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Effects of conantokins on L-3,4-dihydroxyphenylalanine-induced behavior and immediate early gene expression

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Abstract

Conantokins, peptides from *Conus* snails, are *N*-methyl-D-aspartate (NMDA) receptor antagonists. NMDA receptor antagonists potentiate L-3,4-dihydroxyphenylalanine (L-DOPA)-induced rotation in 6-hydroxydopamine-treated rodents, an index of anti-Parkinsonian potential. This study examined the effects of conantokin-G, conantokin-T(G), CGS 19755, and ifenprodil on L-DOPA-induced contralateral rotation and immediate early gene (IEG) expression in 6-hydroxydopamine-treated rats. Rats received unilateral infusions of 6-hydroxydopamine into the medial forebrain bundle. Three weeks later, rats were treated with an NMDA receptor antagonist, followed by an injection of L-DOPA. Contralateral rotations were recorded for 2 h. In addition, the expression of *zif268* and c-*fos* were examined. Conantokin-G, conantokin-T(G), and CGS 19755 potentiated L-DOPA-induced rotation. Conantokin-G and ifenprodil had no effect on L-DOPA-induced IEG expression, whereas conantokin-T(G) and CGS 19755 attenuated expression. These data suggest that conantokins may be useful in treating Parkinson's disease. Furthermore, different NMDA receptor antagonists have distinct effects on striatal gene expression.

Keywords: c-*fos*; *zif268*; 6-Hydroxydopamine; *N*-methyl-D-aspartate (NMDA); L-3,4-dihydroxyphenylalanine (L-DOPA); Striatum

1. Introduction

Parkinson's disease is a neurodegenerative disorder afflicting half a million people in the US alone. Parkinson's disease is characterized by a loss of dopamine input to the medium spiny neurons of the striatum from the substantia nigra pars compacta (Hornykiewicz et al., 1968). Currently, L-3,4-dihydroxyphenylalanine (L-DOPA) is the most frequently used therapy for treating the symptoms of Parkinson's disease. However, loss of efficacy (wearing-off phenomenon) and potential side effects after continued use of L-DOPA have created a situation in which novel therapeutic strategies need to be developed.

Cortical glutamate afferents to medium spiny neurons of the striatum may also be involved in the pathophysiology of Parkinson's disease. For example, an increase in binding to the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor has been observed in dopamine-depleted animals (Ulas et al., 1994). Furthermore, Meshul et al. (1999) have reported increases in basal extracellular levels of glutamate after 6-hydroxydopamine administration, which are correlated with an initial decrease in the density of glutamate immunolabeling in nerve terminals. It has, therefore, been suggested that agents altering glutamate activity may be useful in the treatment of Parkinson's disease (Greenamyre and O'Brien, 1991).

Although glutamate antagonists administered alone have not proven useful in treating Parkinsonian symptoms in animal models of the disease (Starr, 1995a,b for review), glutamate antagonists may be useful adjuncts to L-DOPA or other dopamine receptor agonists (Boldry et al., 1993; Loschmann et al., 1991; Morelli et al., 1992). The 6-hydroxydopamine-treated rodent is a commonly used model of Parkinson's disease. Animals with unilateral dopamine depletions rotate contralateral to the lesioned side after administration of "anti-Parkinsonian" compounds such as L-DOPA (Ungerstedt, 1971). Antagonists of the NMDA receptor have been shown to potentiate the behavioral effects of L-DOPA in animal models of Parkinson's disease, including the potentiation of L-DOPA-induced contralateral rotation in 6-hydroxydopamine-treated rats (Morelli et al., 1992; Starr, 1995b).

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Conantokins are a novel class of peptides (17–25 amino acids) from *Conus* snails that are NMDA receptor antagonists (Hammerland et al., 1992; Mena et al., 1990). This study was designed to test whether two conantokins, conantokin-G and conantokin-T(G), would potentiate L-DOPA-induced contralateral rotation in 6-hydroxydopamine-treated rats. In addition, the effect of the conantokins on L-DOPA-induced expression of the immediate early genes (IEGs), c-*fos* and *zif268*, was also examined.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 225–300 g were used in all experiments. Rats were housed in groups of four in hanging wire-mesh cages in a temperature-controlled room on a 12:12 light/dark cycle. Rats had free access to food

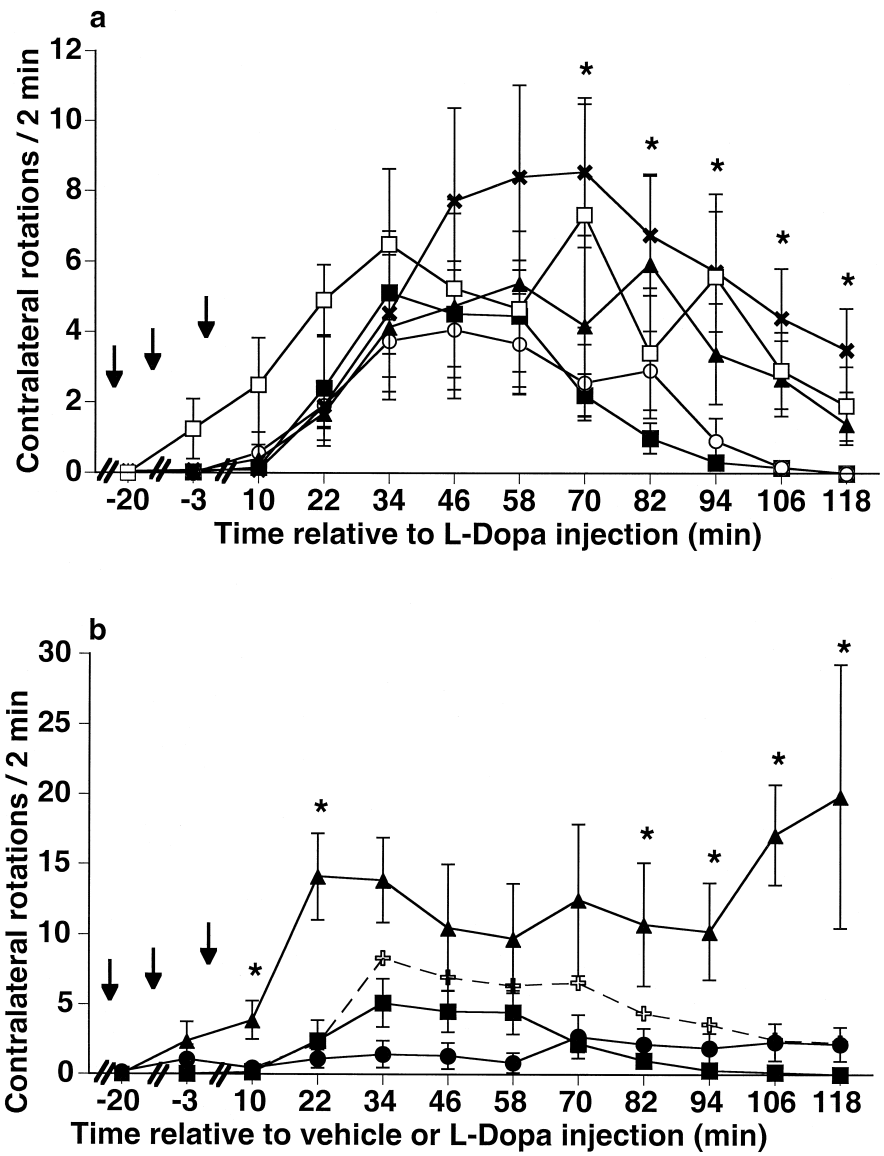

Fig. 1. (a) Effects of i.c.v. administration of conantokin-G (Con-G; ○, 0.05 mM, $n = 6$; ▲, 0.1 mM, $n = 10$; ×, 0.5 mM, $n = 11$; □, 5.0 mM, $n = 6$) on contralateral rotation induced by systemic administration of L-DOPA to dopamine-depleted rats. L-DOPA was administered 15 min after infusion of Con-G (2 μl/2 min). Rats receiving L-DOPA alone (■, 4 mg/kg i.p., $n = 16$) received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. (b) Comparison of the effects of administration of Con-G alone (●, 0.1 mM, $n = 9$), L-DOPA alone (■, 4 mg/kg, $n = 16$), and Con-G + L-DOPA (▲, 0.1 mM and 4 mg/kg, respectively, $n = 6$) on contralateral rotation in dopamine-depleted rats. The dashed line (+) represents how the data would appear if the administration of Con-G and L-DOPA produced an additive effect. Control rats, which received an infusion of aCSF followed 15 min later by an injection of vehicle solution, did not rotate at any time point (data not shown). All rats were sacrificed 2 h after L-DOPA or vehicle administration. Numbers on the *x*-axis indicate time relative to the administration of L-DOPA or vehicle (min). Arrows from left to right indicate the time of injection of benserazide, conantokin-G or aCSF, and L-DOPA or vehicle, respectively. The number of contralateral rotations/2 min is indicated for each time point (±S.E.M.). * Significantly different from L-DOPA alone (a) or L-DOPA alone and Con-G alone (b), $p < 0.05$.

and water. Studies were approved by the Institutional Animal Care and Use Committee at the University of Utah, and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

CGS 19755 was generously donated by Ciba-Geigy (Summit, NJ). Conantokins-G and T(G) were supplied by Cognetix (Salt Lake City, UT). Desmethylimipramine, sodium pentobarbital, 6-hydroxydopamine hydrobromide, (*R*)-apomorphine hydrochloride, benserazide hydrochloride, L-DOPA methyl ester hydrochloride, and ifenprodil tartrate were obtained from Research Biochemicals International (Natick, MA). All doses were calculated as free bases. Desmethylimipramine, sodium pentobarbital, and ifenprodil were dissolved in deionized water, CGS 19755 in phosphate buffered saline, and the conantokins in artificial cerebrospinal fluid (aCSF). The aCSF consisted of (final concentrations): NaCl (144 mM), KCl (2 mM), KH_2PO_4 (0.4 mM), $CaCl_2$ (1.2 mM), and $MgCl_2 \cdot 6H_2O$ (1.2 mM). All other drugs were dissolved in 0.02% ascorbic acid/0.9% NaCl.

2.3. Dopamine depletions and guide cannula implantation

Rats were pretreated with the norepinephrine uptake inhibitor desmethylimipramine (25 mg/kg, i.p.), anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and placed in a stereotaxic instrument. A 31-gauge needle connected to a 25-μl Hamilton syringe on a syringe pump (Instech Model 2000) was lowered into the right medial forebrain bundle at the following coordinates relative to bregma (in millimeter): AP-4.0, ML-1.5, and DV-8.5. The neurotoxin 6-hydroxydopamine was dissolved in 0.02% ascorbic acid/0.9% saline (8 μg/2 μl) and was infused at a rate of 0.4 μl/min for 5 min. The needle was left in place for an additional 5 min after the infusion. Immediately following the infusion of 6-hydroxydopamine, a 26-gauge guide cannula (Plastics One, Roanoke, VA) was lowered into the right lateral ventricle at the following coordinates relative to bregma (in millimeter): AP-0.3, ML-1.2, and DV-3.5. Cranioplastic cement and cranial screws were used to fix the guide cannula to the skull. A dummy cannula was tightened onto the guide cannula in order to prevent post-operative damage or cannula blockage.

2.4. Apomorphine prescreening

Approximately 2.5 weeks after injection of 6-hydroxydopamine and guide cannula implantation, rats were prescreened with the mixed dopamine agonist apomorphine (0.05 mg/kg, s.c.). Rats turning contralateral to the lesioned side after apomorphine administration were used for subsequent experiments.

2.5. Pharmacological manipulations and behavioral testing

Four days after the apomorphine screening, animals were taken from their home cages and placed individually in cylindrical Plexiglass tubs (11″ diameter, 15″ high). The peripheral decarboxylase inhibitor benserazide (25 mg/kg,

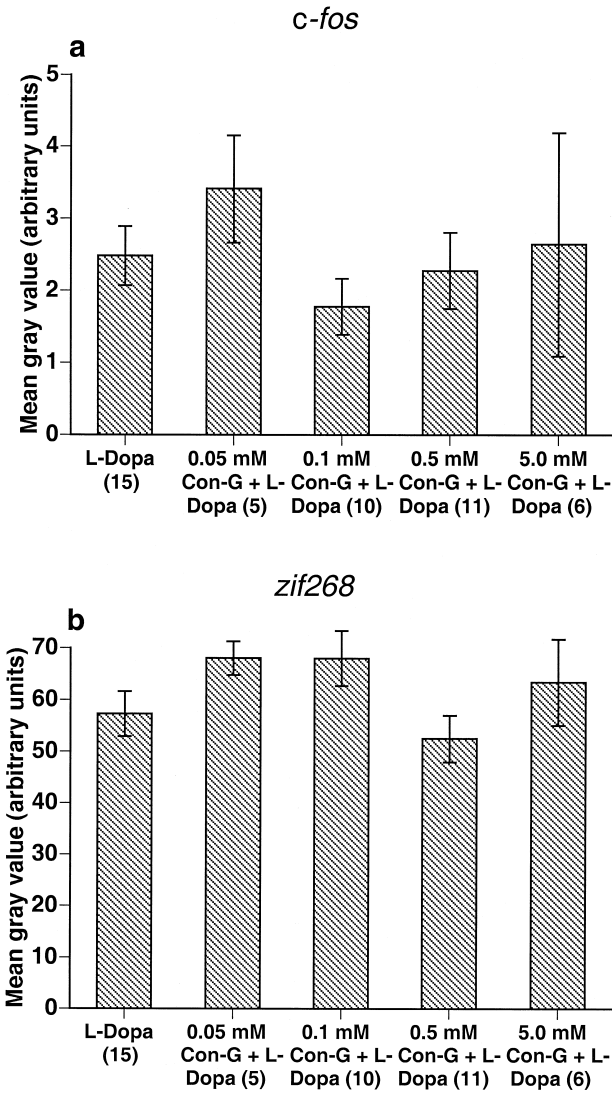

Fig. 2. Effects of i.c.v. administration of conantokin-G (Con-G; 0.05–5.0 mM) on c-*fos* (a) and *zif268* (b) expression induced in mid-striatum (approximately 0.5 mm anterior to bregma) by systemic administration of L-DOPA (4 mg/kg, i.p.) to dopamine-depleted rats. L-DOPA was administered 15 min after infusion of Con-G (2 μl/2 min). Rats receiving L-DOPA alone received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. All rats were sacrificed 2 h after L-DOPA administration. Values are mean gray values (±S.E.M.; arbitrary units) measured in the medial third of the striatum. Data obtained from the central and lateral thirds of the striatum did not differ from those presented here (data not shown). Numbers in parentheses indicate the number of animals/group.

i.p.) was administered 15 min prior to intracerebroventricular (i.c.v.) infusion of an NMDA receptor antagonist. Rats were hand held while the experimenter replaced the dummy cannula with a 33-gauge infusion cannula (1.5 mm projection from the bottom of the guide) connected to a fluid swivel and a 25-μl Hamilton syringe on a syringe pump. The syringe and connecting tubing were backfilled with aCSF or the appropriate NMDA receptor antagonist. The aCSF or the NMDA receptor antagonist was infused through the infusion cannula at a rate of 1 μl/min for 2 min. The concentrations of the NMDA receptor antagonists were 50 μM–5 mM conantokin-G, 500 μM–5 mM conantokin-T(G), 22.4 and 224 μM CGS 19755, and 5.0 μM–25 mM ifenprodil. The infusion cannula was replaced with the dummy cannula after the infusion. Fifteen minutes after the i.c.v. infusion, L-DOPA methyl ester (4 mg/kg, i.p.) was administered. Control animals received vehicle injections instead of benserazide and L-DOPA and an i.c.v.

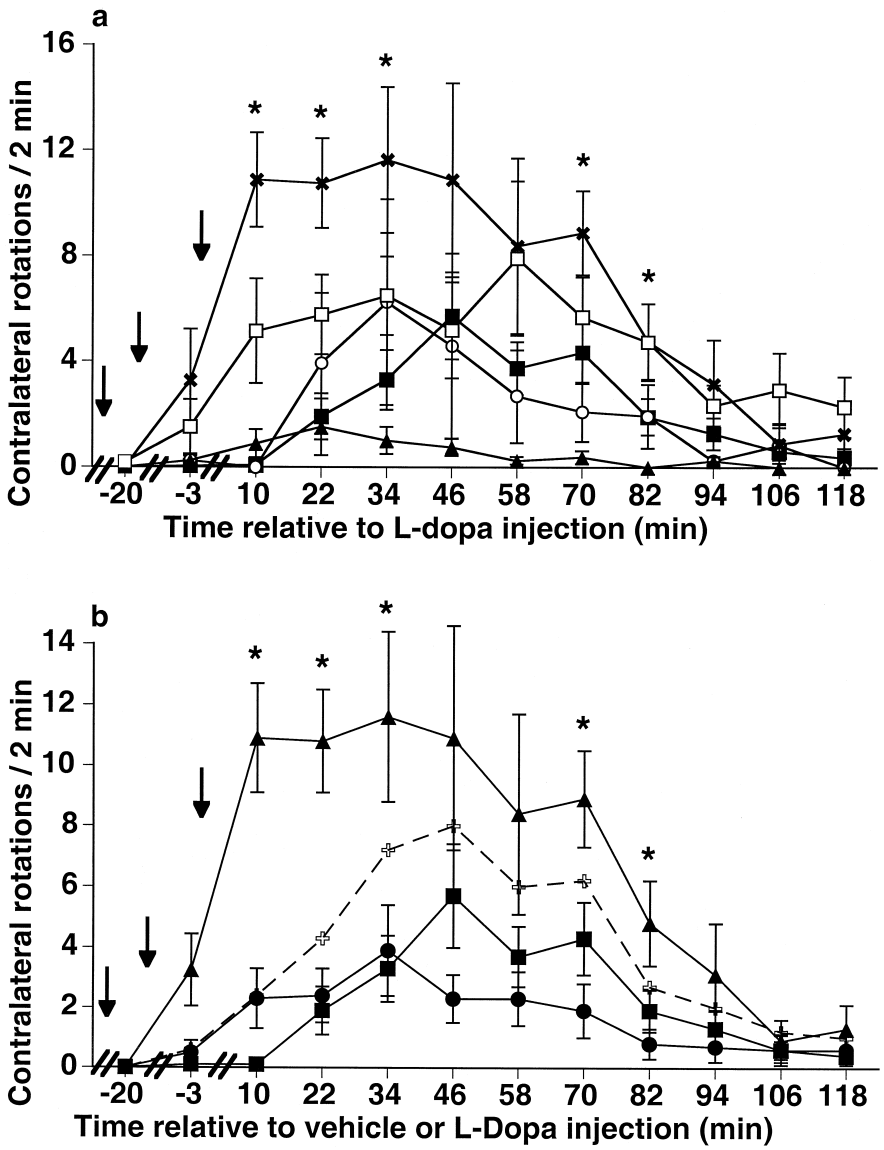

Fig. 3. (a) Effects of i.c.v. administration of conantokin-T(G) (Con-T(G); ○, 0.5 mM, $n = 6$; ▲, 1.0 mM, $n = 4$; ×, 2.5 mM, $n = 4$; □, 5.0 mM, $n = 11$) on contralateral rotation induced by systemic administration of L-DOPA to dopamine-depleted rats. L-DOPA was administered 15 min after infusion of Con-T(G) (2 μl/2 min). Rats receiving L-DOPA alone (■, 4 mg/kg i.p., $n = 19$) received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. (b) Comparison of the effects of administration of Con-T(G) alone (●, 2.5 mM, $n = 9$), L-DOPA alone (■, 4 mg/kg, $n = 19$), and Con-T(G) + L-DOPA (▲, 2.5 mM and 4 mg/kg, respectively, $n = 4$) on contralateral rotation in dopamine-depleted rats. The dashed line (+) represents how the data would appear if the administration of Con-T(G) and L-DOPA produced an additive effect. Control rats, which received an infusion of aCSF followed 15 min later by an injection of vehicle solution, did not rotate at any time point (data not shown). All rats were sacrificed 2 h after L-DOPA or vehicle administration. Numbers on *x*-axis indicate time relative to the administration of L-DOPA or vehicle (min). Arrows from left to right indicate the time of injection of benserazide, conantokin-T(G) or aCSF, and L-DOPA or vehicle, respectively. The number of contralateral rotations/2 min is indicated for each time point (±S.E.M.). * Significantly different from L-DOPA alone (a) or L-DOPA alone and Con-T(G) alone (b), $p < 0.05$.

infusion of aCSF. Animals receiving an infusion of the NMDA receptor antagonist alone received systemic injections of vehicle instead of benserazide and L-DOPA.

Contralateral and ipsilateral rotations were counted for 2 min before the i.c.v. infusion and before L-DOPA administration (precounts 1 and 2). After the L-DOPA or vehicle injection, rotations were recorded for 2 min, every 10 min, for 2 h. The rotations were counted by the experimenter (A.A.) as 180° turns and then corrected to full rotations. Although ipsilateral rotations were counted, no significant effects were found (data not shown).

2.6. In situ hybridization histochemistry

Immediately following the final 2-min rotation count, rats were euthanized by exposure to CO_2 (1 min) and decapitated. The brains were rapidly removed and frozen in isopentane chilled on dry ice. Brains were stored at −20°C until they were cut in 12-μm thick sections in a cryostat (Cryocut 1800, Cambridge Instruments, Germany) and thaw-mounted onto gelatin-chrome alum-subbed slides. Slides were stored at −20°C. Once all brains from an experiment were sectioned, slides were post-fixed in 4% paraformaldehyde/0.9% NaCl, acetylated in fresh 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0), dehydrated in an ascending series of alcohols, delipidated in chloroform, and rehydrated in a descending series of alcohols. Slides were air-dried and stored at −70°C.

For detection of c-*fos* and *zif268* mRNAs, full-length ribonucleotide probes complementary to the mRNAs for c-*fos* (Curran et al., 1987) and *zif268* (Milbrandt, 1987) were synthesized from the cDNAs using ^{35}S-UTP and SP6 (c-*fos*) or T7 (*zif268*) RNA polymerase (Boehringer Mannheim, Indianapolis, IN). Labeled probes were diluted in hybridization buffer to obtain 2×10^6 cpm/100 μl buffer. The ribonucleotide probe was mixed with nuclease-free water and RNA mix (final concentrations: 100 μg/ml salmon sperm DNA; 250 μg/ml yeast total RNA; 250 μg/ml yeast tRNA). The mixture was heated to 65°C for 5 min and then cooled on wet ice for 1 min. Dithiothreitol (100 mM, final concentration), sodium dodecyl sulfate (0.2% w/v, final concentration), sodium thiosulphate (0.1% w/v, final concentration), and hybridization buffer were added to the ribonucleotide mixture. The hybridization buffer contained (final concentrations): Tris buffer (23.8 mM, pH 7.4), EDTA (1.2 mM, pH 8.0), NaCl (357 mM), dextran sulfate (11.9%, w/v), Denhardt's solution (1.2 ×), and formamide (59.5%, v/v). Ninety microliters of probe in hybridization buffer was applied to each slide containing four sections. Slides were coverslipped and hybridized overnight in humid chambers at 55°C. Slides were then washed at room temperature four times in 1 × saline–sodium citrate (SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.2), incubated in ribonuclease A (RNase A; 5–20 μg/ml; Boehringer Mannheim) in buffer containing 0.5 M NaCl, 10 mM Tris (pH 8.0), and 0.25 mM EDTA (pH 8.0) for 15 min at room temperature, and washed four times in 0.2 × SSC at 60°C. Slides were rinsed briefly in deionized water, air dried, and apposed to Kodak Biomax X-ray film (Kodak Biomax MR, Eastman Kodak, NY) for 4 days–2 weeks to obtain film autoradiograms.

For detection of tyrosine hydroxylase mRNA, a 48-base oligonucleotide probe complementary to bases 1441–1488

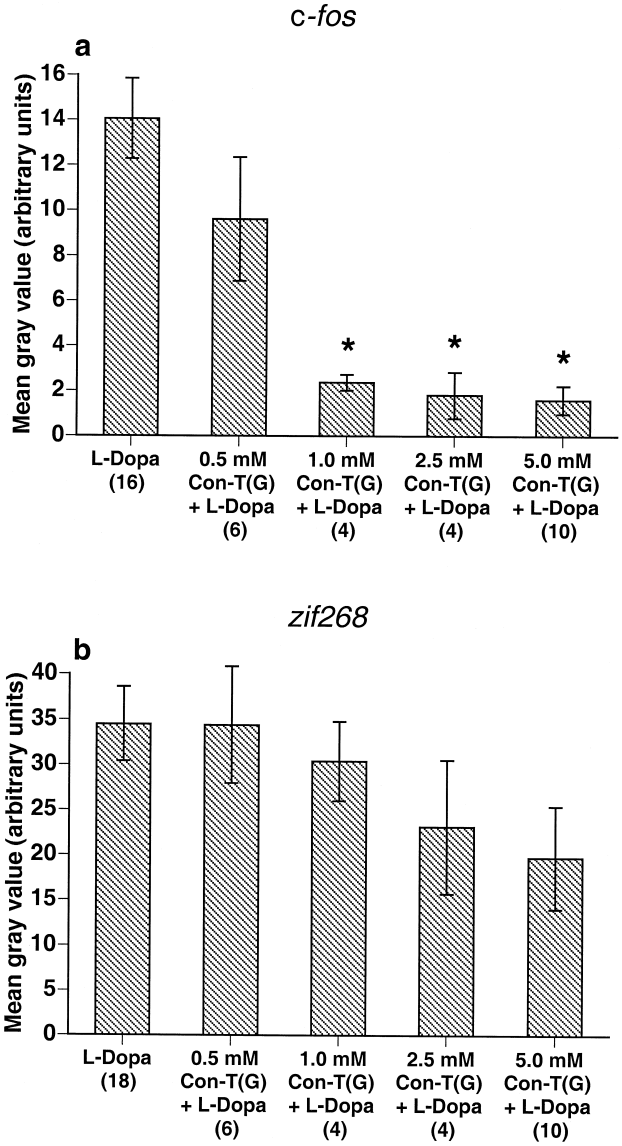

Fig. 4. Effects of i.c.v. administration of conantokin-T(G) (Con-T(G); 0.5-5.0 mM) on c-*fos* (a) and *zif268* (b) expression induced in mid-striatum (approximately 0.5 mm anterior to bregma) by systemic administration of L-DOPA (4 mg/kg, i.p.) to dopamine-depleted rats. L-DOPA was administered 15 min after infusion of Con-T(G) (2 μl/2 min). Rats receiving L-DOPA alone received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. All rats were sacrificed 2 h after L-DOPA administration. Values are mean gray values (±S.E.M.; arbitrary units) measured in the medial third of the striatum. Data obtained from the central and lateral thirds of the striatum did not differ from those presented here (data not shown). Numbers in parentheses indicate the number of animals/group. * Significantly different from L-DOPA alone, $p < 0.05$.

(Grima et al., 1985) was synthesized by the DNA/peptide facility at the University of Utah and end-labeled using terminal deoxynucleotidyl transferase (Boehringer Mannheim) as previously described (Keefe and Gerfen, 1996). The probe was then diluted in hybridization buffer for a final concentration of 1×10^6 cpm/100 μl buffer. The hybridization buffer consisted of (final concentrations): NaCl (0.6 M), Tris buffer (80 mM, pH 7.5), EDTA (4 mM, pH 8.0), sodium pyrophosphate (0.1%, w/v), dextran sulfate (10%, w/v), sodium dodecyl sulfate (0.2%, w/v), heparin sulfate (0.02%, w/v), formamide (50%, v/v), and dithiothreitol (100 mM). Ninety microliters of probe in hybridization buffer was applied to each slide containing four sections. Slides were coverslipped and hybridized overnight in humid chambers at 37°C. After hybridization, slides were washed in four changes of 1 × SSC at room temperature, followed by three washes in 2 × SSC with 50% (v/v) formamide at 40°C, and two washes in 1 × SSC at room temperature. Slides were rinsed briefly in deionized water, air dried, and apposed to X-ray film for 1–3 days.

2.7. Data analysis

Film autoradiograms were analyzed using the Macintosh-based image analysis program, Image (Wayne Rasband, NIH). Images of brain sections were captured with a video camera, digitized, and stored on computer. Images were captured under constant lighting conditions and within the linear range of the system response. Mean gray values were analyzed in medial, central, and lateral thirds of the dopamine-depleted striatum from its dorsal aspect to the anterior commissure ventrally for c-*fos*- and *zif268*-labeled sections, and in the substantia nigra pars compacta (bilaterally) for TH-labeled sections. The average gray value of the white matter was subtracted from the average gray value of the regions of interest to correct for background labeling.

Data from c-*fos* and *zif268* film autoradiograms were analyzed with a one-way analysis of variance for medial, central, and lateral thirds of the striatum. Post hoc analysis was performed with the Tukey–Kramer test. For TH film autoradiograms, the intact side was compared with the lesioned side to obtain a percentage reflective of the extent of dopamine depletion. All rats analyzed had $> 90\%$ depletion. Behavioral data were analyzed with a two-way analysis of variance with repeated measures across time, followed by post hoc analyses using the Students' *t*-test and a Bonferroni correction or the Tukey–Kramer test. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Effects of conantokin-G and conantokin-T(G) on L-DOPA-induced contralateral rotation and IEG expression

The i.c.v. infusion of conantokin-G (0.05–5.0 mM) potentiated L-DOPA-induced contralateral rotations in 6-hydroxydopamine-treated rats in a dose-dependent manner, increasing the peak rate of rotation and prolonging the duration of the response (Fig. 1a). The effect was signifi-

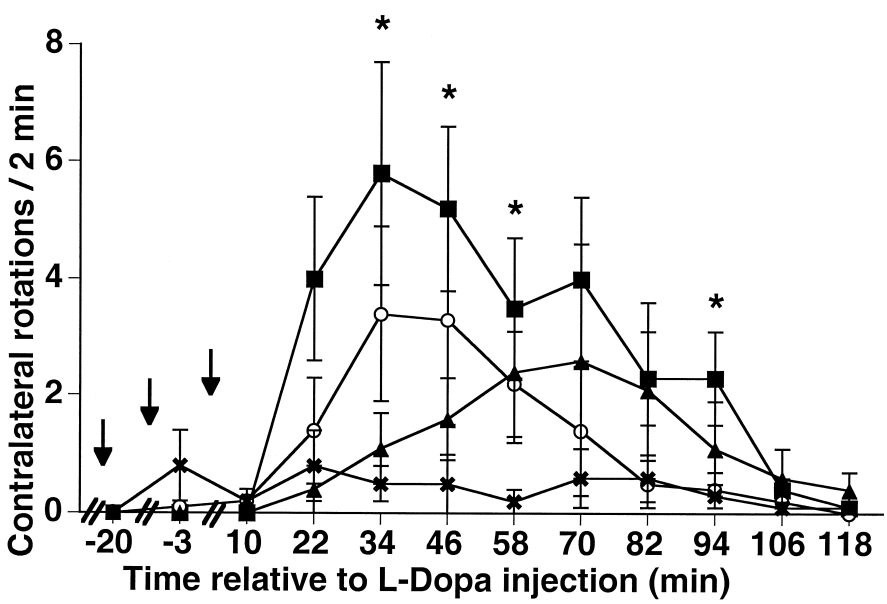

Fig. 5. Effects of i.c.v. administration of ifenprodil (Ifen; ○, 5.0 mM, $n = 5$; ▲, 12.5 mM, $n = 8$; ×, 25.0 mM, $n = 10$) on contralateral rotation induced by systemic administration of L-DOPA to dopamine-depleted rats. L-DOPA was administered 15 min after the infusion of ifenprodil (2 μl/2 min). Rats receiving L-DOPA alone (■, 4 mg/kg i.p., $n = 16$) received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. Arrows from left to right indicate the time of injection of benserazide, ifenprodil or aCSF, and L-DOPA, respectively. All rats were sacrificed 2 h after L-DOPA administration. Numbers on the *x*-axis indicate time relative to the administration of L-DOPA (min). The number of contralateral rotations/2 min is indicated for each time point (±S.E.M.). Statistical significance was set at $p < 0.05$. * Significantly different from 25.0 mM ifenprodil group, $p < 0.05$.

cant at the 0.5 mM dose ($p < 0.05$). At the highest dose tested (5 mM), conantokin-G produced ataxic behaviors, affecting the ability of the animals to rotate at an even higher rate and reversing the potentiation of L-DOPA-induced rotation. Animals were considered ataxic if they were unable to turn due to motor deficits such as incoordination of their limbs and falling over. In addition, in a separate experiment, conantokin-G induced contralateral rotation in 6-hydroxydopamine-treated rats when administered alone at a dose of 0.1 mM ($p < 0.05$) (Fig. 1b). The combined effect of conantokin-G and L-DOPA was more than additive, however, as is shown in Fig. 1b (hypothetical additive result indicated by dashed line). A new sample (batch) of conantokin-G was used in this experiment (Fig. 1b), and the effects of this batch of conantokin-G were more potent than those seen in the initial experiment (Fig. 1a).

Although conantokin-G potentiated the behavioral effects of L-DOPA, it had no effect on L-DOPA-induced c-*fos* (Fig. 2a) or *zif268* (Fig. 2b) expression in any region of striatum. Similarly, when administered alone conantokin-G did not alter striatal c-*fos* or *zif268* expression (data not shown).

Like conantokin-G, conantokin-T(G) (2.5 and 5.0 mM) potentiated L-DOPA-induced contralateral rotation in 6-hydroxydopamine-treated rats (Fig. 3a). The effect was significant at the 2.5 mM dose ($p < 0.05$). As with the high dose of conantokin-G, a 5 mM dose of conantokin-T(G) produced ataxia and reduced the rate of rotation. There was an apparent trend for two lower doses of conantokin-T(G) (0.5 and 1.0 mM) to attenuate L-DOPA-induced contralateral rotation. However, this effect was not statistically significant and rotation values for these animals overlapped with values obtained from animals receiving L-DOPA alone. Conantokin-T(G) produced contralateral rotation when administered alone (main effect for group, $p < 0.03$) (Fig. 3b). Again, however, the combined effect of conantokin-T(G) and L-DOPA was more than additive (hypothetical additive result indicated by the dashed line; Fig. 3b).

Unlike conantokin-G, conantokin-T(G) suppressed the induction of c-*fos* (Fig. 4a) and *zif268* (Fig. 4b) by L-DOPA in a dose-dependent manner ($p < 0.05$). This effect was more prominent on c-*fos* expression than on *zif268* expression, as the effects of conantokin-T(G) on c-*fos* expression were apparent and of greater magnitude at lower doses and were statistically significant ($p < 0.05$), whereas those on *zif268* were not. Conantokin-T(G) did not alter either IEG when administered alone (data not shown).

3.2. Effects of ifenprodil and CGS 19755 on L-DOPA-induced contralateral rotation and IEG expression

We also investigated the effects of ifenprodil and CGS 19755 on L-DOPA-induced rotation and IEG expression. The NR2b-selective NMDA receptor antagonist (Williams, 1993), ifenprodil (5.0–25.0 mM), attenuated L-DOPA-induced contralateral rotation in 6-hydroxydopamine-treated rats in a dose-dependent manner. This effect was significant at the 25.0 mM dose ($p < 0.05$) (Fig. 5). Although ifenprodil had the opposite effect from conantokin-G on L-DOPA-induced contralateral rotation, ifenprodil also had no effect on L-DOPA-induced IEG expression in any region of striatum (Fig. 6).

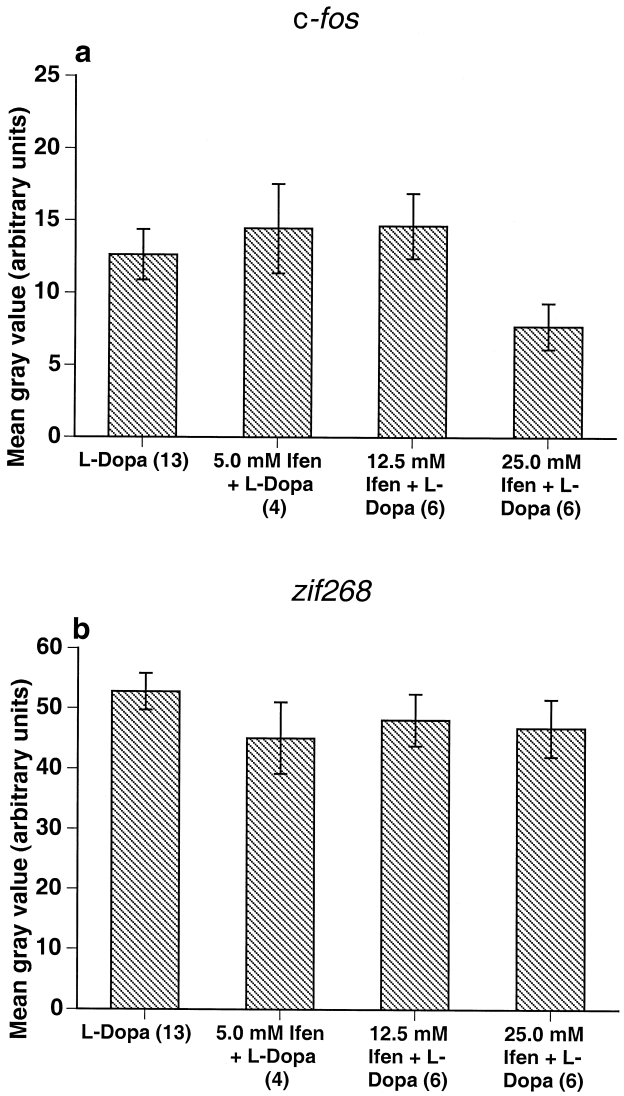

Fig. 6. Effects of i.c.v. administration of ifenprodil (Ifen; 5.0–25.0 mM) on c-*fos* (a) and *zif268* (b) expression induced in mid-striatum (approximately 0.5 mm anterior to bregma) by systemic administration of L-DOPA (4 mg/kg, i.p.) to dopamine-depleted rats. L-DOPA was administered 15 min after the infusion of ifenprodil (2 μl/2 min). Rats receiving L-DOPA alone received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. All rats were sacrificed 2 h after L-DOPA administration. Values are mean gray values (±S.E.M.; arbitrary units) measured in the medial third of the striatum. Data obtained from the central and lateral thirds of the striatum did not differ from those presented here (data not shown). Numbers in parentheses indicate the number of animals/group.

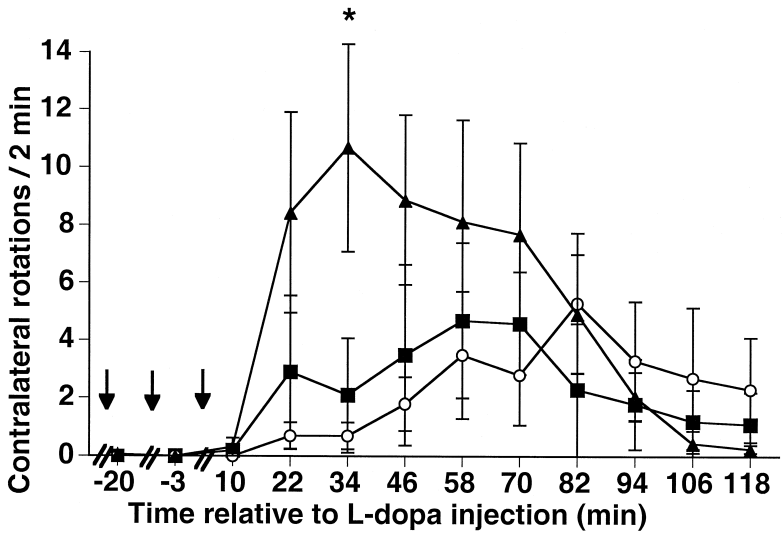

Fig. 7. Effects of i.c.v. administration of CGS 19755 (CGS; ○, 22.4 μM, $n = 5$; ▲, 224 μM, $n = 8$) on contralateral rotation induced by systemic administration of L-DOPA to dopamine-depleted rats. L-DOPA was administered 15 min after infusion of CGS (2 μl/2 min). Rats receiving L-DOPA alone (■, 4 mg/kg i.p., $n = 5$) received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. Arrows from left to right indicate the time of injection of benserazide, CGS 19755 or aCSF, and L-DOPA, respectively. All rats were sacrificed 2 h after L-DOPA administration. Numbers on *x*-axis indicate time relative to the administration of L-DOPA (min). The number of contralateral rotations/2 min is indicated for each time point (±S.E.M.). * Significantly different from L-DOPA alone, $p < 0.05$.

CGS 19755 (22.4 and 224 μM), a non-selective NMDA receptor antagonist (Laurie and Seeburg, 1994), also potentiated L-DOPA-induced contralateral rotation in 6-hydroxydopamine-treated rats at the 224 μM dose ($p < 0.05$; Fig. 7). As was the case for conantokin-T(G), CGS 19755 dose-dependently suppressed L-DOPA-induced c-*fos* expression ($p < 0.05$; Fig. 8a). Although not statistically significant, there was a trend towards an attenuation of *zif268* expression by CGS 19755 (Fig. 8b).

4. Discussion

NMDA receptor antagonists may be useful adjuncts in the treatment of Parkinson's disease, both in prolonging the effects of L-DOPA and reducing the side effects associated with prolonged usage and high dosing (Greenamayre and O'Brien, 1991; Klockgether and Turski, 1990; Starr, 1995b). The conantokins are a novel family of NMDA receptor antagonists (Hammerland et al., 1992; Mena et al., 1990). We have demonstrated here that two of the conantokins, conantokin-G and conantokin-T(G), are able to potentiate the behavioral effects of L-DOPA in 6-hydroxydopamine-treated rats, increasing the rotational response to L-DOPA in a synergistic manner as has been previously demonstrated for other NMDA receptor antagonists (Loschmann et al., 1991; Morelli et al., 1992). Therefore, the conantokins may be useful adjunct compounds for the treatment of Parkinson's disease.

The potentiation of L-DOPA-induced contralateral rotation by the conantokins and CGS 19755 presented here may result from alterations in the function of striatal efferent neurons as a consequence of combined L-DOPA and NMDA receptor antagonist administration. Current models of basal ganglia circuitry (Albin et al., 1989) suggest that L-DOPA likely produces its therapeutic effects in dopamine-depleted animals by increasing the activity of striatonigral neurons and decreasing the activity of striatopallidal neurons through the activation of D1 and D2 subtypes of dopamine receptors, respectively. Blockade of NMDA receptors would augment the L-DOPA-induced suppression of striatopallidal neuron function, while counteracting the increase in striatonigral neuron function by L-DOPA administration. Therefore, potentiation of L-DOPA-induced rotation by NMDA receptor antagonists likely reflects changes in the function of striatopallidal neurons rather than striatonigral neurons. In addition, the functional output of the basal ganglia may be altered by NMDA receptor antagonists acting on NMDA receptors localized to other neuronal populations within the striatopallidal output pathway (i.e., subthalamic nucleus, substantia nigra pars reticulata, and entopeduncular nucleus; Klockgether and Turski, 1990). Presumably, alterations in neuronal function at either of these sites by NMDA receptor antagonism will result in increased thalamocortical drive and subsequent behavioral activation.

Despite our behavioral data with the conantokins and CGS 19755, we were unable to potentiate L-DOPA-induced rotation with ifenprodil. Ifenprodil is a NR2b-selective NMDA receptor antagonist (Williams, 1993). Nash et al. (1999) have shown that systemic administration of ifenprodil results in a dose-dependent increase in locomotor activity in reserpine-treated rats. This is in contrast to our results, in which ifenprodil decreased L-DOPA-induced

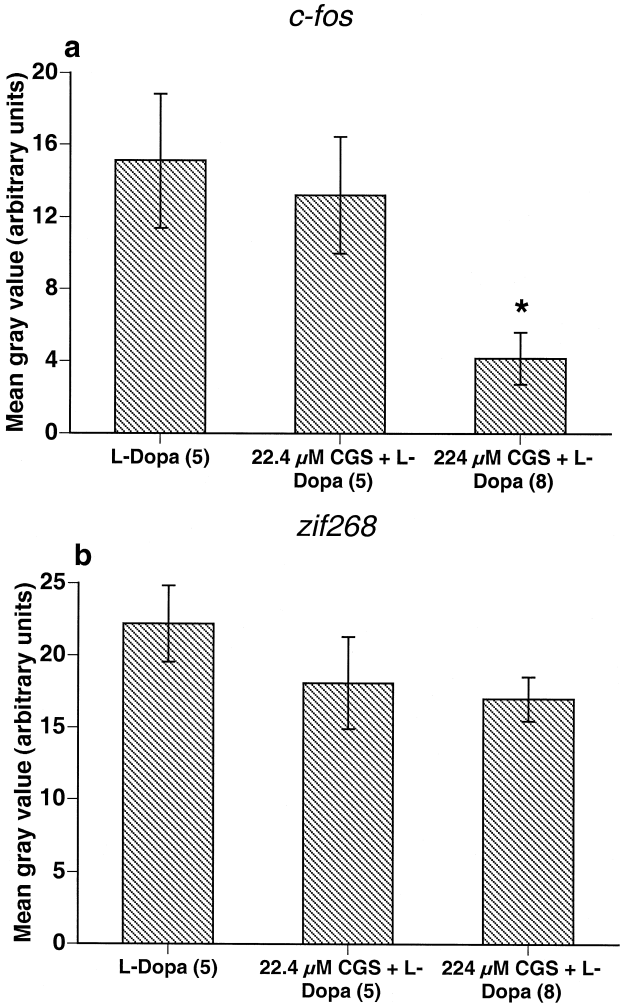

Fig. 8. Effects of i.c.v. administration of CGS 19755 (CGS; 22.4 and 224 μM) on c-*fos* (a) and *zif268* (b) expression induced in mid-striatum (approximately 0.5 mm anterior to bregma) by systemic administration of L-DOPA (4 mg/kg, i.p.) to dopamine-depleted rats. L-DOPA was administered 15 min after infusion of CGS (2 μl/2 min). Rats receiving L-DOPA alone received an i.c.v. infusion of aCSF 15 min prior to L-DOPA administration. All rats were sacrificed 2 h after L-DOPA administration. Values are mean gray values (±S.E.M.; arbitrary units) measured in the medial third of the striatum. Data obtained from the central and lateral thirds of the striatum did not differ from those presented here (data not shown). Numbers in parentheses indicate the number of animals/group. * Significantly different from L-DOPA alone, $p < 0.05$.

contralateral rotation in 6-hydroxydopamine-treated rats in a dose-dependent manner. A direct comparison between these two studies cannot be made, however, because of the differences in experimental design (i.e., parkinsonian model used, route of administration, dose). It is also important to note that ifenprodil has α_1 adrenergic receptor antagonist properties (Chenard et al., 1991). The effects of ifenprodil on adrenergic receptors therefore may have contributed to the attenuation of L-DOPA-induced contralateral rotation that we observed. Wright et al. (1999) have developed two new NR2b-selective antagonists based on the chemical structure of ifenprodil with only weak α_1-adrenoceptor adrenergic antagonist properties. These compounds potentiated L-DOPA-induced contralateral rotations in 6-hydroxydopamine-treated rats without producing any behavioral side effects (Wright et al, 1999). These data suggest that the development of compounds, such as conantokin-G and conantokin-T(G), with NMDA receptor subunit selectivity and weaker activity at other neuronal receptors may lead to a more beneficial treatment for Parkinson's disease.

The NMDA receptor is a multimeric receptor, the pharmacology of which is determined by the subunit composition of the receptor (Laurie and Seeburg, 1994). The NR2 (a–d) subunits couple with the NR1 subunit, which is necessary for the formation of a functional receptor. The NR2a and NR2b subunits are found in medium spiny neurons of the striatum (Landwehrmeyer et al., 1995). As noted above, ifenprodil binds with greater affinity to NR2b-incorporating NMDA receptors (Williams, 1993), whereas CGS 19755 binds NR2a- and NR2b-incorporating NMDA receptors (Laurie and Seeburg, 1994). Interestingly, these antagonists, as well as the two conantokins, had different effects on L-DOPA-induced c-*fos* expression in the dopamine-depleted striatum. Ifenprodil and conantokin-G did not affect c-*fos* expression whereas CGS 19755 and conantokin-T(G) significantly suppressed it.

The induction of IEGs in the dopamine-depleted striatum after L-DOPA administration seems to be mediated primarily by the activation of dopamine D1 receptors on striatonigral neurons (Robertson et al., 1989). These dopamine D1 receptor-mediated effects appear to be more sensitive to NMDA receptor blockade by antagonists that are non-selective for NR2a- and NR2b-containing NMDA receptors. For example, we have previously reported differential regulation of striatal IEG expression by selective versus non-selective NMDA receptor antagonists (Keefe and Ganguly, 1998). In that study, CGS 19755 blocked dopamine D1 receptor agonist-induced c-*fos* and *zif268* expression in the intact striatum, whereas ifenprodil potentiated the expression. In the present study, it was again the case that the non-selective NMDA receptor antagonist CGS 19755 suppressed L-DOPA-induced gene expression, whereas the NR2b-selective NMDA receptor antagonist ifenprodil did not. In addition, conantokin-G, which has been shown to be NR2b-selective (S. Donevan[1]), did not affect L-DOPA-induced IEG expression. Although the subunit selectivity of conantokin-T(G) is unknown, the attenuation of L-DOPA-induced c-*fos* expression in this study suggests that it is a non-selective antagonist. Together, these results suggest that activation of dopamine D1 receptors by L-DOPA induces the expression of IEGs in striatonigral neurons and that NMDA receptors containing the

[1] S.D. Donevan, R.T. McCabe, 2000. Conantokin-G is an NR2b-selective competitive antagonist of *N*-methyl-D-aspartate receptors. Mol. Pharmacol., 58, 614–623.

NR2a subunit are more strongly linked to dopamine D1 receptor agonist-mediated regulation of striatal gene expression.

Dopamine D2 receptor-mediated effects on striatopallidal neuron gene expression, on the other hand, are not differentially sensitive to NMDA receptor antagonists with different subunit selectivities. The induction of IEGs by the dopamine D2 receptor antagonist eticlopride is blocked by both NR2a/NR2b-non-selective and NR2b-selective NMDA receptor antagonists (Keefe and Adams, 1998). This pattern is consistent with the similar effects that CGS 19755, conantokin-G, and conantokin-T(G) had on L-DOPA-induced rotation in the present study. This similarity between the effects of these antagonists on L-DOPA-induced behavior and on dopamine D2 receptor-mediated gene induction provide further support for the notion introduced above that the synergistic behavioral effects of NMDA receptor antagonists and L-DOPA may reflect their interactions in striatopallidal neurons or the indirect pathway. This possibility would further account for the differential effects of the antagonists in the present study on behavior and L-DOPA-induced gene expression, a dopamine D1 receptor-mediated effect.

The basis for such differential coupling of specific subtypes of NMDA receptors to dopamine receptor-mediated processes remains to be determined. One possible basis is differential phosphorylation of NR2a subunits by dopamine D1 receptor activation. Although phosphorylation of NR2 subunits by acute dopamine D1 receptor activation has not yet been reported, chronic administration of the D1 agonist SKF 38393 or L-DOPA to rats with unilateral depletions of dopamine increased serine phosphorylation of the NR2a subunit of the NMDA receptor, and decreased serine phosphorylation of the NR2b subunit (Oh et al., 1999). A second possible basis for the preferential association of specific NMDA receptors with dopamine receptor-mediated processes may lie in the nature of the afferent input mediating the responses. Cortical and thalamic input to striatum tends to synapse onto different post-synaptic elements, with cortical input synapsing more prominently on the heads of dendritic spines (Bouyer et al., 1984) and thalamic input synapsing more on dendritic shafts (Sidibe and Smith, 1996; Sadikot et al., 1992; Smith et al., 1994). Furthermore, the majority of striatal input from the centromedian nucleus of the thalamus appears to synapse onto striatonigral, as opposed to striatopallidal, efferent neurons (Sidibe and Smith, 1996). Studies in the hippocampus indicate that neurons expressing both NR2a and NR2b subunits of the NMDA receptor nonetheless segregate NMDA receptors comprised of NR2a versus NR2b subunits to dendritic regions receiving different excitatory afferents (Ito et al., 1997). Thus, we think that dopamine D1 receptor-mediated gene expression may be dependent on input from the thalamus and dopamine D2 receptor-mediated functions dependent on input from cortex, and that these two inputs may be mediated by distinct subtypes of NMDA receptors. Further experiments clearly are needed to determine the basis of these selective interactions and to fully appreciate the interactions between glutamate and dopamine systems in the regulation of striatal neuron function.

In conclusion, the data presented here demonstrate that the conantokins may be useful adjuncts in the treatment of Parkinson's disease, increasing the effectiveness of L-DOPA. Although the conantokins induced similar behavioral activity, the effects of conantokin-G and conantokin-T(G) on L-DOPA-induced IEG expression in the striatum were different, possibly as a consequence of the NMDA receptor subunit selectivity of the compounds. These data therefore further suggest that NMDA receptors comprised of different subunits may play different roles in the regulation of basal ganglia function.

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