ELSEVIER

Pharmacology, Biochemistry and Behavior 71 (2002) 353–363

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

www.elsevier.com/locate/pharmbiochembeh

Circling behavior induced by microinjection of serotonin reuptake inhibitors in the substantia nigra

José L. Bata-García[a], Francisco J. Heredia-López[a], Fernando J. Alvarez-Cervera[a], Gloria Arankowsky-Sandoval[b], José L. Góngora-Alfaro[a,*]

[a]*Laboratorios de Neurofisiología y,* [b]*Neurobiología, Centro de Investigaciones Regionales, "Dr. Hideyo Noguchi," Universidad Autónoma de Yucatán, Avenida Itzaes No. 490, Mérida, Yucatán, México 97000*

Received 28 March 2001; received in revised form 21 September 2001; accepted 9 October 2001

Abstract

The nigrostriatal dopaminergic neurons of the substantia nigra pars compacta (SNc) and the nondopaminergic neurons of the substantia nigra pars reticulata (SNr) receive a dense synaptic input from the serotonergic neurons of the raphe nuclei. To assess whether serotonin [5-hydroxytryptamine (5-HT)] spontaneously released at the substantia nigra could modulate motor activity, the 5-HT reuptake inhibitors (SRIs), duloxetine (6–12 nmol) and clomipramine (12 nmol), were unilaterally microinjected either into the SNc or the SNr of freely moving rats, and the circling behavior was counted with an automated rotometer. In the SNc, the main effect of the SRIs was a contraversive circling behavior that was not observed when applied at distances ≥ 0.2 mm above the SNc. The circling induced by clomipramine was blocked by microinjection of haloperidol (53 nmol) into the ipsilateral neostriatum, suggesting that the circling elicited by microinjection of the SRIs into the SNc depends on an intact striatal dopaminergic transmission. Microinjection of 5-HT (21 nmol) only produced a significant contraversive circling response when it was coinjected with the SRIs. Pretreatment with methysergide (1 mg/kg ip), a nonselective 5-HT_2 antagonist, did not block the circling elicited by microinjection of clomipramine into the SNc, either alone or in combination with 5-HT. However, microinjection of the 5-HT_2 antagonist mianserin (2 nmol) into the SNc partially inhibited the circling induced by duloxetine (6 nmol), alone or coinjected with 5-HT. Since current theories of circling behavior hypothesize that the animal turns away from the cerebral hemisphere where dopamine neurotransmission predominates, these results suggest that the contraversive circling induced by the unilateral microinjection of SRIs into the SNc could be mediated by a 5-HT-induced increase of firing frequency of nigrostriatal dopaminergic neurons. When applied into the SNr, clomipramine and duloxetine also elicited a contraversive circling behavior and enhanced the circling induced by 5-HT. Systemic methysergide (1 mg/kg ip), but not intranigral mianserin (2 nmol), blocked the circling elicited by microinjection of clomipramine into the SNr, either alone or in combination with 5-HT. These results suggest that 5-HT_2-like receptors are involved in the contraversive circling induced by enhancement of serotonergic transmission in the SNr.

Keywords: Turning behavior; Pars reticulata; Pars compacta; Basal ganglia; Duloxetine; Clomipramine

1. Introduction

The role of nigrostriatal dopaminergic neurons of the substantia nigra pars compacta (SNc) and of GABAergic projection neurons of the substantia nigra pars reticulata (SNr) in the control of motor behavior is well established (Smith et al., 1998). The spontaneous activity of these neurons is modulated by inhibitory and excitatory inputs from many nuclei, among which the dorsal raphe nucleus has emerged as the origin of a serotonergic projection that innervates both the SNc and SNr (Dray et al., 1976; Fibiger and Miller, 1977; Wirtshafter et al., 1987; Corvaja et al., 1993). In fact, the substantia nigra is among the brain nuclei with the highest content of serotonin [5-hydroxytryptamine (5-HT); Palkovitz et al., 1974; Reubi and Emson, 1978; Dewar et al., 1992], which is localized in a dense network of 5-HT-immunoreactive fibers and nerve endings (Mori et al., 1987; Lavoie and Parent, 1990). In the SNc, there is evidence that the serotonergic nerve terminals make synaptic

* Corresponding author. Apdo. Postal 47-8 Urbana Pensiones, Mérida, Yucatán, México 97217. Fax: +52-999-923-6120.

E-mail address: galfaro@tunku.uady.mx (J.L. Góngora-Alfaro).

0091-3057/01/$ – see front matter
PII: S0091-3057(01)00721-3

contact with tyrosine hydroxylase-immunoreactive neurons (Nedergaard et al., 1988; Corvaja et al., 1993; Moukhles et al., 1997), indicating that the dopaminergic neurons are under the modulatory influence of 5-HT. It has been calculated that the density of 5-HT-immunoreactive varicosities in the SNc is in the order of $6 \times 10^6/mm^3$, of which about 50% form synaptic specializations with dendrites (Moukhles et al., 1997). Within the SNr, the density of 5-HT synaptic buttons increases up to $9 \times 10^6/mm^3$, of which about 74% form synaptic specializations with nondopaminergic profiles, presumably the GABAergic projection neurons (Moukhles et al., 1997). This picture gives an idea of the great influence that 5-HT could exert on the activity of dopaminergic and nondopaminergic neurons of the substantia nigra.

With one exception (James and Starr, 1980), studies agree that contraversive circling is the predominant effect following unilateral microinjection of 5-HT or some selective 5-HT_1 agonists into the SNr of the rat (Blackburn et al., 1981; Oberlander et al., 1981) and the guinea pig (Higgins et al., 1991), suggesting that 5-HT receptors within the SNr are functional and participate in some way in motor behavior control. However, to our knowledge, there are no studies that have evaluated whether increasing the serotonergic transmission within the SNc or the SNr has distinct behavioral consequences.

Current theories of circling behavior hypothesize that the animal turns away from the cerebral hemisphere where dopamine neurotransmission predominates (Miller and Beninger, 1991). If 5-HT released in the SNc of freely moving animals modulates the firing rate of nigrostriatal dopaminergic neurons, one might expect that changes in serotonergic transmission within the SNc of one cerebral hemisphere would influence dopamine release in the ipsilateral neostriatum. If the main effect of 5-HT in SNc is excitatory, then contraversive circling should develop. The opposite would occur if inhibition of firing predominates. In the case of the SNr, it has been hypothesized that GABA released from the striatonigral pathway facilitates the motor control through inhibition of the efferent GABAergic SNr neurons that project to the superior colliculus, the ventromedial and mediodorsal thalamic nuclei and the pedunculopontine nucleus (Scheel-Krüger, 1986). According to this model, if excitation is the main effect of 5-HT on SNr GABAergic output neurons, ipsiversive circling should develop. The opposite would occur if inhibition of firing predominates.

Here, we have used the circling behavior model to assess the motor effects of 5-HT spontaneously released from the serotonergic nerve endings that make synapses with the nigrostriatal dopaminergic neurons and the GABAergic projection neurons of the SNr. For this purpose, the 5-HT reuptake inhibitors (SRIs), duloxetine and clomipramine (Wong et al., 1995), were unilaterally microinjected into the SNc or the SNr of freely moving rats, either alone or with 5-HT, and the circling behavior was counted with an automated rotometer. Since 5-HT_2 receptors mediate excitatory effects on SNc and SNr neurons (Pessia et al., 1994; Rick et al., 1995; Góngora-Alfaro et al., 1997b), attempts were made to block the circling induced by microinjection of SRIs into both nigral subdivisions with nonselective 5-HT_2 antagonists, applied locally or systemically. Finally, it was tested whether the circling induced by microinjection of SRIs in the SNc could be inhibited by disruption of striatal dopaminergic transmission by microinjecting the dopamine antagonist haloperidol into the neostriatum. Part of this work has been presented in abstract form (Góngora-Alfaro et al., 1996, 1997a).

2. Experimental procedures

2.1. Animals

Male Wistar-derived rats (260–320 g) bred in our facilities were used throughout the experiments. Animals were housed in individual acrylic cages at constant room temperature (23 ± 1 °C) and maintained on a 12:12-h light/dark cycle (lights on at 07:00 h). Food and water were available ad libitum. All efforts were made to minimize animal suffering according to the recommendations of the *Guide for the Care and Use of Laboratory Animals* (National Research Council, USA, 1996).

2.2. Surgery

Following a dose of atropine sulfate (0.1 mg/kg im) to prevent bronchial secretions, rats were anesthetized with sodium pentobarbital (45 mg/kg ip) and placed in a stereotaxic frame (Stoelting) with the incisor bar placed 3.3 mm below the interaural line (Paxinos and Watson, 1986). Guide cannulae (23-G needle tubing) were implanted into the right cerebral hemisphere, with their tips positioned 2 mm dorsal to the target nuclei. Coordinates for SNc were AP, from -5.3 to -6.04 mm from bregma; L, -2.1 mm from the midline and P, -4.9 mm from the cortex surface. Coordinates for SNr were AP, from -5.3 to -6.04 mm; L, -2.1 mm and P, -5.1 mm. In some animals, a second cannula was implanted at the level of the neostriatum at the coordinates AP, $+0.5$ mm; L, -2.6 mm and P, -2.4 mm. Cannulae were fixed to the skull with stainless-steel screws and dental acrylic, and stainless-steel stylets were inserted into them to avoid lumen occlusion. After surgery, benzathine penicillin (300,000 IU/kg im) was administered to prevent infection. The behavioral experiments were performed 7 days after surgery.

2.3. Circling behavior

Experiments were carried out during light hours (9:00–16:00 h). The circling behavior was measured in a flat circular container (diameter 40 cm). Animals were habituated to the receptacle for 30 min prior to the pharmaco-

logical manipulations. A 30-G stainless-steel infusion cannula with a conic tip was connected via PE10 tubing to a 10-μl microsyringe (Hamilton). To avoid contamination, a different catheter was used for each drug combination. Once inserted, the infusion cannula protruded 2 mm beyond the tip of the guide cannula. Physiological buffered saline (PBS; pH 7.4) or drug solutions in PBS were manually injected in steps of ≈ 0.05 μl/30 s. The volume injected into both the SNc and the SNr was 0.5 μl over 5 min and the volume in the neostriatum was 1 μl over 10 min. After completion of the injection, the cannula was left in place for an additional minute before being withdrawn. During the injection procedure, animals could freely move in the receptacle, and turns that occurred during this period were also recorded. Complete turns (360°) were counted at 5-min intervals with an automated rotometer that discriminates turns in either direction (Heredia-López et al., 1992).

2.4. Experimental design

For comparative purposes, two SRIs with different pharmacological profiles were tested. Clomipramine, a tricyclic compound that is about 16 times more potent to inhibit 5-HT vs. the noradrenaline transporter and also binds with high affinity to histamine H_1, adrenergic α_1 and muscarinic receptors and duloxetine, a SRI that is about three times more selective for the 5-HT transporter but does not have affinity for the indicated receptors (Wong et al., 1995). Both SRIs were tested at doses that are less than 50% of that used in the only available report about circling behavior following microinjection of SRIs in the substantia nigra (James and Starr, 1980). In order to reduce the number of animals used, each rat received two consecutive intracerebral injections. In all cases, no more than 30 min elapsed between the end of the first recording period and the second injection. In the first trial, either PBS (0.5 μl) or a dose of the SRIs, duloxetine (6 or 12 nmol) or clomipramine (12 nmol), was injected into the SNc or the SNr. Between 60 and 90 min later, a single dose of 5-HT (21 nmol) was injected, either alone or in combination with the same dose of the SRI applied before. The purpose of the latter experiments was to assess the extent of the influence of the 5-HT reuptake system in the serotonergic transmission in the substantia nigra (Dewar et al., 1992) and to verify whether the direction of circling induced by the SRIs could be mimicked by exogenously applied 5-HT, thus indirectly supporting that the circling induced by the SRIs was mediated by endogenous 5-HT. The nonselective 5-HT_2 antagonists, methysergide and mianserin (Zifa and Fillion, 1992), were used to evaluate the role of 5-HT_2 receptors in the circling induced by intranigral SRIs. In two groups of rats microinjected with clomipramine, methysergide (1 mg/kg ip) was administered 60 min before the first intranigral injection. In separate groups, mianserin (2 nmol) was coinjected with duloxetine into the SNc or SNr. In double cannula experiments, animals first received an intrastriatal injection of haloperidol (53 nmol) or vehicle [20% 2-hydroxypropyl-β-cyclodextrin (HPβCD) plus 1% lactic acid in distilled water] and then a single dose of clomipramine (12 nmol) into the SNc 60 min later.

2.5. Drugs and solutions

5-HT creatinine sulfate and HPβCD were purchased from RBI (Natick, MA, USA). Clomipramine (HCl) and haloperidol were obtained from Sigma (St. Louis, MO, USA). Duloxetine (HCl) was a generous gift from Lilly Research Laboratories (Indianapolis, IN). Methysergide (bimaleate) and mianserin (HCl) were kind donations from Dr. Daniel Martínez (CINVESTAV-IPN, México). Drug solutions were prepared in PBS within 15 min before use. To prepare the mixture of 5-HT plus the SRIs, the solutions were heated at 50 °C and sonicated for 5 min since the drugs were close to their limit of solubility. PBS was composed of NaCl 136.9 mM, KCl 2.7 mM, Na_2HPO_4 6.25 mM and KH_2PO_4 1.5 mM and was adjusted to pH 7.4. Haloperidol was dissolved in HPβCD (20%) plus lactic acid (1%) in distilled water. Mianserin was dissolved in PBS plus lactic acid (0.1%).

2.6. Histology

After the behavioral test, rats were deeply anesthetized and perfused through the ascending aorta with 200 ml of PBS followed by 200 ml of 4% formaldehyde in PBS. The brain was removed and stored in the fixative solution for at least 48 h before slicing. The positions of the injection sites were verified in 100-μm coronal sections made with a vibroslicer (Vibratome) and stained with cresyl violet (Nissl). Histological inspection revealed that when the cannula tip was placed at ≥ 0.2 mm above the SNc, microinjection of SRIs, either alone or with 5-HT, did not produce a significant circling bias. These experiments were analyzed separately as negative controls (SRIs outside SNc). Experiments with extensive hemorrhage or tissue damage along the cannula track were excluded from the analysis.

2.7. Data analysis

Data were analyzed with nonparametric statistics since Bartlett's test revealed that variances differed substantially between some groups (Godfrey, 1985). The Wilcoxon Signed Rank Test for pairs (two-sided) was used to compare the ipsiversive vs. contraversive turns and the number of turns performed in 60 min after the first and second drug injections in the same animals. The Mann–Whitney *U*-test was used to assess the significance of drug-induced circling between two independent groups. When simultaneous comparisons between three or more independent groups were made, the Kruskall–Wallis test, a nonparametric analog of one-way ANOVA, was applied

to determine whether at least one experimental group differed from the rest (Godfrey, 1985). Afterwards, Dunn's post hoc test was applied to compare all pairs of groups to establish which pairs of groups differed significantly. A value of $P<.05$ was considered to be statistically significant. The number of contraversive turns performed during the 60-min period after starting the injection of drugs into the brain was represented as box plots, since they offer a clear graphical representation of the distribution of values for a given parameter (Tukey, 1977). They also allow a better visualization of values that lie far (outliers) from the bulk of data. The line dividing the box represents the median, with the outer edges of the box (hinges) delimiting the inner quartiles (25–50% and 50–75%) of the data set. The outer quartiles (0–25% and 75–100%) are represented by the lines (whiskers) extending from the hinges. Outside the hinges, the values that are 1.5 and 3.0 times the difference between the upper and lower hinges are defined as the inner and outer fences, respectively (Tukey, 1977). Data points between the inner and outer fences are plotted with asterisks (near outliers). Values beyond the outer fence are plotted with empty circles (far outliers). Values in text are expressed as means ± S.E.M.

3. Results

3.1. Circling behavior induced by microinjection of SRIs into the SNc

Microinjection of the SRIs into the SNc induced a significant contraversive circling bias (Figs. 1A, 2A, and 6B). In most cases, during circling, the rats adopted a tight head-to-tail posture. Following clomipramine (12 nmol; $n=17$), the rats performed 19.5 ± 4.7 contraversive vs. 1.0 ± 0.4 ipsiversive turns/60 min ($P=.0024$, Wilcoxon test). Duloxetine produced a similar effect, although the contraversive circling bias was not dose dependent: 6 nmol ($n=15$), 24.0 ± 7.8 contraversive vs. 2.7 ± 2.5 ipsiversive turns/60 min ($P=.0085$, Wilcoxon test), and 12 nmol ($n=8$) 19.5 ± 5.1 contraversive vs. 0 ipsiversive turns/60 min ($P<.0001$, Wilcoxon test). The rats showed no preference to turn in either direction following microinjection of PBS into the SNc (0.5 μl, $n=20$), 3.6 ± 0.8 contraversive vs. 6.5 ± 2.4 ipsiversive turns/60 min ($P=.7857$, Wilcoxon test), or when the SRIs were applied at distances ≥ 0.2 mm above the SNc, 5.3 ± 2.0 contraversive vs. 3.3 ± 1.1 ipsiversive turns/60 min ($n=13$; pooled data of clomipramine and duloxetine; $P=.5771$, Wilcoxon test). Of the rats

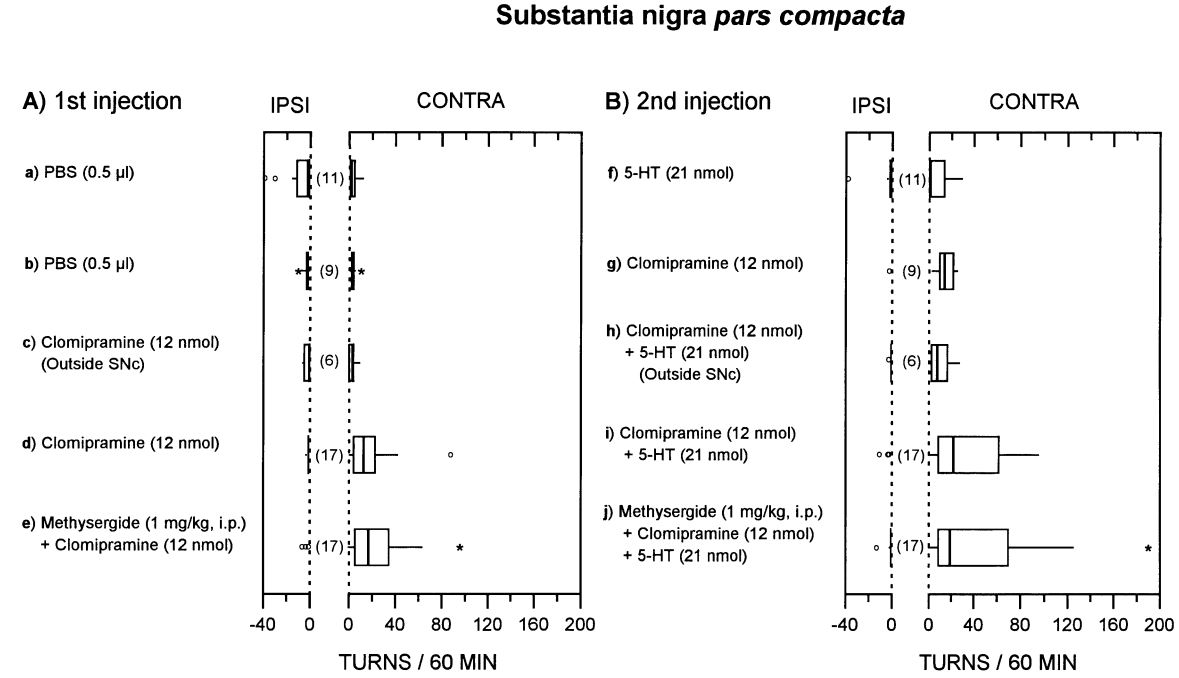

Fig. 1. Contraversive circling induced by microinjection of clomipramine into the SNc. In this and next figures, box plots illustrate the contraversive turns (positive values) and ipsiversive turns (negative values) recorded during 60 min after starting the microinjection of vehicle or drugs into the SNc. Asterisks and open circles are outliers (see text for details). Numbers of observations are in parentheses. (A) For analysis, results of PBS-treated animals were pooled (groups a + b). The Kruskall–Wallis test revealed significant effects only for contraversive circling ($H_3=17.2$, $P<.001$). Clomipramine induced significant contraversive circling only when microinjected into the SNc (group d vs. groups a + b, $P<.05$; Dunn's test) but not when applied at distances ≥ 0.2 mm above this nucleus (group c). Pretreatment with the 5-HT_2 antagonist methysergide did not block the effect of clomipramine but even increased the significance (group e vs. groups a + b, $P<.01$; Dunn's test). (B) Clomipramine and 5-HT act synergically in the SNc to induce contraversive circling (Kruskall–Wallis, $H_4=9.7$, $P<.05$). Post hoc comparisons showed that the effect of 5-HT alone was significantly increased when applied simultaneously with clomipramine (group i vs. group f, $P<.05$; Dunn's test) but not when the mixture was applied at distances ≥ 0.2 mm above this nucleus (group h). Pretreatment with the 5-HT_2 antagonist methysergide did not block the effect of clomipramine plus 5-HT (group j vs. group f, $P<.05$; Dunn's test).

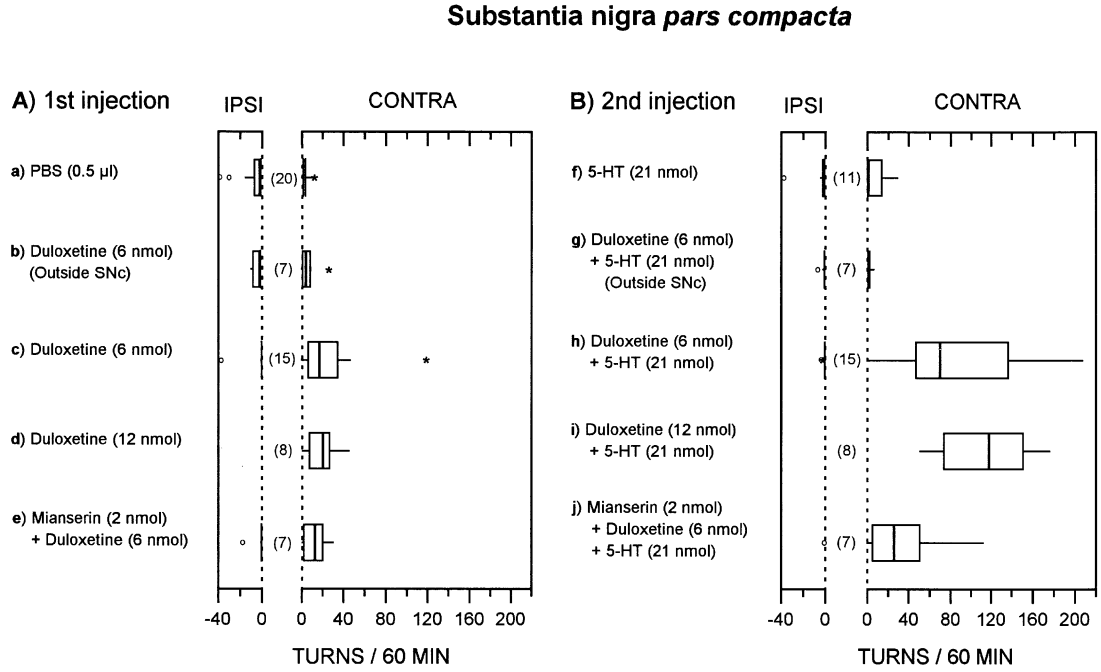

Fig. 2. Contraversive circling induced by microinjection of duloxetine into the SNc. (A) The Kruskall–Wallis test revealed significant effects for contraversive ($H_4 = 14.3$, $P < .01$) and ipsiversive ($H_4 = 16.4$, $P < .01$) circling. Post hoc analysis showed that both doses of duloxetine induced significant contraversive circling only when microinjected into the SNc (groups c and d vs. group a, $P < .05$; Dunn's test) but not when applied at distances ≥ 0.2 mm above this nucleus (group b). Coinjection of duloxetine with the 5-HT_2 antagonist mianserin attenuated the contraversive circling and abolished the significance (group e). At the higher dose tested, microinjection of duloxetine into the SNc induced fewer ipsiversive turns than the group microinjected with PBS (group d vs. group a, $P < .05$, Dunn's test). (B) Duloxetine and 5-HT act synergically in the SNc to induce contraversive circling (Kruskall–Wallis, $H_4 = 29.3$, $P < .0001$). The effect of 5-HT alone was significantly increased when applied simultaneously with duloxetine (group h vs. group f, $P < .01$; group i vs. group f, $P < .001$; Dunn's test) but not when the mixture was applied at distances ≥ 0.2 mm above this nucleus (groups h and i vs. group g, $P < .01$; Dunn's test). Coinjection of duloxetine plus 5-HT with the 5-HT_2 antagonist mianserin (group j) inhibited the contraversive circling bias and abolished the significance vs. 5-HT alone (group f).

microinjected with PBS, nine also received a microinjection of clomipramine (12 nmol) 60–90 min later, and the contraversive circling increased significantly from 4.3 ± 1.2 (PBS) to 14.9 ± 2.7 turns/60 min (clomipramine; $P = .0273$, Wilcoxon test). This last value is close to but not statistically different from the contraversive circling count elicited in rats that received clomipramine as a first injection (19.5 ± 4.7 turns/60 min).

Statistical comparison between independent groups showed that, in rats pretreated with methysergide (1 mg/kg ip), the contraversive circling induced by infusion of clomipramine in the SNc was still significant compared with the group that received PBS (Fig. 1A). On the contrary, microinjection of mianserin (2 nmol) into the SNc partially inhibited and cancelled the significance of the contraversive circling elicited by duloxetine (Fig. 2A).

Microinjection of the mixed $D_2 > D_1$ dopamine receptor antagonist haloperidol (53 nmol) into the neostriatum (Figs. 3 and 6C–D) produced a significant inhibition of the contraversive circling induced by clomipramine (12 nmol) microinjected into the ipsilateral SNc 60 min later (3.0 ± 1.0 contraversive turns/60 min; $n = 9$) as compared with the effect elicited by clomipramine in rats that received an intrastriatal injection of the vehicle (23.7 ± 6.0 contraversive turns/60 min; $n = 9$; $P = .0008$, Mann–Whitney U-test).

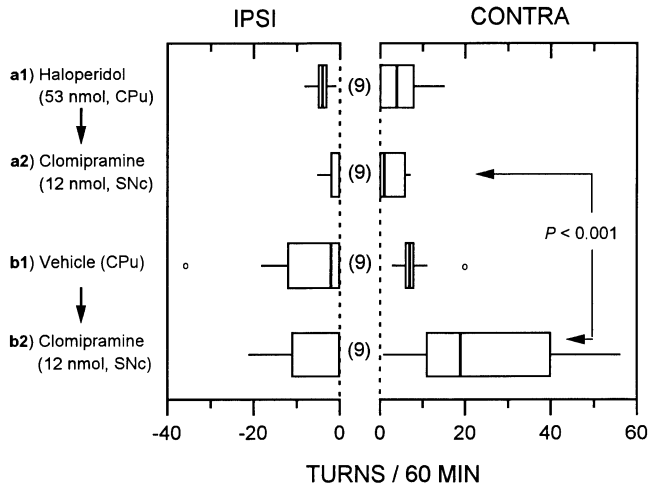

Fig. 3. Intrastriatal haloperidol blocks the contraversive circling induced by microinjection of clomipramine into the SNc. Neither haloperidol (group a1) nor vehicle (group b1: 20% HPβCD plus 1% lactic acid in distilled water) into the neostriatum (CPu) caused a significant turning bias. However, intrastriatal application of haloperidol, but not of vehicle, inhibited the contraversive circling induced by microinjection of clomipramine applied 1 h later into the SNc (group b2 vs. group a2, Mann–Whitney U-test, two-tailed, $U = 5$, $P < .001$).

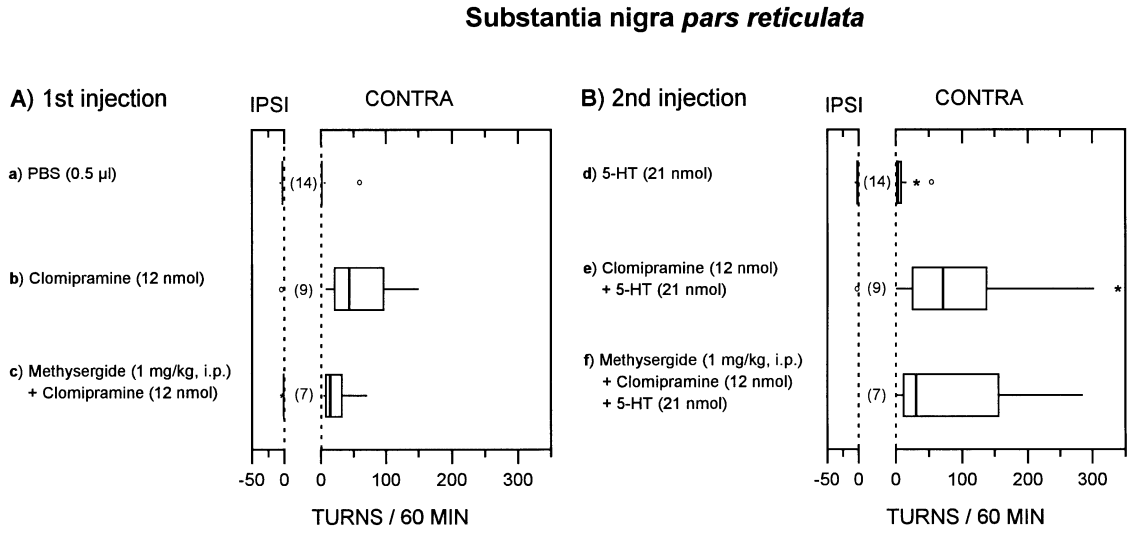

Fig. 4. Methysergide blocks the contraversive circling induced by microinjection of clomipramine into the SNr. (A) The Kruskall–Wallis test showed significant effects only for contraversive circling ($H_2 = 14.1$, $P < .001$). Post hoc comparisons revealed that microinjection of clomipramine into the SNr induced significant contraversive circling (group b vs. group a, $P < .001$; Dunn's test). Pretreatment with the 5-HT_2 antagonist methysergide inhibited the effect of clomipramine (group c). (B) Clomipramine and 5-HT act synergically in the SNr to induce contraversive circling. (Kruskall–Wallis, $H_2 = 11.2$, $P < .005$). Post hoc comparisons revealed that the contraversive circling bias induced by microinjection of 5-HT was enhanced when clomipramine was applied together with 5-HT into the SNr (group e vs. group d, $P < .01$; Dunn's test). Pretreatment with methysergide attenuated the effect of clomipramine plus 5-HT (group f) and abolished the significance vs. 5-HT alone (group d).

Of the rats microinjected with PBS, 11 received a second injection with 5-HT (21 nmol) 60–90 min later (Fig. 1), and the mean number of contraversive turns increased from 3.0 ± 1.1 (PBS) to 8.0 ± 3.0 turns/60 min (5-HT), but this effect was not significant. Also, 5-HT did not produce any significant circling bias (4.7 ± 3.4 ipsiversive turns/60 min; $P = .5771$, Wilcoxon test). However, when the same dose of 5-HT was coinjected with duloxetine into the SNc, the number of turns increased significantly compared with those produced by duloxetine or 5-HT alone (Fig. 2B). Coinjection of 5-HT with the low dose of duloxetine (6 nmol; $n = 15$) elicited 88.7 ± 16.8 contraversive turns/60 min, and

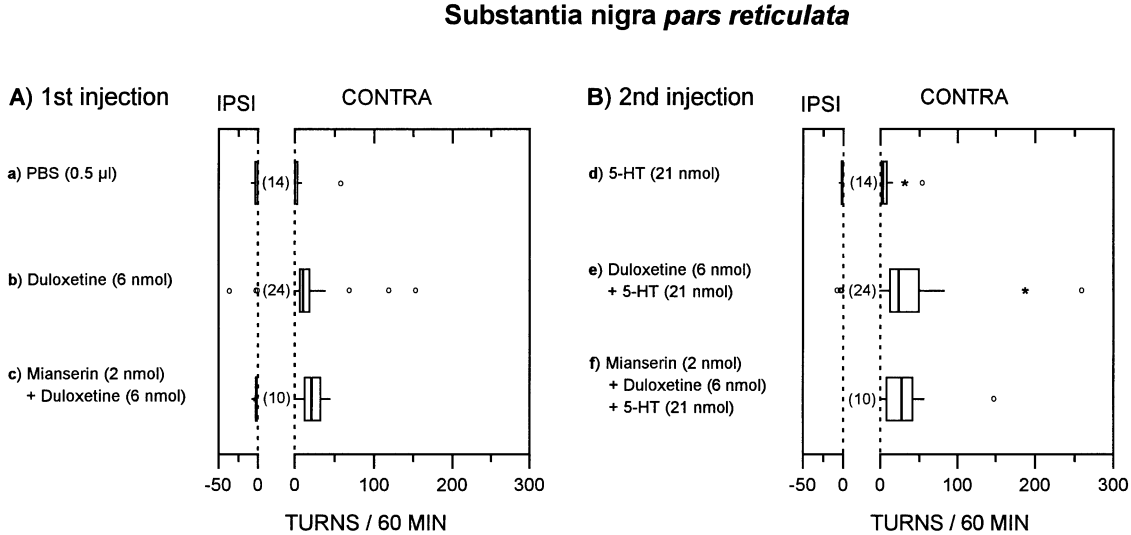

Fig. 5. Mianserin does not block the contraversive circling induced by microinjection of duloxetine into the SNr. (A) The Kruskall–Wallis test showed significant effects only for contraversive circling ($H_2 = 9.9$, $P < .01$). Post hoc comparisons revealed that microinjection of duloxetine into the SNr induced a significant contraversive circling bias (group b vs. group a, $P < .05$; Dunn's test). Intranigral application of the 5-HT_2 antagonist mianserin was unable to block the effect of duloxetine (group c vs. group a, $P < .05$, Dunn's test). (B) Duloxetine and 5-HT act synergically in the SNr to induce contraversive circling. (Kruskall–Wallis, $H_2 = 11.4$, $P < .01$). Post hoc comparisons revealed that the contraversive circling bias induced by microinjection of 5-HT was enhanced when duloxetine was applied together with 5-HT into the SNr (group e vs. group d, $P < .01$; Dunn's test). However, coinjection of mianserin with duloxetine plus 5-HT (group f) induced a contralateral circling that was not statistically significant vs. the effect of 5-HT alone (group d).

with the high dose (12 nmol; $n=8$), this value increased to 114.1 ± 16.0 contraversive turns/60 min. However, although there was a dose-dependency trend, this was not statistically significant. When applied with the 5-HT_2 antagonist mianserin, the effect of 5-HT plus duloxetine was not significant compared with that of 5-HT alone. In the case of clomipramine (12 nmol), the synergism with 5-HT (21 nmol) was less pronounced (18.5 ± 5.4 contraversive turns/60 min) and pretreatment with the 5-HT_2 antagonist methysergide did not block this effect.

3.2. Circling behavior induced by microinjection of SRIs into the SNr

Microinjection of the SRIs into the SNr induced a significant contraversive circling bias (Figs. 4, 5, and 6A) qualitatively similar to that produced after microinjection into the SNc. Following clomipramine (12 nmol; $n=9$), the rats performed 58.8 ± 17.3 contraversive vs. 0.4 ± 0.4 ipsiversive turns/60 min ($P=.0039$, Wilcoxon test). Duloxetine (6 nmol; $n=24$) produced a similar effect, 24.0 ± 7.7 contraversive vs. 1.8 ± 1.5 ipsiversive turns/60 min ($P=.0003$, Wilcoxon test). The contraversive circling bias was not observed following microinjection of PBS (0.5 μl; $n=14$) into the SNc, 7.7 ± 4.0 contraversive vs. 2.1 ± 0.8 ipsiversive turns/60 min ($P=.3757$, Wilcoxon test). The rats microinjected with PBS into the SNr received a second injection with 5-HT (21 nmol) 60–90 min later (Fig. 4B), but the contraversive turns remained essentially unchanged: 7.7 ± 4.0 (PBS) vs. 9.3 ± 4.0 turns/60 min (5-HT).

In contrast with the SNc, pretreatment with methysergide (1 mg/kg ip) was highly effective at blocking the contraversive circling induced by microinjection of clomipramine (12 nmol) into the SNr (Fig. 4A). Methysergide also caused a partial inhibition of the contraversive circling elicited by the coinjection of clomipramine plus 5-HT into the SNr that cancelled the significance as compared with the group treated with 5-HT alone (Fig. 4B). On the contrary, microinjection of mianserin (2 nmol) into the SNr was unable to block the circling induced by duloxetine alone, although it partially reduced the effect of duloxetine plus 5-HT (Fig. 5).

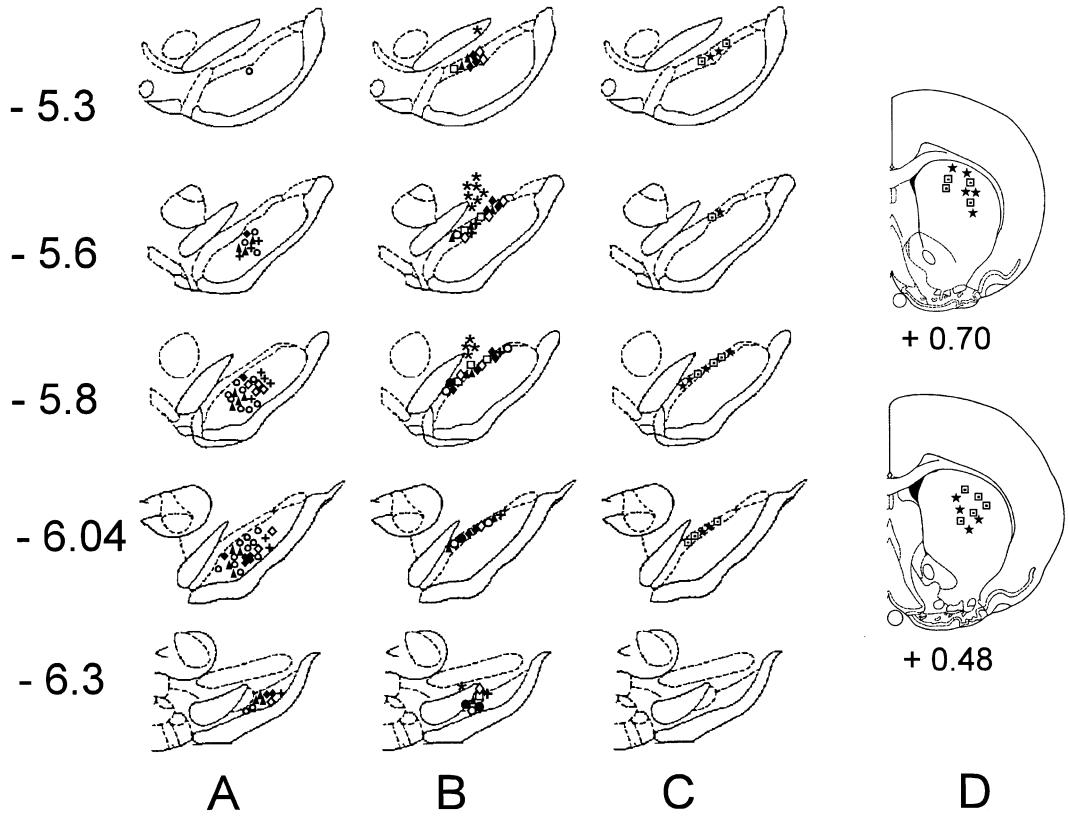

Fig. 6. Anteroposterior plane drawings illustrating the location of cannulae tips. (A) In the SNr. (B) and (C) In the SNc. (D) In the neostriatum. Drawings based on the atlas of Paxinos and Watson (1986). (C) and (D) refer to the double cannula experiments. Numbers are stereotaxic planes from bregma (in mm). Symbols correspond to treatments, as indicated: PBS followed by a second injection of 5-HT (▲); PBS followed by a second injection of clomipramine (□); clomipramine or duloxetine (6 nmol) followed by a second injection of the SRI plus 5-HT outside SNc (✻); clomipramine followed by a second injection of clomipramine plus 5-HT (◆); methysergide pretreatment (intraperitoneal) followed by a first injection of clomipramine and a second injection of clomipramine plus 5-HT (◇); duloxetine (6 nmol) followed by a second injection of duloxetine plus 5-HT into SNc (○); duloxetine (12 nmol) followed by a second injection of duloxetine plus 5-HT (●); mianserin plus duloxetine (6 nmol) followed by a second injection of mianserin plus duloxetine plus 5-HT (+); haloperidol (53 nmol) in neostriatum followed by clomipramine (12 nmol) into SNc (★); vehicle (20% HPβCD plus 1% lactic acid) in neostriatum followed by clomipramine (12 nmol) into SNc (⊡).

4. Discussion

The main finding of the present study was that the SRIs, duloxetine and clomipramine, elicited a contraversive circling bias and potentiated the effect of exogenous 5-HT after microinjection into the SNc or SNr of awake rats. Since no significant circling was produced when the SRIs were applied outside the SNc, it is likely that the effect of the SRIs was a consequence of 5-HT uptake inhibition in the substantia nigra. In fact, it is well documented that the SRIs cause a marked increase in the extracellular concentration of 5-HT when locally infused into the 5-HT innervated areas of the rat brain (Carboni and Di Chiara, 1989; Adell and Artigas, 1991), including the substantia nigra (Thorré et al., 1997). Thus, the most plausible explanation for the circling induced by microinjection of duloxetine and clomipramine into the SNc or SNr is that it was mediated by a local increase of 5-HT in the synaptic cleft, which in turn stimulated the postsynaptic 5-HT receptors located on the cell bodies or on afferents to the nigral dopaminergic and nondopaminergic neurons. It has been reported that microinjection of 5-HT into the substantia nigra elicits contraversive circling only when rats are pretreated systemically with the MAO inhibitor nialamide (Oberlander et al., 1981). Here, we found that the contralateral circling bias induced by infusion of 5-HT either into the SNc or the SNr was enhanced when it was coinjected with clomipramine or duloxetine, indicating that the extracellular concentration of 5-HT is quickly reduced by an efficient 5-HT reuptake system operating within the substantia nigra (Dewar et al., 1992; Iravani and Kruk, 1997).

Since duloxetine and clomipramine are also good inhibitors of norepinephrine uptake (Wong et al., 1995), it could be argued that the contraversive circling was the result of an enhancement of noradrenergic transmission within the substantia nigra. However, it is unlikely that this was the case since the substantia nigra contains very small amounts of norepinephrine (Hornykiewicz,1972; Dray and Straughan, 1976), and the noradrenergic fibers are scarce (Swanson and Hartman, 1975; Nagatsu et al., 1998) compared with those of 5-HT (Mori et al., 1987; Lavoie and Parent, 1990; Moukhles et al., 1997). Moreover, electrophysiological studies have shown that the main effect of norepinephrine is to inhibit the firing rate of nigrostriatal dopaminergic neurons (Collingridge and Davies, 1981). Accordingly, if the circling induced by microinjection of the SRIs into the SNc was mediated by norepinephrine, the circling would have been ipsiversive, not contraversive (see below; Miller and Beninger, 1991). Based on these evidences, we consider that the contraversive circling bias elicited by local application of SRIs into the substantia nigra was mainly mediated by the enhancement of serotonergic neurotransmission.

4.1. Contraversive circling induced by SRIs into the SNc

Prevailing views of circling behavior postulate that the animal turns away from the brain hemisphere in which dopamine neurotransmission is dominant (Miller and Beninger, 1991). This has been supported by microdialysis studies in freely moving rats, showing that unilateral pharmacological manipulations of the SNc that enhance dopamine release in the neostriatum are accompanied by contraversive circling, whereas reductions of dopamine release are associated with ipsiversive circling (Hernández-López et al., 1994). Here, we found that microinjection of the SRIs into the SNc induced a contraversive circling bias that was prevented by microinjection of the $D_2 > D_1$ dopamine receptor antagonist haloperidol into the ipsilateral neostriatum. Taken together, these data suggest that the contraversive circling induced by microinjection of the SRIs into the SNc is mediated by an enhancement of dopamine release into the ipsilateral neostriatum, perhaps consecutive to an increased firing rate of nigrostriatal dopamine neurons. This hypothesis is supported by electrophysiological studies performed in brain slices, showing that 5-HT depolarizes and elicits an inward current in a substantial fraction of dopaminergic neurons of the SNc (Nedergaard et al., 1991; Pessia et al., 1994) and induces firing in previously silent neurons (Nedergaard et al., 1991). Evidence that this excitatory effect can also be evoked in vivo by endogenous 5-HT was given in a study where stimulation of the dorsal raphe nucleus increased the basal firing frequency nearly threefold in 48% of dopaminergic neurons recorded extracellularly in the SNc of anesthetized rats (Trent and Tepper, 1991). In addition to its direct excitatory actions, there is evidence that 5-HT can disinhibit dopaminergic neurons by reducing the amplitude of the inhibitory $GABA_B$ synaptic potential arising from the striatonigral GABAergic afferents (Johnson et al., 1992). These findings jointly support the hypothesis that the contraversive circling bias induced by microinjection of the SRIs into the SNc could be mediated by a direct excitatory action of 5-HT on a subset of nigrostriatal dopaminergic neurons (Pessia et al., 1994), perhaps reinforced by a presynaptic attenuation of GABA release from striatonigral afferents (Johnson et al., 1992).

It has been suggested that the excitatory effects of 5-HT on dopaminergic neurons of the rat substantia nigra are mediated by 5-HT_2 receptors since they are mimicked by the 5-HT_2 agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and blocked by ketanserin, an antagonist of 5-HT_2 receptors (Pessia et al., 1994). Our results are consistent with these observations, since the nonselective 5-HT_2 antagonist mianserin partially blocked the contraversive circling bias when coinfused into the SNc with duloxetine, either alone or with 5-HT. In contrast, the contraversive circling induced by microinjection of clomipramine into the SNc remained unaffected in rats pretreated systemically with methysergide, which has also been classified as an antagonist with very high affinity for 5-HT_2-like receptors (Zifa and Fillion, 1992). Although the lack of effect of methysergide could be ascribed to a low CNS availability after intraperitoneal (ip) administra-

tion, this is unlikely because the circling induced by microinjection of clomipramine into the adjacent SNr was blocked in rats pretreated systemically with the same dose of methysergide (see below). Since there are at least three subtypes of 5-HT_2 receptors, with different pharmacological profiles (Barnes and Sharp, 1999), it is possible that the lack of effect of methysergide could be explained by a lower affinity of methysergide for the 5-HT_{2A} receptor, which has been reported as the predominant 5-HT_2 receptor expressed by midbrain dopaminergic neurons (Doherty and Pickel, 2000; Ikemoto et al., 2000). It should be emphasized, however, that the aim of the present study was not to fully characterize the 5-HT_2 receptor subtype involved in the circling response induced by microapplication of SRIs into the SNc. Moreover, our experimental design does not discard the possible participation of other 5-HT receptor subtypes present in the substantia nigra (e.g. 5-HT_{1B}) that could modulate the circling induced by intranigral SRIs.

4.2. Contraversive circling induced by SRIs into the SNr

Microinjection of SRIs into the SNr produced a contraversive circling bias similar to that induced by 5-HT and serotonergic agonists (Blackburn et al., 1981; Oberlander et al., 1981; Higgins et al., 1991). Within the SNr, all serotonergic axonal varicosities make synaptic contact with dendritic profiles (Moukhles et al., 1997). Thus, it is likely that the contraversive circling induced by SRIs was mediated by the enhancement of 5-HT effects on GABAergic projection neurons, which constitute one of the main output pathways of the basal ganglia (Scheel-Krüger, 1986; Smith et al., 1998). Since the spontaneous firing of SNr projection neurons is inhibited by electrical stimulation of the GABAergic striatonigral pathway (Deniau et al., 1978), and microinjection of the $GABA_A$ agonist muscimol into the SNr induces contraversive circling (Arnt and Scheel-Krüger, 1979), our results suggest that endogenously released 5-HT exerts an effect compatible with a predominantly inhibitory action on the GABAergic output neurons. This effect is supported by electrophysiological studies performed in anesthetized animals, where stimulation of the dorsal raphe nucleus, which is the main source of serotonergic afferents to the substantia nigra (Wirtshafter et al., 1987), evokes inhibitory responses in a high percentage of neurons extracellularly recorded in the SNr of anesthetized rats (Dray et al., 1976; Fibiger and Miller, 1977). However, the effects observed in vivo contrast with the clearly excitatory effects of 5-HT on most SNr neurons recorded in vitro (Rick et al., 1995; Góngora-Alfaro et al., 1997b). Then, according to the model (Scheel-Krüger, 1986), the circling should be ipsiversive, not contraversive. In order to explain these discrepancies, it has been argued that 5-HT released in vivo by stimulation of the dorsal raphe nucleus elicits an excitatory response in a discrete population of SNr neurons, which in turn inhibits a greater number of neighboring SNr cells through GABA release from their extensive axon collaterals (Rick et al., 1995; Stanford and Lacey, 1996). The fact that low doses of the nonselective 5-HT_2 antagonist methysergide inhibited the contraversive circling induced by clomipramine into the SNr nicely fits with the ability of this antagonist to block the excitatory actions of 5-HT on SNr neurons in vitro (see Rick et al., 1995; Góngora-Alfaro et al., 1997b). These findings are consistent with the observation that microinjection of methysergide into the SNr also inhibits the circling behavior induced by 5-HT applied by the same route in rats in which the nigral 5-HT receptors have become supersensitive after unilateral destruction of serotonergic neurons of the dorsal raphe nucleus (Blackburn et al., 1981). Altogether, the above observations support the hypothesis that the contraversive circling behavior induced by enhancement of 5-HT transmission within the SNr is in some way mediated by the excitatory effect of 5-HT on SNr neurons (Rick et al., 1995; Góngora-Alfaro et al., 1997b).

5. Conclusions

The present results strongly suggest that the contraversive circling behavior induced by microinjection of SRIs into the substantia nigra is mediated by a local inhibition of the 5-HT reuptake system that enhances the postsynaptic actions of 5-HT spontaneously released from the serotonergic afferents that synapse with dopaminergic and nondopaminergic neurons. In the SNc, the contraversive circling induced by local application of SRIs depends on an intact striatal dopaminergic transmission, since it is blocked by microinjection of haloperidol into the neostriatum. This suggests that enhancement of 5-HT transmission within the boundaries of the SNc exerts a net excitatory effect on the nigrostriatal dopaminergic neurons that increases dopamine release in the neostriatum. In the SNr, local application of SRIs also produces a contraversive circling that appears to depend on the activation of 5-HT_2-like receptors, because it is blocked by pretreatment with methysergide.

Based on the predominant contraversive circling response induced by the local application of SRIs into both subdivisions of the substantia nigra, it is tempting to propose that the main effect of 5-HT released by the serotonergic afferents to the substantia nigra is to facilitate the processing and output of the basal ganglia during motor control. This hypothesis is supported by a neurochemical study performed in rats trained to turn in circles, showing that the increase of dopamine turnover in the neostriatum contralateral to the circling direction is accompanied by an increase of 5-HT turnover in the substantia nigra ipsilateral to that neostriatum (Yamamoto and Freed, 1984). A possible mechanism could be an enhanced 5-HT release within the SNc and the SNr that acts in concert to enhance dopamine release in the neostriatum and facilitates the output of information through the nigrothalamic pathway.

Acknowledgments

The authors would like to thank the generous gifts of drugs from Ely Lilly USA and from Dr. Daniel Martínez (CINVESTAV-México) This work was sponsored by CONACyT-México grant number 1831-M9211 to J.L.G.A.

References

Adell A, Artigas F. Differential effects of clomipramine given locally or systemically on extracellular 5-hydroxytryptamine in raphe nuclei and frontal cortex. Naunyn-Schmiedeberg's Arch Pharmacol 1991;343: 237–44.

Arnt J, Scheel-Krüger J. Behavioral differences induced by muscimol selectively injected into pars compacta and pars reticulata of substantia nigra. Naunyn-Schmiedeberg's Arch Pharmacol 1979;310:43–51.

Barnes NM, Sharp T. A review of central 5-HT receptors and their function. Neuropharmacology 1999;38:1083–152.

Blackburn TP, Cox B, Heapy CG, Lee TF, Middlemiss DN. Supersensitivity of nigral serotonin receptors and rat rotational behaviour. Eur J Pharmacol 1981;71:343–6.

Carboni E, Di Chiara G. Serotonin release estimated by transcortical dialysis in freely moving rats. Neuroscience 1989;32:637–45.

Collingridge GL, Davies J. The influence of striatal stimulation and putative neurotransmitters on identified neurones in the rat substantia nigra. Brain Res 1981;212:345–59.

Corvaja N, Doucet G, Bolam JP. Ultrastructure and synaptic targets of the raphe-nigral projection in the rat. Neuroscience 1993;55:417–27.

Deniau JM, Hammond C, Riszk A, Feger J. Electrophysiological properties of identified output neurons of the rat substantia nigra (pars compacta and pars reticulata); evidence for the existence of branched neurons. Exp Brain Res 1978;32:409–22.

Dewar KM, Grondin L, Carli M, Lima L, Reader TA. [^{3}H]Paroxetine binding and serotonin content of rat cortical areas, hippocampus, neostriatum, ventral mesencephalic tegmentum, and midbrain raphe nuclei region following *p*-chlorophenylalanine and *p*-chloroamphetamine treatment. J Neurochem 1992;58:250–7.

Doherty MD, Pickel VM. Ultrastructural localization of the serotonin 2A receptor in dopaminergic neurons in the ventral tegmental area. Brain Res 2000;864:176–85.

Dray A, Straughan DW. Synaptic mechanisms in the substantia nigra. J Pharm Pharmacol 1976;28:400–5.

Dray A, Gonye TJ, Oakley NR, Tanner T. Evidence for the existence of a raphe projection to the substantia nigra in rat. Brain Res 1976;113: 45–57.

Fibiger HC, Miller JJ. An anatomical and electrophysiological investigation of the serotonergic projection from the dorsal raphe nucleus to the substantia nigra in the rat. Neuroscience 1977;2:975–87.

Godfrey K. Comparing the means of several groups. N Engl J Med 1985; 313:1450–6.

Góngora-Alfaro JL, Heredia-López FJ, Alvarez-Cervera F, Bata-García JL. Behavioral evidence of serotonergic transmission in the substantia nigra pars compacta of freely moving rats. Soc Neurosci Abstr 1996; 22:612.

Góngora-Alfaro JL, Heredia-López FJ, Alvarez-Cervera F, Bata-García JL. Circling behavior mediated by serotonergic transmission in the substantia nigra. Neural Control Mov Soc Abstr 1997a;2:22.

Góngora-Alfaro JL, Hernández-López S, Flores-Hernández J, Galarraga E. Firing frequency modulation of substantia nigra reticulata neurons by 5-hydroxytryptamine. Neurosci Res 1997b;29:225–31.

Heredia-López FJ, Alvarez-Cervera F, Góngora-Alfaro JL, Mendoza-Camargo L, Chacón-Gutiérrez L. Girómetro, un sistema electrónico automático para contabilizar y discriminar giros completos en ratas. Rev Biomed 1992;3:183–91.

Hernández-López S, Góngora-Alfaro JL, Martínez-Fong D, Rosales MG, Aceves J. Cholinergic stimulation of rostral and caudal substantia nigra pars compacta produces opposite effects on circling behavior and striatal dopamine release measured by brain microdialysis. Neuroscience 1994;62:441–7.

Higgins GA, Jordan CC, Skingle M. Evidence that unilateral activation of 5-HT$_{1D}$ receptors in the substantia nigra of the guinea pig elicits contralateral rotation. Br J Pharmacol 1991;102:305–10.

Hornykiewicz O. Neurochemistry of parkinsonism. In: Lajtha A, editor. Handbook of neurochemistry, pathological chemistry of the nervous system, vol. 7. New York: Plenum, 1972. pp. 465–501.

Ikemoto K, Nishimura A, Okado N, Mikuni M, Nishi K, Nagatsu I. Human midbrain dopamine neurons express serotonin 2A receptor; an immunohistochemical demonstration. Brain Res 2000;853:377–80.

Iravani MM, Kruk ZL. Real-time measurement of stimulated 5-hydroxytryptamine release in rat substantia nigra pars reticulata brain slices. Synapse 1997;25:93–102.

James TA, Starr MS. Rotational behaviour elicited by 5-HT in the rat; evidence for an inhibitory role of 5-HT in the substantia nigra and corpus striatum. J Pharm Pharmacol 1980;32:196–200.

Johnson SW, Mercuri NB, North RA. 5-Hydroxytryptamine$_{1B}$ receptors block the GABA$_B$ synaptic potential in rat dopamine neurons. J Neurosci 1992;12:2000–6.

Lavoie B, Parent A. Immunohistochemical study of the serotoninergic innervation of the basal ganglia in the squirrel monkey. J Comp Neurol 1990;299:1–16.

Miller R, Beninger RJ. On the interpretation of asymmetries of posture and locomotion produced with dopamine agonists in animals with unilateral depletion of striatal dopamine. Prog Neurobiol 1991;36: 229–56.

Mori S, Matsuura T, Takino T, Sano Y. Light and electron microscopic immunohistochemical studies of serotonin nerve fibers in the substantia nigra of the rat, cat and monkey. Anat Embryol 1987;176:13–8.

Moukhles H, Bosler O, Bolam JP, Vallee A, Umbriaco D, Geffard M, Doucet G. Quantitative and morphometric data indicate precise cellular interactions between serotonin terminals and postsynaptic targets in rat substantia nigra. Neuroscience 1997;76:1159–71.

Nagatsu I, Ikemoto K, Takeuchi T, Arai R, Karasawa N, Fujii T, Nagatsu T. Phenylethanolamine-*N*-methyltransferase-immunoreactive nerve terminals afferent to the mouse substantia nigra. Neurosci Lett 1998;245:41–4.

National Research Council of USA. Giuide for the care and use of laboratory animals. Washington, DC: National Academy Press, 1996.

Nedergaard S, Bolam JP, Greenfield SA. Facilitation of a dendritic calcium conductance by 5-hydroxytryptamine in the substantia nigra. Nature 1988;333:174–7.

Nedergaard S, Flatman JA, Engberg I. Excitation of substantia nigra pars compacta neurones by 5-hydroxytryptamine in vitro. NeuroReport 1991;2:329–32.

Oberlander C, Hunt PF, Dumont C, Boissier JR. Dopamine independent rotational response to unilateral intranigral injection of serotonin. Life Sci 1981;28:2595–601.

Palkovitz M, Brownstein M, Saavedra JM. Serotonin content of the brain stem nuclei in the rat. Brain Res 1974;80:237–49.

Paxinos G, Watson C. The rat brain in stereotaxic coordinates. San Diego, CA, USA: Academic Press, 1986.

Pessia M, Jiang ZG, North RA, Johnson SW. Actions of 5-hydroxytryptamine on ventral tegmental area neurons of the rat in vitro. Brain Res 1994;654:324–30.

Reubi JC, Emson PC. Release and distribution of endogenous 5-HT in rat substantia nigra. Brain Res 1978;139:164–8.

Rick CE, Stanford IM, Lacey MG. Excitation of rat substantia nigra pars reticulata neurons by 5-hydroxytryptamine in vitro; evidence for a direct action mediated by 5-hydroxytryptamine$_{2C}$ receptors. Neuroscience 1995;69:903–13.

Scheel-Krüger J. Dopamine–GABA interactions; evidence that GABA transmits, modulates, and mediates dopaminergic functions in the

basal ganglia and the limbic system. Acta Neurol Scand 1986; 73 (Suppl 107):1–54.

Smith Y, Bevan MD, Shink E, Bolam JP. Microcircuitry of the direct and indirect pathways of the basal ganglia. Neuroscience 1998;86:353–87.

Stanford IM, Lacey MG. Differential actions of serotonin, mediated by 5-HT_{1B} and 5-HT_{2C} receptors, on GABA-mediated synaptic input to rat substantia nigra pars reticulata neurons in vitro. J Neurosci 1996; 16:7566–73.

Swanson LW, Hartman BK. The central adrenergic system. An immunofluorescence study of the localization of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. J Comp Neurol 1975;163:467–505.

Thorré K, Sarre S, Ebinger G, Michotte Y. Characterization of the extracellular serotonin release in the substantia nigra of the freely moving rat using microdialysis. Brain Res 1997;772:29–36.

Trent F, Tepper JM. Dorsal raphé stimulation modifies striatal-evoked antidromic invasion of nigral dopaminergic neurons in vivo. Exp Brain Res 1991;84:620–30.

Tukey JW. Exploratory data analysis. Menlo Park, CA, USA: Addison-Wesley, 1977.

Wirtshafter D, Stratford TR, Asin KE. Evidence that serotonergic projections to the substantia nigra in the rat arise in the dorsal, but not the median, raphe nucleus. Neurosci Lett 1987;77:261–6.

Wong DT, Bymaster FP, Engelman EA. Presynaptic regulation of extracellular serotonin concentrations in brain. In: Takada A, Curzon G, editors. Serotonin in the central nervous system and periphery. Amsterdam: Elsevier, 1995. pp. 3–13.

Yamamoto BK, Freed CR. Asymmetric dopamine and serotonin metabolism in nigrostriatal and limbic structures of the trained circling rat. Brain Res 1984;297:115–9.

Zifa E, Fillion G. 5-Hydroxytryptamine receptors. Pharmacol Rev 1992;44: 401–58.