Neurobiology of Disease

The Nociceptin/Orphanin FQ Receptor Antagonist J-113397 and L-DOPA Additively Attenuate Experimental Parkinsonism through Overinhibition of the Nigrothalamic Pathway

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By using a battery of behavioral tests, we showed that nociceptin/orphanin FQ receptor (NOP receptor) antagonists attenuated parkinsonian-like symptoms in 6-hydroxydopamine hemilesioned rats (Marti et al., 2005). We now present evidence that coadministration of the NOP receptor antagonist 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H* benzimidazol-2-one (J-113397) and L-DOPA to 6-hydroxydopamine hemilesioned rats produced an additive attenuation of parkinsonism. To investigate the neurobiological substrates underlying this interaction, *in vivo* microdialysis was used in combination with behavioral measurements (bar test). J-113397 and L-DOPA alone reduced the time on bars (i.e., attenuated akinesia) and elevated GABA release selectively in the lesioned substantia nigra reticulata. J-113397 also reduced nigral glutamate levels, whereas L-DOPA was ineffective. J-113397 and L-DOPA coadministration produced additive antiakinetic effect, which was associated with additive increase in nigral GABA release but no additional reductions in glutamate levels. To investigate whether the increase in nigral GABA release could translate to changes in nigrothalamic transmission, GABA release was monitored in the ventromedial thalamus (one of the main target areas of the nigrothalamic projections). J-113397 and L-DOPA decreased thalamic GABA release and attenuated akinesia, their combination resulting in a more profound effect. These actions were prevented by perfusing the voltage-dependent Na^+ channel blocker tetrodotoxin or the $GABA_A$ receptor antagonist bicuculline in the substantia nigra reticulata. These data demonstrate that J-113397 and L-DOPA exert their antiparkinsonian action through overinhibition of nigrothalamic transmission and suggest that NOP receptor antagonists may be useful as an adjunct to L-DOPA therapy for Parkinson's disease.

Key words: **J-113397; L-DOPA; microdialysis; nociceptin; 6-OHDA; Parkinson's disease**

Introduction

Nociceptin/orphanin FQ (N/OFQ) and its receptor (NOP) are expressed in cortical and subcortical motor areas and, particularly, in the substantia nigra (SN), which contains dopamine (DA) neurons that degenerate in Parkinson's disease (PD). N/OFQ inhibits the activity of DA neurons located in the SN compacta (SNc) (Marti et al., 2004a) and impairs spontaneous (Reinscheid et al., 1995; Devine et al., 1996) and exercise-induced locomotion (Marti et al., 2004a). More recently, we presented evidence that endogenous N/OFQ sustains symptoms and neurodegeneration associated with PD. Indeed, upregulation of N/OFQergic transmission was found in the DA-depleted SN reticulata (SNr) of 6-hydroxydopamine (6-OHDA) hemilesioned (hemiparkinsonian) rats (Marti et al., 2005). Moreover, systemic or intranigral injections of NOP receptor antagonists attenuated akinesia both in hemiparkinsonian and haloperidol-treated rats (Marti et al., 2004b, 2005). Finally, deletion of the NOP receptor or the preproN/OFQ gene conferred mice partial resistance to the cataleptic action of haloperidol and protection against MPTP-induced loss of SNc DA neurons, respectively (Marti et al., 2005). Reduction of glutamate (GLU) release in the SNr may represent the mechanism by which NOP receptor antagonists reverse parkinsonism, because this class of compounds normalized haloperidol-evoked GLU levels in the SNr, an effect that correlated with attenuation of akinesia (Marti et al., 2004b, 2005). To further strengthen the view that NOP receptor antagonists may be useful in PD therapy, we undertook the present study to investigate the ability of the nonpeptide NOP receptor antagonist 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H* benzimidazol-2-one (J-113397) (Kawamoto et al., 1999) to synergize with L-DOPA. L-DOPA still represents the most effective treatment of PD, although chronic treatment almost invariably produces motor fluctuations and

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dyskinesias (Obeso et al., 2000) that cause reduction in the clinical response over time. From a clinical point of view, combining L-DOPA with other antiparkinsonian drugs may allow a reduction in L-DOPA dosage, thereby delaying the onset of L-DOPA side effects.

In the present study, we evaluated L-DOPA action in a full dose range (experiment 1), further testing whether subthreshold (experiment 2) and submaximal (experiment 3) doses of J-113397 and L-DOPA produced additive antiparkinsonian effects. To investigate the mechanisms underlying this interaction, GLU and GABA release was analyzed by microdialysis in the SNr of rats undergoing behavioral testing (bar test; experiment 4). To determine whether changes in SNr neurotransmitter release correlated with changes of activity of nigrothalamic GABAergic neurons (i.e., the basal ganglia motor output), GABA release was also measured in the ventromedial thalamus (VMTh) (experiment 5), one of the main targets of nigrothalamic GABAergic neurons (Di Chiara et al., 1979; MacLeod et al., 1980). Finally, to confirm that both antiakinetic action and the changes in thalamic GABA release after J-113397 and L-DOPA coadministration were attributable to inhibition of nigrothalamic GABAergic transmission, neurochemical and behavioral analysis were performed during reverse dialysis of the voltage-dependent Na^+ channel blocker tetrodotoxin (TTX) (experiment 6) or the $GABA_A$ receptor antagonist bicuculline (experiment 7) in the SNr.

Materials and Methods

Rats used in the study (see below) were kept under regular lighting conditions (12 h light/dark cycle) and given food and water *ad libitum*. The experimental protocols performed in the present study were approved by the Italian Ministry of Health (license number 71-2004-B) and by the Ethics Committee of the University of Ferrara.

Measurement of antiakinetic effects of levodopa and J-113397 in hemiparkinsonian rats

6-OHDA lesion

Unilateral lesion of DA neurons (Marti et al., 2005) was induced in isoflurane-anesthetized male Sprague Dawley rats (150 g; Harlan Italy, S. Pietro al Natisone, Italy). Eight micrograms of 6-OHDA (in 4 μl of saline containing 0.02% ascorbic acid) were stereotaxically injected according to the following coordinates from bregma: anteroposterior (AP), −4.4 mm; mediolateral (ML), −1.2 mm; ventrodorsal (VD), −7.8 mm below dura (Paxinos and Watson, 1982). The rotational model (Ungerstedt and Arbuthnott, 1970) was used to select the rats that had been successfully lesioned. Two weeks after surgery, rats were injected with amphetamine (5 mg/kg, i.p., dissolved in saline) and only those rats performing more than seven ipsilateral turns per minute were enrolled in the study. This behavior has been associated with >95% loss of striatal extracellular DA levels (Marti et al., 2002b). Experiments were performed 6–8 weeks after lesion.

Histological evaluation

The animals were deeply anesthetized with Zoletil 100 (10 mg/kg, i.m.; Virbac Laboratories, Carros, France), transcardially perfused with 20 mM potassium PBS (KPBS) and fixed with 4% paraformaldehyde in KPBS, pH 7.4. The brains were removed, fixed in the fixative overnight, and transferred to 25% sucrose solution in KPBS for cryoprotection. Serial coronal sections of 40 μm thickness were made using a freezing microtome. Every second section in the striatum was selected from the region spanning from bregma −0.5 to +1.5 and processed for tyrosine hydroxylase (TH) immunohistochemistry.

Sections were rinsed three times in KPBS and incubated for 15 min in 3% H_2O_2 and 10% methanol in KPBS to block the endogenous peroxidase activity. After washing in KPBS, the sections were preincubated in blocking serum (5% normal horse serum and 0.3% Triton X-100 in KPBS) for 60 min, followed by incubation in anti-TH mouse monoclonal antibody solution (1:2000; Millipore, Billerica, MA) for 16 h at room temperature. The sections were then rinsed in KPBS and incubated for 1 h in biotinylated horse anti-mouse IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA). After rinsing, sections were incubated with avidin–biotin peroxidase complex (Vector Laboratories) for 30 min at room temperature. After rinsing with KPBS, immunoreactivity was visualized by incubating the sections in a solution containing 0.05% 3,3-diaminobenzidine (DAB) in 0.013% H_2O_2 in KPBS for ~1 min. The sections were rinsed in KPBS, mounted on gelatin-coated slides, dried with ethanol and xylene, and coverslipped with mounting medium. Every section was viewed with a Zeiss Axioskop (Zeiss, Oberkochen, Germany), acquired with Polaroid (Waltham, MA) DMC camera and TH-immunoreactive fiber density analyzed using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). To estimate the TH-density staining, the optical densities were corrected for nonspecific background density, which was measured in the corpus callosum. TH-positive fiber density was calculated as the ratio between optical density in the denervated (ipsilateral) and intact (contralateral) side (Fig. 1).

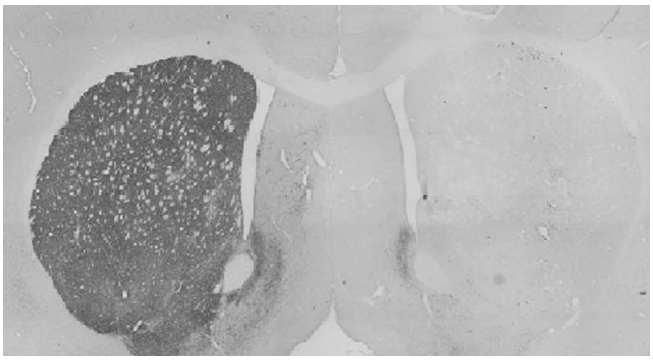

Figure 1. Effect of 6-OHDA injections on the TH-positive fiber density in the striatum. Photomicrograph of TH-positive fibers in the striata of a hemiparkinsonian rat.

Behavioral studies in rats

Motor activity in rats was evaluated by means of three behavioral tests specific for different motor abilities, as previously described (Marti et al., 2005): (1) the bar test (Sanberg et al., 1988), which measures rat ability to respond to an externally imposed static posture; (2) the drag test [modification of the postural adjustment test (Lindner et al., 1999)], which measures rat ability to balance body posture using forelimbs in response to an externally imposed dynamic stimulus (backward dragging); and (3) the rotarod test, which measures rat ability to run on a rotating cylinder (Rozas et al., 1997). The different tests are useful to evaluate akinesia and motor asymmetry under static conditions (bar test), akinesia, bradykinesia, and asymmetry under dynamic conditions (drag test), and overall motor performance (rotarod test) as an integration of coordination, gait, balance, muscle tone, and motivation to run. The bar and the drag tests were performed 10 min after intraperitoneal injections of J-113397, L-DOPA, or their combination, whereas the rotarod test was performed 10 and 60 min after drug injection.

Bar test. Each rat was placed gently on a table, and the contralateral and ipsilateral forepaws were placed alternatively on blocks of increasing heights (3, 6, and 9 cm). Total time (in seconds) spent by each paw on the blocks was recorded (cutoff time, 20 s).

Drag test. Each rat was gently lifted from the tail (allowing forepaws on the table) and dragged backwards at a constant speed (~20 cm/s) for a fixed distance (100 cm). The number of steps made by each paw was counted by two separate observers.

Rotarod test. The fixed-speed rotarod test (Rozas et al., 1997) was used according to a previously described protocol (Marti et al., 2004a). Briefly, rats were trained for 10 d to a specific motor task on the rotarod until their motor performance became reproducible. Rats were tested in a control session at four increasing speeds (10, 15, 20, 25 rpm for hemiparkinsonian rats and 30, 35, 40, and 45 rpm for sham-operated rats; 180 s each), causing a progressive decrement of performance to ~40% of the maximal response (i.e., the experimental cutoff time). Such a protocol

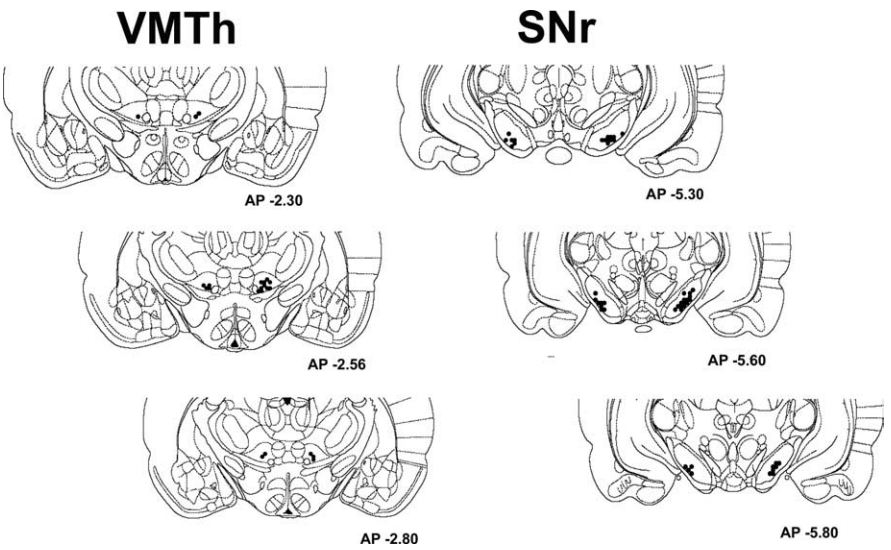

Figure 2. Schematic representation of coronal sections indicating microdialysis probe location in the SNr (AP −5.30, −5.60, and −5.80 from bregma) and VMTh (AP −2.30, −2.56, and −2.80 from bregma). The filled circles represent the location of the tip of the probe (data obtained from 45 animals).

was set to detect both facilitatory and inhibitory drug effects. Two other sessions were repeated 10 and 60 min after drug injection, and drug effect was expressed as total time spent on the rod.

Measurement of GLU and GABA levels in hemiparkinsonian rats by microdialysis

Two microdialysis probes (1 mm dialyzing membrane; AN69; Hospal, Bologna, Italy) were implanted bilaterally in the lesioned and unlesioned SNr (AP, −5.5; ML, ± 2.2; VD, −8.3) or ipsilateral and contralateral VMTh (AP, −2.3; ML, ± 1.4; VD, −7.4) of isoflurane-anesthetized hemiparkinsonsian rats. Twenty-four hours after implantation, probes were perfused (3 μl/min) with a modified Ringer's solution (in mM: 1.2 $CaCl_2$, 2.7 KCl, 148 NaCl, and 0.85 $MgCl_2$) and sample collection (every 15 min) started after a 6–7 h washout. L-DOPA and J-113397 were administered systemically (intraperitoneally) alone or in combination, and GLU and GABA levels were monitored every 15 min up to 3 h. In a separate set of experiments, two microdialysis probes were implanted in the lesioned SNr and ipsilateral VMTh, and GABA was measured in the VMTh. In these experiments, to correlate changes of amino acid dialysate levels with motor activity, rats undergoing microdialysis were challenged in the bar test every 15 min (Marti et al., 2004b, 2005). Routinely, experiments were repeated for 3 d after surgery, and treatments were randomized between groups, because preliminary testing showed that drug effect did not change depending on the day of experiment or treatment received in the preceding days. A notable exception was, however, represented by experiments using TTX because the toxin produced long-lasting effects that prevented additional testing. At the end of the experiments, rats were killed and probe location verified by microscopic examination (Fig. 2).

GLU and GABA levels in the dialysate were measured by HPLC coupled to fluorimetric detection, with minor modifications of the method described previously (Marti et al., 2003). Briefly, 40 μl samples were pipetted into glass microvials and placed in a thermostated (4°C) Triathlon autosampler (Spark Holland, Emmen, The Netherlands). Thirty-five microliters of *o*-pthaldialdheyde/boric acid solution were added to each sample, and 50 μl of the solution was injected onto a Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column was eluted at a flow rate of 0.48 ml/min with a mobile phase containing 0.1 M sodium acetate, 10% ethanol, and 2.5% tetrahydrofuran, pH 6.5; to achieve a good separation, a two-step linear gradient of methanol in aqueous sodium acetate buffer was provided by a Beckman 125 pump (Beckman Instruments, Fullerton, CA). GLU and GABA were detected by means of a fluorescence spectrophotometer RF-551 (Shimadzu, Kyoto, Japan). GLU and GABA retention times were ~4 and ~17 min, respectively, and the sensitivity of the method for both amino acids was 150 fmol/sample.

Data presentation and statistical analysis

Motor performance has been expressed as time on bar or on rod (in seconds) and number of steps (drag test). In microdialysis studies, GLU and GABA release has been expressed as percentage ± SEM of basal values (calculated as mean of the two samples before the treatment). In Figure legends (and in Results), amino acid dialysate levels for each group of rats are also given in absolute values (in nanomolar concentration).

To analyze behavior, statistical analysis was performed (CoStat 6.3; CoHort Software, Monterey, CA) on absolute data by one-way or two-way ANOVA followed by the Newman–Keuls test for multiple comparisons. Drug interaction was studied experimentally according to a 2 × 2 factorial design, and data were analyzed with conventional two-way ANOVA, factor one being L-DOPA and factor two being J-113397. Whenever behavior was analyzed at different time points (e.g., during the rotarod test), repeated-measure ANOVA was performed on absolute data, "within" factor being time and "between" factor being treatment. In the case that ANOVA yielded to a significant *F* score, *post hoc* analysis was performed in contrast analysis to determine group differences. In the case that a significant time × treatment interaction was found, the sequentially rejective Bonferroni test was used (implemented on an Excel spreadsheet) to determine specific differences (i.e., at the single time-point level) between groups. Two-way ANOVA with repeated measures on percentage data was used in neurochemical experiments, within factor being time and between factor being treatment. For each group, two pretreatment and six post-treatment samples were considered. Contrast *post hoc* analysis was used to determine group differences, and the sequentially rejective Bonferroni test was used to determine differences at the single time-point level, as described above. p values <0.05 were considered to be statistically significant.

Materials

Amphetamine, 6-OHDA bromide, methyl L-DOPA hydrochloride, and benserazide were purchased from Sigma (St. Louis, MO), TTX from Alomone Labs (Jerusalem, Israel), and bicuculline from Tocris Neuramin (Bristol, UK). J-113397 was synthesized in our laboratories as reported previously (Marti et al., 2004a). All drugs were freshly dissolved in isoosmotic saline solution just before use.

Results

L-DOPA relieved akinesia in hemiparkinsonian rats (experiment 1)

Hemiparkinsonian rats displayed severe (~98%) loss of TH-positive DA terminals in the striatum ipsilateral to the 6-OHDA injection side (0.02 ± 0.03 ipsilateral/contralateral ratio, $n = 6$) (Fig. 1), whereas sham-operated rats showed no depletion at all (0.99 ± 0.02 ipsilateral/contralateral ratio, $n = 5$). Hemiparkinsonian rats also displayed overall marked akinesia/bradykinesia and motor asymmetry compared with vehicle-injected sham-operated rats (Table 1). L-DOPA (in combination with 15 mg/kg benserazide, i.p.) attenuated akinesia/bradykinesia and normalized motor asymmetry within the 0.1–6 mg/kg dose range, inducing a clear contralateral bias at higher doses (25 mg/kg) (Fig. 3). Lower L-DOPA doses reduced the time spent on the blocks

Table 1. Characterization of motor activity in vehicle-injected (sham-operated) and 6-OHDA-injected (hemiparkinsonian) rats

	Sham-operated		Hemiparkinsonian	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Bar test (s)	0.7 ± 0.2 ($n = 12$)	0.8 ± 0.2 ($n = 12$)	41.6 ± 2.0* ($n = 22$)	50.0 ± 1.5*,** ($n = 22$)
Drag test (steps)	12.3 ± 0.3 ($n = 12$)	12.4 ± 0.5 ($n = 12$)	10.4 ± 0.4 ($n = 17$)	3.8 ± 0.3*,** ($n = 17$)
Rotarod (s)	1044 ± 93 ($n = 11$)		428 ± 30* ($n = 19$)	

Hemiparkinsonian rats displayed an increase in the total time spent on the blocks in the bar test, reduction in the number of steps made by the contralateral forepaw in the drag test, and reduced rotarod performance, compared with sham-operated animals. Data (mean ± SEM) have been obtained from 11 vehicle-injected and 19 hemiparkinsonian rats. *$p < 0.05$ versus sham-operated rats (Student's *t* test or ANOVA followed by the Newman–Keuls *post hoc* test, when appropriate). **$p < 0.05$ versus the ipsilateral forepaw.

($F_{(9,64)} = 54.74$; $p < 0.001$) (Fig. 3*A*), increased the number of steps ($F_{(9,72)} = 45.96$; $p < 0.001$) (Fig. 3*B*), and affected rotarod performance (Fig. 3*C*). In this respect, repeated-measure ANOVA revealed a significant effect of treatment ($F_{(5,22)} = 11.25$; $p < 0.001$), time ($F_{(2,68)} = 6.59$; $p = 0.002$), and time × treatment ($F_{(10,68)} = 50.81$; $p < 0.001$). *Post hoc* analysis revealed that L-DOPA stimulated motor performance in the 0.3–6 mg/kg dose range and inhibited it at 25 mg/kg. Overall, the most sensitive test was the bar test, with L-DOPA significantly attenuating akinesia at 0.1 mg/kg and normalizing motor asymmetry at 6 mg/kg. When L-DOPA was injected at high doses (25 mg/kg), it induced contralateral turning and reversal of motor asymmetry, the contralateral side of the body being more active than the ipsilateral one. Contralateral turning was, however, associated with hampered rotarod performance (see also Rozas et al., 1997; Marti et al., 2004a).

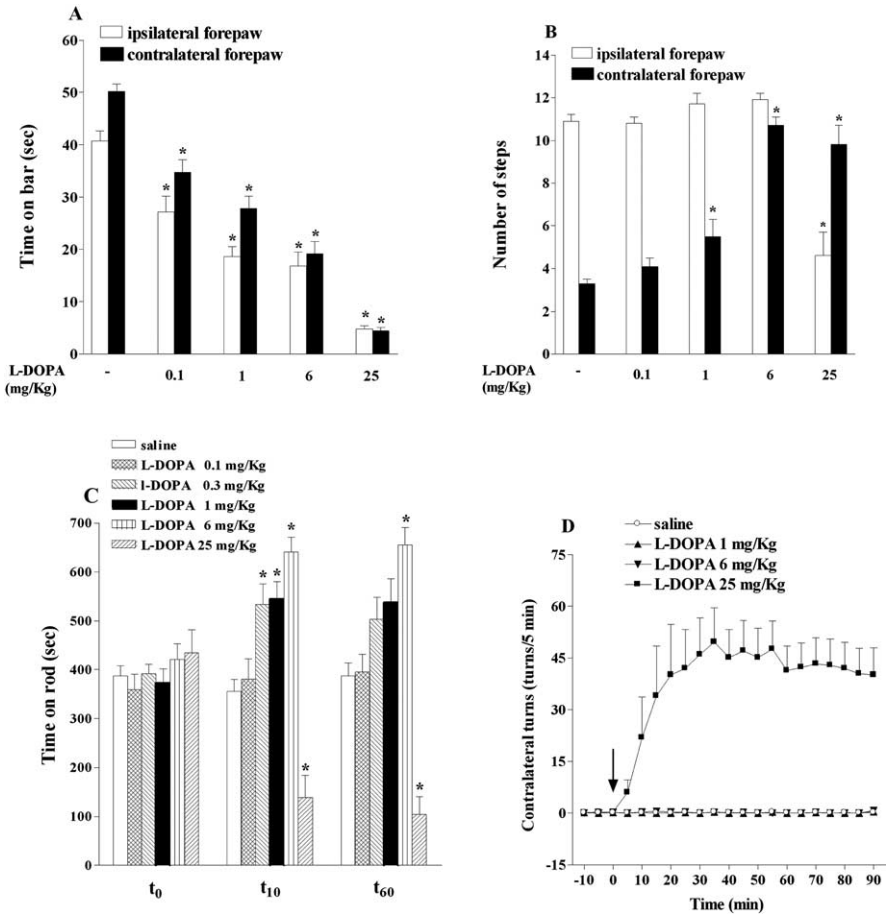

Figure 3. L-DOPA relieved akinesia/bradykinesia in hemiparkinsonian rats. ***A–D***, Systemic administration (intraperitoneal; arrow) of L-DOPA (0.1–25 mg/kg, in combination with 15 mg/kg benserazide) reduced the time spent on the blocks in the bar test (***A***), increased the number of steps of the contralateral forepaw in the drag test (***B***), improved overall motor performance in the rotarod test (***C***), and induced contralateral rotations (***D***). The bar and drag tests were performed 10 min after injection; the rotarod test was performed 10 and 60 min after injection. Motor asymmetry was evaluated by separately measuring activity of the paws ipsilateral and contralateral (parkinsonian) to the lesioned side. Turning behavior (***D***) was assessed by counting the number of rotations in the direction opposite to the injection side (i.e., contralateral) in 90 min. Data are mean ± SEM (6–14 rats per group). Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test (***A***, ***B***) or ANOVA with repeated measures followed by contrast analysis and the sequentially rejective Bonferroni test (***C***). *$p < 0.05$ versus saline-treated rats.

Combination of L-DOPA and J-113397 attenuated parkinsonian-like symptoms in an additive way (experiments 2 and 3)

By using the bar, drag, and rotarod tests, we reported previously (Marti et al., 2005) that systemic (intraperitoneal) injections of J-113397 attenuated akinesia/bradykinesia and attenuated motor asymmetry in hemiparkinsonian rats. Its action fully developed within the 0.1–3 mg/kg range, with 0.1 and 1 mg/kg being the subthreshold and submaximal doses in two of three tests, respectively. We therefore selected subthreshold (0.1 mg/kg) and submaximal (1 mg/kg) doses of J-113397 and L-DOPA to test whether their combination could produce additive effects.

Interaction between subthreshold doses (experiment 2)

In the bar test (Fig. 4*A*), two-way ANOVA revealed the main effect of L-DOPA ($F_{(1,32)} = 11.89$; $p = 0.0016$), but not J-113397 ($F_{(1,32)} = 0.71$; $p = 0.40$), and a significant L-DOPA × J-113397 interaction ($F_{(1,32)} = 14.08$; $p = 0.0007$) at the ipsilateral side. At the contralateral side, two-way ANOVA revealed the main effect of L-DOPA ($F_{(1,30)} = 26.06$; $p < 0.0001$) and J-113397 ($F_{(1,30)} = 26.06$; $p = 0.057$) and a significant L-DOPA × J-113397 interaction ($F_{(1,30)} = 5.41$; $p = 0.0269$). *Post hoc* analysis revealed that L-DOPA and J-113397 reduced the time spent on the bar at both forepaws, the effect of the combination being no greater than that evoked by each compound alone. In the drag test (Fig. 4*B*), no effect was detected, either when compounds were administered alone or together. In the rotarod test (Fig. 4*C*), no main effect of treatment was found ($F_{(3,15)} = 1.84$; $p = 0.18$). Instead, a main effect of time ($F_{(2,42)} = 8.0$; $p < 0.001$) and a significant time × treatment interaction ($F_{(6,42)} = 9.28$; $p < 0.001$) was evident. *Post hoc* analysis revealed that L-DOPA and J-113397 were ineffective, whereas their combination elevated rotarod performance at 60 min after injection.

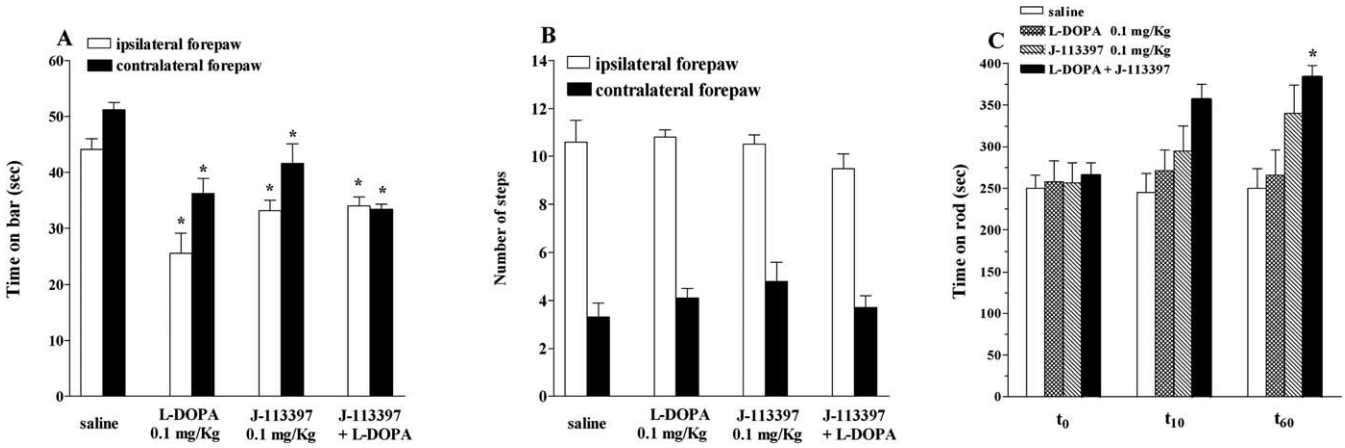

Figure 4. Combination of low (subthreshold) doses of J-113397 and L-DOPA relieved akinesia/hypokinesia in hemiparkinsonian rats. ***A***, Systemic (intraperitoneal) administration of J-113397 (0.1 mg/kg), L-DOPA (0.1 mg/kg plus 15 mg/kg benserazide), or their combination reduced the time spent on the blocks in the bar test. ***B***, No effect was observed in the drag test. ***C***, In the rotarod test, improvement of motor performance was detected only when J-113397 and L-DOPA were combined. Motor asymmetry was evaluated by separate measures at the paws ipsilateral and contralateral (parkinsonian) to the lesioned side. The bar and drag tests were performed 10 min after injection; the rotarod test was performed 10 and 60 min after injection. Data are mean ± SEM (6–14 rats per group). Statistical analysis was performed by conventional two-way ANOVA followed by the Newman–Keuls test (***A***, ***B***) or by two-way ANOVA with repeated measures followed in contrast analysis and the sequentially rejective Bonferroni test (***C***). $^{*}p < 0.05$ versus saline-treated rats.

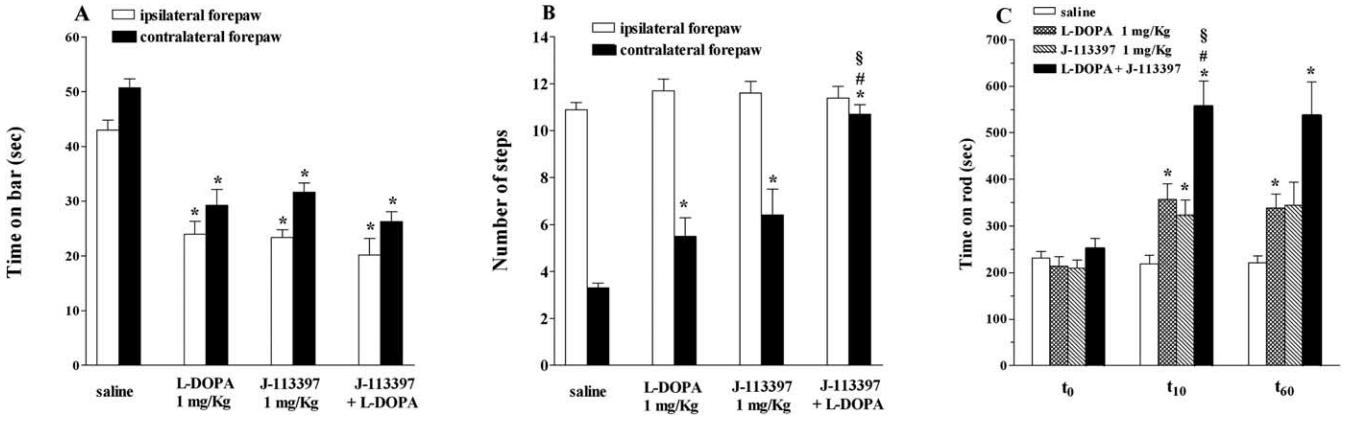

Figure 5. Combination of high (submaximal) doses of J-113397 and L-DOPA relieved akinesia/hypokinesia in hemiparkinsonian rats. ***A–C***, Systemic (intraperitoneal) administration of J-113397 (1 mg/kg), L-DOPA (1 mg/kg plus 15 mg/kg benserazide), or their combination reduced the time spent on the blocks in the bar test (***A***), increased the number of steps made by the contralateral paw in the drag test (***B***), and improved overall motor performance in the rotarod test (***C***). In the drag and rotarod tests, a greater effect (additive) was detected when J-113397 and L-DOPA were combined together. Motor activity was evaluated by separate measures at the paws ipsilateral and contralateral (parkinsonian) to the lesioned side. The bar and drag tests were performed 10 min after injection; the rotarod test was performed 10 and 60 min after injection. Data are mean ± SEM (6–10 rats per group). Statistical analysis was performed by conventional two-way ANOVA followed by the Newman–Keuls test (***A***, ***B***) or by two-way ANOVA with repeated measures followed by contrast analysis and the sequentially rejective Bonferroni test (***C***). $^{*}p < 0.05$ versus saline-treated rats. $^{\#}p < 0.05$ versus L-DOPA alone. $^{\S}p < 0.05$ versus J-113397 alone.

Interaction between submaximal doses (experiment 3)

In the bar test (Fig. 5*A*), the main effect of L-DOPA ($F_{(1,26)} = 29.00$; $p < 0.0001$) and J-113397 ($F_{(1,26)} = 26.77$; $p < 0.0001$) were found together with a significant L-DOPA × J-113397 interaction ($F_{(1,26)} = 15.03$; $p = 0.0006$) at the ipsilateral paw. Main effects of L-DOPA ($F_{(1,23)} = 43.30$; $p = 0.0001$) and J-113397 ($F_{(1,23)} = 21.33$; $p < 0.0001$) and a significant L-DOPA × J-113397 interaction ($F_{(1,23)} = 17.42$; $p = 0.0004$) were also observed at the contralateral paw. At both forepaws, the effects of each compound alone did not differ from that induced by their coadministration.

In the drag test (Fig. 5*B*), neither compound affected motor activity at the side of the body ipsilateral to the lesion. Conversely, two-way ANOVA revealed the main effect of L-DOPA ($F_{(1,29)} = 23.70$; $p < 0.0001$) and J-113397 ($F_{(1,29)} = 13.25$; $p = 0.0011$), although not a significant L-DOPA × J-113397 interaction ($F_{(1,29)} = 0.09$; $p = 0.76$), at the contralateral forepaw. *Post hoc* analysis showed that the coapplication induced a greater effect than each compound alone, leading to restoration of motor activity at the parkinsonian forepaw.

In the rotarod test (Fig. 5*C*), main effects of treatment ($F_{(3,16)} = 9.53$; $p = 0.0007$) and time ($F_{(2,57)} = 32.24$; $p < 0.0001$) and a significant time × treatment interaction ($F_{(10,57)} = 6.91$; $p < 0.0001$) were found. *Post hoc* analysis at 10 min after injection revealed that L-DOPA and J-113397 elevated rotarod performance, and the coapplication produced greater (additive) improvement.

L-DOPA and J-113397 differentially modulated GLU and GABA release in the SNr (experiment 4)

In previous studies, we showed that NOP receptor antagonists inhibited spontaneous (Marti et al., 2002a, 2005) and haloperidol-evoked (Marti et al., 2004b, 2005) GLU release in the SNr and that this effect correlated with relief from akinesia. We

therefore first investigated whether additive antiparkinsonian effects produced by coadministration of submaximal L-DOPA and J-113397 doses were associated with greater reduction of SNr GLU release (Fig. 6*A*,*B*). GLU levels did not differ in the unlesioned (96.1 ± 6.9 nM, $n = 30$) and lesioned (94.4 ± 6.9 nM, $n = 30$) SNr. Neither compounds affected GLU outflow in the unlesioned SNr (Fig. 6*A*). However, repeated-measure ANOVA revealed a main effect of treatment ($F_{(3,18)} = 33.15$; $p < 0.0001$) and time ($F_{(7,168)} = 9.53$; p=<0.0001), as well as a significant time × treatment interaction ($F_{(21,168)} = 3.27$; $p = 0.0045$) in the lesioned SNr (Fig. 6*B*). *Post hoc* analysis showed that L-DOPA did not affect GLU levels, whereas J-113397 reduced them, the combination of the two being no more effective than J-113397 alone. Because these findings did not indicate that changes in GLU levels underlie the additive effect on behavior, we next measured GABA release in the same area (Fig. 6*C*,*D*). GABA levels did not differ in the unlesioned (10.5 ± 0.4 nM, $n = 26$) and lesioned (10.0 ± 0.5 nM, $n = 26$) SNr. Neither compounds affected GABA outflow in the unlesioned SNr (Fig. 6*C*). Conversely, repeated-measure ANOVA showed main effects of treatment ($F_{(3,15)} = 26.46$; $p < 0.0001$) and time ($F_{(7,147)} = 23.42$; $p < 0.0001$) and a significant time × treatment interaction in the lesioned SNr (Fig. 6*D*). *Post hoc* analysis revealed that L-DOPA and J-113397 elevated GABA levels compared with saline, the effect of the combination being greater than that of each compound alone (Fig. 6*D*).

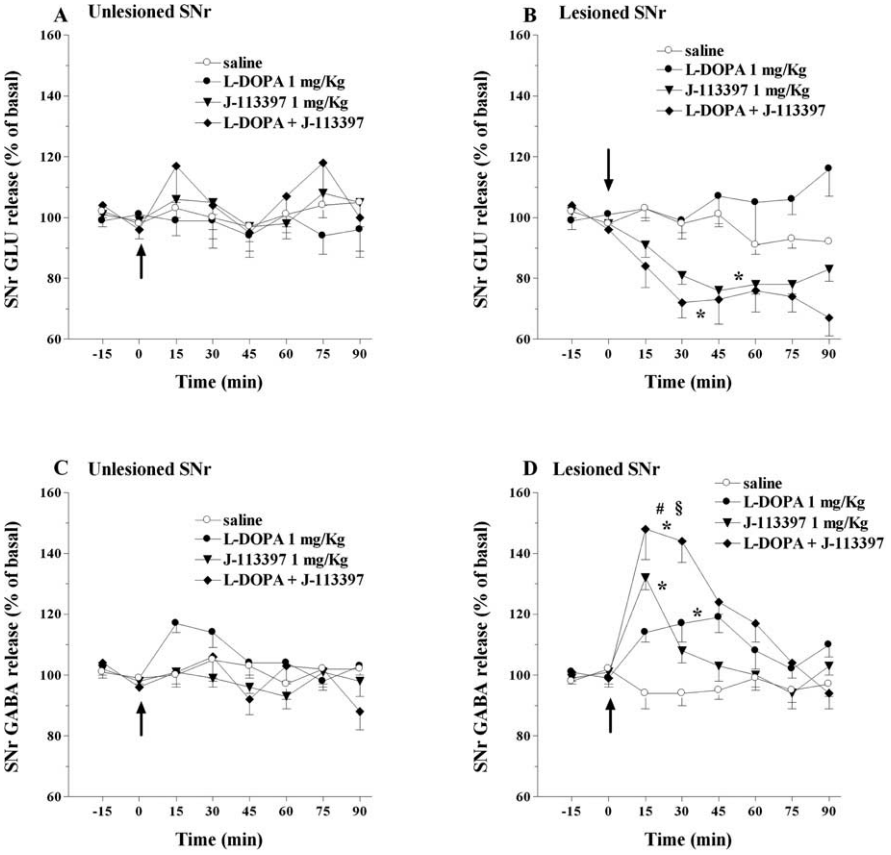

Figure 6. J-113397 and L-DOPA regulated GLU and GABA release in the lesioned SNr. ***A–D***, Two microdialysis probes were bilaterally implanted in the unlesioned (***A***, ***C***) and lesioned (***B***, ***D***) SNr of hemiparkinsonian rats. Rats were treated systemically (intraperitoneally; arrow) with J-113397 (1 mg/kg), L-DOPA (1 mg/kg plus benserazide 15 mg/kg), or their combination. Data are expressed as means ± SEM of *n* experiments. Basal GLU levels (in nanomolar concentration) in the dialysate from the unlesioned and lesioned SNr were, respectively, as follows: 100.2 ± 15.8, 103.7 ± 19.5 (saline, $n = 6$ both); 76.8 ± 15.2, 73.2 ± 14.7 (L-DOPA, $n = 6$ both); 112.4 ± 12.4, 100.9 ± 12.1 (J-113397, $n = 11$ and 12, respectively); 83.4 ± 9.5, 94.7 ± 9.9 (L-DOPA + J-113397, $n = 7$ both). Basal GABA levels (in nanomolar concentration) in the dialysate from the unlesioned and lesioned SNr were, respectively, as follows: 9.4 ± 0.6, 9.2 ± 0.9 (saline, $n = 6$ both); 10.9 ± 0.9, 9.5 ± 1.0 (L-DOPA, $n = 6$ both); 10.5 ± 1.2, 11.6 ± 0.9 (J-113397, $n = 7$ both); 11.0 ± 0.7, 9.6 ± 1.3 (L-DOPA + J-113397, $n = 7$ both). Statistical analysis was performed by two-way ANOVA with repeated measures followed by contrast analysis and the sequentially rejective Bonferroni test. $^{*}p <$ 0.05 versus saline-treated rats. $^{\S}p < 0.05$ versus J-113397 alone. $^{\#}p < 0.05$ versus L-DOPA alone.

L-DOPA and J-113397 reduced GABA release in the VMTh (experiment 5)

SNr is known to send GABAergic projections to the VMTh (Di Chiara et al., 1979; MacLeod et al., 1980), which represents the motor output of the basal ganglia. Thus, we hypothesized that the attenuation of motor disabilities could be associated with inhibition of nigrothalamic transmission. To this purpose, we first investigated whether J-113397 and L-DOPA administration evoked changes in thalamic GABA release (Fig. 7*A*,*B*). GABA levels in the dialysate from the VMTh ipsilateral to the lesioned SNr (18.9 ± 1.0 nM; $n = 27$) were ~23% higher compared with those collected from the contralateral one (14.6 ± 1.0 nM; $n = 25$; $p <$ 0.05, Student's *t* test). Neither L-DOPA or J-113397 affected GABA release in the VMTh contralateral to the lesioned SNr (Fig. 7*A*). However, repeated-measure ANOVA revealed main effects of treatment ($F_{(3,15)} = 18.48$; $p < 0.0001$) and time ($F_{(7,132)} = 13.55$; $p < 0.0001$) and a significant time × treatment interaction ($F_{(21,132)} = 2.24$; $p = 0.003$) in the VMTh ipsilateral to the lesioned SNr (Fig. 7*B*). *Post hoc* analysis revealed that L-DOPA and J-113397 inhibited thalamic GABA release compared with saline, and the combination produced greater inhibition compared with that evoked by each compound alone.

Simultaneous behavioral testing was performed in animals subjected to microdialysis, and motor activity was assessed separately at the contralateral and ipsilateral forepaw by using the bar test (Fig. 7*C*,*D*). At the contralateral paw, the main effects of treatment ($F_{(3,32)} = 34.23$; $p < 0.0001$) and time ($F_{(7,300)} = 95.22$; $p < 0.0001$) and a significant time × treatment interaction ($F_{(21,300)} = 14.06$; $p < 0.0001$) were found (Fig. 7*C*). The main effects of treatment ($F_{(3,32)} = 8.44$; $p < 0.0001$) and time ($F_{(7,300)} = 51.07$; $p < 0.0001$) and a significant time × treatment interaction ($F_{(21,300)} = 8.50$; $p < 0.0001$) were also observed at the ipsilateral paw (Fig. 7*D*). *Post hoc* analysis showed that L-DOPA and J-113397 reduced to the same extent the time spent on the blocks at both the contralateral and ipsilateral forepaw, although only at the contralateral paw the combined administration of L-DOPA and J-113397 produced greater effect than each compound alone.

Reverse dialysis of TTX in the SNr prevented changes in thalamic GABA release and motor behavior by L-DOPA and J-113397 combination (experiment 6)

To demonstrate that ongoing neuronal activity in the SNr was essential for both the reduction of GABA release in the VMTh

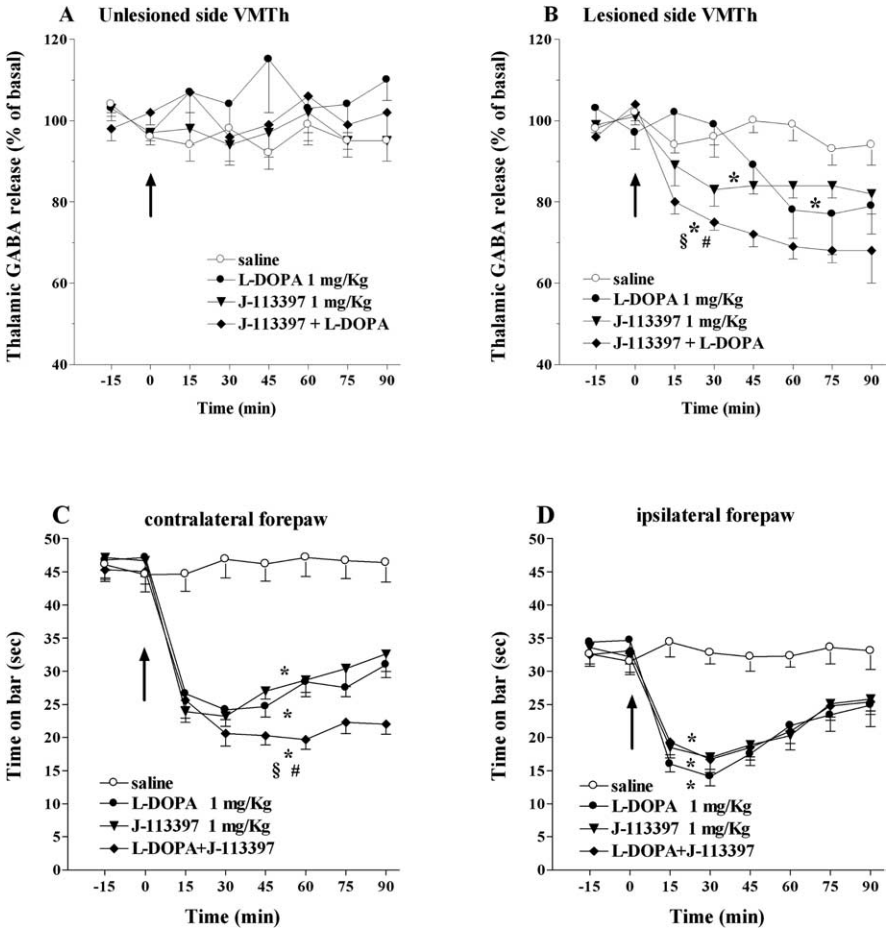

Figure 7. J-113397 and L-DOPA produced inhibition of GABA release in the VMTh and reduction of the time spent on the blocks. ***A***, ***B***, Two microdialysis probes were bilaterally implanted in the VMTh contralateral (unlesioned side; ***A***) and ipsilateral (lesioned side; ***B***) to the lesioned SNr of hemiparkinsonian rats. Rats were treated systemically (intraperitoneally; arrow) with J-113397 (1 mg/kg), L-DOPA (1 mg/kg plus 15 mg/kg benserazide), or their combination. Data are expressed as means ± SEM of *n* experiments. Basal GABA levels (in nanomolar concentration) in the dialysate from the VMTh at the unlesioned and lesioned side were, respectively, as follows: 12.5 ± 1.0, 15.8 ± 2.3 (saline, $n = 6$ both); 16.4 ± 1.9, 20.4 ± 0.4 (L-DOPA, $n = 6$ both); 15.4 ± 1.2, 20.9 ± 1.7 (J-113397, $n = 6$ both); 15.3 ± 3.2, 22.7 ± 1.4 (L-DOPA + J-113397, $n = 6$ both). ***C***, ***D***, Hemiparkinsonian rats implanted in the SNr (Fig. 6) or VMTh were challenged in the bar test. Akinesia was evaluated (every 15 min for up to 90 min) by using the bar test separately at the contralateral (***C***) and ipsilateral (***D***) paws (described in Materials and Methods). L-DOPA and J-113397 attenuated akinesia at both paws although greater effect was observed at the contralateral paw when both compounds were applied together. Data are means ± SEM of *n* experiments (8–12 per group). Statistical analysis was performed by two-way ANOVA with repeated measures followed by contrast analysis and the sequentially rejective Bonferroni test. $^{*}p < 0.05$ versus saline-treated rats. $^{\#}p < 0.05$ versus L-DOPA alone. $^{\S}p < 0.05$ versus J-113397 alone.

and the antiakinetic action, L-DOPA and J-113397 were coadministered systemically while perfusing the voltage-operated Na^+ channel blocker TTX in the lesioned SNr (Fig. 8).

Repeated-measure ANOVA on GABA levels in the lesioned SNr (Fig. 8*A*) revealed a main effect of treatment ($F_{(3,14)} = 46.40$; $p < 0.0001$) and time ($F_{(7,125)} = 9.50$; $p < 0.0001$) and a significant time × treatment interaction ($F_{(21,125)} = 7.52$; $p < 0.0001$). *Post hoc* analysis showed that SNr perfusion with TTX did not affect spontaneous SNr GABA levels and prevented the elevation of GABA release induced by combined administration of J-113397 and L-DOPA. Repeated-measure ANOVA on ipsilateral VMTh GABA levels (Fig. 8*B*) revealed main effect of treatment ($F_{(3,14)} = 77.50$; $p < 0.0001$) and time ($F_{(7,125)} = 2.55$; $p = 0.017$) and a significant time × treatment interaction ($F_{(21,125)} = 4.63$; $p < 0.0001$). *Post hoc* analysis showed that SNr perfusion with TTX reduced VMTh GABA release and prevented the inhibitory effect induced by coapplication of J-113397 and L-DOPA.

From a behavioral point of view, repeated-measure ANOVA analysis at the contralateral paw revealed a main effect of treatment ($F_{(3,12)} = 15.40$; $p = 0.0002$) and time ($F_{(7,120)} = 13.61$; $p < 0.0001$), as well as a significant time × treatment interaction ($F_{(21,120)} = 6.92$; $p < 0.0001$) (Fig. 8*C*). A similar response was observed at the ipsilateral paw (Fig. 8*D*). *Post hoc* analysis showed that TTX significantly reduced the time spent on the bar and prevented the inhibition induced by coapplication of J-113397 and L-DOPA.

Reverse dialysis of bicuculline in the SNr prevented changes in thalamic GABA release and motor behavior by L-DOPA and J-113397 combination (experiment 7)

To demonstrate that activation of $GABA_A$ receptors in the SNr was responsible for overinhibition of nigrothalamic neurons and antiparkinsonian action associated with it, L-DOPA and J-113397 were coadministered systemically while perfusing the $GABA_A$ receptor blocker bicuculline in the lesioned SNr (Fig. 9).

Repeated-measure ANOVA on GABA levels in the lesioned SNr (Fig. 9*A*) revealed a main effect of treatment ($F_{(3,14)} = 27.75$; $p < 0.0001$) and time ($F_{(7,125)} = 11.05$; $p < 0.0001$) and a significant time × treatment interaction ($F_{(21,125)} = 5.17$; $p < 0.0001$). *Post hoc* analysis showed that perfusion with bicuculline in the SNr did not affect basal GABA levels and left unchanged the elevation of GABA release induced by combined administration of J-113397 and L-DOPA. In the VMTh (Fig. 9*B*), main effects of treatment ($F_{(3,14)} = 10.10$; $p = 0.0008$), but not time ($F_{(7,125)} = 1.58$; $p = 0.14$), and a significant time × treatment interaction ($F_{(21,125)} = 2.29$; $p = 0.0025$) were found. *Post hoc* analysis revealed that bicuculline did not affect spontaneous GABA release and prevented the inhibitory influence induced by J-113397 and L-DOPA combination.

From a behavioral point of view, repeated-measure ANOVA at the contralateral paw (Fig. 9*C*) revealed a main effect of treatment ($F_{(3,12)} = 36.33$; $p < 0.0001$) and time ($F_{(7,112)} = 8.12$; $p < 0.0001$), as well as a significant time × treatment interaction ($F_{(21,112)} = 8.93$; $p < 0.0001$). At the ipsilateral paw (Fig. 9*D*), repeated-measure ANOVA revealed a main effect of treatment ($F_{(3,12)} = 4.51$; $p = 0.024$) and time ($F_{(7,112)} = 2.16$; $p = 0.043$) and a significant time × treatment interaction ($F_{(7,112)} = 2.82$; $p < 0.0001$). *Post hoc* analysis revealed that bicuculline did not affect the time spent on the bar but prevented the inhibitory effect induced by J-113397 and L-DOPA combination.

To study the relevance of GLU release inhibition in the antiakinetic response to combined application of L-DOPA and J-113397, GLU levels were analyzed in the lesioned SNr during perfusion of bicuculline (Fig. 10). Repeated-measure ANOVA

revealed a main effect of treatment ($F_{(3,14)} = 18.80$; $p < 0.0001$) and time ($F_{(7,125)} = 15.46$; $p < 0.0001$) and a significant time × treatment interaction ($F_{(21,125)} = 5.47$; $p < 0.0001$). *Post hoc* analysis showed that perfusion with bicuculline in the SNr did not affect basal GLU levels. However, it delayed the reduction in GLU release induced by combined administration of J-113397 and L-DOPA.

Discussion

Systemic administration of an NOP receptor antagonist (J-113397) or L-DOPA alone dose-dependently attenuated parkinsonian-like symptoms in hemiparkinsonian rats, whereas their combination evoked additive effects depending on the dose and the test used. Both compounds exerted their antiakinetic actions through a common "effector" as they produced additive elevation of SNr GABA and inhibition of VMTh GABA release. This effect was dependent on ongoing neuronal activity in the SNr (TTX sensitive) and activation of SNr $GABA_A$ receptors (bicuculline sensitive), suggesting that J-113397 and L-DOPA acted through overinhibition of nigrothalamic GABAergic neurons.

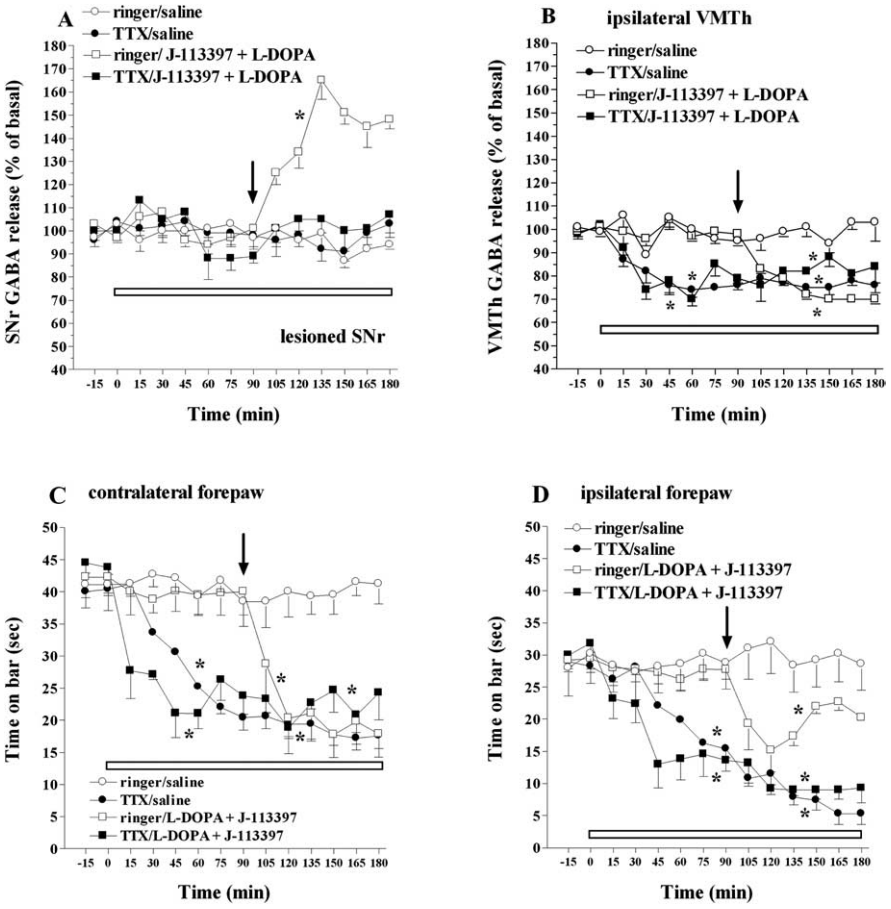

Figure 8. TTX prevented the reduction in VMTh GABA release and immobility time induced by L-DOPA and J-113397 coadministration. Two microdialysis probes were implanted in the lesioned SNr and ipsilateral VMTh of hemiparkinsonian rats. Perfusion with TTX (1 μM; open bar) in the SNr started 90 min before systemic (intraperitoneal) coadministration (arrow) of J-113397 (1 mg/kg) and L-DOPA (1 mg/kg plus 15 mg/kg benserazide) and continued until the end of experiment. ***C***, ***D***, In the same rats, akinesia was evaluated (every 15 min for up to 180 min) by using the bar test separately at the contralateral (***C***) and ipsilateral (***D***) paws (described in Materials and Methods). Statistical analysis was performed by two-way ANOVA with repeated measures followed by contrast analysis and the sequentially rejective Bonferroni test. Data are means ± SEM of *n* experiments. ***A***, ***B***, Basal GABA levels (in nanomolar concentration) in the dialysate from the lesioned SNr (***A***) and ipsilateral VMTh (***B***) were, respectively, as follows: 9.1 ± 0.6 and 9.7 ± 1.1 (washout/saline, $n = 5$); 7.0 ± 0.5 and 11.6 ± 0.8 (TTX/saline, $n = 6$); 7.3 ± 0.7 and 13.5 ± 0.9 (washout/J-113397 + L-DOPA, $n = 5$); 9.0 ± 0.5 and 10.9 ± 2.1 (TTX/J-113397 + L-DOPA, $n = 5$). *$p < 0.05$ versus saline-treated rats.

J-113397/L-DOPA interaction on behavior

Unilateral lesion of SNc DA neurons caused dramatic bilateral increase in the immobility time, with the contralateral ("parkinsonian") forepaw being more severely affected than the ipsilateral ("good") one. Although puzzling, this observation is consistent with reports that unilateral DA depletion also affected posture (Whishaw et al., 2003), stepping time, and stepping length (Olsson et al., 1995) at the ipsilateral paw. Interestingly, when the animal was forced to move (drag test), motor activity at the ipsilateral paw was only slightly impaired, suggesting that the bar and drag test provide information on different aspects of motor program. The bar test essentially measures the time to initiate a movement (akinesia), whereas the drag test measures both the time to initiate and to execute it (bradykinesia). The degree of motor asymmetry in the drag test (~65%) was consistent with that reported by Wessell et al. (2004) (~75%) and in line with that found with the stepping [~90% (Olsson et al., 1995); 80–90% (Winkler et al., 2002); ~70% (Tillerson et al., 2001); ~75% (Tseng et al., 2005); 50–90% (Kelsey et al., 2004)] or the postural adjustment [~75% (Lindner et al., 1996); >95% (Chang et al., 1999)] tests. Powerful, dose-dependent attenuation of parkinsonism was produced by increasing L-DOPA doses: reduction of akinesia at both the ipsilateral and the contralateral forepaw (0.1 mg/kg), improvement of exercise-induced motor performance (0.3 mg/kg), and reversal of motor asymmetry both under resting (bar test) and dynamic (drag test) conditions (6 mg/kg). Similar findings were reported by using the stepping (Olsson et al., 1995; Chang et al., 1999; Winkler et al., 2002; Kelsey et al., 2004), postural adjustment (Lindner et al., 1996), and "wheelbarrow" [i.e., forward dragging (Schallert et al., 1979)] tests. It is noteworthy that L-DOPA exerted an antiparkinsonian action at doses (0.1–6 mg/kg) lower than those eliciting contralateral rotations (25 mg/kg), strengthening the view that ethological tests may be more sensitive than analysis of pharmacologically induced (e.g., by dopamine agonists) turning behavior in screening for antiparkinsonian drugs.

The NOP receptor antagonist J-113397 reproduced L-DOPA action, although less effectively because it did not reverse fully motor asymmetry in the drag test (Marti et al., 2005). However, combination of subthreshold doses (0.1 mg/kg) of J-113397 and L-DOPA (ineffective per se) stimulated rotarod performance to the same extent as L-DOPA 0.3 mg/kg, whereas combination of submaximal doses (1 mg/kg) was as effective as L-DOPA 6 mg/kg in the drag test (i.e., it fully reversed asymmetry) and evoked a sustained antiakinetic response in the bar test (see Results). Most interestingly, combination of submaximal doses evoked supramaximal rotarod performance, suggesting that L-DOPA and

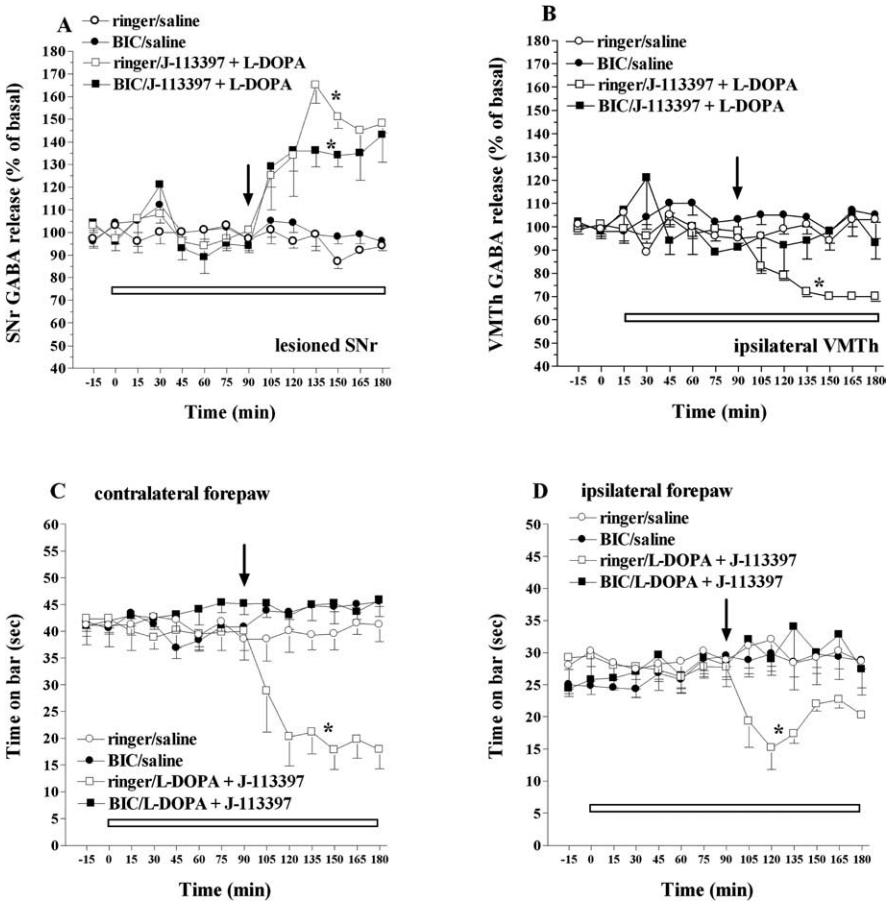

Figure 9. Bicuculline (BIC) prevented the reduction in VMTh GABA release and immobility time induced by L-DOPA and J-113397 coadministration. Two microdialysis probes were implanted in the lesioned SNr and ipsilateral VMTh of hemiparkinsonian rats. Perfusion with BIC (10 μM; open bar) in the SNr started 90 min before systemic (intraperitoneal) coadministration (arrow) of J-113397 (1 mg/kg) and L-DOPA (1 mg/kg plus 15 mg/kg benserazide) and continued until the end of experiment. ***C***, ***D***, In the same rats, akinesia was evaluated (every 15 min for up to 180 min) by using the bar test separately at the contralateral (***C***) and ipsilateral (***D***) paws (described in Materials and Methods). Statistical analysis was performed by two-way ANOVA with repeated measures followed by contrast analysis and the sequentially rejective Bonferroni test. Data are means ± SEM of *n* experiments. ***A***, ***B***, Basal GABA levels (in nanomolar concentration) in the dialysate from the lesioned SNr (***A***) and ipsilateral VMTh (***B***) were, respectively, as follows: 9.1 ± 0.6 and 9.7 ± 1.1 (washout/saline, $n = 5$); 7.8 ± 0.9 and 11.9 ± 1.8 (BIC/saline, $n = 5$); 7.3 ± 0.7 and 13.5 ± 0.9 (washout/J-113397 + L-DOPA, $n = 5$); 9.2 ± 1.5 and 12.1 ± 2.3 (BIC/J-113397 + L-DOPA, $n = 5$). *$p < 0.05$ versus saline-treated rats.

J-113397 activated independent neuronal pathways rather than a common, "saturable" mechanism. These data suggest that an NOP receptor antagonist may be a good candidate for a combination therapy. From a clinical point of view, combination therapy may benefit early PD patients by lowering L-DOPA dosage and delaying dyskinesia onset, or it may benefit advanced PD patients who require more than one medication to adequately control PD symptoms.

Neurobiological substrates underlying J-113397/L-DOPA interaction

Massive GLUergic innervation to the SNr is provided by the subthalamic nucleus, which becomes overactive under parkinsonian conditions (DeLong, 1990). Consistently, haloperidol evoked catalepsy and elevated SNr GLU release (Marti et al., 2004b, 2005). NOP receptor antagonists attenuated haloperidol-induced akinesia and normalized SNr GLU levels (Marti et al., 2004b, 2005), prompting us to suggest that inhibition of SNr GLU release underlies their antiparkinsonian action. SNr injections of UFP-101 [[Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH$_2$] (Marti et al., 2004b) reproduced the effects of systemic injections of J-113397 (Marti et al., 2005), further pointing to the SNr as their site of action. Reverse dialysis of N/OFQ in the SNr elevated local GLU release via GABA- and DA-mediated mechanisms (Marti et al., 2002a). Thus, NOP receptor antagonists may reduce SNr GLU release in the DA-depleted SNr by blocking tonic inhibition of DA and GABA neurons by endogenous N/OFQ. Indeed, N/OFQ inhibits DA and GABA transmission in mesencephalic areas (Zheng et al., 2002; Marti et al., 2004a), whereas NOP receptor antagonists elevate SNr GABA release (present study) and nigrostriatal DA transmission (Marti et al., 2004a). However, bicuculline did not affect the increase in SNr GABA and only delayed the reduction in GLU release evoked by L-DOPA and J-113397, suggesting that GABAergic inhibition is minimally involved in this phenomenon. In the same animals, bicuculline prevented the motor effects of combined L-DOPA and J-113397, further indicating that inhibition of GLU release may not be sufficient to explain the antiakinetic action of J-113397. In this respect, J-113397 also increased GABA release in the lesioned SNr and lowered GABA release in the ipsilateral VMTh. L-DOPA shared this action, although it did not affect SNr GLU levels. This suggests that DA receptor stimulation and NOP receptor blockade activated independent neuronal pathways, converging on a common "effector". According to the current model of basal ganglia functional organization (DeLong et al., 1990), reduction of the subthalamonigral GLUergic excitatory drive and/or increase of the GABAergic inhibitory influence on nigrothalamic GABAergic neurons leads to disinhibition of thalamocortical GLUergic projections and movement initiation (Deniau and Chevalier, 1985). Thus, this "effector" is likely represented by nigrothalamic GABAergic neurons. More evidence endorses this view. TTX perfusion in the SNr produced per se a rapid decline in VMTh GABA release. This indicates that spontaneous VMTh GABA release partly reflects impulse-dependent neuronal activity and is consistent with the fact that nigrothalamic GABA neurons provide a tonic input to the thalamus (Guyenet and Aghajanian 1978; Waszczak et al., 1980). This input is enhanced after DA lesion (Burbaud et al., 1995), which may explain why GABA levels were higher in the VMTh ipsilateral to the lesioned side. Interestingly, reduction of VMTh GABA release induced by TTX was associated with attenuation of akinesia. This is consistent with our previous finding that SNr perfusion of higher TTX concentrations (10 μM) evoked contralateral rotations in naive rats (Morari et al., 1996) and strengthens the view that inhibition of nigrothalamic GABAergic neurons results in disinhibition of locomotion (Starr et al., 1983; Deniau and Chevalier, 1985). Nigrothalamic GABAergic neurons express $GABA_A$ receptors (Nicholson et al., 1995), which drive both tonic (Rick

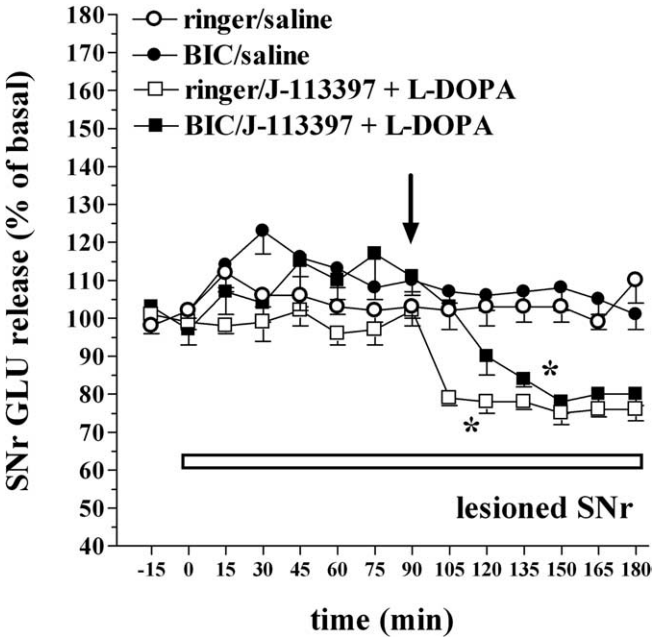

Figure 10. Bicuculline (BIC) delayed the reduction in SNr GLU release induced by L-DOPA and J-113397 coadministration. Perfusion with BIC (10 μM; open bar) in the SNr started 90 min before systemic (intraperitoneal) coadministration (arrow) of J-113397 (1 mg/kg) and L-DOPA (1 mg/kg plus 15 mg/kg benserazide) and continued until the end of experiment. Statistical analysis was performed by two-way ANOVA with repeated measures followed by contrast analysis and the sequentially rejective Bonferroni test. Basal GLU levels (in nanomolar concentration) in the dialysate from the lesioned SNr were, respectively, as follows: 115.3 $\pm$ 6.1 (washout/saline, $n = 5$); 104.2 $\pm$ 7.4 (BIC/saline, $n = 6$); 105.3 $\pm$ 6.7 (washout/J-113397 + L-DOPA, $n = 5$); 129.9 $\pm$ 14.8 (BIC/J-113397 + L-DOPA, $n = 5$). *$p < 0.05$ versus saline-treated rats.

and Lacey, 1994) and phasic (*in vivo*) (Deniau and Chevalier, 1985) inhibition. Thus, bicuculline may prevent both the attenuation of akinesia and the decrease in VMTh GABA release by blocking those postsynaptic $GABA_A$ receptors. Likewise, bicuculline prevented the reduction in VMTh GABA release induced by systemic methamphetamine (Mark et al., 2004). Alternatively, blockade of inhibitory $GABA_A$ receptors on (residual) nigral DA cells may lead to increased DA release (Westerink et al., 1992) and attenuation of the inhibitory control driven by $GABA_A$ receptors onto nigrothalamic GABAergic neurons (Waszczak and Walters, 1986), which may also result in attenuation of the antiakinetic effect of L-DOPA.

The mechanisms through which systemic L-DOPA increased SNr GABA release also remain to be investigated. L-DOPA could stimulate D_1 receptors on striatonigral neurons (Gerfen et al., 1990), so to activate the "direct" pathway (Robertson and Robertson, 1989; Carta et al., 2005), or D_1 receptors on striatonigral nerve terminals (Starr, 1987; Aceves et al., 1991). The fact that TTX prevented the L-DOPA effect rules out this latter hypothesis, although it does not exclude an intranigral action of L-DOPA because this agent could stimulate GABAergic interneurons [dendritic location of D_1 receptors has been detected in the SNr (Huang et al., 1992)]. Finally, the possibility that L-DOPA increases SNr GABA release by modulating the subthalamonigral GLUergic projection cannot be discarded because L-DOPA elevated SNr GLU levels. However, this possibility appears remote in view of the mismatch between the dynamics of GABA and GLU levels.

Concluding remarks

Combined administration of an NOP receptor antagonist (J-113397) and L-DOPA produced additive attenuation of parkinsonian-like symptoms through an increase in SNr GABA release and consequent overinhibition of SNr GABAergic neurons projecting to the VMTh. These data strengthen the role of the VMTh in the modulation of parkinsonism (Wolfarth et al., 1985; Kolasiewicz et al., 1988), emphasizing its role as a relay nucleus for impulses ascending from the SNr to the cortex (Klockgether et al., 1986). This appears relevant also in humans, in which motor improvement induced by deep brain stimulation in the subthalamic nucleus was associated with lowered GABA release in the motor thalamus (Stefani et al., 2006). Overall, the present study provides novel insights into the mechanisms underlying the antiparkinsonian action of J-113397 and L-DOPA and suggests that an NOP receptor antagonist may be used alone or as an adjunct to L-DOPA in the therapy of PD.

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