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Gene transfer of constitutively active protein kinase C into striatal neurons accelerates onset of levodopa-induced motor response alterations in parkinsonian rats

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Abstract

Alterations in motor response that complicate levodopa treatment of Parkinson's disease appear to involve sensitization of striatal ionotropic glutamate receptors. Since protein kinase C (PKC)-mediated phosphorylation regulates glutamatergic receptors of the α -amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA) subtype and has been linked to several forms of behavioral plasticity, activation of PKC signaling in striatal spiny neurons may also contribute to the motor plasticity changes associated with chronic levodopa therapy. To evaluate this possibility, we sought to augment PKC signaling by using Herpes Simplex Virus type 1 vectors (pHSVp Δ) to directly transfer the catalytic domain of the PKC β II gene into striatal neurons of parkinsonian rats.

Microinjection of pHSVp Δ vectors lead to the persistent expression of Pk Δ (35% loss over 21 days) in medium spiny neurons together with an increase in serine 831 phosphorylation on AMPA receptor GluR1 subunits and hastened the appearance of the shortened response duration produced by chronic levodopa treatment ($P < 0.05$). In pHSVp Δ -infected animals, intrastriatal injection of the PKC inhibitor NPC-15437 (1.0 μ g) attenuated both the increased GluR1 phosphorylation ($P < 0.01$) and the accelerated onset of the levodopa-induced response modifications ($P < 0.01$).

However, in rats that received levodopa treatment for 21 days without the gene transfer, intrastriatal NPC-15437 had no effect on the response shortening or on GluR1 S831 phosphorylation. The results suggest that an increase in PKC-mediated signaling, including, in part, phosphorylation of AMPA receptors, on striatal spiny neurons may be sufficient to promote the initial appearance, but not necessary the ultimate expression, of the levodopa-induced motor response changes occurring in a rodent model of the human motor complication syndrome.

Keywords

Chronic levodopa administration; 6-Hydroxydopamine lesion; AMPA receptor; Herpes Simplex Vector type 1 vector; Phosphorylation; Basal ganglia

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1. Introduction

A hallmark of Parkinson's disease (PD) is striatal dopamine depletion due to degeneration of the nigrostriatal dopaminergic pathway. Initially, treatment with either the dopamine (DA) precursor levodopa or a direct dopamine receptor agonist ordinarily confers substantial clinical benefit. Within a few years, however, these drugs begin to produce increasing difficulties, including response alterations such as motor fluctuations and dyskinesias [1,3,34]. Parkinsonian rats [25,60] or nonhuman primates [59] treated once or twice daily with levodopa manifest similar changes, including a shortening in response duration that gives rise in humans to motor fluctuations of the wearing-off type [53]. Current evidence suggests that these disabling complications involve, at least in part, signaling changes in striatal medium spiny neurons due to the chronic nonphysiological stimulation of their dopaminergic receptors [12, 15,35,42,84].

Intermittent high-intensity stimulation of dopamine receptors on striatal medium spiny neurons in parkinsonian rats has been implicated in the activation of dendritic signaling cascades that promote the selective phosphorylation of co-expressed glutamatergic receptors [13,15,21,22, 55–57]. Regarding *N*-methyl-D-aspartate (NMDA) receptors, serine/threonine phosphorylation appears to involve the activity of such kinases as cyclic AMP-dependent protein kinase (PKA) [55,72] and calcium/calmoduline-dependent protein kinase II (CaMK II) [19,57], while tyrosine phosphorylation is mediated by as yet unidentified kinases, presumably including those of the src and fyn families [36,50,57,75]. As a result, synaptic efficacy apparently becomes enhanced, in view of the potent ability of NMDA receptor antagonists to prevent or palliate the characteristically altered motor responses to dopaminergic stimulation [5,8,14, 16,48,51,52,58,79]. A similar sensitization may also involve other glutamatergic receptors including those of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) class, since drugs that selectively block them also reverse levodopa-induced response alterations in parkinsonian rodents and non-human primates [38,47,48].

AMPA receptors, like those of the NMDA class, are highly expressed by striatal medium spiny neurons, especially within the postsynaptic density at tips of their dendritic spines [6,11,70]. The localization and function of AMPA receptors is tightly regulated by protein phosphorylation, particularly at sites along their intracellular carboxy termini [10,31,81]. Protein kinase C (PKC), increasingly linked to various forms of synaptic plasticity [32,33, 41,49,63,71], occurs at high levels in spiny neurons [6,70] and regulates AMPA channel function [18,20,68], in part via phosphorylation of GluR1 subunits at serine residue 831 (S831) [9,11,35,43,64]. Conceivably, a rise in the synaptic efficacy of striatal AMPA receptors by long-term stimulation of dopaminergic receptors may contribute to the development of motor response plasticity in parkinsonian animals that attends chronic dopaminomimetic therapy. To evaluate this possibility, we studied the effects of the direct intrastriatal gene transfer of constitutively active PKC by herpes simplex virus type 1 (HSV-1) [83,86], as well as those produced by the pharmacologic inhibition of PKC, on the phosphorylation state of striatal GluR1 subunits (S831) and the development of motor response alterations in levodopa-treated hemiparkinsonian rats.

2. Materials and methods

2.1. Vector construction and packaging

Construction of HSV-1 was performed by standard recombinant DNA procedures [44,86]. Using the CMV immediate early promoter, pHSVlac or pHSVpkc Δ vectors were constructed to regulate expression of the LacZ or PKC Δ , respectively. pHSVlac virus was included as a control vector which supports the expression of β -galactosidase in multiple cell types [86]. To genetically activate the PKC pathway, HSV-1 vectors were designed to express a PKC β II

deletion encoding the aa 285 to C terminus fused with codons encoding the flag epitope tag [61,69,85]. The gene product was designated PkcΔ. Vectors were then packaged into HSV-1 particles using a helper virus-free packaging system [26,28,77] by a modified protocol to improve efficiency [74]. Vector stocks, following purification and concentration [40], were titered by counting the number of either 5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside (X-Gal)-positive cells or flag immunoreactivity-positive cells [69,77,82] obtained 1 day after infection of BHK cells. Titers of the vector stocks were 2.4×10^6 infectious vector particles (IVP)/ml pHSVpkcΔ and 1.2×10^6 IVP/ml pHSVlac. The efficiency of gene transfer was calculated as the average number of X-gal or flag cells divided by the IVP of vector injected.

2.2. Animals and behavioral assessment

Male Sprague–Dawley rats weighting 200–250 g were housed with free access to food and water. Under sodium pentobarbital anesthesia (50 mg/kg, i.p.), the nigrostriatal pathway was unilaterally lesioned by administering 6-OHDA HCl (8 μg in 4 μl of saline with 0.02% ascorbate) into the left medial forebrain bundle (AP −5.0, L 1.3, V 8.0 from the dura matter). Three weeks later, animals having >100 contralateral turns/h in response to apomorphine (0.05 mg/kg, s.c.) began levodopa (25 mg/kg with 6.25 mg/kg of benserazide, i.p.) treatment by twice-daily injection for 21 days.

To assess the effect of the selective PKC antagonist NPC-15437 dihydrochloride (*S*-2,6-diamino-*N*-[[1-(1-ox-octadecyl)-2-piperidinyl]methyl]-hexanamide dihydrochloride) (NPC-15437; RBI, Natick, MA, USA), a competitive inhibitor of the Ca^{2+} -induced activation of PKC [73], on the rotational response to an acute levodopa challenge following chronic levodopa administration, animals showing >15% reduction in response duration on day 22 were randomly assigned to receive intrastratial vehicle (saline; $n=5$), 1.0 μg NPC-15437 (dissolved in saline; $n=5$), or 5.0 μg NPC (also dissolved in saline; $n=6$) 5 min before acute levodopa challenge. On day 23, NPC or an equal volume of saline was administered into the striatum ipsilateral to the lesion under halothane anesthesia by means of a syringe microinfusion pump (Harvard Apparatus) at a rate of 0.5 μl/min for 4 min (total volume of 2.0 μl) at coordinates A +0.5, L 2.5, V 4.6. Following recovery, animals were immediately placed in a rotometer and given an acute injection of levodopa (20 mg/kg with 5.0 mg/kg benserazide, i.p.).

The duration of the rotational response was considered the time between the first 5 min interval when turning exceeded 20% of its maximal rate and the first interval when it fell below 20% maximal rate; peak rotational intensity was taken as the maximum number of turns occurring in any 5 min interval during a given session.

2.3. Stereotactic injections and behavioral testing

PKC gene transfer was achieved by the intrastratial administration of pHSVlac vector stock (2.0×10^5 IVP/2 μl/site), pHSVpkcΔ vector stock (2.0×10^5 IVP/2 μl/site), or an equal volume of saline vehicle at pH 7.4. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. Vector stocks in 2 μl of solution or saline vehicle were delivered by a syringe pump (Hamilton) into the left striatum (ipsilateral to the 6-OHDA lesion) at a rate of 0.5 μl/min for 4 min at two sites: coordinates A +0.5, L 3.5, V 4.6 and A +0.5, L 3.5, V 3.6. The total amount of vector injected/rat was 4.0×10^5 IVP for pHSVlac and pHSVpkcΔ (two sites, 2 μl/site, for 4 μl of total injection volume for both vectors). After injection, the syringe was kept in place for an additional 3 to 4 min to allow diffusion of the drug solution. Rats received the vector stock or saline vehicle 1 day prior to the first levodopa dose and subsequently received twice-daily levodopa injections for 1, 3, 7, 14, 21 or 28 days. On each levodopa treatment day, some animals were tested for their motor response to an acute

dopaminomimetic challenge (25 mg/kg of levodopa with 6.25 mg/kg of benserazide, i.p.) and some were sacrificed for striatal tissue analysis.

To assess the time course of the levodopa-induced motor response alterations following striatal gene transfer, rats were examined for their rotational response to acute levodopa challenge (25 mg/kg with 6.25 mg/kg of benserazide, i.p.) after 1, 3, 7, 14, 21 and 28 days of levodopa treatment.

To evaluate the effect of Pkc Δ on the rotational response to either a D1 (SKF 38393) or D2 dopamine receptors agonist (quinpirole), some rats were microinjected with pHSVlac and pHSVpkc Δ vectors into the striatum 1 day prior to initiation of levodopa treatment. Both groups of animals then received levodopa twice daily for 1, 7 or 21 days. Following a 3-day drug washout, all were tested for rotational behavior in response to either SKF 38393 (1.5 mg/kg, s.c.) or quinpirole (0.1 mg/kg, i.p.). Rats microinjected with saline prior to chronic levodopa therapy served as controls.

To evaluate the effect of NPC-15437 on the motor response to acute levodopa challenge following gene transfer with pHSVpkc Δ , rats that received gene transfer and had >15% reduction in response duration on levodopa treatment day 7 compared to day 1 were randomly assigned to receive either intrastriatal saline vehicle ($n=3$) or 1.0 μ g NPC-15437 ($n=4$) just prior to the acute levodopa challenge on day 8.

On day 8, NPC-15437 or an equal volume of saline was administered into the striatum ipsilateral to the side of gene transfer and the animals were tested for their rotational behavior as described above. Some animals were sacrificed 30 min after onset of levodopa-induced rotation to harvest striatal tissue for subsequent assay.

2.4. Sample preparation and Western blot analysis

Three weeks after 6-OHDA lesioning alone or plus 21 days of levodopa treatment (25 mg/kg i.p. plus 6.25 mg/kg benserazide), rats were sacrificed by decapitation and their brains rapidly removed and cooled in ice-cold buffer containing 126 mM NaCl, 5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgCl₂ and 10 mM D-glucose at pH 7.4. Striatal tissues from the lesioned (ipsilateral) side and intact (contralateral) side were excised from 1 mm thick coronal brain slices using a 12-gauge needle [78]. In animals receiving intrastriatal gene transfer, brains were removed 8 days after intrastriatal microinjection and striatal tissue around the gene transfer site (ipsilateral) and corresponding tissue from the intact non-injected (contralateral) side were removed 12 h after the last levodopa injection. In NPC-15437-treated rats, brains were removed 30 min after onset of levodopa-induced rotation. Striatal tissues were homogenized by sonication and an aliquot taken for protein determination by the Lowry method. Another aliquot containing 100 μ g of striatal tissue was heated for 5 min at 90 °C in cold sonication buffer [1.0% ultrapure SDS, 20 mM HEPES (pH 7.4), 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 25 mM NaF, 10 mM NaPPi, 1.0 mM Na₃VO₄, 10 μ g/ml of leupeptin, aprotinin, pepstatin A, and ddH₂O) containing 10% glycerol, 1.0 mM phenylmethylsulfonyl fluoride, 1.5 mM MgCl₂, and 1 mM ZnCl₂.

For Western blot analysis, striatal homogenates (14 μ g of protein per lane) were subjected to SDS-PAGE on 12% polyacrylamide gels and transferred to a nitrocellulose membrane (Novex, San Diego, CA, USA). Immunoblotting was performed [50] with antibodies against PKC β II (Sigma, St. Louis, MO, USA), tyrosine hydroxylase, GluR1 (TH and GluR1; Chemicon, Temecula, CA, USA), or P-GluR1 (S831) receptors (New England Biolabs, Beverly, MA, USA). Following immunoreactivity detection by ECL chemiluminescence (Tropix, Bedford, MA, USA), ECL-exposed films were scanned with an image analyzer (NIH Image 1.60 software) and densitometric quantification of immunopositive bands carried out. Values, after

background subtraction, are expressed as the mean percent of intact striatum (contralateral side) for each immunoblot.

2.5. Histochemical analysis

Three weeks after 6-OHDA lesioning followed by twice-daily levodopa treatment for 3, 7, 14 or 21 days, animals with pHSVlac or pHSVpkcΔ gene transfer or with saline microinjection were anesthetized with 50 mg/kg nembutal, placed on a bed of ice, and perfused with 30 ml of cold PBS (pH 7.6), followed by 60 to 70 ml of cold PBS containing 4% paraformaldehyde and 0.2% picric acid. Brain perfusion began approximately 12 h after the final dopaminomimetic drug injection (25 mg/kg levodopa with 6.25 mg/kg benserazide, i.p.). Brains were removed, post-fixed overnight in the paraformaldehyde–picric acid solution and then immersed overnight in 30% sucrose before being sectioned on a sliding microtome. Coronal sections (30 μm) were cut on a freezing microtome and histological staining or immunohistochemistry was performed on free-floating sections.

For pHSVlac-transfected animals, expression of β-galactosidase was detected by incubation with X-gal [24]. Briefly, the X-gal reaction was carried out for 3 h at room temperature at pH 7.9. For pHSVpkcΔ-transfected rats, brains were perfused for immunohistochemical analysis with antibodies against flag (Sigma) and P-GluR1 (S845). For immunohistochemistry, floating sections were washed three times in PBS containing 0.3% Triton X-100 and blocking serum for 45 min. Tissue sections were then rinsed in several washes of PBS and transferred to solutions of a monoclonal antibody against flag or P-GluR1 diluted in 0.1% sodium azide PBS 1:1000 and 1:100, respectively. Sections were incubated in primary antibody solutions for 1–2 days at room temperature with moderate agitation. Sections were then rinsed three times in PBS and incubated in secondary antibody solutions diluted 1:200 in PBS using a Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA). After 2 h incubation, sections were rinsed several times in PBS and transferred to solutions of 0.3% H₂O₂ in PBS for 30 min, rinsed and placed into solutions of biotin–avidin–peroxidase for 2 h. Sections were subsequently rinsed in several washes of PBS and reacted for 15–20 min in PBS containing 0.05% diaminobenzidine 0.01% H₂O₂ and 2.5% nickel ammonium sulfate. As controls, some tissue sections were prepared with the primary antibodies omitted.

Medium spiny neuronal expression was evaluated by morphological analysis (including cell size, shape and staining intensity) as well as by co-labeling with neuronal specific nuclear protein (NeuN) antibody (Chemicon, Temecula, CA, USA) and flag antibody following intrastriatal infection with pHSVlac or pHSVpkcΔ, respectively. The cell-type specificity of β-galactosidase or Flag expression was evaluated 8 days following pHSVlac or pHSVpkcΔ striatal gene transfer. To evaluate AMPA receptor phosphorylation in pHSVpkcΔ-infected cells, sections were incubated in solutions containing anti-flag (anti-mouse) plus anti-P-GluR1 (anti-rabbit) antibodies, and later labeled with secondary antibodies labeled with rhodamine (anti-mouse) and fluorescein (anti-rabbit), respectively. To evaluate transfection of neuronal cells in pHSVpkcΔ-infected cells, sections were incubated in solutions containing anti-flag (anti-rabbit) plus anti-NeuN (anti-mouse) antibodies, and later labeled with secondary antibodies labeled with fluorescein (anti-rabbit) and rhodamine (anti-mouse), respectively. Finally, for most of the rats that received gene transfer, tissue sections were processed for Nissl staining to assess tissue damage.

2.6. Quantitative histology

For quantification, X-gal-, flag-, or P-GluR1-positive cells were counted on three coronal sections located approximately 260, 500 and 740 μm rostral to the bregma where striatal neurons are prominent. Cell numbers were determined by direct counts using an Olympus Vanox microscope and neurons were identified using the Bioquant Image Analysis System

and microdensitometry software (R&M Biometrics, Nashville, TN, USA). X-gal- or flag-positive neurons were counted only if they had a diameter $\geq 10 \mu\text{m}$ along the major axis. For each animal, three coronal sections from the gene transferred side were analyzed at $10\times$ magnification and the results presented as mean number of cells counted $\pm$ S.E.M. P-GluR1-immunopositive neurons were counted if they had a regular shape (shape factor ≥ 0.85) with diameter $\geq 5 \mu\text{m}$ along the major axis. For each animal, neurons were counted on both the ipsilateral and contralateral sides on three standard coronal sections at $20\times$ magnification and the results presented as mean number of cells counted $\pm$ S.E.M.

2.7. Statistical analysis

Data, reported as means $\pm$ S.E.M., were analyzed by analysis of variance (ANOVA) followed by Scheffe's post-hoc comparison tests. Statistical significance was taken as $P < 0.05$ for all analyses.

3. Results

3.1. Detection of pHSVlac or pHSVpkc Δ gene transfer

Many striatal sections from rats that received pHSVlac contained X-gal-positive cells displaying neuronal morphology 4, 8, 15 or 22 days after striatal gene transfer (Fig. 1A). Most X-gal-positive cells were distributed over a distance of about $1400 \mu\text{m}$ in the anteroposterior direction from the injection site. Consistent with previous results [27], A helper virus-free HSV-1 vector-mediated gene transfer produced minimal cell damage or cell infiltration as assessed by Nissl staining (Fig. 2C). Cell counts from two coronal sections from gene transferred rats indicated that the injected striatum contained 175 ± 10 , 169 ± 14 , 156 ± 18 , and $104 \pm 7^*$ X-gal-positive cells 4, 8, 15 and 22 days after gene transfer, respectively ($n=3-4$; $*P < 0.05$ compared with 4 and 8 days after gene transfer). Striata from animals given intrastriatal saline vehicle contained few if any X-gal-positive cells. Four days after gene transfer, three rats injected with pHSVlac or pHSVpkc Δ contained approximately 1760 ± 90 X-gal-positive cells or 1640 ± 110 flag-positive cells in the striatum, respectively. The efficiency of gene transfer was approximately 1.0% for both pHSVlac and pHSVpkc Δ .

For pHSVlac gene transfer, the X-gal-positive cells in animals 22 days after gene transfer were 59% of the cells in animals 4 days after gene transfer ($P < 0.04$ compared with 4 days post-gene transfer group; Fig. 1B). For pHSVpkc Δ gene transfer, the X-gal-positive cells in rats 22 days after gene transfer were 65% of the cells in rats 4 days after gene transfer ($P < 0.05$ compared with 4 days post-gene transfer group; Fig. 2B). No X-gal staining was observed in the striatum of rats that received saline.

Pkc Δ at a $M_r \sim 50,000$ band was observed only in striatal tissue infected with pHSVpkc Δ , but not in tissue infected with pHSVlac (Fig. 2A). Flag immunopositivity was seen predominantly in the cell bodies and proximal processes [Fig. 2B(a)–(c)] 7, 14, and 21 days after pHSVpkc Δ gene transfer, respectively) of cells having the characteristic morphology of medium spiny neurons. On the other hand, only about 60% of the NeuN-positive cells appeared to co-localize with flag (54 ± 3 colocalized cells per $84 \pm 5^*$ flag-positive cells per coronal section; $*P < 0.04$; $n=3$). Finally, most of the cells stained with Nissl were still detectable 7, 14, and 21 days after the pHSVpkc Δ gene transfer [Fig. 2C(a)–(c), respectively], suggesting no major tissue damage as a result of the gene transfer [85,86].

3.2. Effect of Pkc Δ gene transfer on striatal GluR1 phosphorylation

Immunoblotting of rat striatal homogenate with GluR1 or P-GluR1 antibodies showed a major immunoreactive band at ~ 96 and ~ 106 kDa, respectively. Unilateral 6-OHDA-induced destruction of the nigrostriatal followed by subsequent chronic levodopa treatment augmented

P- GluR1 immunoreactivity on the lesioned side (104 ± 4 , 94 ± 3 , and $*187\pm5\%$ of the intact striatum for naïve, 6-OHDA-lesioned, and 6-OHDA-lesioned plus levodopa-treated animals, respectively; $n=3-4$; $*P<0.001$ compared with naïve and 6-OHDA-lesioned rats; Fig. 3A). The total amount of GluR1 protein in striatal homogenates following 6-OHDA lesioning and following subsequent levodopa therapy was not altered (103 ± 5 , 98 ± 3 , and $103\pm4\%$ of the intact striatum for naïve, 6-OHDA-lesioned, and 6-OHDA-lesioned plus levodopa-treated animals, respectively; $n=3-4$, Fig. 3A).

Immunohistochemical analysis of striatal tissue from 6-OHDA rats that received chronic levodopa treatment for 21 days further revealed that this enhancement of P-GluR1 immunoreactivity may result largely from the increased accumulation as well as number of P-GluR1-positive staining in striatal medium spiny neurons [Fig. 3B; frame (e) compared with frame (f)]. In 6-OHDA-lesioned animals, such enhancement in P-GluR1 immunoreactivity was not observed [Fig. 3B, frame (c) compared with (d)]. The number of P-GluR1-expressing neurons in the dorsolateral striatum was augmented in 6-OHDA-lesioned rats that received 21 days of twice-daily levodopa followed by a single levodopa challenge 30 min prior to sacrifice [423 ± 32 and 319 ± 21 neurons for the lesioned and intact sides, respectively; $P<0.05$; $n=3$; Fig. 3B(e) and (f)]. The number of striatal P-GluR1-positive neurons on the side ipsilateral to the 6-OHDA lesion did not change compared with that on the intact side in rats that did not receive 21 days of twice-daily levodopa treatment [312 ± 23 and 346 ± 29 neurons for the lesioned and intact sides, respectively; $P>0.05$; $n=3$; Fig. 3B(c) and (d)].

3.3. Effect of PkcΔ gene transfer on levodopa-induced motor response alterations

After 3 weeks of levodopa treatment, the duration of the motor response decreased by 32, 30 and 28% in control, pHSVlac and pHSVpkcΔ, respectively ($P<0.04$; Fig. 4A). While parkinsonian rats microinjected with saline or pHSVlac vector showed no significant change in response duration for the initial 2 weeks of levodopa treatment (Fig. 4A), animals that received pHSVpkcΔ vector evidenced a substantial reduction in their response duration at the end of the first week of levodopa therapy (37% decrease from levodopa day 1; $P<0.03$; Fig. 4A). In another control experiment, pHSVpkcΔ gene transfer did not alter the motor response duration in rats given twice-daily saline, instead of levodopa, injections for 21 days (102 ± 5 and 113 ± 8 for day 1 and day 22, respectively, in rats with no gene transfer; 102 ± 7 and 115 ± 6 for day 1 and day 22, respectively, in rats with pHSVpkcΔ gene transfer; $n=3-4$).

Three weeks of levodopa treatment to parkinsonian rats shortened the response duration to the acute injection of the D1-preferring agonist SKF 38393 (153 ± 14 and $92\pm9^*$ min for day 1 and day 22, respectively, $n=5$, $*P<0.001$ compared with day 1; Fig. 4B), and prolonged the response to an injection of the D2-preferring agonist quinpirole (157 ± 16 and $203\pm11^*$ min for day 1 and day 21, respectively, $n=6$, $*P<0.005$ compared with day 1; Fig. 4B). Animals that received pHSVpkcΔ gene transfer evidenced changes of similar magnitude when challenged with SKF 38393 or quinpirole by levodopa treatment day 7 ($P<0.005$; Fig. 4B). In contrast, rats that received control vector pHSVlac, when given the same D1- or D2-agonist challenge on day 7, did not manifest any significant change compared with the day 1 response (Fig. 4B). In both pHSVlac and pHSVpkcΔ groups, levodopa treatment for 21 days shortened the response duration to SKF 38393 challenge and extended the response duration to quinpirole administration, changes that were not significantly different from those occurring in saline-injected animals (Fig. 4B).

P-GluR1 immunoreactivity in striatal sections from rats that received unilateral gene transfer with pHSVpkcΔ on the lesioned side indicated increased ipsilateral phosphorylation of AMPA receptor subunit GluR1 just 8 days after gene transfer [Fig. 4C(d)–(f), 8, 15, 22 days after pHSVpkcΔ gene transfer, respectively]. On the other hand, striatal sections from rats that received unilateral gene transfer with control vector pHSVlac revealed increased

immunoreactivity against the P-GluR1 on that side only 22 days after gene transfer [Fig. 4C (a)–(c), 8, 15, 22 days of twice-daily levodopa injections, respectively].

Striatal P-GluR1 (S831)-positive cells co-stained with flag antibody appeared much more pronounced in pHSVpkcΔ-infected animals than in animals infected with control vector pHSVlac 8 and 15 days following gene transfer. Immunohistochemical double staining with antibodies against flag [Fig. 4D(a)] and P-GluR1 [S831; Fig. 4D(b)] confirmed that most flag-positive cells also contained P-GluR1 immunoreactivity [Fig. 4D(b)]. Similarly, striatal NeuN-positive cells co-stained with flag antibody appeared much more pronounced in pHSVpkcΔ-infected animals than in animals infected with control vector pHSVlac 8 and 15 days following gene transfer. Immunohistochemical double staining with antibodies against flag [Fig. 4D(c)] and NeuN [Fig. 4D(d)] showed that most flag-positive cells also contained NeuN immunoreactivity [Fig. 4D(d)].

3.4. Effects of PKC inhibition on motor responses to levodopa and striatal GluR1 phosphorylation

The intrastriatal injection of the PKC inhibitor NPC-15437 (1.0 μg), but not of saline, reversed the shortened response duration to levodopa occurring in rats given the pHSVpkcΔ gene after just 7 days of levodopa treatment ($n=4$, $P<0.04$, Fig. 5A, pHSVpkcΔ). In control rats that received saline, instead of NPC-15437, on day 8, the levodopa-induced response shortening remained unchanged (113 ± 10 , $75\pm5^*$, and $80\pm5^*$ min for day 1, 7 and 8, respectively; $*P<0.02$ compared with day 1; $n=4$).

On the other hand, in rats that did not receive pkcΔ gene transfer but were only treated with levodopa for 21 days, the same dose of NPC-15437 (1.0 μg) had no effect on the levodopa-induced response shortening ($n=4$; Fig. 5A, no pHSVpkcΔ). A higher dose of NPC-15437 (5.0 μg) was similarly ineffective in reversing the levodopa-induced response shortening in these animals (78 ± 6 min; $n=6$; $P<0.01$ compared with day 1). Similarly, in control rats that received saline on day 22, the levodopa-induced response shortening was not altered (112 ± 6 , 80 ± 5 and 82 ± 6 min for day 1, 21, and 22, respectively; $*P<0.05$ compared with day 1; $n=4$).

The intrastriatal infusion of NPC-15437 prior to acute levodopa administration also diminished the enhanced serine phosphorylation of striatal GluR1 receptors induced by pHSVpkcΔ gene transfer ($110\pm6\%$ of contralateral side, $n=3$; $P<0.001$ compared with pHSVpkcΔ animals that received vehicle infusion; Fig. 5B). The total amount of GluR1 protein in striatal homogenates following the intrastriatal infusion of NPC-15437 was not changed (108 ± 5 and $104\pm5\%$ of contralateral side for intrastriatal vehicle and NPC injection, respectively; $n=3$, Fig. 5B). The attenuation by NPC-15437 of the enhanced serine phosphorylation of GluR1 (Fig. 5B) occurred at a dose (1.0 μg) that was effective in reversing the shortened motor response to levodopa. However, the striatal administration of vehicle prior to levodopa injection on levodopa treatment day 8 failed to diminish augmented striatal P-GluR1 receptor immunoreactivity ($185\pm5\%$ of intact side; $n=3$; $P<0.001$; Fig. 5B).

4. Discussion

Both genetic and pharmacologic interventions were employed in this study to evaluate the participation of striatal PKC-mediated signaling in the pathogenesis of the response changes attending levodopa treatment of parkinsonian rats. The results suggest that PKC augmentation as a consequence of the intrastriatal transfer of pHSVpkcΔ can hasten the onset of the characteristically shortened motor response duration produced by levodopa therapy. In animals that received the pHSVpkcΔ gene, it took just 1 week of treatment to develop the degree of shortening in response duration ordinarily associated with 3 weeks of levodopa treatment. The ability of the intrastriatal gene transfer of pHSVpkcΔ to accelerate the initial appearance of

levodopa-induced changes in motor response appeared specific. The gene transfer failed to elicit a motor response or to modify the magnitude of the response in control rats that had received chronic saline treatment. Moreover, alterations in PKC signaling appeared to occur in both D1- and D2-expressing medium spiny neurons, since pHSVpkcΔ gene transfer affected the motor response both to SKF 38393 and to quinpirole challenge after 1 week of levodopa therapy. However, since the motor response to D1 and D2 agonists can be modified as a result of downstream changes from the striatum, it is also possible that only D1-, D2-, or interneurons were directly affected by PKC gene transfer. Our results further suggest that a CMV promoter-induced genetic intervention strategy [86] making use of a HSV-1 helper virus-free vector system [28,37,74,77,82,83,85,86] can support long-term (up to 3 to 4 weeks in our study) striatal gene expression, without producing substantial cytopathic effects as revealed by the Nissl staining profile [26,69]. As reported in previous studies, the helper virus-free vectors efficiently infected rat neural cells in the brain with minimal immune or inflammatory responses [26,69,74]. This is similar to that previously demonstrated with TH promoter-induced gene expression in the substantia nigra [69] as well as with NFH promoter-induced transfection in the striatum [74], and could thus prove useful in future studies of basal ganglia function [28,86].

PkcΔ is analogous to constitutively active PKCs and has structural and functional homology to the naturally occurring catalytic domain of PKCs produced by calpain [65]. Moreover, PkcΔ and rat brain PKC exhibit similar substrate specificities, and the catalytic domains of all PKC isoforms are highly homologous and have a comparable profile of substrate interactions [76]. In the present experiments, PkcΔ, as revealed by flag immunostaining, was detected in the cell bodies and in some processes of striatal cells that met histological criteria for medium sized spiny neurons [23,74]. Since most cells were found to co-label with flag and a neuronal marker NeuN [27,86], the flag-positive cells are probably the medium spiny neurons which account for more than 90% of striatal neuronal cells [29,66]. On the other hand, since not all of the NeuN- positive cells appeared to co-localize with flag, transfection of striatal cells other than medium spiny neurons may have occurred.

PkcΔ accumulation by these neurons presumably contributed to the observed changes in levodopa-associated cellular and motor plasticity. Manipulation of signal transduction pathways involving PKC in a relatively restricted striatal region by local gene transfer thus appears to be effective in modulating motor response changes characteristically associated with chronic dopaminomimetic therapy of parkinsonian animals. The presence of PkcΔ in a relatively small percentage (~1.0%) of striatal neurons, of which most are GABA-ergic medium spiny neurons, appears sufficient to alter levodopa-induced motor response. Consistent with this view, the microinfusion of certain kinase inhibitors such as Rp-cAMPS and KN-93 in the dorsolateral striatum also substantially altered motor behavior in levodopa-treated hemiparkinsonian animals [55,57]. Perhaps, because the transfected cells are clustered into groups (see Fig. 4C), a relatively small number can be sufficient to produce both the localized changes in striatal GluR1 phosphorylation and the alteration in levodopa-induced motor response. Similar results have been observed in apomorphine-induced rotational behavior in rats following direct gene transfer of PkcΔ into nigrostriatal neurons [69,74,86].

Increasing evidence now suggests that an augmentation in the sensitivity of AMPA receptors may contribute to the pathogenesis of the characteristic motor response alterations associated with levodopa treatment [17,38,45,47]. The present study suggests that the observed effect of PkcΔ gene transfer on motor responses may, in part, be related to an increase in the phosphorylation state of striatal AMPA receptors. Immunoblotting of striatal homogenates from animals receiving Pkc gene transfer revealed a major immunoreactive band at 106 kDa, previously identified as P-GluR1 subunits [64]. Our immunohistochemical results further indicated that the increase in P-GluR1 immunoreactivity occurred only in flag-positive medium

spiny neurons. Since the synaptic efficacy of AMPA receptors is affected by the phosphorylation state of its serine residues [30,43,81], it is reasonable to assume that the increased serine phosphorylation of GluR1 subunits by Pkc Δ observed here may have contributed to enhancing the synaptic strength of glutamatergic inputs to the striatum. Thus it is not inconceivable that the PKC-mediated phosphorylation of an AMPA receptor subunit acts to accelerate the onset of these altered motor responses. In further support of this possibility, we found that PKC inhibition by NPC-15437 attenuated both the shortened response duration and the GluR1 subunit phosphorylation increase produced by 1 week of levodopa treatment in rats that had received Pkc Δ gene transfer. Moreover, nigrostriatal denervation and subsequent levodopa treatment augmented striatal P-GluR1 immunoreactivity. Since there was no associated change in basal protein concentrations of GluR1 following 6-OHDA lesioning and subsequent chronic levodopa treatment, the observed serine phosphorylation increase could not have occurred on this basis.

On the other hand, since pkc Δ can have multiple other effects on striatal neuron function, the possibility that factors other than AMPA receptor-mediated transmission contribute to the development of levodopa-associated motor response changes cannot be excluded. In this regard, three major PKC substrates, growth associated proteins (GAP)-43/B-50, neurogranin RC3 and the myristoylated alanine-rich C kinase substrate (MARCKS) [62], as well as PKC-mediated phosphorylation of NMDA receptor channels [72], have been linked to activity-dependent changes in CNS synaptic plasticity. In terms of NMDA channel receptors, activation of PKA, CaMKII and tyrosine kinase signaling cascades in association with an enhanced phosphorylation of NMDA receptor subunits in rat striatal neurons has also been implicated in the development of the altered responses associated with levodopa therapy [4,8,15]. The present results add to the converging behavioral, biochemical, and pharmacologic evidence suggesting that changes in the phosphorylation state of S831 serine residues on striatal GluR1 receptor subunits may, in part, be involved in the initial expression of motor response alterations associated with levodopa administration.

In contrast to our gene transfer data showing that early onset of motor response alterations is favored by the activation of striatal PKC signaling, a PKC-mediated mechanism does not appear to be essential for the later expression of the motor response alterations associated with chronic levodopa treatment in the absence of PKC gene transfer. Following 3 weeks of levodopa administration, striatal PKC inhibition by NPC-15437 failed to attenuate the shortened response duration occurring in parkinsonian animals that did not receive pHSVpkc Δ gene transfer. In this case, PKC-independent mechanisms, such as those involving PKA, CaMKII [1,2,39,54,55,57,67] or tyrosine kinase [56], may be primarily responsible for the long-term maintenance of the motor response changes associated with levodopa therapy as well as for the enhanced phosphorylation of GluR1. Involvement of striatal cells other than medium spiny neurons, including cholinergic interneurons [7], also cannot be excluded. Nevertheless, the weight of current evidence suggests that an abundance of constitutively active PKC in the striatum may be sufficient to promote the initial appearance of levodopa-induced motor response alterations.

In relation to P-GluR1 involvement, twice-daily treatment with levodopa as well as with PKC gene transfer increased the phosphorylation of this receptor. This suggests that AMPA receptor alterations induced by chronic, intermittent, levodopa treatment and the concomitant motor response alterations are PKC dependent. The foregoing observations, together with increasing evidence demonstrating that the characteristic motor response alterations associated with levodopa therapy are sensitive to AMPA antagonists [38,45,47], suggest that the striatal PKC-dependent activation of AMPA receptors contributes to the expression of this phenomenon. However, a full understanding of the causal and functional relationships between the striatal AMPA receptor changes and the motor response alterations requires further investigation.

In conclusion, while PKC activation in striatal spiny neurons by PkcΔ gene transfer in association, in part, with a rise in AMPA GluR1 subunit phosphorylation appears sufficient to cause the early onset of levodopa-induced response alterations, PKC-mediated mechanisms do not appear necessary for the ultimate expression of these motor response changes. The contribution of AMPA receptor regulation changes to the initiation of levodopa-induced motor response alterations is not inconsistent with earlier findings demonstrating the beneficial effects of AMPA receptor antagonists in parkinsonian rodent and non-human primate models of PD [38,46,47,80] and suggests that the pharmaceutical targeting of striatal AMPA-mediated mechanisms could prove useful in the treatment of this disorder.

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Fig. 1.

(A) Schematic representation of coronal section from rat brain with gene transfer with pHSVlac vector, indicating approximate area of striatal injection [A: +0.7 from bregma; panel A(a)]. The coordinate represents the largest injected area upon examining the corresponding brain sections after X-gal histological procedures. The framed area in panels (a) and (b) denotes the approximate position of the photographs in Fig. 1B, Fig. 2B and C, Fig. 3B, and Fig. 5C and D. Frames (b) and (c) are high-power photomicrographs of β -gal-positive somata in the striatum. Observe the perikaryal X-gal-positive reaction products as well as the predominant presence of label in proximal processes. Scale bars in panels (b) and (c) equal 100 and 25 μ m, respectively. AC, anterior commissure; CC, corpus callosum; HDB, horizontal limb of the diagonal band of Broca; MS, medial septum; Par, parietal cortex; RS, retrosplenial cortex; Str, striatum; VDB, vertical limb of the diagonal band of Broca. (B) High-power photomicrographs of X-gal-stained cells with striatal neuronal morphology at 4, 8, 15, and 22 days [(a)–(d)] after gene transfer with pHSVlac vector stock. X-gal-positive somata staining on the injection side (ipsilateral side to the 6-OHDA lesion) was evident in animals 4, 8, 15, and 22 days following gene transfer. X-gal-positive processes staining was evident until 15 days, but was substantially diminished 22 days after pHSVlac gene transfer. Scale bar in panel (a) equals 100 μ m and applies also to frames (b), (c), and (d).

Fig. 2.

(A) Western blot analysis of PKC β II (top panel) and tyrosine hydroxylase (TH; bottom panel) in hemiparkinsonian rat striatum 8 days after pHSVpkc Δ vector microinjection into striatum. Aliquots of striatal tissue from rats treated with levodopa for 7 days following gene transfer were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblotting analysis with antibodies against PKC β II. Lanes 1–2, 3–4, 5–6: extracts from striatal tissue receiving intrastratial saline vehicle, pHSVlac, or pHSVpkc Δ gene transfer, respectively. A total of 14 μ g of protein was loaded per lane. I, striatum ipsilateral to the lesion; C, striatum contralateral to the lesion. (B) Flag-immunopositive cells at 8, 15, and 22 days [(a) and (d); (b) and (e); (c) and (f), respectively] after striatal gene transfer with pHSVpkc Δ . Flag-positive somata and processes staining on the injection side (ipsilateral side to the 6-OHDA lesion) was evident in animals 8, 15, and 22 days following gene transfer. The framed area in panels (a), (b), and (c) denotes the approximate position of the photographs in panels (d), (e), and (f), respectively. Frames (d), (e), and (f) are enlarged photomicrographs of panels (a), (b), and (c), respectively. Scale bar in frame (a) equals 100 μ m and also applies to frames (b) and (c). Scale bar in frame (d) equals 50 μ m and also applies to frames (e) and (f). I, striatum ipsilateral to the lesion. (C) Nissl-stained cells at 8, 15, and 22 days [(a)–(c)] after striatal gene transfer with pHSVpkc Δ . Nissl-positive somata around the injection site (ipsilateral side to the 6-OHDA lesion) were evident in animals 8, 15, and 22 days following gene transfer. The framed area in panels (a), (b), and (c) denotes the approximate position of the photographs in panels (d), (e), and (f), respectively. Frames (d), (e), and (f) are scaled-up photomicrographs of panels (a), (b), and (c), respectively. Scale bar in frame (a) equals 200 μ m and also applies to frames (b) and (c). Scale bar in frame (d) equals 100 μ m and also applies to frames (e) and (f). I, striatum ipsilateral to the lesion.

Fig. 3.

(A) Western blot analysis with P-GluR1 (top panel), GluR-1 (middle panel), and tyrosine hydroxylase (TH; bottom panel) of striatal homogenates from control naïve (lanes 1 and 2), 6-OHDA (lanes 3 and 4), and 6-OHDA-lesioned plus levodopa-treated (lanes 5 and 6) rats. Unilateral 6-OHDA lesioning and subsequent levodopa treatment increased P-GluR1 immunoreactivity on the lesioned side (I) of rat striatum. A total of 14 μ g of protein was loaded per lane. I, striatum ipsilateral to the lesion; C, striatum contralateral to the lesion. (B) Photomicrographs of P-GluR1-positive neurons in the striatum of control naïve [panels (a) and (b)], 6-OHDA [panels (c) and (d)], and 6-OHDA-lesioned plus levodopa-treated [panels (e) and (f)] rats. Unilateral 6-OHDA lesioning and subsequent levodopa treatment increased P-GluR1 immunoreactivity on the lesioned side (I) of rat striatum. Photographs were taken from coronal sections near the site of injection. P-GluR1-positive neurons appeared substantially elevated on the 6-OHDA-lesioned side in animals receiving chronic levodopa treatment. Scale bar in frame (e) equals 100 μ m and also applies to all frames [(a)–(d) and (f)]. I, striatum ipsilateral to the lesion; C, striatum contralateral to the lesion.

Fig. 4.

(A) Time course of mean rotational response duration ($\pm$ S.E.M.; $n=4-6$) of hemiparkinsonian rats to acute levodopa challenge (25 mg/kg with 5.0 mg/kg benserazide, i.p.) that received intrastratial saline (●), control vector (pHSVlac; ○), or pHSVpkcΔ (▲) 1 day prior to initiation of chronic twice-daily levodopa treatment (25 mg/kg with 5.0 mg/kg benserazide, i.p., twice daily). Acute levodopa-induced rotational behavior was measured 1, 3, 7, 14, 21, and 28 days following levodopa treatment. * $P<0.04$ and # $P<0.03$ compared with levodopa day 1 and day 3 animals, respectively. ^a $P<0.03$ and ^b $P<0.03$ compared with saline and control vector group, respectively. (B) Rotational response of rats to D1 (lightly hatched) and D2 (darkly hatched) dopamine agonist following gene transfer. One day prior to levodopa treatment day 1, control vector (pHSVlac; top panel), or pHSVpkcΔ (bottom panel) was injected into the striatum ipsilateral to the 6-OHDA lesion. Mean rotation duration ($\pm$ S.E.M.; $n=5$) to dopamine agonists is compared on days 1, 7, and 21 of levodopa treatment after a 3 day drug washout. * $P<0.04$ and # $P<0.02$ compared with chronic levodopa day 1 and day 7, respectively. (C) P-GluR1 immunostaining in the striatum of animals 8, 15, and 22 days after gene transfer with pHSVlac [(a)–(c)] or pHSVpkcΔ [(d)–(f)] vectors. An increased accumulation of P-AMPA-R staining on the injected side was evident at 8, 15, and 22 days after intrastratial infection with pHSVpkcΔ [panels (d)–(f), respectively]. An increased accumulation of P-GluR1 staining on the injected side was evident only at 22 days after intrastratial infection with pHSVlac [panel (c)]. Intrastratial microinjections of control vector pHSVlac failed to enhance the phosphorylation of striatal AMPA receptors at levodopa treatment day 8 or 15 [panels (a) and

(b), respectively]. Peak immunostaining with P-GluR1 correlated with the appearance of shortening of response duration associated with chronic levodopa treatment. Scale bar in panel (f) is 100 μm and also applies to (a)–(e). (D) Phosphorylated GluR1-immunopositive neurons (FITC) co-stained with antibody against flag (Texas Red) in hemiparkinsonian rats 8 days after gene transfer with pHSVpkc Δ . Viral stock was microinjected into the striatum and 8 days later striatal sections were co-stained for P-GluR1 and flag antibody [panels (a) and (b), respectively]. Filled arrows (b) indicate immunostained P-GluR1 cells without flag immunoreactivity, and open arrows (a,b) indicate striatal neuronal profiles co-stained with P-GluR1 and flag antibody produced by pHSVpkc Δ gene transfer. Flag-positive neurons (FITC) co-stained with antibody against NeuN (Texas Red) in hemiparkinsonian rats 8 days after gene transfer with pHSVpkc Δ . Viral stock was microinjected into the striatum and 8 days later striatal sections were co-stained for flag and NeuN antibody [panels (c) and (d), respectively]. Filled arrows [panel (d)] indicate immunostained NeuN cells without flag immunoreactivity, and open arrows [panels (c) and (d)] indicate striatal neuronal profiles co-stained with flag and NeuN antibody produced by pHSVpkc Δ gene transfer. Scale bar, 200 μm .

Fig. 5.

(A) Mean rotational response duration ($\pm$ S.E.M.) to acute levodopa (20 mg/kg with 5.0 mg/kg benserazide, i.p.) challenges in 6-OHDA-lesioned rats that received pHSVpkc Δ gene transfer on day 1 and treated with levodopa for 7 days (gray bars; pHSVpkc Δ) or 6-OHDA-lesioned animals that did not receive the gene transfer but were treated with levodopa for 21 days (gray bars; no pHSVpkc Δ). On day 8 or day 22 (darkly stippled bars), 1.0 μ g NPC-15437 was administered intrastrially, ipsilateral to the nigrostriatal system lesion, immediately prior to levodopa challenge. NPC-15437 attenuated motor response alterations associated with Pkc Δ gene transfer plus 7 days levodopa therapy (pHSVpkc Δ), but not with just 21 days levodopa therapy (no pHSVpkc Δ). * P <0.05 and # P <0.04 compared to levodopa treatment day 1 and day 7, respectively. (B) P-GluR 1 (top panel) and GluR1 (bottom panel) Western blot analysis of striatal homogenate samples from the vehicle-treated (lanes 1 and 2) or NPC-treated (1.0 μ g; lanes 3 and 4) animals 5 min prior to acute levodopa challenge. Enhanced serine phosphorylation of striatal GluR1 associated with pHSVpkc Δ gene transfer in rats that received levodopa treatment for 7 days (lanes 1 and 2) was attenuated by intrastrially administered NPC-15437 (lanes 3 and 4). The bar graph represents optical density quantified and expressed as percent of intact striatum for bands corresponding to P-GluR1 on Western blot. For each panel: I, striatum ipsilateral to the lesion; C, striatum contralateral to the lesion. * P <0.001 compared to striatal vehicle on levodopa day 8.