4–5 days with 0.25% trypsin/EDTA. Antibiotics were not used in the growth medium. The cells were kept at 37 °C in a humidified 5% CO_2 incubator. Tissue culture media and sera were obtained from Gibco BRL (Grand Island, NY, USA). Cationic lipid transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Cells were studied 48 h following transfection unless otherwise stated.

Primary neuronal culture

Timed-pregnant Sprague–Dawley rats were anesthetized by CO_2 inhalation. Fetuses were removed at E18, anesthetized by cooling on ice, and decapitated. The procedure was approved by the Institutional Animal Care and Use Committee at the University of Arizona and conformed to the guidelines of the National Institutes of Health. The number of animals used and their suffering was minimized. The procedure followed the previously published protocol (Falk et al., 2006). Cultures were used between day 8 and 12 in culture. Viral transductions were carried out by diluting the virus to the required titer in 2 ml of complete growth medium and adding the solution to the 35 mm culture plates for 48 h. Thus the concentration of pfu/dish is twice that of the pfu/ml titers indicated. In initial experiments, we found that removal of serum from the culture medium did not improve transduction rates, nor did the prolongation of the duration of exposure to the viral incubation solution to greater than 2 days. For immunocytochemistry experiments using anemone toxin II (Alomone Laboratories, Jerusalem, Israel), a final concentration of 1 μM was achieved by the bolus application of a stock solution (0.5 mM) to the culture plate 180 min prior to fixation.

Immunocytochemical methods

Immunocytochemical methods for labeling and semi-quantitative measurement of immunofluorescence in striatal cultures were performed as described earlier (Falk et al., 2006) including semi-quantitative measurement of immunofluorescence. The D_1 receptor monoclonal antibody was produced in rat (Sigma-Aldrich, St. Louis, MO, USA; clone 1-1-F11 s.E6) and was used at a dilution of 1:500. The secondary antibody was biotin-labeled goat anti-rat Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and was used at a dilution of 1:250. The secondary antibody was then visualized with streptavidin-linked AlexaFluor 555 at 1:10,000 dilution (Molecular Probes, Inc., Eugene, OR, USA). The rabbit anti-eGFP antibody was a gift from Dr. W. D. Stamer (The Univ. of Arizona, Tucson, AZ, USA). It was visualized using AlexaFluor 488 conjugated goat anti-rabbit IgG (Invitrogen, Molecular Probes™, Carlsbad, CA, USA) at 1:500 dilution.

Flow cytometry

NS20Y cells were grown to 80% confluency in six well plates (Corning, Corning, NY, USA) and transfected with plasmid DNA using cationic lipids (Lipofectamine 2000>) according to the manufacturer's protocol. We found that the optimum DNA:lipid ratio was 1:3. After 48 h, cells were dissociated with 0.25% trypsin-EDTA (Invitrogen) removed with a Teflon scraper and gently triturated. For adenovirus experiments, virus was added to the cultures at the indicated titer 48 h before flow cytometry analysis and cells were fixed in 4% paraformaldehyde after trituration. Striatal cultures were dissociated with 0.25% Trypsin–EDTA (Invitrogen) for 5 min. Flow cytometry was performed on a FACScan (BD Biosciences, San Jose, CA, USA) with a 15 mW argon laser tuned to 488 nm. Side and forward scatter was monitored and a region of interest (ROI) was determined for subsequent fluorescent analysis. We found that the fluorescence analysis was robust and was not systematically influenced by selection of the initial ROI.

Western blot analysis

Striatal cultures or HEK 293 cells were incubated with adenovirus for 48 h prior to harvesting. Membrane protein was isolated as follows. Cells were washed three times in PBS then lysed in 150 μl