Further Insights into the Molecular Mechanisms of Action of the Serotonergic Neurotoxin 5,7-Dihydroxytryptamine

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Autoxidation and various enzyme-mediated oxidations of the serotonergic neurotoxin 5,7-dihydroxytryptamine (1) give 5-hydroxytryptamine-4,7-dione (2) and 6,6′-bi(5-hydroxytryptamine-4,7-dione) (3) as the major products. When administered into the ventricular system of mice 2 and 3 are general toxins. The LD_{50} values for 2 (29.6 ± 0.04 μg) and 3 (25.4 ± 0.30 μg) are lower than that for 1 (51.8 ± 0.28 μg). In the presence of cellular reductants (glutathione, cysteine, ascorbate) and molecular oxygen, or when incubated with rat brain homogenate, 2 and 3 redox cycle and form superoxide radical anion, $O_2^{\bullet-}$, as a byproduct. The lethal effects of 2 and 3 when introduced into the brain may in part be due to such redox cycling reactions which deplete oxygen levels and, as a result of Haber-Weiss chemistry deriving from $O_2^{\bullet-}$, form the cytotoxic hydroxyl radical ($HO^\bullet$). Intraventricular administration of 2 and 3 to mice causes only relatively minor and transient (ca. 1 h to 1 day) changes in whole brain levels of dopamine, 5-hydroxytryptamine (from both 2 and 3), acetylcholine, and choline (from 2 only). These changes differ from the profound and long-lasting serotonergic deficit evoked by 1. On the basis of these results a hypothesis has been formulated which proposes that the selective neurotoxicity of 1 derives from its rapid uptake into serotonergic neurons where it is oxidatively converted to 2 and 3. Redox cycling reactions of 2 and 3 then result in the depletion of intraneuronal oxygen and concomitant formation of $O_2^{\bullet-}$. Dismutation of $O_2^{\bullet-}$ gives H_2O_2 which, as a result of transition metal ion-catalyzed Haber-Weiss chemistry, yields $HO^\bullet$. Thus, neuronal damage and death might result from the combined effects of hypoxia and $HO^\bullet$ formation.

Central administration of 5,7-dihydroxytryptamine (1) to laboratory animals results in the selective destruction of serotonergic neurons.[1-3] Histopathological evidence indicates that 1 rapidly accumulates inside serotonergic neurons,[4] and the drug also strongly inhibits the uptake of the indolic neurotransmitter 5-hydroxytryptamine (5-HT; serotonin).[3,5] Taken together such evidence indicates that the selectivity of 1 derives from its high affinity uptake into serotonergic neurons. However, little is known about the fate of 1 once it is inside these neurons or of the molecular mechanisms responsible for its neurodegenerative effects. It is known that pretreatment of rats with monoamine oxidase inhibitors (pargyline, iproniazid, and pheniprazine) has no effect on the ability of 1 to deplete brain levels of 5-HT,[6] indicating that intraneuronal oxidative deamination of the drug is not a prerequisite for neurotoxicity. Following intracerebroventricular (icv) administration of [^{14}C]-1 to the rat, protein-bound radioactivity has been detected in various brain regions.[7] This observation and the known ease of autoxidation of 1[8-10] has led to the rather widely-held assumption that autoxidation of 1 generates an electrophilic quinone imine intermediate which alkylates nucleophilic thiol residues of intraneuronal proteins leading to compromise of function and, ultimately, cell death. However, other investigators have suggested that byproducts of the autoxidation of 1 are cytotoxic reduced oxygen species.[8,11,12] Klemm et al.[13] have proposed that the putative quinone imine intermediate interferes with the electron-transport chain resulting in cell death. However, kinetic studies of the autoxidation of 1 do not support the transformation of 1 into a quinone imine.[13,14] Work from this laboratory has demonstrated that the major products of autoxidation of 1 are 5-hydroxytryptamine-4,7-dione (2) and 6,6′-bi(5-hydroxytryptamine-4,7-dione) (3).[15] Mechanistic investigations[14,16] indicate that in the absence of any catalytic influences the autoxidation reaction proceeds by initial attack of O_2 on the C(4)-centered carbanion 1a to give a free-radical superoxide complex 4 which subsequently decomposes to 2 as shown in Scheme I. Dimer 3 is formed as a result of the initial reaction of *p*-quinone 2 with the C(6)-centered carbanion 1b (Scheme I).[16] Under such uncatalyzed conditions, autoxidations of 1 to 2 and 3 are very slow and cytotoxic reduced oxygen species are probably not formed as byproducts. Trace concentrations of transition metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}, and Mn^{2+}) exert a catalytic effect on the autoxidation of 1 by reaction pathways in which hydroperoxide 6 is a key intermediate in at least two catalytic cycles.[16] These reactions lead ultimately to 2 and 3 as the major products and superoxide radical anion $O_2^{\bullet-}$, H_2O_2, and hydroxyl radical $HO^\bullet$ are formed as byproducts. In the presence of molecular O_2, several enzyme systems (peroxidase/H_2O_2, ceruloplasmin, ferricytochrome c, rat brain mitochondria) oxidize 1 predominantly to 2 and 3.

These recent investigations[14-16] provide insights into the fundamental oxidation chemistry and biochemistry of 1 but do not directly address possible mechanisms by which the drug evokes its neurodegenerative effects in vivo. However, a role for a quinone imine intermediate in the neurodegenerative process seems unlikely. Furthermore, neither the uncatalyzed autoxidation nor the enzyme-mediated oxidations of 1 appear to generate significant amounts of cytotoxic reduced oxygen species as byproducts. The transition metal-catalyzed autoxidation of the indolamine does yield $O_2^{\bullet-}$, H_2O_2, and $HO^\bullet$. However, the rate of these reactions in the presence of micromolar concentrations of catalyst are still quite slow

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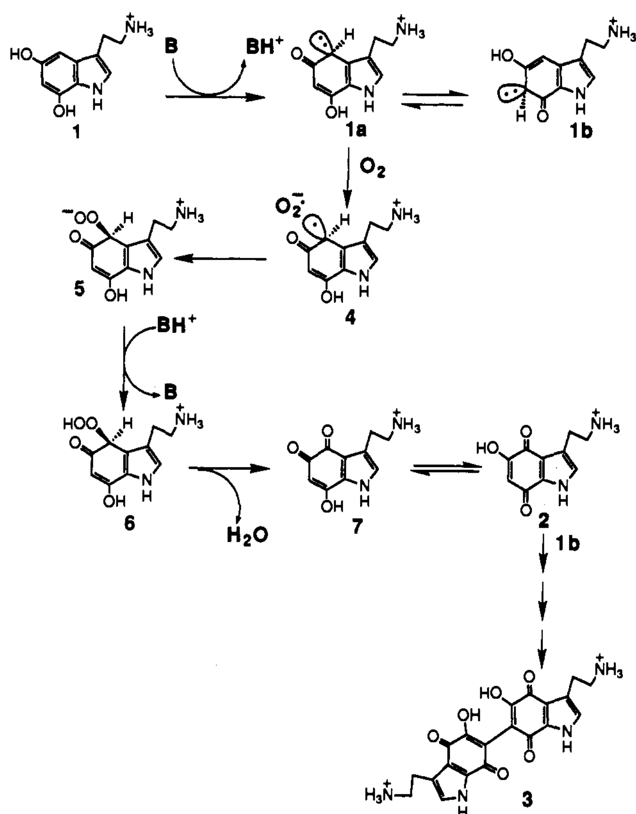
Scheme I



and hence are unlikely to produce concentrations of these toxic byproducts sufficient to overwhelm normal cellular defense systems. In view of this information it seems reasonable to suggest that the major products of the autoxidation or enzyme-mediated oxidations of 1 might be responsible for expressing the neurotoxic properties of the drug. In this article it is demonstrated that 2 and 3 are general toxins when centrally administered to the mouse and are lethal at doses significantly lower than for 1. Both 2 and 3 effectively redox cycle in the presence of typical cellular reductants at physiological pH and when incubated with rat brain homogenate. The rapid consumption of O_2 and formation of cytotoxic reduced oxygen species as a result of such redox cycling might explain in part the general toxicity of 2 and 3. The effects of centrally administered 2 and 3 on the levels of several neurotransmitter/metabolite systems in whole mouse brain are described. The results from these studies have been employed to advance a hypothesis for the chemical mechanisms by which 1 expresses its neurodegenerative effects in the central nervous system.

Results

Autoxidation of 1 in the Presence of Glutathione (GSH). In the absence of endogenous cellular nucleophiles and reductants, the autoxidation of 1 at physiological pH yields 2 and 3 as the major products in approximately equimolar yield.[15] However, intraneuronal oxidation of 1 would necessarily occur in the presence of several nucleophiles, the most abundant being GSH.[17,18] A series of HPLC chromatograms recorded during the incubation of 1 (1.5 mM) and GSH (7.5 mM) in pH 7.4 phosphate buffer (μ = 0.1, 37 °C) are shown in Figure 1. Under these conditions, three major products were formed 2, 3, and 6-*S*-glutathionyl-5-hydroxytryptamine-4,7-dione (8). Compound 8 was isolated and its structure elucidated using spectroscopic methods (FAB-MS, ^{1}H and ^{13}C NMR; see the Experimental Section). In the absence of GSH the time required for complete autoxidation of 1 (1.5 mM) to 2 and 3 was 6.5 ± 0.5 h. In the presence of 4.5 and 7.5 mM GSH, under otherwise identical conditions, the time required to completely oxidize 1 was 14 ± 0.5 and 17.5 ± 0.5 h, respectively. With increasing molar excesses of GSH the yields of 2 and 3 systematically decrease, and correspondingly, the yield of 8 increases.

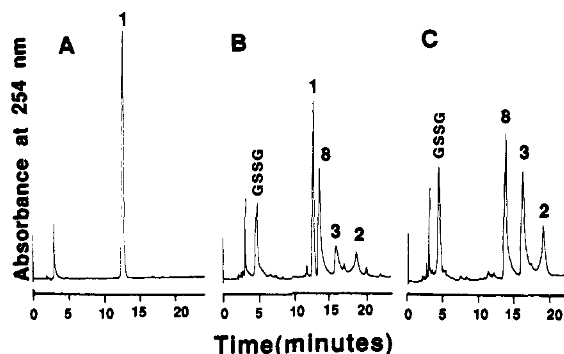


Figure 1. HPLC chromatograms recorded during the autoxidation of 5,7-dihydroxytryptamine (1.5 mM) in pH 7.4 phosphate buffer (μ = 0.1; 37 °C) in the presence of glutathione (7.5 mM): (A) immediately after preparing the reaction solution; (B) after incubation for 6 h, and (C) after incubation for 18 h. Chromatography employed HPLC Method III; injection volume was 200 μL.

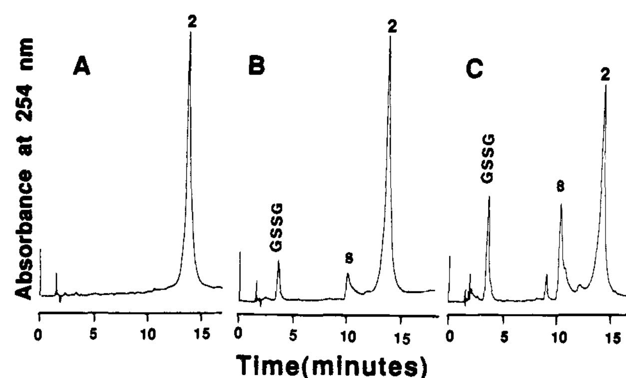


Figure 2. HPLC chromatograms recorded when 5-hydroxytryptamine-4,7-dione (2; 0.5 mM) was incubated with glutathione (2.5 mM) in pH 7.4 phosphate buffer (μ = 0.1; 37 °C): (A) immediately after preparing the reaction solution, (B) after 80-min incubation, and (C) after 400-min incubation. Chromatography employed HPLC Method III; injection volume was 200 μL.

Incubation of 2 (0.5 mM, pH 7.4 phosphate buffer, μ = 0.1, 37 °C) with GSH (2.5 mM) resulted in formation of 8 (Figure 2). After 6.5 h approximately 30% of 2 was converted into 8. Incubation of 3 (0.5 mM) with GSH (2.5 mM) under the same experimental conditions did not result in the formation of 8 or any new products. A byproduct of the reactions between 1 and 2 with GSH was the oxidized form of the latter tripeptide, i.e., GSSG (Figures 1 and 2).

Redox Properties of 2, 3, and 8. Information bearing on the redox properties of 2, 3, and 8 at pH 7.4 was obtained from cyclic voltammetry at a pyrolytic graphite electrode (PGE; Figure 3). At a sweep rate (v) of 200 mV/s 2, 3, and 8 (0.5 mM in pH 7.4 phosphate buffer) exhibited voltammetric reduction peaks at peak potentials (E_p) of −0.61, −0.62, and −0.53 V vs SCE, respectively. These peaks are due to the 2e − 2H$^+$ electrochemical reduction of 2, or the residues of 2 in 3 and 8, to the corresponding 4,5,7-

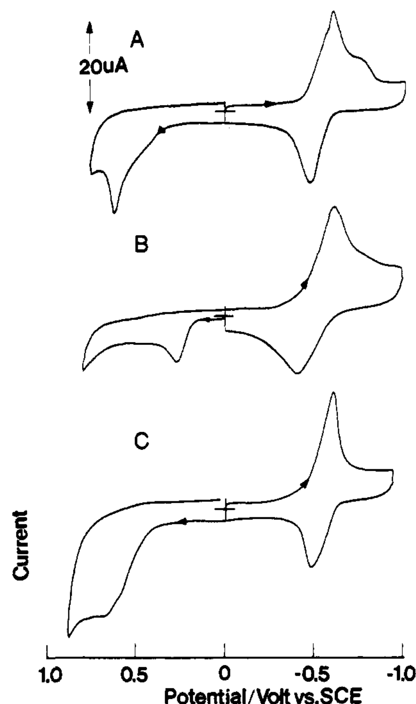
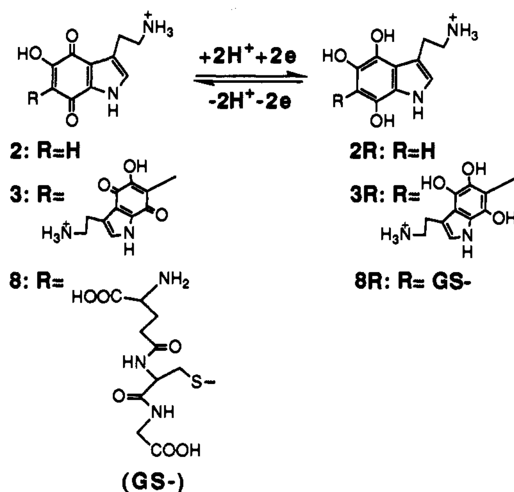


Figure 3. Cyclic voltammograms at the PGE of 0.5 mM solutions of (A) 5-hydroxytryptamine-4,7-dione (2), (B) 6,6'-bi(5-hydroxytryptamine-4,7-dione) (3), and (C) 6-*S*-glutathionyl-5-hydroxytryptamine-4,7-dione (8) in pH 7.4 phosphate buffer (μ = 0.1; 25 °C). Sweep rate was 200 mV/s.

Scheme II



trihydroxytryptamine moieties (Scheme II). After scan reversal, the cyclic voltammograms of all three compounds show quasi-reversible oxidation peaks (Figure 3) corresponding to the electrochemical oxidations of the 4,5,7-trihydroxytryptamine residues, formed at the electrode surface on the initial sweep, back to residues of 2 (Scheme II). The $E^{\circ\prime}$ values for the **2**/**2R**, **3**/**3R**, and **8**/**8R** couples at pH 7.4 were determined from the cyclic voltammograms shown in Figure 3 to be −0.54, −0.51, and −0.54 V vs SCE, respectively, using the expression:

$$E^{\circ\prime} = [(E_p)_{red} + (E_p)_{ox}]/2$$

In this expression $(E_p)_{red}$ and $(E_p)_{ox}$ refer to the E_p values for the voltammetric reduction and oxidation peaks, respectively.

Although **2**, **3**, and **8** exhibited similar cyclic voltammetric behaviors in the vicinity of −0.6 V, each compound showed quite characteristic irreversible oxidation peaks

Table I. Initial Oxygen Consumption Rates Measured upon Incubation of 5-Hydroxytryptamine-4,7-dione (2) or 6,6'-Bi(5-hydroxytryptamine-4,7-dione) (3) with Physiological Reductants at pH 7.4[a]

reactant concn, mM	reducing agent (concn, mM)	initial rate of oxygen[b] consumption, nmol of O_2/min
	5-Hydroxytryptamine-4,7-dione (2)	
0.1	GSH (1.0)[c]	1.87 ± 0.42
0.1	(2.0)	2.02 ± 0.44
0.2	(1.0)	2.47 ± 0.34
0.1	cysteine (1.0)[d]	1.79 ± 0.62
0.1	(2.0)	2.20 ± 0.32
0.2	(1.0)	2.44 ± 0.42
0.1	ascorbic acid (1.0)[e]	3.43 ± 0.40
0.1	(2.0)	4.39 ± 0.37
0.2	(1.0)	7.81 ± 0.23
0.2	(2.0)	10.45 ± 0.48
0.2	(4.0)	18.56 ± 0.74
0.2	(6.0)	21.43 ± 0.51
0.1	rat brain homogenate[f]	23.9 ± 6.5
0.2		43.8 ± 6.3
	6,6'-Bi(5-hydroxytryptamine-4,7-dione) (3)	
0.1	GSH (1.0)[c]	1.48 ± 0.21
0.1	(2.0)	1.48 ± 0.12
0.2	(1.0)	1.52 ± 0.38
0.2	(2.0)	1.56 ± 0.20
0.1	cysteine (1.0)[d]	2.22 ± 0.43
0.1	(2.0)	2.64 ± 0.30
0.2	(1.0)	2.71 ± 0.24
0.1	ascorbic acid (1.0)[e]	6.20 ± 0.15
0.1	(2.0)	8.48 ± 0.74
0.2	(1.0)	8.81 ± 0.09
0.2	(2.0)	11.69 ± 0.14
0.2	(4.0)	28.01 ± 2.41
0.2	(6.0)	33.46 ± 1.19
0.1	rat brain homogenate[f]	41.6 ± 8.8

[a] Except for rat brain homogenate preparations all solutions were prepared in pH 7.4 phosphate buffer (μ = 0.1) at 37 °C. [b] Measured with a Clark-type oxygen electrode assembly. Reported values, corrected for oxygen uptake by reductants employed in the absence of 2 or 3, are the mean of at least three replicate measurements ± standard deviation. [c] Initial oxygen consumption rates for GSH in the absence of 2 or 3 were as follows: 1.0 mM, 0.78 ± 0.14; 2.0 mM, 1.04 ± 0.10 nmol of O_2/min. [d] Initial oxygen consumption rates for cysteine in the absence of 2 or 3 were as follows: 1.0 mM, 0.74 ± 0.04; 2.0 mM, 0.98 ± 0.09 nmol of O_2/min. [e] Initial oxygen consumption rates for ascorbic acid in the absence of 2 or 3 were as follows: 1.0 mM, 2.11 ± 0.18; 2.0 mM, 3.40 ± 0.41; 4.0 mM, 8.90 ± 0.62; 6.0 mM, 11.23 ± 0.92 nmol of O_2/min. [f] Rat brain (ca. 2 g) homogenized in 17 mL of a medium consisting of 140 mM NaCl, 5 mM KCl, 10 mM Tris-Hepes, 5 mM $NaHCO_3$, 1.3 mM $NaHPO_4$, pH 7.4. The initial oxygen consumption rate for this homogenate in the absence of 2 or 3 was 126 ± 3.5 nmol of O_2/min.

at more positive potentials. E_p values for these oxidation peaks of **2**, **3**, and **8** at pH 7.4 (phosphate buffer, v = 200 mV/s, 22 °C) were 0.62, 0.27, and 0.60 V, respectively.

The $E^{\circ\prime}$ values for the **2**/**2R** and **3**/**3R** couples (ca. −0.54 V vs SCE or ca. −0.30 V vs NHE) at pH 7.4 suggested that both **2** and **3** should be reducible by physiological reducing agents to **2R** and **3R**, respectively, which in turn would be expected to be oxidized by molecular oxygen. Such redox cycling reactions were investigated by incubating **2** or **3** with representative cellular reductants (GSH, cysteine, and ascorbate) in pH 7.4 phosphate buffer at 37 °C. Redox cycling activity was monitored by measuring initial oxygen consumption rates with a Clark-type polarographic oxygen electrode assembly. The results (Table I) reveal that in the presence of GSH, cysteine, and particularly ascorbic acid, both **2** and **3** evoke appreciable levels of oxygen consumption. Similarly, **2** and **3** caused a very significant increase in the rate of oxygen consumption by a rat brain homogenate preparation (Table I). Indeed, in the latter preparation it was noted that the initially bright purple

Table II. Effects of Central Administration of 5-Hydroxytryptamine-4,7-dione (2) and 6,6′-Bi(5-hydroxytryptamine-4,7-dione) (3) on Whole Mouse Brain Neurotransmitter/Metabolite Levels[a]

compound	time[b]		n	NE[c]	DA	DOPAC	HVA	5-HT	5-HIAA	ACh	Ch
5-hydroxytrypt-amine-4,7-dione[d]	1 hr										
		controls	12	100 ± 16	100 ± 6	100 ± 27	100 ± 20	100 ± 16	100 ± 17	100 ± 4	100 ± 5
		expt'ls	12	80 ± 12	92 ± 4	54 ± 11	147 ± 37	89 ± 14	159 ± 32	100 ± 13	153 ± 24*
	1 day										
		controls	12	100 ± 5	100 ± 3	100 ± 12	100 ± 15	100 ± 3	100 ± 8	100 ± 6	100 ± 4
		expt'ls	11	84 ± 10	79 ± 14****	107 ± 21	92 ± 21	72 ± 5****	82 ± 9	65 ± 3****	57 ± 4****
	1 week										
		controls	15	100 ± 9	100 ± 12	100 ± 12	100 ± 14	100 ± 11	100 ± 10	100 ± 16	100 ± 10
		expt'ls	15	114 ± 9	114 ± 10	117 ± 11	112 ± 14	104 ± 9	106 ± 8	107 ± 20	106 ± 12
6,6′-bi(5-hyhydroxy-tryptamine-4,7-dione)[e]	1 hr										
		controls	12	100 ± 16	100 ± 5	100 ± 21	100 ± 17	100 ± 16	100 ± 17	100 ± 4	100 ± 5
		expt'ls	12	95 ± 15	97 ± 7	74 ± 10	102 ± 17	88 ± 12	118 ± 9	83 ± 10	126 ± 15
	1 day										
		controls	17	100 ± 10	100 ± 5	100 ± 13	100 ± 18	100 ± 10	100 ± 18	100 ± 7	100 ± 7
		expt'ls	17	82 ± 11	85 ± 2***	116 ± 20	85 ± 22	75 ± 6*	76 ± 12	78 ± 11	78 ± 11
	1 week										
		controls	17	100 ± 10	100 ± 6	100 ± 9	100 ± 27	100 ± 8	100 ± 27	100 ± 16	100 ± 10
		expt'ls	18	89 ± 13	108 ± 9	101 ± 12	152 ± 43	103 ± 9	152 ± 43	123 ± 25	115 ± 18

[a] Results are expressed as percent of controls ± sem. [b] Time between drug administration and sacrifice. [c] NE: norepinephrine. DA: dopamine. DOPAC: 3,4-dihydroxyphenylacetic acid. HVA: homovanillic acid. 5-HT: 5-hydroxytryptamine. 5-HIAA: 5-hydroxyindole-3-acetic acid. ACh: acetylcholine. Ch: choline. [d] Experimental animals were treated with 30 μg (free base) of 2 delivered in 5 μL of isotonic saline solution containing 1 mg/mL of ascorbic acid. Administered intracranically in the vicinity of the left-lateral ventrical. [e] Experimental animals were treated with 25 μg (free base) of 3 delivered as in footnote *d*. Asterisks are designated as follows: *, $P < 0.05$ compared to controls. **, $P < 0.01$ compared to controls. ***, $P < 0.005$ compared to controls. ****, $P < 0.001$ compared to controls.

color of the solutions due to 2 or 3 faded significantly as the oxygen level decreased during the course of the experiment. When air was reintroduced into the solution the initial bright purple color reappeared.

Biological Studies. The in vivo effects evoked by central administration of 2 and 3 were assessed using adult male mice (Harlan Sprague-Dawley Hsd:ICR albino strain, 25–30 g). Drugs were administered intracranially in the vicinity of the left lateral ventricle in 5 μL of vehicle (isotonic saline, 0.9% NaCl in deionized water, containing 1.0 mg/mL of ascorbic acid). LD_{50} values, defined as the weight of the injected compound (expressed as free base) which caused death of 50% of the treated animals within 1 h following drug administration, were determined using the statistical methods of Dixon.[19,20] Both 2 and 3 were extremely toxic having experimental LD_{50} values of 29.6 ± 0.04 μg (mean ± standard deviation on a log microgram scale) and 25.4 ± 0.30 μg, respectively. The LD_{50} for 1, measured for comparative purposes, was 51.8 ± 0.28 μg.

The behavioral responses evoked following central administration of 2 and 3 to mice were virtually identical. The behavior responses described below followed administration of 30 μg of 2 and 25 μg of 3. Between approximately 5 and 30–45 min after drug administration the following behavioral patterns were observed: (1) at ca. 5 min, animals walked in a circular path contralateral to the site of injection; (2) at ca. 10–15 min, animals exhibited noticeable extensions of forelimbs and hindlimbs, periodically ran about the cage very rapidly, and rolled over along the body–tail axis completing 7–8 revolutions within a few seconds in a typical episode; (3) at ca. 10–20 min, animals lay on their backs against the side of the cage and vigorously rubbed the sides of their faces against the bars resulting, frequently, in bleeding from the mouth; (4) after 45 min or longer, all surviving animals exhibited behavior which was indistinguishable from that of controls. Death, if it occurred, always took place in phase 2 noted above. Animals treated with 5 μL of vehicle alone exhibited none of the above behavioral responses. Indeed, following recovery from anesthesia the behaviors of controls were indistinguishable from those of untreated animals.

Whole Brain Neurotransmitter/Metabolite Analyses. Levels of the biogenic amines dopamine (DA), norepinephrine (NE) and 5-HT, acetylcholine (ACh), and appropriate metabolites in whole mouse brain were measured 1 h, 1 day, and 1 week following central administration of 2 (30 μg) and 3 (25 μg) delivered in 5 μL of vehicle. Concentrations of NE, DA, 5-HT and associated metabolites,[21] and of ACh and choline (Ch)[22] were measured using standard LCEC methods.[23] The results obtained (Table II) revealed that 1 h after administration of 2 the only significant difference between experimentals and controls was a rise in the level of Ch. After one day, reductions in the whole brain levels of DA, 5-HT, ACh, and Ch were noted. One week after treatment with 2, however, the measured transmitter/metabolite levels of experimentals became indistinguishable from controls. Central administration of 3 resulted in a decrease in the levels of both 5-HT and DA one day after treatment (Table II). However, 1 week after drug administration the levels of all transmitters and metabolites measured were indistinguishable from controls.

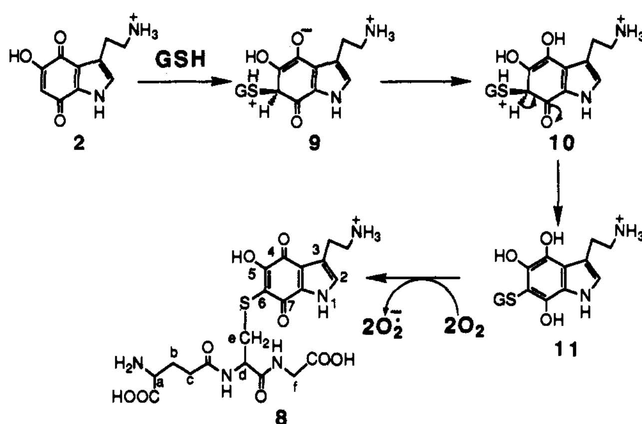
Discussion

In the absence of catalytic influences deriving from trace levels of transition metal ions, $O_2^{\bullet-}$, or oxidative enzymes, the autoxidation of 1 is very slow. For example, complete autoxidation of 1 (1.0 mM) at pH 7.4 and 37 °C requires 17 ± 0.5 h.[16] The uncatalyzed autoxidation of 1 forms equimolar 2 and 3 (Scheme I) but does not result in the formation of significant levels of cytotoxic reduced oxygen species. In the presence of molecular O_2 and enzyme systems such as peroxidase/H_2O_2, ceruloplasmin, ferricytochrome c, and rat brain mitochondria the oxidation of 1 to 2 and 3 is more rapid than the uncatalyzed oxidation. Again, however, $O_2^{\bullet-}$, H_2O_2, or $HO^\bullet$ are not formed as significant byproducts. Accordingly, reduced oxygen species resulting *directly* from the uncatalyzed autoxidation or enzyme-mediated oxidations of 1 to 2 and 3 cannot play a role in the neurotoxic effects evoked by the indolamine. Trace concentrations of Fe^{2+}, Fe^{3+}, Cu^{2+}, and Mn^{2+} catalyze the autoxidation of 1 and $O_2^{\bullet-}$, H_2O_2, and

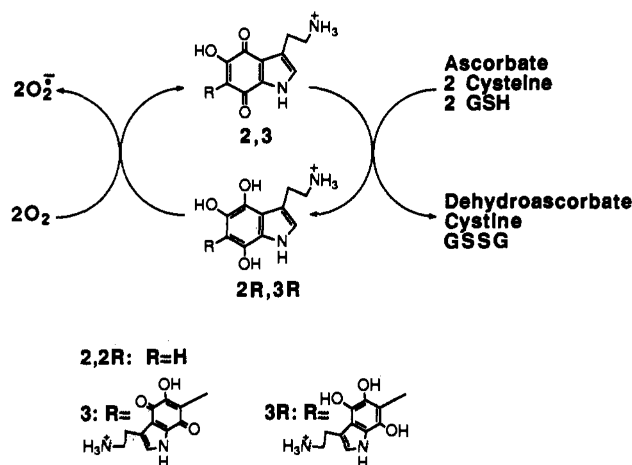
$HO^{\bullet}$ are formed as byproducts.[16] However, complete autoxidation of 1 (1 mM) at pH 7.4 and 37 °C in the presence of 50 μM Fe^{2+} or Fe^{3+} (i.e., relatively high catalyst concentrations) requires 4.5 ± 0.5 and 5.0 ± 0.5 h, respectively. It seems improbable that such rates of oxidation could generate concentrations of $O_2^{\bullet-}$ and H_2O_2 (and hence $HO^{\bullet}$) sufficient to overwhelm the protective capacities of intraneuronal antioxidants and enzymes such as glutathione peroxidase and superoxide dismutase and inflict cellular damage and death.[24]

Under aerobic conditions all chemical and enzyme-mediated oxidations of 1 which have been investigated give 2 and 3 as the major reaction products. Furthermore, both 2 and 3 are general toxins, having experimental LD_{50} values lower than 1 when centrally administered to mice. These observations might be interpreted to indicate that in order for 1 to express its neurotoxic effects it must be first oxidatively transformed to 2 and 3. And, furthermore, that it is the latter compounds which are the actual neurotoxic substances. However, central administration of 2 or 3 to mice evokes neurochemical effects which are quite different to those caused by 1. For example, intraventricular infusions of 1 to mice and rats result in long-lasting and profound depletion of brain 5-HT levels and, to a somewhat lesser extent, depletion of NE.[3] However, 1 causes no observable alteration in the brain levels of DA, ACh, or several other transmitter systems.[25] By contrast, 2 evokes a rapid (≤1 h) increase in the level of Ch and, one day after administration, decreases in whole brain levels of DA, 5-HT, and Ch (Table II). Compound 3 evokes a decrease in whole brain levels of DA and 5-HT one day after administration (Table II). However, one week after administration of 2 or 3 all the transmitter/metabolite levels return to control values. Thus, since neither 2 nor 3 evoke any long-term disruption of any of the transmitter systems studied they cannot themselves be neurotoxins. In particular, 2 and 3 cause no long-lasting effects on the levels of 5-HT, suggesting that they are unable to enter serotonergic nerve terminals. Accordingly, if oxidation of 1 to 2 and 3 is of relevance to the neurodegenerative properties of the indoleamine then the oxidation reactions must occur intraneuronally, i.e., after 1 has been taken up into serotonergic neurons. The results obtained in this investigation suggest that if 2 and 3 are formed within serotonergic neurons by oxidation of 1 they could evoke neurodegenerative effects by at least two chemical mechanisms. For example, 2, which is the precursor of 3 (Scheme I), has been shown to react with the intraneuronal nucleophile GSH to form the 6-S-glutathionyl conjugate 8. A likely pathway leading from 2 to 8 is presented in Scheme III. This reaction serves as a model for similar reactions between 2 and the sulfhydryl residues of intraneuronal proteins. Alkylation of such proteins by 2 would be expected to compromise their biological function leading to cellular damage or death. That such reactions occur in vivo might be inferred from the work of Klemm and Baumgarten[26] who administered ^{14}C-labeled 1 to rats via the intracerebroventricular route and found that after 2 h the level of protein-bound radioactivity was about 30% of the total activity, a value which increased to ca. 50% after 20 h. However, the reaction between 2 (0.5 mM) and excess GSH (2.5 mM) at pH 7.4 is rather slow (approximately 30% complete after 6 h). This observation suggests that reactions between 2 and protein nucleophiles probably have little or no relevance to the toxicity of 1.

Scheme III



Scheme IV



A more plausible chemical mechanism by which intraneuronal 2 and 3 might evoke toxic and/or degenerative effects derives from their redox cycling reactions in the presence of typical cellular reductants and brain homogenate (Table I). Optimum activity of cellular redox cycling agents should occur when the redox potential ($E^{\circ\prime}$) of the agent is intermediate between that of cellular reductants (ca. −0.4 V vs NHE) and that of the $O_2/O_2^{\bullet-}$ couple (ca. −0.2 V vs NHE)[27] at physiological pH.[28] The $E^{\circ\prime}$ values for the 2/**2R** and 3/**3R** couples are −0.30 and −0.27 V vs NHE, respectively. Thus, the $E^{\circ\prime}$ values of these couples, which might be generated intraneuronally as a result of oxidation of 1, occur within the optimum range for redox cycling. General reactions associated with the chemical redox cycling of 2 and 3 in the presence of cellular reductants and molecular O_2 are shown in Scheme IV. Thus, GSH, cysteine, and ascorbate chemically reduce 2 or 3 to the corresponding trihydroxytryptamines **2R** or **3R**. Concomitantly GSSG, cystine, or dehydroascorbate, respectively, are formed. Reduced compounds **2R** and **3R** are very easily oxidized by molecular O_2 to 2 and 3, respectively. Previous studies[16] have demonstrated that the latter reactions generate $O_2^{\bullet-}$ as a byproduct. Spontaneous dismutation of $O_2^{\bullet-}$ yields H_2O_2 which in the presence of trace concentrations of transition metal ions generates $HO^{\bullet}$ as a result of the Haber–Weiss reaction:[29,30]

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\bullet}$$

Spin-trapping experiments in conjunction with electron paramagnetic resonance spectroscopy have confirmed that

$HO^{\bullet}$ is formed as a result of the oxidation of **2R** to **2** by molecular oxygen.[16] The hydroxyl radical is the most reactive of all reduced oxygen species and is widely believed to be responsible for much of the cellular damage caused by redox cycling reactions, particularly peroxidation of membrane lipids[31] and damage to proteins.[29,32] Cytotoxicity deriving from the redox cycling reactions conceptualized in Scheme IV depends upon intraneuronal generation of $O_2^{\bullet-}$ at rates which exceed the capacity of protective enzyme systems and antioxidants to destroy this reduced oxygen species. Whether the rates of O_2 consumption (and hence $O_2^{\bullet-}$ production) measured when **2** or **3** are incubated with GSH, cysteine, and ascorbate (Table I) are sufficiently rapid to exceed intraneuronal protective mechanisms is presently unanswerable. However, a brain homogenate clearly evokes a significant catalytic effect on the redox cycling reactions of both **2** and **3** (Table I). Indeed, the redox potentials of **2** and **3** are such that their reduction should be catalyzed by intraneuronal flavoprotein enzymes of relatively low substrate specificity.[31] Such enzyme-mediated redox cycling mechanisms would be expected to greatly accelerate the rate of production of cytotoxic reduced oxygen species.

Conclusions

Centrally administered **1** is rapidly taken up by serotonergic neurons.[3-5] The intraneuronal fate of **1** remains to be determined although biochemical studies in vivo[11] and in vitro[26] suggest that it is oxidized. Autoxidation and many in vitro enzyme-mediated oxidations of **1** give **2** and **3** as the major products.[15,16] The present investigation has demonstrated that **2** and **3** are potent general toxins following intraventricular administration to mice. However, the neurotransmitter/metabolite changes which occur in whole mouse brain following intracerebral administration of **2** or **3** are transient ($\leq$1 h to ca. 1 day), disappear after one week, and are different to those caused by similar administration of **1**. In particular, neither **2** nor **3** cause the profound and prolonged depletion of 5-HT which occurs following infusions of **1**. Thus, it is quite apparent that extraneuronal oxidation of **1** to **2** and **3** cannot occur to any significant extent. Taken together, these observations and conclusions lead to the hypothesis that oxidative conversion of **1** to **2** and **3** occurs after the transport of the indoleamine into serotonergic neurons. Furthermore, it is proposed that **2** and **3** are at least partially responsible for the resulting degeneration of serotonergic neurons. The most probable chemical mechanism by which **2** and **3** could evoke toxic effects inside such neurons involves redox cycling reactions (Scheme IV) which generate $O_2^{\bullet-}$ and, as a result of transition metal ion-catalyzed Haber–Weiss chemistry, cytotoxic $HO^{\bullet}$. Furthermore, increased oxygen consumption resulting from redox cycling reactions of **2** and **3** would lead to hypoxic conditions which are known to grossly change intermediary metabolism.[31] Indirect support for the above hypothesis is provided from the observation that $HO^{\bullet}$ scavengers protect sympathetic neurons against destruction by **1**.[8] A second, potentially cytotoxic mechanism derives from the ability of **2** to alkylate protein nucleophiles. However, the slow rate of the reaction of **2** with GSH argues against this reaction being an important contributor to the neurotoxic events deriving from **1**.

Compounds **2** and **3** are both quite potent general toxins when administered into the mouse brain. However, it seems unlikely that these drugs are taken up into serotonergic, catecholaminergic, or cholinergic neurons because if this occurred followed by intraneuronal redox cycling a neurotoxic effect would be expected, i.e., a profound and long-lasting neurotransmitter deficit. The fact that such effects were not observed, therefore, suggests that **2** and **3** are unable to enter the terminals of such neurons. The basic mechanisms underlying the transient behavioral and neurochemical changes evoked by intraventricular administration of **2** or **3** are presently unknown. However, it seems reasonable to tentatively conclude that the lethal effects of **2** and **3** are related to redox cycling reactions and concomitant hypoxia.

The hypothesis advanced above is based on the proposition that the neurotoxic properties of **1** derive from intraneuronal reactions of **2** and **3** formed as a result of oxidation of the indoleamine following its selective uptake into serotonergic neurons. The hypothesis therefore neglects the actual process of intraneuronal oxidation of **1** and hence the roles of intermediates in the process in expressing the neurodegenerative properties of the indoleamine. However, if the intraneuronal oxidation of **1** is catalytically assisted by trace levels of transition metal ions, $O_2^{\bullet-}$, or enzymes, then a previous study indicates that a variety of organic radicals deriving from the indoleamine are probably formed.[16] Indeed, using a spin-trapping agent, it has been possible to detect at least one such radical using EPR spectroscopy[16] although its structure has not yet been elucidated. Such radicals derived from **1** could interact with and damage a variety of intraneuronal constituents and hence contribute to the neurotoxic properties of the indoleamine.

Experimental Section

5,7-Dihydroxytryptamine (**1**, creatinine sulfate salt), glutathione (GSH), the oxidized form of GSH (GSSG), acetylcholine (ACh) chloride, acetylthiocholine (ATCH) chloride, choline (Ch) chloride, norepinephrine (NE) hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA) hydrochloride, 5-hydroxyindole-3-acetic acid (5-HIAA), 5-HT (creatinine sulfate monohydrate salt), homovanillic acid (HVA), disodium ethylenediaminetetraacetic acid (Na_2EDTA), ascorbic acid, and guaiacol were obtained from Sigma (St. Louis, MO) and were used without additional purification. Compounds **2** and **3** were synthesized by autoxidation of 3 mM **1** in pH 7.4 phosphate buffer (μ = 0.1). The reaction solution was stirred for 7–8 h at room temperature. The procedures employed to separate and purify **2** and **3** have been described in detail previously.[15]

Preparative high-performance liquid chromatography (HPLC) employed a Gilson (Middleton, WI) system consisting of two Model 305 pumps equipped with 25-mL pump heads, a Rheodyne (Cotati, CA) Model 7125 loop injector fitted with a 10-mL sample loop, and a Gilson Holochrome variable-wavelength UV detector set at 254 nm. The system was controlled by an IBM PS/2 computer. A preparative reversed-phase column (J. T. Baker, Phillipsburg, NJ; RP-18, 10 μm particle size, 250 × 21 mm) and a short guard column (50 × 10 mm) packed with the same material were used. Preparative HPLC Method I employed a binary gradient solvent system. Solvent A was prepared by adding 8.0 mL of HPLC grade methanol (MeOH) and 7.0 mL of concentrated ammonia solution (NH_4OH) to 1000 mL of deionized water and adjusting the pH to 3.80 by addition of 85% formic acid (HCOOH). Solvent B was MeOH. The gradient employed was as follows: 0–14 min, linear gradient from 100% solvent A to 7% solvent B with a corresponding linear increase of the flow rate from 10 to 12 mL/min; 14–25 min, 7% solvent B and a linear increase of flow rate to 15 mL/min; 25–28 min, 7% solvent B, flow rate 15 mL/min; 28–42 min, linear increase to 30% solvent B, flow rate 15 mL/min; 42–50 min, linear increase to 40% solvent B, flow rate 15 mL/min. The mobile phase was then linearly returned to 100% solvent A over 10 min at a flow rate of 15

mL/min. Preparative HPLC Method II, used for desalting purposes, employed solvents B (MeOH) and C. Solvent C was prepared by adding 0.5 mL of concentrated HCl to 1000 mL of deionized water. The following binary gradient was employed: 0–7 min, linear gradient from 100% solvent C to 7% solvent B at a constant flow rate of 10 mL/min; 7–9 min, linear gradient to 20% solvent B and a linear increase of flow rate to 16 mL/min; 9–30 min, 20% solvent B, flow rate 16 mL/min.

Analytical HPLC Method III, used to monitor the course of reactions, employed a Gilson System 42 equipped with dual Model 302 pumps controlled by an Apple IIe computer and a Waters (Milford, MA) Model 440 UV detector (254 nm). A Rheodyne Model 7125 loop injector was equipped with 2.0-mL or 200-μL sample loops. A reversed-phase column (Brownlee Laboratories, San Jose, CA, RP-18, 5-μm particle size, 250 × 7 mm) was employed which was protected by a short guard column (Brownlee, RP-18, 5 mm, OD-GU, 50 × 5 mm). A binary gradient mobile phase with solvents A and B was used as follows: 0–1 min, 100% solvent A; 1–60 min, linear gradient to 50% solvent B. The flow rate was constant at 2.5 mL/min.

Low- and high-resolution fast-atom bombardment mass spectrometry (FAB-MS) was performed on a VG Instruments (Manchester, UK) Model ZAB-E spectrometer. ^{1}H and ^{13}C NMR spectra were obtained with a Varian model 300XL spectrometer. UV–visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. Cyclic voltammetry was carried out with either a conventional operational amplifier-based instrument or with a BAS Model 100A (Bioanalytical Systems, West Lafeyette, IN) electrochemistry system. Voltammograms were corrected for iR drop. Cyclic voltammograms were obtained at a pyrolytic graphite electrode (PGE; Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 4 mm^2. The preparation of the PGE has been described elsewhere.[33] A conventional three-electrode electrochemical cell was employed containing a platinum counter electrode and a saturated calomel reference electrode (SCE). All potentials are referred to the SCE at ambient temperature (22 ± 2 °C) except where otherwise noted. Test solutions were thoroughly deoxygenated with a vigorous stream of nitrogen for about 3 min before cyclic voltammograms were recorded.

Oxygen consumption rates were measured with a Clark-type oxygen electrode assembly (YSI, Yellow Springs, OH; Model 5300). In order to measure oxygen consumption, 3.0 mL of test solutions, containing 7 or 8 and various reducing agents in pH 7.4 phosphate buffer having an ionic strength (μ) of 0.1, were incubated at 37 °C.

Isolation and Identification of 6-*S*-Glutathionyl-5-hydroxytryptamine-4,7-dione (8). GSH (7.5 mM) and 1 (1.5 mM) were incubated in 50 mL of pH 7.4 phosphate buffer (μ = 0.1) at 37 °C for 18 h. After this time repetitive 10-mL aliquots of the bright pink-purple solution were injected into a preparative HPLC system using method I. Compound 8 eluted at a retention time (t_R) of 26 min as a bright pink solution. The fractions containing 8 were combined and then concentrated by freeze drying. The resulting solution was desalted using preparative HPLC method II. Under these conditions a peak due to ammonium formate eluted at t_R = 7 min, and that due to 8 at t_R = 19 min. The desalted product solution was orange-pink and, after freeze drying, yielded an orange-brown solid. The UV–visible spectrum of 8 was very characteristic with λ_{max} (pH 3.8, HPLC mobile phase): 520, 350 (sh), 296, 260, and 238 nm; in pH 7.4 phosphate buffer, 526, 350 (sh), 300, 260, and 232 nm. FAB-MS (thioglycerol matrix) showed m/e = 512 (MH^+, 6.8%) and 534 (MNa^+, 8.1%). Exact mass measurements on MH^+ gave m/e = 512.1506 ($C_{20}H_{26}N_5O_9S$; calcd. m/e = 512.1451). Thus, 8 has a molar mass of 511 g and a molecular formula $C_{20}H_{25}N_5O_9S$ and, hence, consisted on one residue each of 2 and GSH. ^{1}H NMR (Me_2SO-d_6): δ 12.60 (s, 1 H, N(1)H), 8.40–8.20 (m, 5 H, OH, 2×NH, NH_2), 7.95 (bs, 3 H, NH_3^+), 7.06 (s, 1 H, C(2)H), 4.20 (dd, 1 H, J = 4.5 Hz, J = 4.8 Hz, C(d)H), 3.94 (bs, 1 H, C(a)H), 3.69 (t, 1 H, J = 17.5 Hz, 2 H, C(f)H_2), 3.30 (dd, 2 H, J = 4.5 Hz, J = 4.8 Hz, C(e)H_2), 3.04 (m, 2 H, C(α)H_2), 2.94 (m, 2 H, C(β)H_2), 2.40–2.25 (m, 2 H, C(c)H_2), 2.06–1.97 (m, 2 H, C(b)H_2); in D_2O δ 6.96 (s, 1 H, C(2)H), 4.42 (dd, J = 5.2 Hz, J = 2.7 Hz, 1 H, C(d)H), 3.98 (t, J = 6.5 Hz, 1 H, C(a)H), 3.80 (s, 2 H, C(f)H_2), 3.24–3.15 (m, 4 H, C(e)H_2, C(β)H_2), 2.96 (t, J = 6.9 Hz, 2 H, C(α)H_2), 2.52 (t, J = 7.5 Hz, 2 H, C(c)H_2), 2.15 (m, 2 H, C(b)H_2). The above assignments were based on comparisons of the spectrum of 8 with those of 2 and GSH and by the use of homonuclear decoupling experiments. ^{13}C NMR (Me_2SO-d_6 + D_2O): δ 179.46 (C=O), 178.67 (C=O), 174.19 (C=O), 172.66 (C=O), 172.32 (C=O), 172.20 (C=O), 163.78, 133.37, 124.90, 120.65, 119.51, 107.48, 53.03, 52.80, 41.04, 39.22, 34.01, 31.05, 25.55, 23.32.

Animals. Brain homogenates used for polarographic oxygen consumption measurements were prepared from male Wistar rats. Following sacrifice by decapitation, the brain was rapidly removed and homogenized in 17 mL of a medium consisting of Krebs Henseleit–Hepes buffer containing 140 mM NaCl, 5 mM KCl, 10 mM Tris–Hepes, 5 mM $NaHCO_3$, 1.3 mM $MgSO_4$, 1 mM $NaHPO_4$, pH 7.4. Homogenization was accomplished with 10 strokes of a Teflon-coated glass Potter homogenizer. The resulting homogenate was equilibrated by stirring in air for 3–4 min.

In vivo experiments employed outbred adult male mice of the Hsd:ICR albino strain (Harlan Sprague-Dawley, Madison, WI) weighing 25–35 g. These mice were housed 10 per cage, allowed access to Purina Rat Chow and water ad libitum, and maintained on a 12 h light/dark cycle with lights on at 7:00 a.m. No mice were used in experiments until at least 7 days following receipt from the supplier. The experimental animals used in the reported studies were treated with 5 μL of isotonic saline (0.9% NaCl) containing 1.0 mg/mL of ascorbic acid administered intracranially in the vicinity of the left lateral ventricle, after light ether anesthesia, containing various doses of 2 and 3. All doses of these drugs refer to the free base. Control animals were treated with 5 μL of vehicle alone under otherwise identical conditions.

In experiments designed to determine the effects of 2 and 3 on neurotransmitter/metabolite levels, animals were sacrificed after predetermined periods of time following drug administration using 250 ms of 7.0 kW microwave radiation concentrated on the head and delivered by an NJE-2603-10kW Microwave Irradiator (New Japan Radio, Tokyo, Japan).[34] After sacrifice the brain was rapidly removed from the skull cavity, weighed, and homogenized. The homogenization solution (1.00 mL/brain) contained 0.50 M acetic acid, 0.50 M sodium acetate, 0.40 M $NaClO_4$, 4.70 nmol/mL of guaiacol, and 40.0 nmol/mL of ATCh. The latter two compounds were employed as internal standards for the HPLC analyses for the biogenic amines and metabolites, and ACh/Ch, respectively. Homogenization was performed in a Kontes ground-glass Duall apparatus using 20 up/down strokes with the pestle attached to a Fisher Dynamix motor at a setting of 10. The homogenate was centrifuged using a Beckman L8-80 centrifuge at 50 000g and 4 °C for 1 h; the supernate was filtered through a 0.45 μm BAS (West Lafeyette, IN) polyacetate filter with the help of low-speed centrifugation, and captured in a 1.5 mL polypropylene tube. The filtrate so obtained was stored at −80 °C until required. Aliquots (20.0 μL) of this filtrate were employed to measure the concentrations of ACh and Ch in whole mouse brain using a modification of the HPLC method with electrochemical detection (LCEC) described by Eva et al.[22] The concentrations of NE, DA, 5-HT, and related metabolites in whole mouse brain were measured using 5.0 μL aliquots of the filtrate and a modification of the LCEC method in Lin et al.[21] The exact experimental procedures employed to measure ACh and Ch and the biogenic amines and their metabolites have been recently described in detail elsewhere.[23] The results of all endogenous neurochemical determinations are reported as the average plus or minus the standard error of the mean, with both quantities expressed as percent of controls. Student's t test was used to assess statistical significance, with $P \leq 0.05$ being taken as a significant difference. Typical neurochemical concentrations found in the whole brain analyses for controls, expressed in nmol/g wet tissue weight, were as follows: NE 3.43 ± 0.55; DOPAC, 1.16 ± 0.14; DA, 6.30 ± 0.40; 5-HIAA, 1.36 ± 0.23; HVA, 2.20 ± 0.59; 5-HT, 4.07 ± 0.12; ACh, 33.5 ± 5.2; Ch, 60.0 ± 2.9.

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