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Dopaminergic denervation enhances susceptibility to hydroxyl radicals in rat neostriatum

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Summary. To determine if greater amounts of hydroxyl radical (•OH) are formed by dopamine (DA) denervation and treatment with L-dihydroxyphenylalanine (L-DOPA), the neostriatum was DA denervated (99% reduction in DA content) by 6-hydroxydopamine treatment (134 μg icv, desipramine pretreatment) of neonatal rats. At 10 weeks the peripherally restricted dopa decarboxylase inhibitor carbidopa (12.5 mg/kg i.p.) was administered 30 min before vehicle, L-DOPA (60 mg/kg i.p.), or the known generator of reactive oxygen species, 6-hydroxydopa (6-OHDOPA) (60 mg/kg i.p.); and this was followed 30 min later (and 15 min before termination) by the spin trap, salicylic acid (8 μmoles icv). By means of a high performance liquid chromatographic method with electrochemical detection, we found a 4-fold increase in the non-enzymatically formed spin trap product, 2,3-dihydroxybenzoic acid (2,3-DHBA), with neither L-DOPA nor 6-OHDOPA having an effect on 2,3-DHBA content of the neostriatum. Basal content of 2,5-DHBA, the enzymatically formed spin trap product, was 4-fold higher vs. 2,3-DHBA in the neostriatum of untreated rats, while L-DOPA and 6-OHDOPA each reduced formation of 2,5-DHBA. We conclude that DA innervation normally suppresses •OH formation, and that the antiparkinsonian drug L-DOPA has no effect (2,3-DHBA) or slightly reduces (2,5-DHBA) •OH formation in the neostriatum, probably by virtue of its bathing the system of newly formed •OH.

Keywords: Amino acids – Denervation – Dopamine – 6-Hydroxydopa – 6-Hydroxydopamine – Hydroxyl radicals – Levodopa – Parkinson's disease – Reactive oxygen species

Introduction

Starting from the 1950s, dopamine (DA) has been viewed not only as a neurotransmitter but as a putative neurotoxic species that, over time, may

auto-destruct those nerves in which it is synthesized. From this vantage, either alone or by interaction with neuromelanin and iron, DA has been implicated as an auto-toxin in *pars compacta* substantia nigra neurons, namely those dopaminergic neurons that give rise to nigroneostriatal fibers and are preventive of the onset of Parkinson's disease.

Most neuronal DA is ultimately metabolized by monoamine oxidase (MAO) via a process that generates one molecule of hydrogen peroxide (H_2O_2) for each molecule of DA that is enzymatically converted to dihydroxyphenylacetic acid (DOPAC). In the presence of iron (Fenton reaction), H_2O_2 can lead to formation of superoxide anion ($O_2^{-\cdot}$), which through the Haber-Weiss reaction leads to formation of hydroxyl radical ($^{\bullet}OH$). Each of these reactive oxygen species (ROS) is potentially cytotoxic, with $^{\bullet}OH$ being the most unstable and most reactive (see Kaur and Halliwell, 1996). The involvement of neuromelanin in this schema is uncertain. Generally, neuromelanin is considered to be neuroprotective by virtue of its ability to sequester and inactivate *o*-quinone (Wilczok et al., 1999), but yet when there is excessive oxidative stress the most-highly pigmented neurons in *pars compacta* are thought to be those that first disappear in Parkinson's disease (see Smythies, 1999b).

In addition, DA can auto-oxidize (i.e., a misnomer for reaction with molecular O_2) to form several potentially cytotoxic species, including DA-semiquinone, DA-hydroquinone, DA-*o*-quinone, DA-*p*-quinone, and dopaminochrome (Bindoli et al., 1999; Graham, 1978; Segura-Aguilar, 1999; Senoh et al., 1959a; Senoh and Witkop, 1959a, 1959b; Sulzer and Zecca, 1999). There is also evidence that DA can auto-oxidize to 6-hydroxydopamine (6-OHDA) (Senoh et al., 1959b), a widely used neurotoxin for catecholamine neurons (see Kostrzewa, 1999). All of these described species are cytotoxic.

There is widespread demonstration of DA toxicity for a variety of neuronal and non-neuronal cultures in vitro (Galzigna et al., 1999). In these cells, DA produces lipid peroxidation, DNA base damage and cell death (Masserano, 1999). It also is relevant that anti-oxidants like ascorbic acid and α-tocopherol confer cytoprotection, as do scavengers of ROS.

For all of the above reasons, DA has been implicated as a causative agent in neurodegenerative disorders, such as Parkinson's disease, and in neuropsychiatric disorders (see Metodiewa and Kośka, 1999; Smythies, 1999b).

The objective of the following study was to determine if dopaminergic denervation had a promoting or suppressing effect on ROS. In our study we focused on the most cytotoxic of these ROS, namely $^{\bullet}OH$. A secondary objective was to determine if L-dihydroxyphenylalanine (L-DOPA) enhanced or suppressed $^{\bullet}OH$; and its effects were compared to that of an analogue, 6-hydroxydopa (6-OHDOPA), a recognized neurotoxin (Jacobowitz and Kostrzewa, 1971; Kostrzewa, 1998; Sachs and Jonsson, 1972a, 1972b) and known generator of ROS (Rodriguez-Lopez et al., 1992).

Experimental methods and design

Destruction of DA innervation of neostriatum in neonatal rats

Litters born to timed-pregnant rats were randomized among the dams. At three days after birth rats were treated with desipramine (20 mg/kg ip, 1 hr; to prevent damage to noradrenergic neurons), then anesthetized with methoxyflurane (Metofane) and placed on a flat surface under a bright light, so that cranial sutures were visible through a transparent dermis. A 30-gauge needle with polyethylene sleeve up to 2 mm from the tip was positioned 1.5 mm anterior and 2 mm lateral to the sagittal suture. Exactly 5 μl of vehicle [saline (0.85%)-ascorbic acid (0.1%)] or 6-OHDA (67 μg, base) was administered into each lateral ventricle (Kostrzewa and Gong, 1991). The chosen total dose of 6-OHDA HBr was expected to deplete DA in neostriatum by 99% (Gong et al., 1993).

As evident from the above description, injection of 6-OHDA did not entail a surgical procedure. The 6-OHDA lesion in neonatal rats does not produce aphagia, adipsia, or akinesia, as it would in adult rats; and in the absence of definitive behavioral assessments, neonatally 6-OHDA-lesioned rats are indistinguishable from controls. Also, the degree of DA depletion is well correlated with the degree of DA denervation of the neostriatum (Kostrzewa et al., 1998). The effect on DA innervation is practically invariable in its effect on DA content of neostriatum (Gong et al., 1992) and much greater than that attainable with a 6-OHDA lesion of adult rats (see Breese and Breese, 1998). Administered in this way and at this time, 6-OHDA has no effect on animal survival. This experimental protocol was approved by the Animal Care Committee of East Tennessee State University and is in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals in Research.

Administration of L-DOPA, 6-OHDOPA, and salicylic acid

At ten weeks after birth, control and 6-OHDA-lesioned rats were pretreated with the peripherally-restricted DOPA decarboxylase inhibitor carbidopa (12.5 mg/kg i.p.), 30 min before administration of either saline vehicle, L-DOPA (60 mg/kg i.p.), or 6-OHDOPA (60 mg/kg i.p.). Rats were then anesthetized with ketamine (60 mg/kg) plus xylazine (7.5 mg/kg), for surgical implantation of a cannula guide into the left lateral ventricle, at A-4.0, L2.0, D3.0 (coordinates for 280–320 g rats; Pellegrino et al., 1979). Exactly 30 min after the last injection, and 15 min before termination, salicylic acid (10 μl, saturated solution, 8 micromoles) was administered into the lateral ventricle. [Salicylate, administered icv, is a more reliable indicator of •OH in brain than salicylate administered i.p. (Giovanni et al., 1995).] All rats were decapitated without awakening from anesthesia, and the neostriatum was rapidly dissected from the brain and frozen on dry ice, stored at −80°C, and analyzed within two weeks.

Assessment of DA, 5-HT and DHBA contents of neostriatum

Rat neostriatum was sonicated on ice in 0.01 N HCl–0.1 N $HClO_4$, and homogenates were centrifuged at 4°C at 12,000 × g for 10 min. Aliquots were injected via a 20 μl loop onto a Microdialysis MD-150 × 1 Microbore analytical column, using a mobile phase consisting of 1.7 mM 1-octanesulfonic acid sodium, 25 μM EDTA, 10% acetonitrile, and 0.01% triethylamine in 75 mM phosphate buffer at pH 3.0 and a flow rate of 0.6 ml/min. A guard cell (250 mV) and flow-through microdialysis cell (75 mV) were used for the analysis, with a Coulochem data analysis sytem to integrate peak areas. In the above study isoproterenol was the internal standard for analysis of DA, dihydroxyphenylacetic acid (DOPAC), serotonin (5-hydroxytryptamine, 5-HT), 5-hydroxyindoleacetic acid (5-HIAA), 2,3-DHBA, and 2,5-DHBA. Representative spectra are shown as Figs. 1 and 2. DA and 5-HT (Fig. 1), present in amounts much higher than newly formed 2,3- and 2,5-DHBA (Fig. 2), were analyzed from diluted homogenates. The spectrum includes peaks for norepinephrine (NE), L-DOPA, DOPAC, homovanillic acid (HVA), and 5-HIAA.

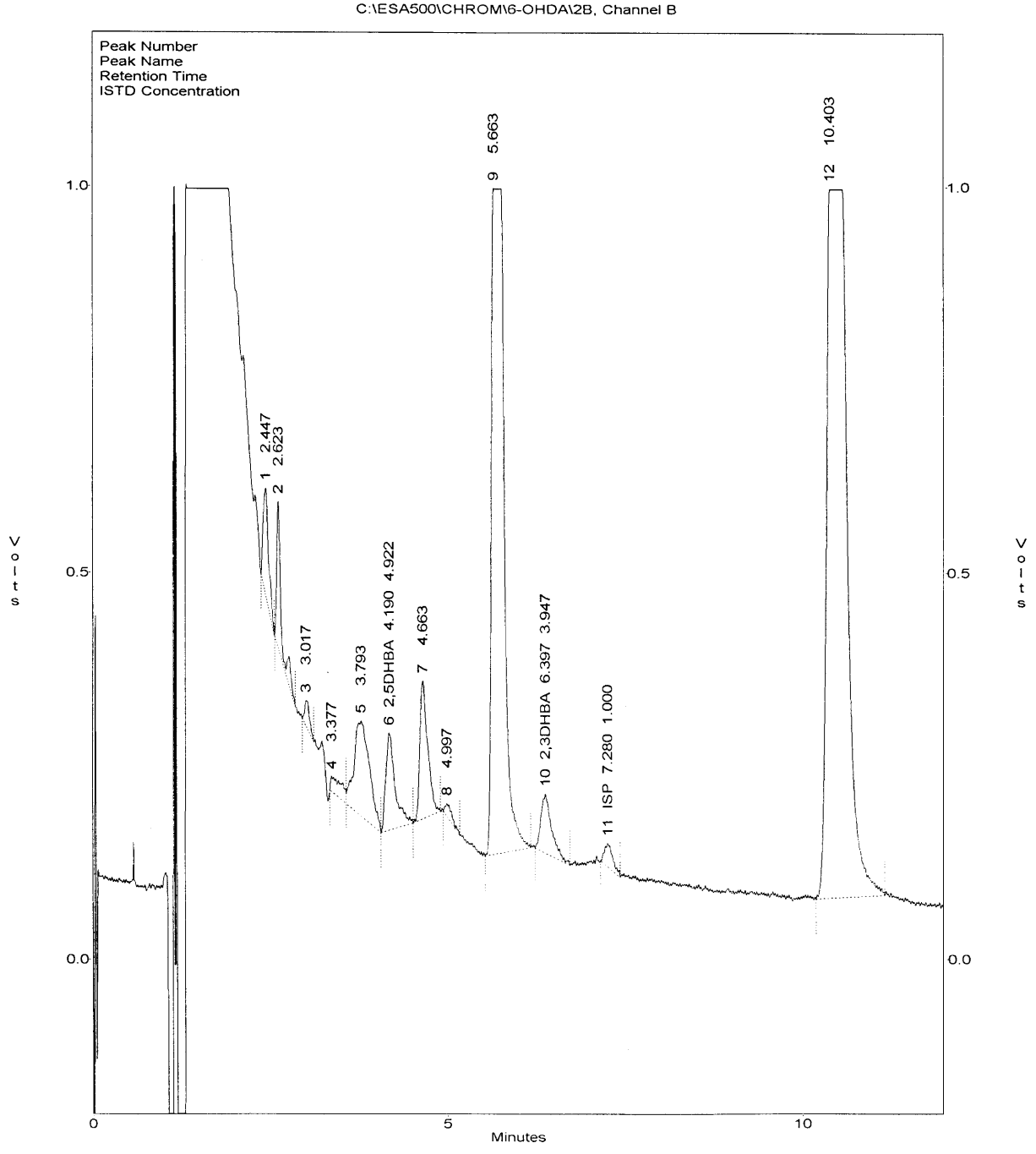

Fig. 1. HPLC chromatogram from analysis of monoamines and their metabolites; and the salicylate spin trap products, 2,3- and 2,5-dihydroxybenzoic acid (2,3- and 2,5-DHBA) in homogenates of control neostriatum

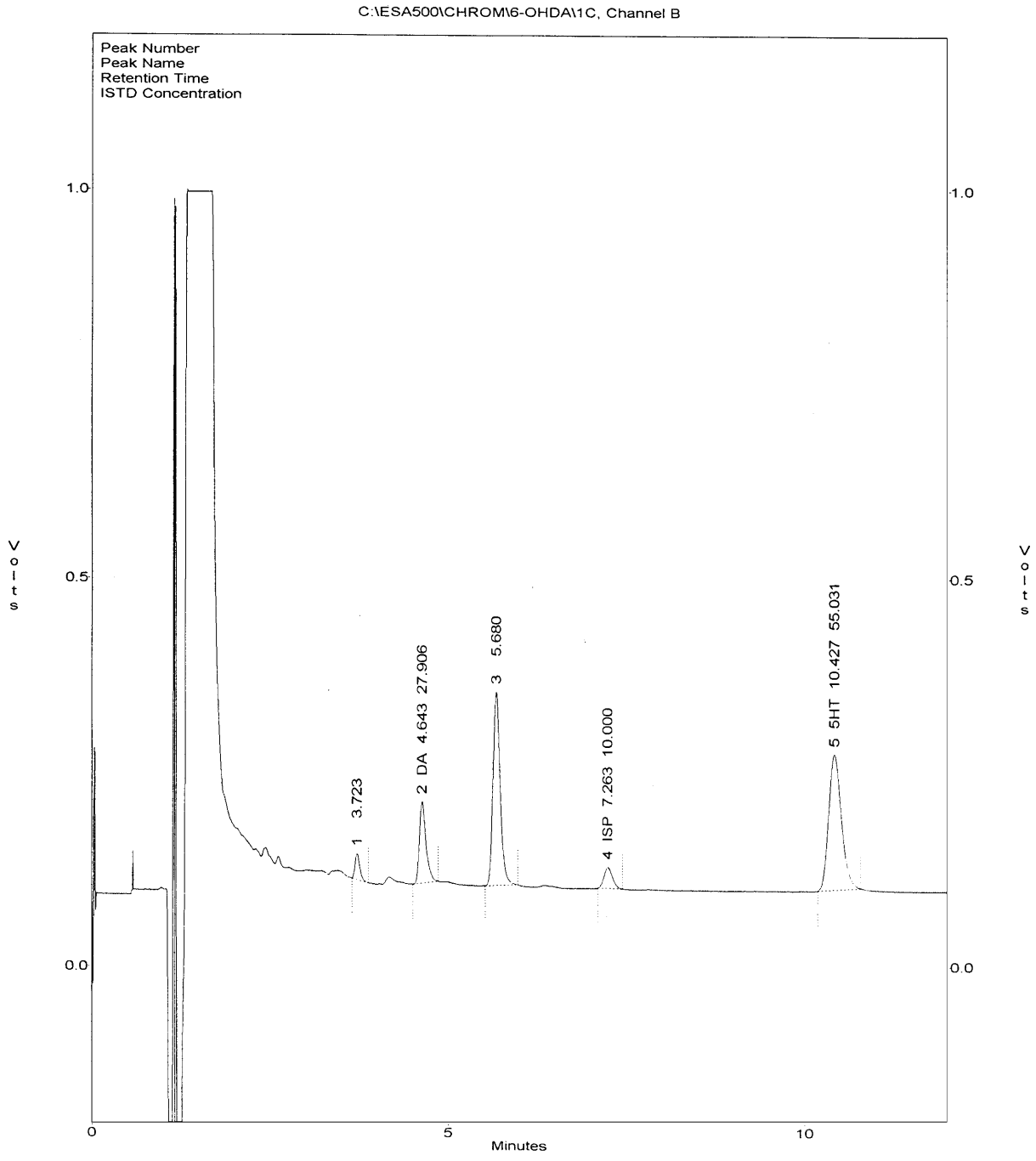

Fig. 2. HPLC chromatogram from analysis of monoamines and their metabolites; and the salicylate spin trap products, 2,3- and 2,5-DHBA in homogenates of DA-denervated neostriatum

The high sensitivity of the assay is discernable from low levels of 2,3-DHBA and 2,5-DHBA (pmol/g) that were easily detectable; and from the reliable analysis of DA, even when the neostriatum was DA depleted by 99.3% after 6-OHDA treatment.

Chemicals and drugs

Carbidopa, L-DOPA, 6-OHDOPA and HPLC standards (NE, DA, DOPAC, HVA, 2,3-DHBA, 2,5-DHBA, 5-HT, 5-HIAA) were obtained from RBI (Natick, MA); ketamine, from Fort Dodge Laboratories (Fort Dodge, IA); and xylazine, from Lloyd Laboratories (Shenandoah, IA); desipramine and salicylic acid from Sigma Chemical Co. (St. Louis, MO). The HPLC components of the mobile phase, were purchased from the following sources: acetonitrile from EM Science (Gibbstown, NJ), triethylamine from Aldrich Chemical Co. (Milwaukee, WI); mono- and di-sodium phosphates from JT Baker (Phillipsburg, NJ). All chemicals not specified were ACS grade or better, and most were obtained from Sigma Chemical Co.

Data analysis

A one-way analysis of variance (ANOVA), followed by the post-ANOVA Neuman-Keuls test was used to analyze mean ± SEM for groups of rats with DA-denervated and fully DA-innervated neostriatum.

Results

Determination of 2,3-DHBA and 2,5-DHBA

Under conditions used for analysis of monoamines, the R_f values for 2,3-DHBA and 2,5-DHBA were such that they appeared on the HPLC chromatogram as distinct peaks in the absence of interfering peaks. Typical spectra are shown for the analysis (Figs. 1 and 2). Also, with the flow-through microbore electrode, sensitivity for 2,3- and 2,5-DHBA was <1 pmol/gram of neostriatum.

Effect of neonatal 6-OHDA treatment on neostriatal content of DA and 5-HT

In rats treated at three days after birth with 6-OHDA (67 μg in each lateral ventricle; desipramine pretreatment, 20 mg/kg i.p., 1 hr), the DA content of the neostriatum was reduced by 99.3% at 10 weeks ($F_{5,30} = 5{,}175$, $P < .0001$, ANOVA; $P < .001$ Newman Keuls test) (Fig. 3, 1st and 4th bars). The principle DA metabolite, DOPAC was reduced to a similar extent, by 97.4% ($F_{5,30} = 210.4$, $P < .0001$; $P < .001$ Newman Keuls test) (Fig. 4, 1st and 4th bars).

As is well-known, ontogenetic destruction of DA innervation of neostriatum is accompanied by the eventual sprouting and hyperinnervation of neostriatum by 5-HT fibers originating from the medial raphe nucleus (Berger et al., 1985; Snyder et al., 1986). In our study, 5-HT content of neostriatum was elevated by 78% ($F_{5,30} = 28.0$, $P < .0001$; $P < .001$ Newman Keuls test) (Fig. 5, 1st and 4th bars), and the principle 5-HT metabolite 5-HIAA was elevated by 46.7% ($F_{5,30} = 23.46$, $P < .0001$; $P < .001$ Newman Keuls test) (Fig. 6, 1st and 4th bars).

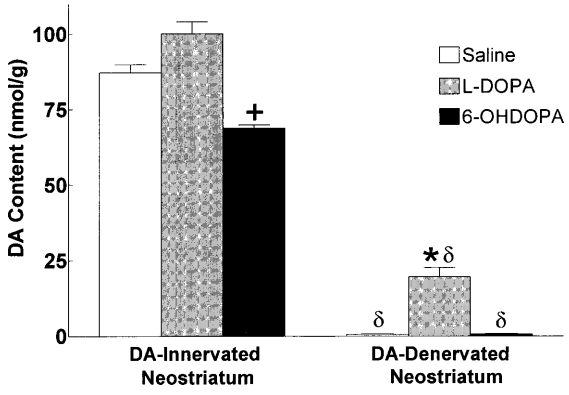

Fig. 3. Effects of L-DOPA and 6-OHDOPA treatments on DA content of DA-innervated and DA-denervated neostriatum of rats. + indicates difference from vehicle-treated, DA-innervated group, $P < .01$. * indicates difference from other DA-denervated groups, $P < .01$. δ indicates difference from respective DA-innervated group, $P < .001$

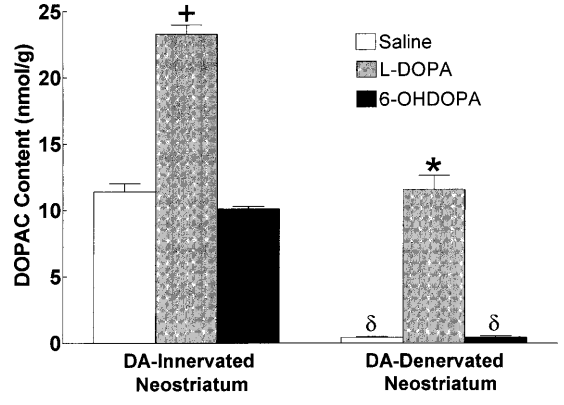

Fig. 4. Effects of L-DOPA and 6-OHDOPA treatments on DOPAC content of DA-innervated and DA-denervated neostriatum of rats. + indicates difference from vehicle-treated DA-innervated group, $P < .001$. * indicates difference from other DA-denervated groups, $P < .01$. δ indicates difference from respective DA-innervated group, $P < .001$

Effect of L-DOPA treatment on neostriatal contents of DA and 5-HT

In control rats with complete DA-innervation of the neostriatum, acute L-DOPA treatment (60 mg/kg i.p.; carbidopa pretreatment, 12.5 mg/kg i.p., 30 min), 45 minutes before termination, resulted in a slight increase (15%) in DA content (Fig. 3, 1st and 2nd bars), and a 104% increase in DOPAC content ($P < .001$) (Fig. 4, Newman Keuls test) (Fig. 4, 1st and 2nd bars) in the neostriatum of rats. In DA-denervated rats L-DOPA-induced changes in DA and DOPAC were much greater. In these rats L-DOPA treatment was followed by greater than a 30-fold elevation in DA ($P < .001$) (Fig. 3, 4th and 5th bars) and 28-fold elevation in DOPAC ($P < .001$) (Fig. 4, 4th and 5th bars) content of neostriatum. DOPAC content, in this instance, was identical to that

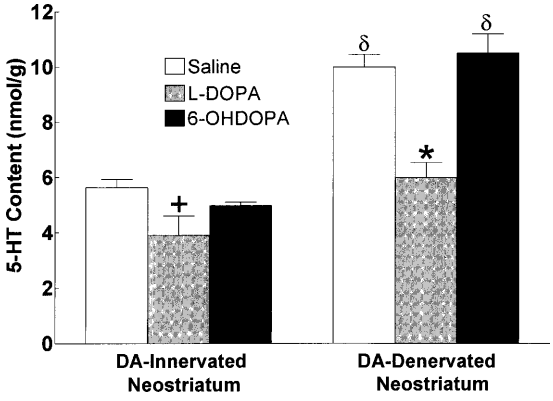

Fig. 5. Effects of L-DOPA and 6-OHDOPA treatments on 5-HT content of DA-innervated and DA-denervated neostriatum of rats. + indicates difference from vehicle-treated DA-innervated group, $P < .05$. * indicates difference from other DA-denervated groups, $P < .01$. δ indicates difference from respective DA-innervated group, $P < .001$

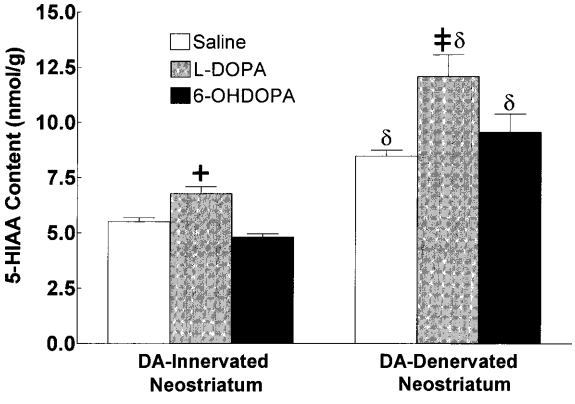

Fig. 6. Effects of L-DOPA and 6-OHDOPA treatments on 5-HIAA content of DA-innervated and DA-denervated neostriatum of rats. + indicates difference from vehicle-treated DA-innervated group, $P < .05$. ‡ indicates difference from other DA-denervated groups, $P < .01$. δ indicates difference from respective DA-innervated group, $P < .001$

in untreated controls (Fig. 4, 1st and 5th bars), while DA content was still reduced by a factor of 4.4 (Fig. 3, 1st and 5th bars).

L-DOPA-induced changes in neostriatal 5-HT content were opposite to that for DA. In fully DA-innervated control rats, L-DOPA treatment was associated with a 31% reduction in endogenous 5-HT content of neostriatum ($P < .01$) (Fig. 5, 1st and 2nd bars). Conversely, after L-DOPA treatment, 5-HIAA content was increased by 23% ($P < .05$) (Fig. 6, 1st and 2nd bars) relative to 5-HIAA content of neostriatum of untreated control rats.

In the neostriatum of 6-OHDA-lesioned rats (i.e., DA-denervated neostriatum), L-DOPA produced a 40% reduction in 5-HT content (Fig. 5, 4th and 5th bars) ($P < .01$) and 42% elevation in 5-HIAA content (Fig. 6, 4th and

5th bars) ($P < .05$) of neostriatum. These changes were many-fold greater than those produced in DA-innervated neostriatum after L-DOPA treatment.

Effect of 6-OHDOPA treatment on DA and 5-HT contents of neostriatum

In fully DA-innervated control rats, acute 6-OHDOPA treatment (60 mg/kg i.p.; carbidopa pretreatment, 12.5 mg/kg i.p., 30 min), produced a 22% reduction in DA content ($P < .01$) (Fig. 3, 1st and 3rd bars), with no change in DOPAC content (Fig. 4, 1st and 3rd bars) in the neostriatum of rats. In rats in which the neostriatum was largely DA-denervated, 6-OHDOPA had virtually no effect on DA content (Fig. 3, 4th and 6th bars) and DOPAC content (Fig. 4, 4th and 6th bars). This was not surprising, in considering that DA content of the neostriatum was reduced by 97% or more, so that neurochemical effects of this neurotoxin would be blunted.

6-OHDOPA had virtually no effect on 5-HT content in fully DA-innervated (Fig. 5, 1st and 3rd bars) and in DA-denervated neostriatum (Fig. 5, 4th and 6th bars). Likewise, 6-OHDOPA failed to alter neostriatal 5-HIAA content in DA-innervated neostriatum (Fig. 6, 1st and 3rd bars) and in DA-denervated neostriatum (Fig. 6, 4th and 6th bars).

Effect of DA-denervation on neostriatal contents of 2,3-DHBA and 2,5-DHBA

Basal levels of 2,3- and 2,5-DHBA, spin trap products from interaction of •OH with salicylate, were substantially altered in the neostriatum after DA-denervation. 2,3-DHBA content was increased more than 4-fold (Fig. 7, 1st and 4th bars) ($F_{5,30} = 8.18$, $P < .0001$; $P < .001$, Newman-Keuls test) and 2,5-DHBA content was increased 2.5-fold (Fig. 8, 1st and 4th bars) ($F_{5,30} = 12.79$,

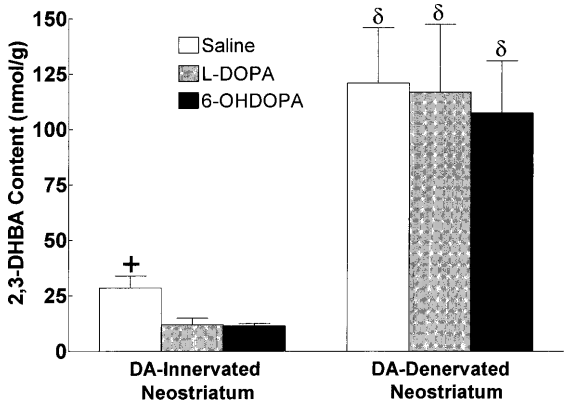

Fig. 7. Effects of L-DOPA and 6-OHDOPA treatments on 2,3-DHBA content of DA-innervated and DA-denervated neostriatum of rats. + indicates difference from other DA-innervated groups, $P < .05$. δ indicates difference from respective DA-innervated group, $P < .01$

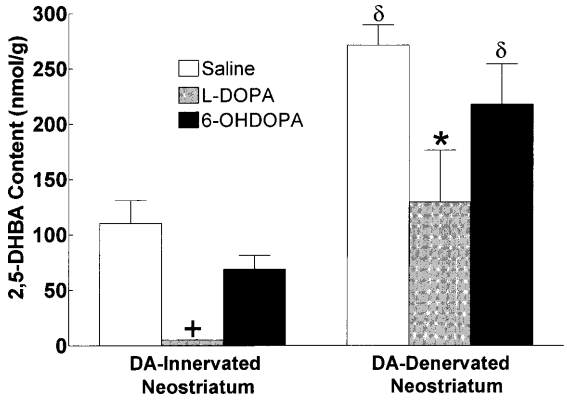

Fig. 8. Effects of L-DOPA and 6-OHDOPA treatments on 2,5-DHBA content of DA-innervated and DA-denervated neostriatum of rats. + indicates difference from other DA-innervated groups, $P < .01$. * indicates difference from vehicle-treated DA-denervated group, $P < .05$. δ indicates difference from respective DA-innervated group, $P < .001$

$P < .0001$; $P < .001$, Newman-Keuls test) in DA-denervated neostriatum of rats acutely treated with vehicle.

Effect of L-DOPA treatment on neostriatal contents of 2,3-DHBA and 2,5-DHBA

In rats with a fully DA-innervated neostriatum, acute L-DOPA treatment was accompanied by a 60% reduction in 2,3-DHBA content (Fig. 7, 1st and 2nd bars) ($P < .05$) and 95% reduction in 2,5-DHBA content of neostriatum (Fig. 8, 1st and 2nd bars) ($P < .001$). In rats with a largely DA-denervated neostriatum, the effects of L-DOPA were much lessened. In these rats L-DOPA treatment failed to alter 2,3-DHBA content (Fig. 7, 4th and 5th bars), but did reduce 2,5-DHBA content of neostriatum by 50% ($P < .05$).

Effect of 6-OHDOPA treatment on neostriatal contents of 2,3-DHBA and 2,5-DHBA

In control rats acute 6-OHDOPA treatment reduced neostriatal 2,3-DHBA content by 60% (Fig. 7, 1st and 3rd bars) ($P < .05$) and neostriatal 2,5-DHBA content by 40% (Fig. 8, 1st and 3rd bars) ($P > .05$). In 6-OHDA-lesioned rats, acute 6-OHDOPA treatment did not alter either 2,3-DHBA content (Fig. 7, 4th and 6th bars) or 2,5-DHBA content of neostriatum (Fig. 8, 4th and 6th bars).

Discussion

Effectiveness of DA-denervation

In this study ontogenetic 6-OHDA treatment was associated with a 99% reduction in endogenous DA content of neostriatum at 10 weeks.

Quantitative immunocytochemistry for the marker enzyme tyrosine hydroxylase and quantitative autoradiographic analysis of DA transporters have established that the magnitude of reduction in DA content is correlated with the extent of DA fiber denervation of neostriatum (see Breese and Breese, 1998; Kostrzewa et al., 1998).

In 6-OHDA-treated rats in this study, 5-HT content was elevated by 78%. This alteration is also in accord with previous findings associated with ontogenetic 6-OHDA treatment, namely hyperinnervation of neostriatum by 5-HT fibers (Berger et al., 1985; Snyder et al., 1986).

Methodologic considerations relating to assessment of •OH

The indirect, salicylate trap-HPLC method for assessing •OH, is reliable and extremely sensitive. Of the products from the salicylate trap, 2,3-DHBA and 2,5-DHBA, the 2,3-DHBA is the more reliable indicator of non-enzymatically formed product (Carney and Floyd, 1991; Halliwell et al., 1991; Ingelman-Sundberg et al., 1991). In the absence of a change in 2,3-DHBA, 2,5-DHBA has been used as an index of cytochrome P450 (CYT 450) metabolism (Dajas-Bailador et al., 1998).

Associations between DA innervation and ROS, including •OH

Free radicals have been implicated not only in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, but in psychiatric conditions like schizophrenia, and in movement disorders like tardive dyskinesia (see Metodiewa and Kośka, 1999; Smythies, 1999a, 1999b). Catechols uniquely have the ability to both generate and scavenge free radicals, and to chelate iron, which tends to promote ROS formation. A measure of radical scavenging ability, suppression of peroxidation of linoleic acid, indicates the DA is equipotent with α-tocopherol and more potent than noradrenaline (Yen et al., 1997). Similarly, the DA-mimetic apomorphine, a DA D_2 agonist, suppresses iron-induced lipid peroxidation in rat brain mitochondria *in vitro* (IC_{50}, 0.3 μM) and inhibits MAO-A and MAO-B (IC_{50}, 93 μM and 241 μM, respectively). This antioxidant ability confers on apomorphine, the ability to protect rat pheochromocytoma (PC12) cells from 6-OHDA (150 μM) and H_2O_2 (0.6 mM); and to protect mice in vivo from destruction of nigrostriatal dopaminergic fibers by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Grunblatt et al., 1999). Part of the protective effect may be related to both inhibition of DA turnover and D_2 stimulation with induction of new synthesis of radical-scavenging proteins (Sawada et al., 1998).

A supplementary neuroprotective process relates to DA-induction of antioxidant enzymes, possibly catalase, in the glutamate synapse, thereby altering the balance between neurodestructive pro-oxidant and neuroprotective antioxidant processes which ultimately govern synaptic plasticity with associated learning and memory (Smythies, 1999b).

Conversely, DA mediates generation of extraneuronal •OH and other ROS (Obata, 1999). Similarly, DA can be oxidized to a reactive quinone that covalently reacts with cellular macromolecules, particularly in the presence of manganese (Mn) and iron (Fe) (Stokes et al., 1999; Velez-Pardo et al., 1998). Accordingly, it is evident that catechols, DA in particular, are able to promote and protect from ROS. In cultures of cortical neurons (Alagarsamy et al., 1997), striatum (McLaughlin et al., 1998) or in neuroblastoma SH-SY5Y cells (Lai and Yu, 1997) or the N18-RE-105 neuroblastoma-retina hybridoma cell line (Duffy and Murphy, 1998) or in cultured pheochromocytoma (PC12) cells (Velez-Pardo et al., 1998), DA dose-dependently produces neuronal cell death, particularly if there is additional inhibition of mitochondrial function as after methyl malonate (McLaughlin et al., 1998).

Biologic and clinical significance of alterations in •OH reported in current study

Because DA-denervation is associated with enhanced •OH production after N-methyl-D-aspartate (NMDA) excitotoxic insult (Lancelot et al., 1995), and because of the pro-oxidant (above discussion) and neurotoxic properties of DA per se (Hastings et al., 1996), we expected that DA-denervation might be associated with a reduction in spontaneous •OH formation. Contrary to this hypothesis, the findings clearly indicate that DA-denervation of neostriatum, the major extrapyramidal motor control center, is associated with greater spontaneous •OH formation. The implication for untreated Parkinsonians is that there is greater susceptibility of the neostriatal neuropil to increased damage by spontaneously formed •OH as the disease progresses and as the neuropil becomes more DA-denervated.

Biologic and clinical significance of L-DOPA effects in current study

Our findings clearly show that L-DOPA, the major and most effect antiparkinsonian agent, actually suppresses •OH formation in neostriatum. This is an important finding, particularly in light of the suggestion that L-DOPA accelerates progression of Parkinson's disease. However, a clear distinction must be made between L-DOPA effects in a DA-innervated target, neostriatum, vs. perikarya in which DA is produced. It is conceivable that L-DOPA can suppress •OH formation by non-dopaminergic neurons (i.e., glutamatergic), while exacerbating •OH formation in neurons in which DA is made, as in pars compacta cells of substantia nigra. The current study does not address this possibility.

In an earlier study it was shown that L-DOPA treatment (25 mg/kg; benserazide, 6.25 mg/kg pretreatment) did not alter •OH level (i.e., 2,3- and 2,5-DHBA content) in the hippocampal microdialysate of Dark Agouti rats (Colado et al., 1999). However, one must be cautious to differentiate between extraneuronal and intraneuronal indices of free radical production. Increased methamphetamine-induced neurotoxicity in heterozygous DA transporter

knockout mice was accompanied by reduced extraneuronal free radical content, but probably greater intraneuronal levels (Fumagalli et al., 1999). Also, L-DOPA (100 mg/kg) did not alter levels of malondialdehyde, a marker of oxidative stress, in ventral midbrain at 8 hr (Loeffler et al., 1998). In contrast to these findings, •OH levels were elevated via iron-mediated Fenton chemistry after DOPA and inhibited by *O*-methyldopa (Miller et al., 1996; Nappi and Vass, 1998). And L-DOPA is known to be neurotoxic to cultured dopamine neurons (Mena et al., 1997a, 1997b) and neuroblastoma SH-SY5Y cells (Lai and Yu, 1997). Carbidopa pretreatment appears to suppress L-DOPA (0.1 mM, 1 μl/min microdialysate)-induced •OH generation by striatum (Obata and Yamanaka, 1996), indicating that formation of DA from L-DOPA may be a critical event. Finally, one caveat is that ROS per se may not be the major element involved in DA neurotoxicity, because thiols that effectively prevent cellular depletion of GSH are cytoprotective to PC12 cells, while the antioxidant vitamins C and E are not cytoprotective (Offen et al., 1996). In addition to forming •OH and other ROS, both L-DOPA and DA react with ROS, tending to neutralize these species. Accordingly, there is a complex mix of pro- and anti-oxidant properties of L-DOPA and DA, with the local environ and metal ions affecting their effects (Spencer et al., 1996).

Significance of 6-OHDOPA alterations, relative to L-DOPA actions

In the current study, 6-OHDOPA suppressed •OH formation. This is contrary to what might be expected, as 6-OHDOPA is a known generator of ROS. The implication is that it is the mere bathing of the neostriatal neuropil with an abundant •OH scavenger that results in reduction in •OH. Both L-DOPA and 6-OHDOPA would thus act similarly. Any •OH that formed in the neostriatal neuropil would likely interact with these chemical scavengers in rats that had been treated with either substance. Also, even though 6-OHDOPA might generate ROS like •OH, the 6-OHDOPA would similarly scavenge so-formed ROS and thereby reduce detectable levels of •OH. The implication for human Parkinsonians is 1) there would be more •OH formed in DA-denervated basal ganglia, but that 2) L-DOPA treatment would afford protection of the neuropil. We cannot make a statement about possible effects of L-DOPA on •OH formation in pars compacta substantia nigra cells which synthesize DA.

One minor point, related to the rationale for using 6-OHDOPA as an L-DOPA analogue in this study, is that 6-OHDOPA has little effect, and short-lived, on DA neurons (Jacobowitz and Kostrzewa, 1971; Kostrzewa, 1978). Therefore, 6-OHDOPA was useful because of its known action as a generator of ROS, but suitable because of its low neurotoxic potential for DA nerves that are under study.

Conclusions

Findings from this study demonstrate that a DA-denervated neuropil in brain is associated with a higher basal level of •OH. The implication is that DA-

denervated neuropils lack antioxidants that are normally within DA nerve fibers; or that DA per se acts largely as an antioxidant (neuroprotectant); or that the normal inhibitory control exerted by DA nerves on pro-toxic neurotransmitters, like GLU and other EAAs, is absent. Accordingly, DA should be viewed as a neuroprotectant. Also, because L-DOPA is associated with reduced levels of tissue $^{\bullet}OH$, L-DOPA can also be viewed as a neuroprotectant, a finding with reassuring value to treated Parkinsonians.

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