

Persistent Spontaneous Oral Dyskinesias in Haloperidol-Withdrawn Rats Neonatally Lesioned with 6-Hydroxydopamine: Absence of an Association with the B_{\max} for [^3H]Raclopride Binding to Neostriatal Homogenates¹

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Accepted for publication August 2, 1996

ABSTRACT

To investigate the influence of dopamine (DA) nerves on haloperidol (HAL)-induced oral dyskinesias, rats were first injected at 3 days after birth with 6-hydroxydopamine HBr (200 μg i.c.v., salt form; 6-OHDA) or vehicle, after desipramine HCl (20 mg/kg i.p., 1 hr) pretreatment. Two months later HAL (1.5 mg/kg/day, 2 days a week for 4 weeks, then daily for 10 months) was added to the drinking water of half the rats. Numbers of vacuous chewing movements, recorded in 1-min increments every 10 min for 1 hr, increased from <5 to about 17 oral movements per session in intact rats, 14 weeks after instituting HAL ($P < .01$ vs. intact rats drinking tap water). In HAL-treated 6-OHDA-lesioned rats, oral activity increased to >30 oral movements per session ($P < .01$ vs. HAL-treated intact rats). These levels of oral activity persisted in intact and 6-OHDA-lesioned rats as long as HAL was administered. After 11 months of HAL treatment, but 8 or

9 days after HAL withdrawal, DA was found to be reduced 97%, whereas serotonin was increased 29% in the striatum of 6-OHDA-lesioned rats. In HAL-treated intact and lesioned rats the B_{\max} for DA D_2 binding sites was elevated about 70%. With reverse transcription polymerase chain reaction, the mRNA level for DA D_{2L} but not D_{2S} receptors was also found to be elevated about 70%. In a fraction of 6-OHDA-lesioned rats that were observed for 8 months after HAL withdrawal, oral activity persisted without decrement and was not accompanied by a change in the B_{\max} or mRNA level for DA D_2 receptors. These findings demonstrate that in rats largely DA-denervated as neonates, long-term HAL treatment produces an unusually high number of oral movements that persists for 8 months after HAL withdrawal and is not accompanied by an increase in DA D_2 receptor expression.

Tardive dyskinesia, an extrapyramidal syndrome caused by neuroleptics in the treatment of psychiatric disorders, is a disorder for which there is no satisfactory treatment. The prevalence of TD has increased steadily by about 1% per year during the past two decades (Casey, 1987; Jeste and Caligiuri, 1993). Spontaneous oral dyskinesias constitute the most common symptom of TD. In numerous studies delving into the mechanisms underlying the neural regulation of oral activity, it has been determined that abnormal oral movements in rodents are induced by agonist (SKF 38393 or A 77636) stimulation of DA D_1 receptors or by antagonist inhibition of DA D_2 receptors (Rosengarten *et al.*, 1983; Huang

and Kostrzewa, 1994a). An imbalance in the functional ratio of DA D_1/D_2 receptors is considered to be an important element related to oral activity behavior (Rosengarten *et al.*, 1983).

Cholinergic systems are also known to have a prominent regulatory action on oral activity. Acute systemic treatments with pilocarpine, a muscarinic agonist, or physostigmine, a cholinesterase inhibitor, produce oral activity in rats. The same effect is produced when pilocarpine is injected directly into the ventrolateral striatum. Long-term treatment with physostigmine similarly increases oral activity. These many actions are attenuated by the muscarinic receptor antagonist, scopolamine (Rupniak *et al.*, 1985b; Salamone *et al.*, 1990).

Bilateral microinfusion of the GABA receptor antagonist bicuculline and GABA-depleting agent isoniazid into the sub-

Received for publication December 20, 1995.

¹ This work was supported by Grant NS 29505 from the National Institute of Neurological Disorders and Stroke.

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ABBREVIATIONS: DA, dopamine; 6-OHDA, 6-hydroxydopamine; 5-HT, serotonin; SKF 38393, (\pm)1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol; SCH 23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; TD, tardive dyskinesia; HVA, homovanillic acid; NE, norepinephrine; bp, basepair; RT-PCR, reverse transcription and polymerase chain reaction; GABA, γ -aminobutyric acid; i.c.v., intracerebroventricular; HAL, haloperidol.

stantia nigra greatly increases oral activity of rats (Gunne *et al.*, 1988). GABA agonists attenuate oral activities in cat, monkey and rats and have beneficial effects on TD patients (Lloyd *et al.*, 1985; Mithani *et al.*, 1987; Tamminga *et al.*, 1979).

5-HT neurons represent one other neurochemical system that prominently regulates oral activity behavior. In intact and DA-denervated rats (neonatal 6-OHDA treatment) oral activity was greatly enhanced by the 5-HT_{2A/2C} receptor agonist *m*-chlorophenylpiperazine but not by agonists acting at other 5-HT receptor subtypes (Gong and Kostrzewa, 1992). The effect of *m*-chlorophenylpiperazine was attenuated only by the predominately 5-HT_{2C} receptor antagonist mianserin, not by the 5-HT_{1A/1B} receptor antagonist pindolol or 5-HT_{2/3} receptor antagonist MDL-72222 (3-tropanyl-3,5-dichlorobenzoate) (Gong *et al.*, 1992). Accordingly, 5-HT_{2C} receptors appear to be the single most important of the 5-HT receptor isoforms involved in regulating oral activity. All these studies suggest that central DA systems, central cholinergic and GABA systems and central 5-HT systems are integrated in the regulation of oral activity.

Mechanisms underlying oral dyskinesias in TD patients and the pathophysiology of TD are still unknown. Previously DA receptor supersensitivity alone was considered to be the explanation of TD (Klawans, 1973; Chiu *et al.*, 1981). Conflicting data (Christensen *et al.*, 1976; Crow *et al.*, 1982) have led to the widely accepted conclusion that TD is an abnormality or imbalance of multineuronal systems such as DA, 5-HT, cholinergic, GABA and neuropeptide systems (Casey, 1987; Waddington, 1990; Gong *et al.*, 1992; Jeste and Caligiuri, 1993; Knable *et al.*, 1994; Egan *et al.*, 1995; Kostrzewa, 1995). In either the DA receptor supersensitivity or multineuronal theory of TD, DA systems are considered to have a major role.

An early study by Gunne *et al.* (1982), in which neuroleptic-induced oral activity appeared to be much enhanced after partial damage to the frontal cortex of rats, was particularly intriguing to us. On the basis of that study, it was hypothesized that selective damage to DA neurons might predispose an animal to an accentuation of neuroleptic-induced oral dyskinesias. In this report we show that chronic haloperidol treatment induced late-onset (14 weeks) spontaneous oral dyskinesias that persisted long (8 months) after drug withdrawal in a portion of neonatal 6-OHDA-lesioned rats. This is analogous to what occurs in humans treated long-term with neuroleptics. The persistence of high numbers of spontaneous oral movements is not related to an up-regulation of DA D₂ receptors.

Methods

Subjects

Timed-pregnant CR albino rats were purchased from Charles River Laboratories (Research Triangle, NC) and single-housed in clear Perspex cages in a room with constant temperature (21 ± 1°C) and 12-hr light-dark cycles (lights on at 7:00 A.M.). Animals had free access to food and water. Litters were reassigned at birth, so that each reconstituted litter consisted of equal numbers of pups from 10 different litters.

Neonatal 6-OHDA Treatment

At 3 days after birth all rat pups were pretreated with desipramine hydrochloride (20 mg/kg i.p., base form; Sigma Chemical Co.,

St. Louis, MO), 1 hr before bilateral i.c.v. administration of 6-OHDA HBr (100 µg, salt form, on each side; RBI, Natick, MA) or saline (0.85%-ascorbic acid (0.1%) vehicle. This procedure has been described in detail (Kostrzewa and Gong, 1991). Rats were weaned at 28 days and were then group housed in wire cages. Only male rats were used in this study.

Adult Treatment and Testing

Starting 2 months after birth about half the rats in the 6-OHDA and vehicle groups received HAL (1.5 mg/kg/day; Sigma Chemical Co.) *via* drinking water (2 days a week for 4 weeks, then daily for 10 months). Thus, the study consisted of four groups: (1) nonlesioned rats with tap water as drinking water (intact + tap water), (2) nonlesioned rats with haloperidol in drinking water (intact + HAL), (3) neonatal 6-OHDA-lesioned rats with tap water as drinking water (6-OHDA + tap water) and (4) neonatal 6-OHDA-lesioned rats with haloperidol in drinking water (6-OHDA + HAL). Rats were weighed once a week to adjust the dose of haloperidol. Except for 9 of the rats treated with both 6-OHDA and haloperidol, all groups of rats were sacrificed by decapitation 8 or 9 days after haloperidol withdrawal, for neurochemical assessment, determination of binding constants for DA D₂ binding sites, and quantification of the mRNA level for D₂ receptors in the neostriatum. The surviving 6-OHDA + HAL rats continued to be observed weekly for another 8 months, to determine whether spontaneous oral dyskinesias would persist.

Observation of Oral Activity

For assessing oral activity rats were placed in individual clear Perspex cages (48 × 26 × 36 cm) with steel grid floors in a quiet, well-ventilated and well-lighted room. Rats were allowed to acclimate to the new environment for at least 30 min. Testing occurred between 9:00 A.M. and 4:00 P.M. All observation sessions were conducted with 9 rats per group except when deaths reduced the pool size in 6-OHDA-lesioned haloperidol-withdrawn rats. Rats were observed one at a time, for 1 min every 10 min during a 60-min session. Numbers of oral movements were counted by an experienced observer who was unblinded to group assignment of each rat. This procedure has been described in detail (Kostrzewa and Gong, 1991). Oral activity represents spontaneous chewing (vacuous chewing) which is not directed onto any physical material (Rupniak *et al.*, 1983; Waddington, 1990; Kostrzewa and Gong, 1991; Huang and Kostrzewa, 1994a,b).

The respective DA D₁-, muscarinic- and GABAergic-receptor antagonists, SCH 23390, scopolamine and muscimol (all from RBI), are known to alter oral activity in rats. These substances were tested for their ability to attenuate spontaneous oral activity in intact and 6-OHDA-lesioned rats after HAL or tap water had been administered for 39 to 44 consecutive weeks. Similarly, these substances were tested on 6-OHDA-lesioned rats after HAL had been withdrawn for 13 to 14 weeks. There was an interval of at least 1 week between tests of any two drugs during the HAL treatment phase; and at least one-half week during the HAL withdrawal phase. Saline-treated base-line oral activity was obtained after acute saline treatment, immediately before administering any test drug. Each rat in each group was tested with any one of the antagonists, or vehicle, within a 10-day interval or less.

Neurochemical Analysis of Neostriatum

After decapitation each brain was rapidly removed and the striata were dissected free, frozen on dry ice and stored at -70°C until the time of analysis. Neostriatal concentrations of DA, DOPAC, HVA, 5-HT, 5-HIAA and NE were determined by liquid chromatography with electrochemical detection, using a Bioanalytical Systems LCEC Analyzer as follows. Each sample of caudal neostriatum was sonicated in 1.0 ml of 0.10 M trichloroacetic acid containing 0.10 mg/ml of cysteine as a stabilizing agent and 5-hydroxyindole carboxylic acid (0.20 nmol/ml) as an internal standard. Homogenates were centri-

fused at $12,000 \times g$ for 5 min. A Waters WISP automatic sample injector with a refrigerated sample compartment (samples kept at 5°C) was used to deliver 30 μ l of each supernatant onto an Econosphere C18 analytical column (5 μ m, 4.6×150 mm), having a mobile phase of 0.10 M monochloroacetic acid, 1 mM EDTA, 220 mg/l of Na octanesulfonic acid, 8% acetonitrile, pH 2.6, with a flow rate of 1.3 ml/min and temperature of 40°C (Gong *et al.*, 1992). A Hewlett-Packard HP1000 chromatography data system was used to calculate peak heights and to determine concentrations of DA, DOPAC, HVA, 5-HT, 5-HIAA and NE.

Assessment of Neostriatal DA D₂ Receptor Binding Profile

A modification of the procedure of Dewar *et al.* (1989, 1990) was used to assess DA D₂ receptors in rat neostriatum. Left rostral neostriata were homogenized with a Teflon-on-glass mortar and pestle in 1:100 ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were then centrifuged at $35,100 \times g$ for 10 min at 4°C. The supernatants were discarded and the pellets were washed four times in the Tris buffer. Final pellets were resuspended in 200 volumes of Tris buffer.

Saturation curves were obtained in the following way. Aliquots of homogenate (2 mg membrane in 400 μ l) were added to a series of incubation tubes with 10 different concentrations (93–8245 pM) of the DA D₂ receptor antagonist [³H]raclopride (specific activity: 82.4 Ci/mmol; DuPont NEN, Boston, MA). The incubate consisted of Tris buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl and 40 nM ketanserin (final concentrations). Samples were incubated in a final volume of 2.5 ml at 25°C for 60 min. Incubation was terminated by rapidly filtering samples on Whatman GF/F glass fiber filters using a Millipore filtration unit followed by three washes with 5 ml ice-cold Tris-salt solution. Filters were then placed into scintillation counting vials and dried overnight. After adding scintillation cocktail to each vial, tritium activity was determined in a Beckman LS 9800 liquid scintillation spectrometer.

A GraphPad program (GraphPad Software Inc., San Diego, CA) was used to construct the binding curves and calculate receptor binding parameters B_{\max} and K_d by fitting the data to a double rectangular hyperbola equation [$Y = A \cdot X / (B + X) + C \cdot X / (D + X) + E \cdot X$]. Factor B in this equation was set as a constant of zero because there is only one binding site for D₂ receptors (Sequential F test, $P > .05$).

Determination of mRNA Levels for DA D_{2L}, D_{2S} and 5-HT_{2C} Receptors in Rat Neostriatum

RNA extraction and purification. Total RNA was isolated and purified with an Ultraspec™-II RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX). In brief, right rostral neostriata were homogenized in 1 ml of Ultraspec RNA reagent with a Tekmar Tissumizer (setting 5, 25 s). Homogenates were transferred to 1.5-ml polypropylene microcentrifuge tubes and kept at 4°C for 5 min to allow for dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, followed by vigorous shaking for 15 s. After 5 min on ice, homogenates were centrifuged at $12,000 \times g$ for 15 min, and the aqueous phase which contained RNA was carefully transferred to a fresh tube. Each tube was vortexed for 30 s after the addition of 0.5 volumes of isopropanol and 0.05 volumes of RNA Tack™ resin. After 1 min centrifugation, supernatants were discarded and pellets were washed twice with 1 ml of 75% ethanol by vortexing for 30 s and centrifuging for 1 min. The supernatants were discarded and samples were desiccated for 5 min in a vacuum centrifuge (VirTis Co., Gardiner, NY). Total RNA was eluted from pellets with diethyl pyrocarbonate-treated water and quantified by UV spectrophotometry at 260 nm.

RT-PCR. RT-PCR assay was performed with Perkin Elmer GeneAmp RNA PCR Kit (Roche Molecular Systems, Inc., Branchburg, NJ). One microgram of total RNA from each sample was transcribed

into cDNA with MuLV reverse transcriptase and random hexamers. Two oligonucleotides (24-mer) flanking the alternative spliced exon 6 of DA D₂ receptor cDNA were used to amplify the mRNA of two DA D₂ receptor isoforms, D_{2L} and D_{2S}. The sequences of these two primers (Martres *et al.*, 1992; Della Vedova *et al.*, 1992) are: primer 1, 5'-TTCAGAGCCAACCTGAAGACACCA-3' (nucleotides 694–717); primer 2, 5'-GCTTTCTGCGGCTCATCGTCTTAA-3' (nucleotides 1067–1090). The amplified fragment of D_{2L} and D_{2S} receptors were 397-bp and 310-bp, respectively. The 5-HT_{2C} receptor primers were 5'-ACACCGAGGAGGAAGTGGCTAATAT-3' and 5'-GACCACAT-TAGAGGGGTTGACTGGC-3' which define a 601-base-long DNA fragment.

β -actin mRNA content was determined and served as internal standard. Primers (Martres *et al.*, 1992) for β -actin mRNA were 5'-GATGGTGGGTATGGGTCAGAAGGA-3' (nucleotides 129–152) and 5'-GCTCATTGCCGATAGTGATGACCT-3' (nucleotides 737–760). Amplification was carried out for 35 cycles using a Perkin Elmer DNA thermal cycler (GeneAmp PCR 9600 system, Perkin Elmer, Roche Molecular systems, Inc., Branchburg, NJ). Each cycle consisted of a 45-s denaturation step at 95°C, a 30-s annealing step at 58°C and a 45-s extension step at 72°C. The final cycle included an extension step (10 min at 72°C) to ensure full extension of the products. The amplified products were fractionated by electrophoresis on 2% Metaphor™ agarose gel (FMC BioProducts, Rockland, ME) and stained with ethidium bromide. Two bands of 397-bp and 310-bp for D_{2L} and D_{2S}, a band of 601-bp for 5-HT_{2C} and a 632-bp band for β -actin mRNA were visualized with UV illumination and photographed with Polaroid film (type 665). The optical density and area of each band were analyzed by a Bio Image Analysis System (Millipore Corporation Imaging Systems, Ann Arbor, MI). All values obtained with the specific DA D₂ and 5-HT_{2C} primers were corrected for slight differences in RNA sample loading by normalizing to the values obtained with the β -actin primers.

Data Analysis

One-way analysis of variance (ANOVA), followed by the *post hoc* test of Newman-Keuls, was used for data analysis except that Student's paired t test was used to determine differences within a group before and after drug treatment when receptor-specific drugs were tested for their ability to attenuate oral activities. A P value $< .05$ was considered to be statistically significant.

Results

Spontaneous Oral Activity

In intact and 6-OHDA-lesioned rats that received tap water for the duration of the study the incidence of oral activity did not change overtly. In intact rats, oral activity remained at five oral movements or less per session (fig. 1, open circles); in 6-OHDA-lesioned rats, about 10 oral movements per session (fig. 1, open squares). This level of oral activity in 6-OHDA-lesioned rats was not significantly greater than that observed in intact rats at any single time. For the first 2 months after HAL administration, spontaneous oral activity of intact and lesioned rats remained at the level observed before HAL treatment (fig. 1, filled symbols). However, in the 12th week of HAL treatment, spontaneous oral activity increased nearly 3-fold to almost 30 oral movements in 6-OHDA-lesioned rats ($P < .01$; fig. 1). In 6-OHDA-lesioned rats spontaneous oral activity ranged between 27.6 and 41.6 movements for the duration of HAL administration (fig. 1).

In intact rats HAL produced a gradual increase in spontaneous oral activity, with the maximal effect occurring when HAL had been administered for about 4 months (fig. 1, filled circles). Starting from the 14th week of HAL treatment, the incidence of spontaneous oral activity in these rats ranged

Effects of Haloperidol Treatment on Spontaneous Oral Activity of Intact and Neonatal 6-OHDA-Lesioned Rats

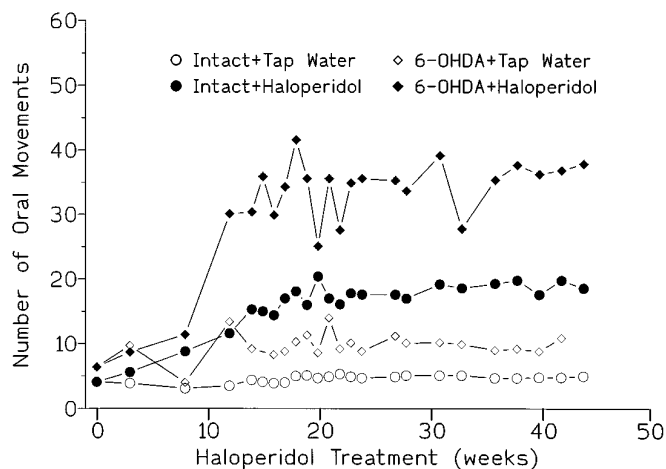


Fig. 1. Time-course effect of chronic HAL treatment on spontaneous oral activity of intact and 6-OHDA-lesioned rats. Rats were treated at 3 days after birth with 6-OHDA (100 μ g salt form in each lateral ventricle; desipramine pretreatment, 20 mg/kg i.p., base, 1 hr) or saline (0.85%-ascorbic acid (0.1%) vehicle. HAL was added to the drinking water starting at 2 months after birth (1.5 mg/kg/day on 2 consecutive days per week for 4 weeks; then daily for 10 months). Spontaneous oral activity was observed at 1 or 2 week intervals during the 11 months of HAL administration, in sessions in which rats were observed for 1 min every 10 min for 60 min. Each point represents the mean number of vacuous chewing episodes in one session ($n = 9$ /group). After HAL had been administered for 12 weeks in 6-OHDA-lesioned rats and for 14 weeks in intact rats, the number of spontaneous oral movements was significantly elevated ($P < .01$) in intact and 6-OHDA-lesioned rats that received HAL.

from 14.4 to 20.4, a level that was consistently higher than that of intact rats that were maintained on tap water (fig. 1). This represented a 3- to 4-fold increase over that of intact rats on tap water ($P < .01$; fig. 1).

The incidence of spontaneous oral activity in HAL-treated 6-OHDA-lesioned rats was about twice as great as that of HAL-treated intact rats ($P < .01$; fig. 1). At 44 weeks when HAL was withdrawn, nine HAL-withdrawn 6-OHDA-lesioned rats continued to be observed for spontaneous oral activity during the subsequent 8 months. The high incidence of oral activity that had been observed before removing HAL, persisted during the HAL-withdrawal period, remaining stable (range of 28.5 to 38.5 oral movements per session) until the 8th month when rats were sacrificed (fig. 2).

The reported incidence of oral activity in 6-OHDA-lesioned rats was recorded from the same rats (a) before instituting HAL administration, (b) during HAL administration and (c) after withdrawal of HAL. Immediately before withdrawing HAL, however, spontaneous oral activity was determined in all rats that received HAL for the previous 10 months. We observed that 3 of the 21 rats had 7 or fewer oral movements in a session; 3 rats had 17 to 19 oral movements; 8 rats had 21 to 26 oral movements; 5 had 35 to 48 oral movements; and 2 rats had more than 60 oral movements.

Effects of Receptor-Specific Agonists and Antagonists on Oral Activity

After HAL had been administered for 39 to 40 weeks, the muscarinic receptor antagonist scopolamine HCl (0.1 mg/kg, salt form, i.p.) was tested for its ability to attenuate oral

Spontaneous Oral Activity of Haloperidol-Withdrawn Neonatal 6-OHDA-Lesioned Rats

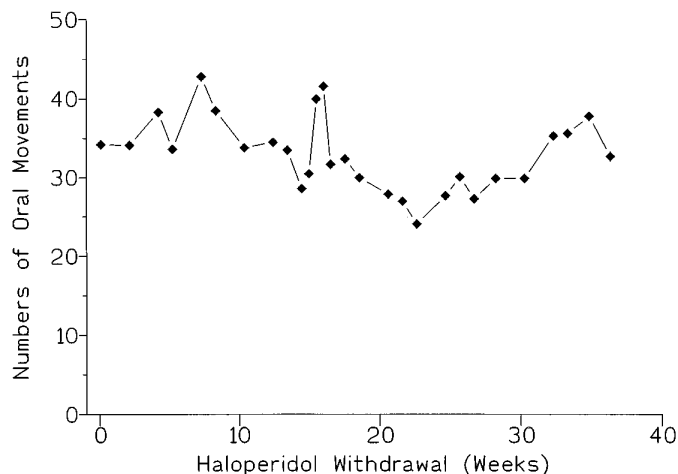


Fig. 2. Spontaneous oral activity in 6-OHDA-lesioned rats after HAL withdrawal. Oral activity was observed as in figure 1. Every 1 or 2 weeks a neurochemical agonist or antagonist was administered acutely, in an attempt to attenuate oral activity (not shown). The number of spontaneous oral movements remained elevated to the same extent as that observed before withdrawal of HAL. Each point represents the mean number of oral movements of 6 to 9 rats.

activity. Scopolamine was found to have no effect on spontaneous oral activity of intact ($P = .653$) and 6-OHDA-lesioned rats ($P = .295$) maintained on tap water (fig. 3, 4th vs. 1st and 8th vs. 5th bars). However, scopolamine significantly attenuated spontaneous oral activity in both intact ($P = .007$) and 6-OHDA-lesioned rats ($P = .008$) receiving HAL (fig. 3, 9th vs. 12th and 13th vs. 16th bars). In rats that had been withdrawn from HAL for 13 weeks, scopolamine had no effect (fig. 3, 17th vs. 20th bars, $P = .405$).

Rats were tested with the DA D_1 receptor antagonist SCH

Effects of SCH 23390, Muscimol and Scopolamine on Oral Activity of Rats

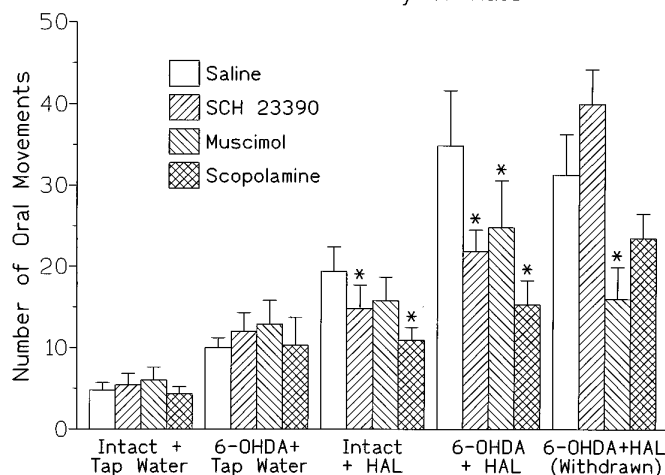


Fig. 3. Effects of SCH 23390, muscimol and scopolamine on spontaneous oral activity of rats. Rats received 6-OHDA and HAL treatments as described in figure 1. The effects of SCH 23390 HCl (1.0 mg/kg i.p., 1 hr), muscimol (3.0 mg/kg i.p., 1 hr), scopolamine HCl (0.1 mg/kg i.p., 1 hr) and saline (0.9%) vehicle on oral activity were determined in rats before and after HAL administration was discontinued. Each bar represents the mean (\pm S.E.M.) number of oral movements for 9 rats. * $P < .05$ vs the saline vehicle group.

23390 HCl (1.0 mg/kg i.p., salt form, 1 hr) after rats had received HAL for 42 weeks. SCH 23390 HCl had no effect on oral activity of intact ($P = .352$) and 6-OHDA-lesioned rats ($P = .321$) maintained on tap water (fig. 3, 1st vs. 2nd and 5th vs. 6th bars). However, in HAL-treated intact ($P = .041$) and 6-OHDA lesioned rats ($P = .008$) that displayed high levels of spontaneous oral activity, SCH 23390 produced a significant reduction of oral activity (fig. 3, 9th vs. 10th and 13th vs. 14th bars). After rats had been withdrawn from HAL for 14 weeks, and despite a continued high incidence of spontaneous oral activity, SCH 23390 had no effect (fig. 3, 17th vs. 18th bar, $P = .173$).

Rats were tested with the GABA_A receptor agonist muscimol (3.0 mg/kg i.p., 1 hr) after HAL had been administered for 43 weeks. Muscimol had no effect on spontaneous oral activity of intact ($P = .211$) and 6-OHDA-lesioned rats ($P = .292$) maintained on tap water (fig. 3, 3rd vs. 1st and 7th vs. 5th bars). Also, there was no effect of muscimol on intact rats maintained on HAL for 43 weeks (fig. 3, 11th vs. 9th bars, $P = .292$). However, muscimol significantly reduced spontaneous oral activity of 6-OHDA-lesioned rats receiving HAL (fig. 3, 15th vs. 13th bars, $P = .033$). The effect of muscimol persisted even after HAL had been withdrawn for 12 weeks (fig. 3, 19th vs. 17th bars, $P = .013$).

Effects of Neonatal 6-OHDA and Prolonged Adult HAL Treatments on Tissue Concentrations of Monoamines and Metabolites in Rat Neostriatum

DA, DOPAC and HVA contents. In rats treated at 3 days after birth with 6-OHDA, the neostriatal concentrations of DA and HVA were reduced by 97% at 1 year, whereas DOPAC concentration was reduced 89% (all $P < .01$; table 1). Although chronic HAL administration had no influence on DA, DOPAC and HVA concentrations in the neostriatum of 6-OHDA-treated rats, chronic HAL administration reduced the concentrations of DOPAC and HVA in intact rats by 30% and 33%, respectively (each $P < .05$; table 1).

5-HT, 5-HIAA and NE concentrations in rat neostriatum. In rats treated neonatally with 6-OHDA, the neostriatal concentrations of 5-HT and its principal metabolite 5-HIAA were elevated by 29% and 23%, respectively (each $P < .01$; table 1). Chronic HAL administration had no influence on 5-HT and 5-HIAA concentrations in the neostriatum of intact rats. Neither 6-OHDA nor HAL treatment altered the concentration of NE in rat neostriatum.

Changes in the DA D₂ Receptor Binding Profile

As revealed by [³H]raclopride binding to homogenates of rat striata, chronic HAL treatment produced a significant

increase of DA D₂ receptor density (B_{max}) in rostral striatum of both intact and neonatal 6-OHDA-lesioned rats. The K_d was not significantly altered. Eight months after HAL withdrawal, the B_{max} of neonatal 6-OHDA-lesioned rats returned to control levels (table 2).

Changes in mRNA Levels of DA D₂ and 5-HT_{2C} Receptors

In an attempt to correlate the functional changes with molecular mechanisms, the relative mRNA levels of DA D_{2L} and D_{2S} receptors, two isoforms of D₂ receptors generated by alternative splicing of the DA D₂ receptor gene, and the mRNA level of 5-HT_{2C} receptors were assessed by RT-PCR.

As shown in figure 4, striatal mRNA levels of D_{2L} receptors in both groups of rats receiving HAL were significantly increased, compared with rats without HAL treatment (fig. 4, 3rd and 4th bars vs. 1st and 2nd bars, $P < .01$). However, 8 months after HAL withdrawal, the mRNA level of D_{2L} receptors in neonatal 6-OHDA-lesioned rats returned to control level (fig. 4, 4th bar vs. 5th bar, $P < .01$). The D_{2S} mRNA levels of neonatal 6-OHDA-lesioned rats, regardless of whether HAL was administered, were slightly but not significantly increased when compared with other treatment groups (fig. 4, 6th to 10th bars, $P > .05$). This may indicate that the changes of D_{2L} subtype of DA D₂ receptors are more closely related to the effects of long-term HAL treatment. There was no significant change in 5-HT_{2C} receptor mRNA levels among different treatment groups (fig. 4, 11th to 15th bars, $P > .05$).

Discussion

In the present study, in agreement with others (Breese and Traylor, 1971, 1972; Breese *et al.*, 1984), there was an extensive reduction in the neostriatal content of DA (97%), HVA (98%) and DOPAC (89%) in 6-OHDA-treated rats, regardless of HAL treatment (table 1). Neostriatal NE content was unaltered, probably reflecting effectiveness of desipramine pretreatment in protecting noradrenergic neurons from 6-OHDA destruction. As expected, neostriatal concentrations of 5-HT and 5-HIAA were significantly elevated by neonatal 6-OHDA treatment (table 1, $P < .01$), reflecting the known proliferative sprouting of 5-HT fibers in the forebrain of rats lesioned neonatally with 6-OHDA (Stachowiak *et al.*, 1984; Berger *et al.*, 1985; Snyder *et al.*, 1986).

Spontaneous oral activity of intact rats remained at a consistently low level for the entire duration of the experiment (fig. 1). Also, neonatal 6-OHDA treatment alone did not increase the level of spontaneous oral activity (fig. 1). The HAL dose (1.5 mg/kg/day) used in this study has been shown

TABLE 1

Effects of neonatal 6-OHDA and chronic HAL treatment on concentrations of monoamines and their metabolites in the rat neostriatum

Rats were treated at 3 days after birth with 6-OHDA HBr (100 μg each side i.c.v., salt form; desipramine pretreatment, 1 hr) or its vehicle. From 2 months after birth, HAL were given *via* drinking water (1.5 mg/kg/day, 2 days/week for 1 month, then daily for 10 months). Striata were removed for assay 8 or 9 days after the withdrawal of HAL. Values are mean nanomoles per gram of tissue ± S.E.M. Eight samples were analyzed from each group.

Treatment Group	Monoamines or Metabolite					
	DA	DOPAC	HVA	5-HT	5-HIAA	NE
Intact + tap water	52.8 ± 5.7	7.6 ± 0.9	4.0 ± 0.2	4.1 ± 0.2	5.2 ± 0.2	1.1 ± 0.1
6-OHDA + tap water	1.6 ± 0.5**	0.8 ± 0.1**	0.1 ± 0.1**	5.3 ± 0.2**	6.4 ± 0.2**	1.0 ± 0.1
Intact + HAL	46.8 ± 3.7	5.3 ± 0.5*	2.7 ± 0.3*	4.3 ± 0.2	5.4 ± 0.2	1.1 ± 0.1
6-OHDA + HAL	1.9 ± 0.3**	0.8 ± 0.1**	0.1 ± 0.1**	4.8 ± 0.3	6.4 ± 0.2**	1.2 ± 0.1

* $P < .05$; ** $P < .01$ when compared with vehicle control group.

TABLE 2

Effects of 6-OHDA and chronic HAL treatments on [³H]raclopride binding to homogenates of rat rostral neostriatum

Rats received vehicle or 6-OHDA (100 µg salt form, each side, i.c.v.; 20 mg/kg desipramine pretreatment i.p., 1 hr) 3 days after birth; plus HAL (1.5 mg/kg per day for 2 consecutive days per week for 4 weeks; then daily for 10 months via drinking water) or tap water from 2 months of age for an 11-month period. Rostral striata were removed 9 days or 8 months after withdrawing HAL. Saturable curves for striatal homogenates were constructed, using binding data ($n = 5$) from 10 concentrations of [³H]raclopride (93–8250 pM). The B_{max} and K_d for [³H]raclopride binding was determined with a computer program (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean (95% confidence interval).

Treatment Group	B_{max} fmol/mg protein	K_d pM
Intact + tap water	204 (174–233) ^a	658 (492–817)
6-OHDA + tap water	260 (219–303) ^b	809 (668–994)
Intact + HAL	339 (259–418) ^c	986 (776–1197)
6-OHDA + HAL	350 (301–399) ^d	896 (822–966)
6-OHDA + HAL (withdrawn)	230 (172–287) ^e	704 (524–873)

^a versus ^c and ^a versus ^d: $P < .01$.

^e versus ^c and ^e versus ^d: $P < .05$.

mRNA Levels of D_{2L}, D_{2S} and 5-HT_{2C} Receptors in Rat Striatum after Long-Term Haloperidol Treatment

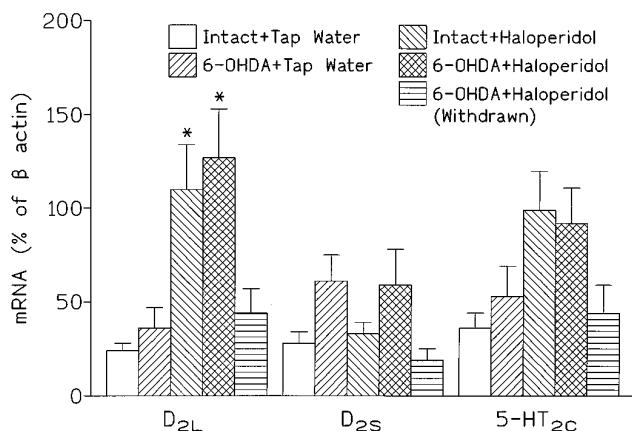


Fig. 4. Messenger RNA levels for DA D_{2L}, D_{2S} and 5-HT_{2C} receptors in neostriatum of intact and neonatal 6-OHDA-lesioned rats after 44 weeks of HAL treatment. mRNA levels were determined by RT-PCR. Each bar represents the mean (\pm S.E.M.) mRNA level, expressed as a percentage of β -actin mRNA ($n = 6$). * $P < .05$ vs D_{2L} content of neostriatum of rats that received tap water or were withdrawn from HAL.

to produce a plasma HAL level that is nearly equivalent to the plasma level in HAL-treated TD patients (Tamminga *et al.*, 1990). The levels of oral activity for both intact and 6-OHDA-lesioned rats were increased markedly by HAL treatment, but only after 12 to 14 weeks of treatment. There was no early emergence of increased spontaneous oral activity or any other abnormal behavior during haloperidol treatment. Long-term HAL treatment produced a 3-fold increase in the number of spontaneous oral movements in intact rats. In these rats there were about 17 oral movements per session. When HAL was administered to rats that had been lesioned neonatally with 6-OHDA, there were 30 to 40 oral movements observed in each session (fig. 1). Therefore, long-term HAL treatment has an additive or synergistic effect with a 6-OHDA lesion in inducing spontaneous oral activity (Huang and Kostrzewa, 1994b).

In other studies in which intact rats were treated long-term with HAL, the level of spontaneous oral activity de-

clined rapidly in the subsequent 2 to 8 weeks after discontinuing HAL treatment (Tamminga *et al.*, 1990; Gunne *et al.*, 1986; Mithani *et al.*, 1987). In the present study there were inadequate numbers of rats to pursue this question in intact rats. However, because of a death rate lower than expected in the HAL-treated 6-OHDA-lesioned group, we were able to maintain 9 rats in this group beyond the time when HAL was discontinued. In this group the high level of spontaneous oral activity, induced by long-term HAL treatment, persisted unabated for 8 months beyond the date when HAL treatment was discontinued (fig. 2). This persistence of elevated oral activity after discontinuance of neuroleptics is the longest ever described. Taking into account the life span of rats, it is clear that the high level of spontaneous oral dyskinesias reported here is present for a major portion of the life of these rats and is probably a permanent effect. In contrast to the 36 to 55% spontaneous remission rate of TD symptoms in humans during neuroleptic withdrawal (Jeste *et al.*, 1988), the level of spontaneous oral activity did not decline in any rat during the HAL-withdrawal phase. The HAL-free period, with elevated levels of oral activity, is especially suited for testing the ability of drugs to attenuate oral activity behavior (Huang and Kostrzewa, 1994b).

The reliability of DA receptor antagonists for attenuating oral activity in humans with TD is still uncertain, despite extensive testing (Levin *et al.*, 1989; Lublin *et al.*, 1993). In this study in rats, the DA D₁ receptor antagonist SCH 23390 (1.0 mg/kg) significantly attenuated spontaneous oral activity of intact and 6-OHDA-lesioned rats before withdrawal of HAL (fig. 3), but had no significant effect after withdrawal of HAL (fig. 3).

In human as well as animal studies GABA agonists like muscimol and progabide effectively attenuate oral activity. Tamminga *et al.* (1979) and Cassady *et al.* (1992) find that muscimol (5–9 mg oral dose) attenuates involuntary movements of TD patients. Coadministration and acute challenge with progabide also significantly reduces neuroleptic-induced oral dyskinesias (Mithani *et al.*, 1987; Kaneda *et al.*, 1992). In this study, muscimol (3.0 mg/kg) markedly attenuated the oral activity during and after HAL treatment.

Central cholinergic systems are involved in the regulation of oral activity in animals (Rupniak *et al.*, 1983; Salamone *et al.*, 1990; Kostrzewa and Neely, 1993), but the role of cholinergic systems in TD is still uncertain. In some cases cholinergic antagonists aggravate TD symptoms (Jeste and Wyatt, 1982; Casey, 1987; Klawans and Rubovits, 1974), whereas in other cases cholinergic antagonists alleviate the early emergence of oral dystonias or even improve symptoms of late-onset oral dyskinesia in TD. This would suggest that excess activity of cholinergic systems is associated with TD (Klawans and Rubovits, 1974). The response to scopolamine has been used as a criterion to evaluate the involvement of cholinergic systems in animal models of TD (Klawans and Rubovits, 1974; Rupniak *et al.*, 1983; Casey, 1987). In the present study, scopolamine significantly attenuated oral activity of rats (fig. 3) during the phase of HAL treatment but lacked any significant effect during the phase of HAL withdrawal (fig. 4). Because scopolamine inhibits the early emergence of oral dystonias, whereas cholinergic agonists improve the symptoms of TD (Rupniak *et al.*, 1984; Waddington, 1990), the failure of scopolamine to inhibit oral activity after HAL withdrawal may suggest that the character of oral activity

after HAL withdrawal is more analogous to the oral dyskinesic activity of TD patients. The opposite effects of SCH 23390 and scopolamine on spontaneous oral activity before and after HAL withdrawal indicate that oral activity is regulated differently in these two phases.

Radja *et al.* (1993) found that neonatal 6-OHDA HBr treatment (75 μg salt form i.c.v.) increased [^3H]raclopride binding to all parts of caudal neostriatum and most parts of rostral neostriatum of rats. In the current study a neonatal 6-OHDA lesion *per se* did not produce changes in DA D_2 receptor binding parameters (table 2). Duncan *et al.* (1987) found that 15-day treatment with HAL led to an increase in the B_{max} for the DA D_2 receptor class in the nucleus accumbens of intact and adult 6-OHDA-lesioned, but not in neonatal 6-OHDA HBr-lesioned rats (150 μg salt form i.c.v. at 5 days after birth). However, the neostriatum was not analyzed in that study.

Chronic neuroleptic treatment causes a reversible up-regulation of the D_2 class of DA receptors (D_2 , D_3 , D_4) in rat neostriatum (Duncan *et al.*, 1987; Laruelle *et al.*, 1992; Marin and Chase, 1993; Schoots *et al.*, 1995). This increased B_{max} for the DA D_2 receptor class returns to control levels 2 weeks to 2 months after discontinuing neuroleptic treatment (Clow *et al.*, 1980; Dewey and Fibiger, 1983). In agreement, chronic HAL treatment increased the B_{max} for the DA D_2 class of receptors in intact rats (table 2). The radioligand used in our study, [^3H]raclopride, has high affinity for DA D_2 receptors, but will also bind to DA D_3 receptors (Schoots *et al.*, 1995; Sokoloff *et al.*, 1990). Accordingly, an increase in the B_{max} for [^3H]raclopride is reflective of an increase in numbers of DA D_2 and/or D_3 receptors in the neostriatum. Clow *et al.* (1980) and Rupniak *et al.* (1985a) found that neuroleptic-induced up-regulation of the DA D_2 receptor class in nucleus accumbens was transient, returning to control levels, despite continued neuroleptic treatment for 3 to 9 months.

In HAL-treated 6-OHDA-lesioned rats the elevated B_{max} for the neostriatal DA D_2 receptor class was at control levels 8 months after discontinuing haloperidol, while the level of spontaneous oral activity was still increased (table 2 and fig. 2). Therefore, the level of oral activity in HAL-withdrawn rats is dissociated from a change in the B_{max} for the DA D_2 receptor class. Knable and associates (1994) have reported that vacuous chewing movements are not correlated with binding of [^3H]raclopride in striatum and nucleus accumbens.

Alternative splicing of DA D_2 receptor genes gives rise to two variants of mRNA that code for two DA D_2 receptor isoforms termed D_{2L} and D_{2S} , with amino acid sequences of 444 and 415, respectively. Although these two isoforms have the same ligand-binding properties, they may couple to different G proteins in signal transduction processes because there are 29 more amino acids in the third intracellular loop of DA D_{2L} receptors vs. DA D_{2S} receptors (Giros *et al.*, 1989; Monsma *et al.*, 1989; Selbie *et al.*, 1989; Martres *et al.*, 1992). Others have found that chronic HAL treatment can either increase (Fishburn *et al.*, 1994; Buckland *et al.*, 1993; Rogue *et al.*, 1991) or lack an effect (Srivistava *et al.*, 1990) on the mRNA level of rat neostriatal DA D_2 , D_{2L} or D_{2S} receptors. It seems that an increase in mRNA levels is not necessary for DA D_2 receptor up-regulation after long-term HAL treatment. Long-term HAL-induced DA D_2 receptor up-regulation may occur because of altered posttranscriptional mecha-

nisms (Srivistava *et al.*, 1990). In the present study we found that long-term HAL treatment increased striatal mRNA levels of DA D_{2L} receptors in both intact and neonatal 6-OHDA-lesioned rats (fig. 4), whereas the mRNA levels for DA D_{2S} and 5-HT $_{2C}$ receptors remained unchanged (fig. 4). These findings suggest that transcriptional processes are of major importance in regulating the expression of DA D_{2L} , D_{2S} and 5-HT $_{2C}$ receptor genes in the neostriatum of rats treated long-term with HAL. In accord with the increase in B_{max} for the DA D_2 receptor class in the neostriatum of HAL-treated 6-OHDA-lesioned rats, the elevated level of DA D_{2L} receptor mRNA returned to control level 8 months after discontinuing HAL treatment (fig. 4).

Because of the inherent difficulty of ethically conducting experiments in humans with TD (Crossman, 1987; Waddington, 1990), animal models are used extensively in studies designed to determine the pathophysiological basis of TD. To adequately model TD, oral dyskinesias in animals should develop only after many weeks of treatment (late-onset). This phenomenon is seen in some studies (Ellison and See, 1989), whereas in others there is early emergence of spontaneous oral activity (Rupniak *et al.*, 1985b) or even a lack of any drug effect (Levy and Ellison, 1987). An increased neuroleptic dose reduces, whereas the withdrawal of a neuroleptic exacerbates TD symptoms in humans. These two manipulations have no effect on spontaneous oral activity of chronic neuroleptic-treated rats (Jeste and Wyatt, 1982; Egan *et al.*, 1995). There are also conflicting findings on whether oral dyskinesias are able to persist, a syndrome that is characteristic of a majority of TD patients (Jeste and Wyatt, 1982). Although it was reported that enhanced oral activity persisted for up to 2.5 months in trifluoperazine-treated rats (Waddington *et al.*, 1983), a gradual diminution in the number of oral movements after neuroleptic-withdrawal was found by many others (Mithani *et al.*, 1987; Gunne and Haggstrom, 1983; Gunne *et al.*, 1982). Actually, even in these instances Waddington (1990) has discussed the high probability that a residual depot of neuroleptic continued to influence oral activity. In no study did oral movements persist beyond several weeks when orally administered drug was withdrawn (Waddington, 1990). Because of the various outcomes of current TD animal models, it is difficult to draw firm evidence for the pathophysiological basis of TD. Therefore, we had interest in determining whether DA-lesioned rats might be candidates for animal modeling of TD.

The rationale was that TD occurs when neuroleptics are given to people with neuropsychiatric disorders, rather than to healthy people. Waddington (1990) has suggested that rats with undefined brain injury may be more appropriate than intact rats as a model for TD. Increasingly more evidence suggests that a brain dysfunction contributes to the vulnerability of TD (Kane *et al.*, 1985; Owens, 1985; Waddington *et al.*, 1989; Waddington, 1990).

A neonatal 6-OHDA lesion is known to supersensitize DA D_1 receptors (Hamdi and Kostrzewa, 1991; Kostrzewa and Hamdi, 1991; Huang and Kostrzewa, 1994a) and 5-HT $_{2C}$ receptor-mediated oral activity (Gong *et al.*, 1992). In either the DA supersensitization or the multineurotransmitter theory of TD, DA systems are a major element and possibly even the central element. Either chronic HAL treatment or neonatal 6-OHDA treatment sensitizes central DA receptors in rats. The exogenous alteration of the balance in central DA

systems produced by neonatal 6-OHDA-lesion may enable these rats to serve as an improved TD animal model under chronic HAL treatment.

In summary, for the first time, neonatal 6-OHDA-lesioned rats were chronically treated with HAL to induce oral dyskinesia. Nearly one year's treatment with HAL produced a level of spontaneous oral activity that was twice as great as for the HAL-treated intact group of rats. The B_{\max} for [^3H]raclopride binding and the level of DA D_{2L} receptor mRNA in striatum were also elevated by long-term HAL treatment. These changes, however, were not closely correlated with oral activity, because high levels of oral activity persisted for 8 months after ceasing HAL treatment, despite a decline to control levels of both the B_{\max} for [^3H]raclopride binding and mRNA level of D_{2L} receptors. The dissociation of oral dyskinesias from changes in DA D_{2L} mRNA level and DA D_2/D_3 receptor up-regulation (*i.e.*, sites to which raclopride binds), suggests that these changes are not critical for emergence of oral dyskinesias in rats. Finally, the long-lasting elevation of oral dyskinesias in 6-OHDA-lesioned rats after HAL withdrawal provides a mean that is useful for testing new drugs in subjects cleared of neuroleptics.

Acknowledgments

The authors thank Mr. David Neely for preparing illustrations and Ms. Lottie Winters for preparing the manuscript.

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